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Exaggerated Glucagon Responses to Hypoglycemia in Women with Polycystic Ovary Syndrome

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Abstract

Context—Premenopausal women have blunted counter-regulatory hormone responses (CRR) to hypoglycemia compared to men. Postmenopausal women have CRR similar to men; the premenopausal pattern can be restored by estrogen. However, glucagon and pancreatic polypeptide (PP) responses remain lower in postmenopausal women than in men. Since hyperandrogenemia contributes to the metabolic phenotype of polycystic ovary syndrome (PCOS), we hypothesize that CRR to hypoglycemia especially of glucagon and PP is exaggerated in premenopausal women with PCOS compared to premenopausal control women.

Study Subjects and Methods—Ten obese women with PCOS and 9 control women of similar ethnicity, age and BMI underwent determination of CRR in response to hypoglycemia during 180-min 60 mU/m^2/min insulin dose hypoglycemic clamp with isotopic assessment of endogenous glucose production (EGP). To assess CRR to hypoglycemia, glucagon, cortisol, growth hormone (GH), epinephrine, norepinephrine, PP, lactate, free fatty acid (FFA), β-hydroxybutyrate, and glycerol levels were sampled at 15-min intervals throughout the clamp.

Main Findings—Incremental glucagon levels were ~3-fold higher during hypoglycemia (P=0.03) in PCOS. Postabsorptive, steady-state and incremental GH, cortisol, epinephrine, norepinephrine, PP, FFA, glycerol and β-hydroxybutyrate did not differ. At target glucose levels of ~52 mg/dl, insulin mediated glucose disposal (IMGD) was decreased by ~40% (P=0.02) in PCOS, compared to control women, despite ~20% higher steady-state insulin levels (P=0.03). Neither postabsorptive nor steady-state EGP differed. However, postabsorptive lactate levels were ~50% higher (P=0.02). PCOS status (P=0.038) and IMGD (P=0.024) predicted the differential glucagon

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response to hypoglycemia in separate regression models, however, neither parameter remained an independent predictor in a combined model.

Principal Conclusions—Glucagon responses were increased in PCOS, whereas other CRR did not differ. Women with PCOS were insulin resistant under hypoglycemic conditions and higher postabsorptive lactate levels in PCOS were consistent with this finding. Insulin resistance may have contributed to exaggerated glucagon response to hypoglycemia in PCOS.

Keywords

counter-regulatory hormones; polycystic ovary syndrome; hypoglycemia

Introduction

Polycystic ovary syndrome (PCOS) is a common endocrinopathy; the classic syndrome of hyperandrogenism and anovulation affects 5–10% of reproductive aged women [1]. Women with the classic phenotype are at markedly increased risk for type 2 diabetes due to defects in both insulin action and secretion [1]. Under euglycemic conditions, affected women have 30–40% decreases in insulin-mediated glucose disposal (IMGD), which primarily reflects skeletal muscle insulin resistance [2], independent of obesity [3]. In contrast, there is a synergistic deleterious effect of obesity *per se* on hepatic insulin action [1] so that abnormalities in endogenous glucose production (EGP) are seen only in obese affected women. Although some of these defects appear to be intrinsic [1], hyperandrogenemia contributes to insulin resistance in PCOS [4, 5].

Sex differences exist in counter-regulatory responses (CRR) to hypoglycemia. Premenopausal women have blunted CRR compared to men [6–8]. Premenopausal women also have reduced EGP compared to men during hypoglycemia [6, 9], which may be a consequence of decreased responses to hypoglycemia in women of epinephrine and other counter-regulatory hormones that increase hepatic glucose output [6, 7, 9]. After menopause, most CRR in women are similar to those in men; a premenopausal response pattern in postmenopausal women can be restored with estrogen administration [10]. However, glucagon and pancreatic polypeptide (PP) responses remain lower in postmenopausal women than in men [10]. These differences suggest that both estrogen-dependent and estrogen-independent, such as direct androgen action, mechanisms contribute to sex differences in CRR. Since women with PCOS have elevated circulating androgen levels, it is possible that they may have greater CRR to hypoglycemia compared to premenopausal women without PCOS, similar to what has been observed in men. If such exaggerated responses were present, they could contribute to the metabolic abnormalities frequently observed in PCOS. However, there has been very limited investigation of CRR or insulin action during hypoglycemia in PCOS [11, 12]. We performed this study to test the hypothesis that women with PCOS have exaggerated CRR to hypoglycemia compared to premenopausal women without PCOS.

Subjects and Methods

Subjects

Participants were healthy Caucasian women of European ancestry, between the ages of 18–40 years and BMI ≥30 kg/m^2. None of the women was taking any medications affecting gonadal function or glucose metabolism for at least one month prior to the study, except for contraceptive steroids, which were stopped at least three months prior to the study. PCOS was diagnosed by the NIH criteria of hyperandrogenism and ≤8 menses per year with exclusion of other hyperandrogenic disorders of the ovaries, adrenals and pituitary [1]. All women with PCOS had elevated total and/or bioavailable T levels obtained at the initial screening visit [13]. Control women had normal reproductive histories, menses every 27–35 days, no clinical evidence for hyperandrogenism and normal circulating androgen levels.

Ethical Approval

The study was approved by the Institutional Review Board of Feinberg School of Medicine at Northwestern University, Chicago, IL. Written informed consent was obtained from all participants.

Data Collection

All women were studied in the Clinical Research Unit of the Feinberg School of Medicine. After a 3-day 300-g/d carbohydrate diet and overnight fast, all women underwent a 75-g oral glucose tolerance test in the morning between 0830-0930-h. Control women with fasting glucose levels ≥100 mg/dl or 2-h postchallenge glucose levels ≥140 mg/dl and affected women with 2-h postchallenge glucose levels ≥200 mg/dl were excluded from the study [14].

Hypoglycemic Clamp

After a 3-day 300-g/d carbohydrate diet, subjects were admitted to the Clinical Research Unit. Control women were studied in the follicular phase of the menstrual cycle. Further, a serum progesterone level in the follicular range on the day prior to study confirmed the absence of recent ovulation in all women. At 0600 h, after a 10-h fast, a primed, continuous infusion of [6,6-^{2}H]-glucose (Cambridge Isotope Laboratories, Andover, Massachusetts), 0.065 mg/kg/min, was initiated (−180-min). At 0900-h (0-min), a primed continuous infusion of insulin, 60 mU/m^2/min, was initiated for 180-min. Twenty-percent dextrose solution enriched to 1.6% [6,6-^{2}H]-glucose was infused to maintain a target glucose level of ~52 mg/dl; women who did not achieve a steady-state glucose of 50–55 mg/dl were excluded from the analysis (4 PCOS).

Blood pressure and heart rate were measured every 15 min. Neuroglycopenic symptoms were assessed every 15 min as reported [10]. Arterialized venous blood samples were obtained as reported [15] at −30, −15, and 0-min and every 5-min for the duration of the study. Blood glucose levels were determined in every sample using a YSI Glucose Analyzer (YSI Inc, Yellow Springs, OH). Samples for glucagon, cortisol, GH, epinephrine, norepinephrine, PP, lactate, FFA, β-hydroxybutyrate, and glycerol levels were taken every

15-min for later assay; also measured off-line were plasma specific glucose activity on samples taken at −30, −15, 0, 120, 150 and 180-min of the study.

Analytic Techniques

Glucose, [6,6 ^{2}H]-glucose specific activity, total and bioavailable T, sex hormone binding globulin, dehydroepiandrosterone sulfate, insulin, C-peptide, epinephrine, and norepinephrine levels were determined as reported [15, 16]. Cortisol and GH assays were performed at the University of Virginia Center for Research in Reproduction Ligand Assay and Analysis Core on Siemens Immulite 2000 immunoassay system (Siemens Healthcare Diagnostics, LA, CA). The sensitivity of the assay for cortisol is 1 μg/dL with an intra-assay CV of 4.8% and an inter-assay CV of 7.7%. The sensitivity for GH assay is 0.5 ng/mL with an intra-assay CV of 2.5% and inter- assay CV of 2.8%. PP assay was performed at the Diabetes Research and Training Center, University of Chicago using a radioimmunoassay (Alpco, Salem, NH) with the limit of sensitivity of 3 pmol/L with an intra-assay coefficient of variation of less than 3%. Glucagon, lactate, β-hydroxybutyrate, FFA and glycerol assays were performed at the Institute for Clinical and Translational Research Analytic Biomarker Research Core, Albert Einstein College of Medicine, Bronx, NY. All of these assays were run on a Beckman Coulter AU400 chemistry autoanalyzer with the exception of glucagon, which was run on a Perkin Elmer Wizard2 gamma counter. Glucagon was assayed using glucagon radioimmunoassay by ALPCO Immunoassays (Salem, NH) with an intra-assay CV of 4.8%. β-hydroxybutyrate was assayed using Pointe Scientific enzymatic reagent set by Pointe Scientific, INC. (Canton, MI) with an intra-assay CV of 1.7%. Lactate was assayed using system reagent for quantitative determination on Beckman Coulter AU analyzers (Brea, CA) with an intra-assay CV of 2.1%. Glycerol was assayed using Sigma reagent kit by Sigma-Aldrich (St. Louis, MO). FFA was assayed using HR Series NEFA-HR an in vitro enzymatic colorimetric method by Wako (Wako Pure Chemical Industries, LTd. Osaka, Japan) with minimum detectable level of mEq/L. Postabsorptive glucagon (1 PCOS), IMGD and EGP (1 control), as well as norepinephrine and epinephrine samples (4 PCOS, 3 control) were missing due to technical difficulties.

Calculations

Steady-state [6,6 ^{2}H]-glucose specific activity was confirmed between -30-0 and 150-180 min of the hypoglycemic clamp. Accordingly, postabsorptive as well as clamped EGP were calculated using steady-state tracer kinetics [17]. IMGD and the metabolic clearance of insulin (MCRI) were calculated as reported [3]. Postabsorptive and steady-state levels were the mean of −30, −15 and 0-min and 150, 165 and 180-min samples, respectively. Incremental levels were the difference between steady-state and postabsorptive levels.

Statistical Analysis

As a conservative approach due to the relatively small sample size, a Gaussian distribution was not assumed and nonparametric Wilcoxon rank sum tests were implemented using SAS Software for Windows, Version 9.3 (SAS Institute, Cary, NC) with α set at 0.05. Data was expressed as the median [25th–75th interquartile range]. Simple linear regression analyses were performed to assess the intercorrelation between variables of interest. Significant factors identified by these regression analyses were then included as independent variables

in a multivariate regression model to assess the individual impact of each variable effect on various outcome measures. Postabsorptive glucagon (1 PCOS), IMGD and EGP (1 control), as well as norepinephrine and epinephrine values (4 PCOS, 3 control) were missing due to technical difficulties.

Results

Age and BMI did not differ between PCOS and control women by design (Table 1). Levels of T and bioavailable T were increased in PCOS, as expected (Table 1). Three PCOS women had impaired glucose tolerance and one PCOS woman had both impaired fasting glucose and impaired glucose tolerance [14]. However, neither fasting nor 2-h postchallenge glucose levels differed between PCOS and control women (Table 1).

Target glucose levels of ~52 mg/dl were achieved at 45-min [30–60] in PCOS vs. 30-min [25–35] in control women (P=0.08, Figure 1). Postabsorptive (29 [19–31] PCOS vs 16 [13–19] Control μU/ml, P=0.01) and steady-state insulin (151 [125–182] PCOS vs 127 [121–141] Control μU/ml, P=0.03) levels were higher in PCOS. Postabsorptive C-peptide levels were higher in PCOS (2.88 [2.41–3.59] PCOS vs 2.17 [1.81–2.46] Control ng/dl, P=0.03) but steady-state C-peptide levels did not differ (0.51 [0.38–0.56] PCOS vs 0.52 [0.45–0.52] Control ng/dl, P=0.66). The MCRI did not differ between groups (data not shown). Postabsorptive (data not shown) and steady-state EGP (Figure 1) did not differ between groups. IMGD was decreased by ~40% in PCOS (Figure 1).

Neither heart rate, blood pressure nor neuroglycopenic symptom scores differed between groups (data not shown). Postabsorptive and steady-state glucagon levels did not differ (Table 1) but incremental glucagon levels were ~275% higher in PCOS (Figure 2). Postabsorptive lactate levels were ~50% higher in PCOS (Table 1) but steady-state and incremental lactate levels did not differ. There was a trend toward decreased steady-state and incremental cortisol levels (both P=0.09, Figure 2) in PCOS. Postabsorptive, steady-state and incremental norepinephrine, epinephrine, PP, GH, FFA, β-hydroxybutyrate and glycerol levels did not differ between groups (Table 1).

PCOS status and IMGD were significantly associated with incremental glucagon response in the simple linear analysis (Table 2). However, in a multivariate linear regression model, neither PCOS status nor IMGD remained significantly associated with glucagon response (Table 2). There was a trend toward a significant association between PCOS status and cortisol response (Table 2) in the simple linear regression analysis. Neither age nor BMI were significantly associated with glucagon or cortisol response. BMI was not was not significantly associated with IMGD (data not shown, P=0.848).

Discussion

Glucagon responses to hypoglycemia were significantly increased in PCOS, a pattern similar to that in normal men [10]. Cortisol response to hypoglycemia was borderline reduced in PCOS. Other CRR did not differ in PCOS compared to control women. Women with PCOS had ~40% lower IMGD under hypoglycemic conditions, despite higher steady-state insulin levels in affected women. Further, this decrease was similar to the one that we have

previously found under euglycemic conditions in PCOS [3]. Postabsorptive lactate levels were increased in PCOS, providing additional evidence for insulin resistance [18]. EGP did not differ during hypoglycemia in affected women, despite higher insulin levels and glucagon responses. It is possible that the action of insulin to suppress hepatic glucose production was antagonized by the opposite effect of glucagon to stimulate this parameter [2, 19]. The higher insulin levels in PCOS during the hypoglycemic clamp were most likely due to decreases in insulin clearance, despite similar MCRI in the two groups. The observation that steady-state C-peptide levels did not differ during the clamp supports the hypothesis that decreased clearance rather than increased secretion accounted for the higher steady-state insulin levels in PCOS.

Premenopausal women have blunted CRR [6–8] and EGP increases during hypoglycemia [6, 9] compared to men. Most of these sex differences are lost after menopause and can be restored by estrogen administration suggesting that the difference is a consequence of circulating estradiol levels [10]. Circulating estradiol levels in PCOS are in midfollicular range [20]; our findings suggest that these levels were adequate to maintain the premenopausal CRR pattern for most counter–regulatory hormones. Although glucagon responses increase in postmenopausal women, they remain blunted compared to those in men [10]. In contrast, blunted PP responses in women compared to men are unaffected by menopausal status [10]. These findings suggest that there is an estrogen-independent component to the sex difference in these responses. Hyperandrogenemia is a cardinal feature of PCOS and was an inclusion criterion for PCOS in our study [1]. In women with PCOS, there is evidence that T acting via the androgen receptor causes resistance of the hypothalamic-pituitary axis to the normal feedback effects of estradiol and progesterone to slow GnRH pulses in affected women [21]. Thus, it is possible that a similar direct action of T accounts for the male pattern glucagon responses to hypoglycemia we found in PCOS.

We did not directly assess the impact of hyperandrogenism on glucagon and cortisol responses. However, the simple linear regression analyses suggested that PCOS status was a significant predictor of both glucagon and cortisol responses. IMGD was also independent significant predictor of glucagon response suggesting that insulin resistance or an unmeasured factor associated with insulin resistance contributed to the exaggerated response. BMI was not significantly associated with IMGD or glucagon response. However, PCOS and control women had similar degrees of obesity so a relationship with BMI may have been obscured. Further, these conclusions should be interpreted with caution due to the relatively small sample size. Additionally, we have evaluated multiple endpoints in this study so there is a chance of false positive results. Thus, our findings should be viewed with caution. Future investigations of larger sample size are needed to directly assess the impact of hyperandrogenism, insulin resistance and hyperinsulinemia on both glucagon and cortisol response to hypoglycemia in PCOS.

There have been very few studies of responses to hypoglycemia in PCOS [11, 12, 22, 23]. Gennarelli and colleagues [11] reported CRR responses to uncontrolled hypoglycemia after an iv insulin bolus in PCOS and control women stratified by obesity. The diagnosis of PCOS was based on ovarian morphology and a history of oligo- or amenorrhea. However, it was unclear whether all PCOS women fulfilled NIH criteria by also having clinical or

biochemical evidence of hyperandrogenism [11]. Norepinephrine responses and hypoglycemic symptoms were blunted in the obese PCOS women, while GH responses were increased in nonobese affected women [11]. There were no differences in basal or incremental glucagon or cortisol responses in PCOS compared to control women in either BMI category [11]. Differences in body weight as well as the duration and mechanisms of hypoglycemia likely accounted for the discrepant findings between the present compared to this previous study [11]. In a separate publication on pituitary-adrenal axis responses to hypoglycemia [23] in what appears to be the same group of women and study protocol, Genarelli and colleagues found that there was a more rapid decline in cortisol levels in response to uncontrolled hypoglycemia in PCOS compared to control women.

Ludwig and colleagues [12] assessed insulin action and leptin responses but not CRR in PCOS diagnosed according to NIH criteria and control women using a sequential hyperinsulinemic clamp to vary glucose levels in a stepwise pattern from hyperglycemia to hypoglycemia in the same range as our study. IMGD was significantly decreased under hypoglycemic conditions analogous to our findings. However, the study may have been constrained by the independent effect of adiposity on insulin sensitivity since the PCOS women tended ($P=0.08$) to be heavier, mean BMI 28.0 kg/m^2, than the control women, mean BMI 21.6 kg/m^2 [12]. Cortisol and other steroid responses to these glycemic changes were reported in a second publication from Ludwig and colleagues [22]. Cortisol levels were lower at baseline and remained lower throughout the study in PCOS compared to control women [22].

Our sample size was relatively small, although similar in size to other studies that assessed sex differences in CRR [7, 8, 9, 10, 11, 22, 24, 25]. The basal and incremental glucagon responses to hypoglycemia in our study were also similar to those reported in healthy women in the literature [8, 10, 24, 25]. The findings of lower cortisol levels in PCOS women in previous studies [22, 23], including overweight and obese women, were consistent with our finding of a trend toward decreased steady-state and incremental cortisol levels in PCOS. Most hypoglycemic clamp studies in normal women have been in nonobese, reproductive-age cohorts [7, 8, 9, 25]. However, there is no evidence that either obesity [11] or age [26, 27] have an independent impact on CRR to hypoglycemia. Since women with the additional PCOS phenotypes diagnosed by the Rotterdam criteria [28] are less insulin resistance than those who fulfill the NIH criteria [29] their CRR to hypoglycemia may differ too. Accordingly, our findings are relevant only to obese women with PCOS diagnosed by the NIH criteria.

In conclusion, obese women with PCOS diagnosed by the NIH criteria have similar and substantial decreases in insulin action during hypoglycemia to those that we have previously reported during euglycemia [1, 3]. The increased postabsorptive lactate levels are consistent with insulin resistance in PCOS. Most CRR did not differ in affected compared to reproductively normal premenopausal women. The clinical relevance of this finding is that women with PCOS would be expected to have the same blunted symptomatic and counter-regulatory responses to hypoglycemia observed in healthy premenopausal women compared to hypoglycemic symptoms and CRR in men [6, 7, 8, 9, 10]. However, glucagon responses, which are partially estrogen-independent, were increased in PCOS. This finding is another

distinctive feature of the PCOS metabolic phenotype. It is possible that hyperandrogenemia, insulin resistance or an unmeasured factor associated with PCOS contribute to this finding [1, 3].

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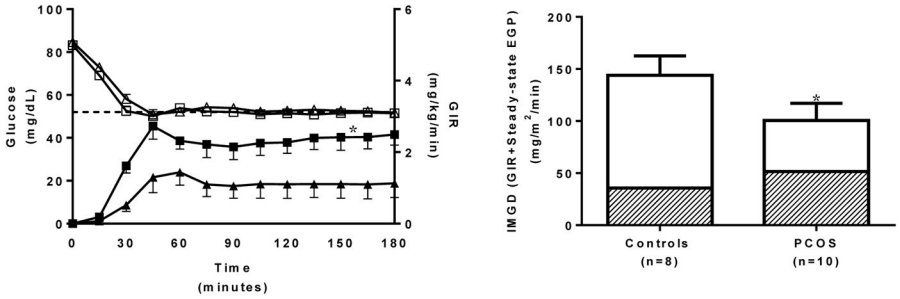

Figure 1. Hypoglycemic clamp glucose levels and glucose infusion rates, and IMGD

Left Panel: target glucose levels (52 mg/dL, dotted line) did not differ in Control (open squares) and PCOS (open triangles) women. Steady-state glucose infusion rates (GIR) were significantly lower in PCOS (closed triangles) compared to Control (closed squares) women (*P<0.05). Right Panel: steady-state endogenous glucose production (EGP, hatched bars) did not differ while GIR (open bars) differed significantly. Insulin mediated glucose disposal (IMGD), the sum of GIR and steady-state EGP, was significantly decreased in PCOS compared to Control women (*P<0.05) due to significant differences in GIR. The differences in IMGD were due to significant differences in GIR (P <0.01) rather than differences in steady-state EGP (P=0.23).

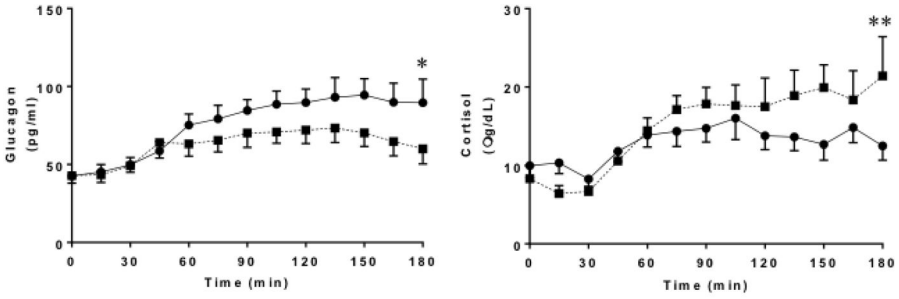

Figure 2. Incremental glucagon and cortisol response to hypoglycemia

Left Panel: Incremental glucagon response was higher in PCOS (solid lines, closed circles) compared to control (dotted lines, closed squares) women (*P=0.03). Right Panel: Incremental cortisol response trended to be reduced in PCOS (solid lines, closed circles) compared to control (dotted lines, closed squares) women (**P=0.09).

Table 1

Clinical and Biochemical Features and Counter-regulatory Responses to Hypoglycemia

	Control (n=9)	PCOS (n=10)	*P*
Age (years)	32 (30–35)	32 (31–33)	0.74
BMI (kg/m^2)	33.2 (31.7–37.3)	35.2 (31.9–38.4)	0.72
Total T (ng/dL)	22 (18–30)	65 (50–72)	0.0002
Bioavailable T (ng/dL)	8 (7–9)	24 (20–28)	0.00002
DHEAS (ng/mL)	1360 (1002–1495)	1740 (1360–2905)	0.10
SHBG (mmol/L)	38 (30–72)	28 (22–60)	0.21
Fasting Glucose (mg/dL)	91 (86–92)	89 (86–96)	0.95
2–h postchallenge Glucose (mg/dL)	110 (107–125)	109 (99–167)	0.98
Glucagon (pg/mL)			
Postabsorptive	38.4 (34.0–52.6)	43.2(37.6–47.0)[a]	0.86
Increment	15.9 (–0.47–32.7)	44.8 (26.2–69.6)	0.03
Cortisol (μg/dL)			
Postabsorptive	7.0 (5.2–9.9)	9.3 (6.6–12.8)	0.23
Increment	6.9 (5.9–19.8)	1.1 (0.0–8.7)	0.09
Growth Hormone (ng/mL)			
Postabsorptive	0.6 (0.3–1.1)	0.3 (0.1–0.6)	0.24
Increment	6.9 (2.4–9.2)	4.8 (2.1–6.5)	0.53
Norepinephrine (pg/mL)			
Postabsorptive	170 (156–187)[b]	161 (150–237)[b]	1.00
Increment	84 (71–156)[b]	125 (108–210)[b]	0.24
Epinephrine (pg/mL)			
Postabsorptive	233 (14–314)[b]	184 (28–378)[b]	0.70
Increment	118 (103–267)[b]	236 (148–352)[b]	0.18
Pancreatic Polypeptide (pmol/L)			
Postabsorptive	17 (12–26)	18 (10–24)	0.59
Increment	42 (16–108)	90 (59–129)	0.25
Lactate (mg/dL)			
Postabsorptive	6.1 (5.1–7.2)	9.5 (8.4–10.7)	0.02
Increment	5.9 (2.2–7.1)	2.1 (–1.2–3.8)	0.11
Free Fatty Acids (mmol/L)			
Postabsorptive	0.40 (0.39–0.51)	0.55 (0.35–0.59)	0.21
Increment	−0.29 (−0.43–−0.22)	0.41 (−0.52–−0.20)	0.45
β-hydroxybutyrate (mg/dL)			
Postabsorptive	0.7 (0.6–0.9)	0.8 (0.6–0.9)	0.89
Increment	0.3 (−0.5−0.2)	0.3 (−0.5–−0.2)	0.92
Glycerol (mmol/L)			

	Control (n=9)	PCOS (n=10)	*P*
Postabsorptive	0.05 (0.05–0.06)	0.06 (0.05–0.07)	0.20
Increment	0.02 (−0.03–−0.01)	0.03 (−0.04–−0.02)	0.11

Data expressed as median (25^{th}–75^{th} interquartile range);

[a] n=9;

[b] n=6;

DHEAS, Dehydroepiandrosterone sulfate; SHBG, Sex hormone binding globulin; Increment =Steady-State-Postabsorptive

Table 2

Predictors of change in glucagon and cortisol with hypoglycemia

	Increment Glucagon			Increment Cortisol	
Predictor	**Regression Coefficient**	**P value**	**Δ Regression Coefficient (P value)***	**Regression Coefficient**	**P value**
Age	0.43	0.515	--	0.10	0.762
BMI	2.62	0.277	--	0.92	0.352
PCOS	5.12	0.038	1.68 (0.216)	3.87	0.065
IMGD	6.31	0.024	2.77 (0.118)	0.001	0.970

* Model includes both PCOS and IMGD as predictors