***In vitro* evidence that hyperglycemia stimulates tumor necrosis factor- α release in obese women with polycystic ovary syndrome**

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Abstract

Women with polycystic ovary syndrome (PCOS) are often insulin resistant and have chronic low-level inflammation. The purpose of this study was to determine the effects of hyperglycemia *in vitro* on tumor necrosis factor (TNF)- α release from mononuclear cells (MNC) in PCOS. Twelve reproductive-age women with PCOS (six lean, six obese) and 12 age-matched controls (six lean, six obese) were studied. Insulin sensitivity (IS_{HOMA}) was estimated from fasting levels of glucose and insulin and percent truncal fat was determined by dual energy absorptiometry (DEXA). TNF α release was measured from MNC cultured under euglycemic and hyperglycemic conditions. IS_{HOMA} was higher in obese women with PCOS than in lean women with PCOS (student's *t*-test; 73.7 ± 14.8 vs 43.1 ± 8.6 , $P < 0.05$), but similar to that of obese controls. IS_{HOMA} was positively correlated

with percent truncal fat ($r = 0.57$, $P < 0.04$). Obese women with PCOS exhibited an increase in the percent change in TNF α release from MNC in response to hyperglycemia compared with obese controls (10 mM, $649 \pm 208\%$ vs $133 \pm 30\%$, $P < 0.003$; 15 mM, $799 \pm 347\%$ vs $183 \pm 59\%$, $P < 0.04$). The TNF α response directly correlated with percent truncal fat ($r = 0.45$, $P < 0.03$) and IS_{HOMA} ($r = 0.40$, $P < 0.05$) for the combined groups, and with plasma testosterone ($r = 0.60$, $P < 0.05$) for women with PCOS. MNC of obese women with PCOS exhibit an increased TNF α response to *in vitro* physiologic hyperglycemia. MNC-derived TNF α release may contribute to insulin resistance and hyperandrogenism, particularly when the combination of PCOS and increased adiposity is present.

Journal of Endocrinology (2006) **188**, 521–529

Introduction

Polycystic ovary syndrome (PCOS) is one of the most common female endocrinopathies, affecting 4–10% of reproductive-age women (Knochenhauer *et al.* 1998, Dunaif 1999). The disorder is characterized by hyperandrogenism, chronic oligo- or anovulation, and polycystic ovaries, with two out of these three findings required to diagnose PCOS (Rotterdam Group 2004a, 2004b). As many as 70% of women with PCOS exhibit insulin resistance, with the compensatory hyperinsulinemia considered to be the cause of the hyperandrogenism (Burghen *et al.* 1980, Nestler *et al.* 1998, Goodarzi & Korenman 2002, Rotterdam Group 2004a, 2004b). In addition, women with PCOS are often obese, a condition strongly associated with insulin resistance and hyperglycemia (Kolterman *et al.* 1980, Ciaraldi *et al.* 1981).

Hyperglycemia can contribute to the development of insulin resistance and impaired insulin secretion in a phenomenon known as 'glucose toxicity' (Rossetti *et al.* 1990, Yki-Jarvinen 1992). It is recognized that these effects may be the exaggeration of normal regulatory responses

to increases in circulating glucose. We have shown that in PCOS, hyperglycemia causes an increase in reactive oxygen species (ROS) generation from peripheral blood mononuclear cells (MNC) (González *et al.* 2006). ROS-induced oxidative stress is a known activator of nuclear factor κB (NF κB), a proinflammatory transcription factor that promotes tumor necrosis factor- α (TNF α) gene transcription (Barnes & Karin 1997, Mohanty *et al.* 2000, Evans *et al.* 2002). TNF α is an established mediator of insulin resistance (Hotamisligil *et al.* 1994). Thus, increased TNF α release from MNC in response to hyperglycemia may be an underlying mechanism for insulin resistance in PCOS.

In vitro studies have shown that TNF α can truncate insulin receptor signaling in all insulin-sensitive tissues (Feinstein *et al.* 1993, Hotamisligil *et al.* 1994, Del Aguila *et al.* 1999). In obesity-related diabetic syndromes, TNF α is overexpressed in adipose tissue and causes increased serine phosphorylation of insulin receptor substrate-1 (IRS-1) (Hotamisligil *et al.* 1993, 1995, Rui *et al.* 2001). This leads to decreased expression of GLUT 4, the insulin-sensitive glucose transport protein (Stephens &

Pekala 1991). Insulin resistance in PCOS is also a post-receptor defect, and increased serine phosphorylation is implicated as the cause of decreased insulin-stimulated IRS-1 activation and decreased GLUT 4 expression (Rosenbaum *et al.* 1993, Dunaif *et al.* 2001, Li *et al.* 2002, Corbould *et al.* 2005). Thus, the ability of TNF α to stimulate increased serine phosphorylation makes it an ideal candidate for initiating these molecular events in PCOS.

We have previously reported that circulating levels of TNF α are elevated in PCOS (González *et al.* 1999). A likely source of excess circulating TNF α in obese women with PCOS is adipose tissue, but the source remains unknown in lean women with the disorder. MNC are known to migrate into adipose tissue to activate adipocyte TNF α production (Weisberg *et al.* 2003, Wellen & Hotamisligil 2003). It is now clear that the major source of TNF α in adipose tissue of the obese is MNC-derived macrophages present in the stromal-vascular compartment (Weisberg *et al.* 2003, Xu *et al.* 2003, Fain *et al.* 2004a, 2004b). Thus, MNC may be an additional source of excess circulating TNF α in PCOS.

In the present study, we evaluated an *in vitro* model of hyperglycemia to determine the effect of direct exposure to hyperglycemia on TNF α release from MNC of women with PCOS. We hypothesized that TNF α release from MNC is increased in women with PCOS compared with weight-matched controls in response to hyperglycemic conditions, and that there is a relationship between measures of adiposity and MNC-derived TNF α release.

Materials and Methods

Subjects

Twelve women with PCOS (six lean and six obese) aged 21–34 years and 12 weight-matched control subjects (six lean and six obese) aged 20–38 years volunteered to participate in the study. The women with PCOS were diagnosed on the basis of oligoamenorrhea and hyperandrogenemia after excluding nonclassic congenital adrenal hyperplasia, Cushing's syndrome, hyperprolactinemia and thyroid disease. Polycystic ovaries were present on ultrasound in all subjects with PCOS. All control subjects were ovulatory as evidenced by regular menses and a luteal phase serum progesterone level greater than 5 ng/ml. All control subjects exhibited normal circulating androgen levels and the absence of polycystic ovaries on ultrasound.

All subjects were screened for diabetes or inflammatory illnesses, and none were taking medications that affect carbohydrate metabolism or immune function for at least 6 weeks prior to study participation. None of the subjects were involved in any regular exercise program for at least 6 months before the time of testing. All of the subjects provided written, informed consent in accordance

with the Case Western Reserve University and Metro-Health Medical Center guidelines for the protection of human subjects.

Study design

All study subjects underwent the oral glucose tolerance test (OGTT) on days 5 and 8 after the onset of menses. Before the OGTT, they were provided with a healthy diet consisting of 50% carbohydrate, 35% fat and 15% protein for 3 consecutive days (days 1–3) before the test. The test was performed on the morning of day 4 after an overnight fast of ~12 h. All subjects also underwent body composition assessment on the same day the OGTT was performed.

Oral glucose tolerance test (OGTT)

Fasting baseline blood samples (5 ml each) were drawn for glucose and insulin determination. A 75 g glucose beverage was subsequently ingested over 10 min. Blood samples (5 ml each) were again drawn for glucose and insulin determination 2 h after glucose ingestion. Upon completion of the test, subjects were fed a high-carbohydrate snack. Plasma glucose concentrations were assayed immediately from the blood samples collected. Additional plasma was isolated from the fasting blood samples and stored at –70 °C until assayed for C-reactive protein (CRP) and TNF α . Glucose tolerance was assessed by the WHO criteria with normal glucose tolerance defined as a 2-h glucose-stimulated value less than 140 mg/dl, impaired glucose tolerance as a 2-h value of 140–199 mg/dl, and type 2 diabetes mellitus defined as a 2-h value of 200 mg/dl or greater (Modan *et al.* 1989). Insulin sensitivity was estimated by IS_{HOMA} by the following formula (Matthews *et al.* 1985): fasting glucose $\times$ fasting insulin/22.5.

Body composition assessment

Height without shoes was measured to the nearest 1.0 cm. Body weight was measured to the nearest 0.1 kg. Waist circumference was measured at the level of the umbilicus and used to estimate abdominal adiposity (Kohrt *et al.* 1993). In addition, all subjects underwent dual energy absorptiometry (DEXA) to determine percent total body fat and percent truncal fat with the QDR 4500 Elite model scanner (Hologic, Waltham, MA, USA). Truncal fat content was defined as the area between the dome of the diaphragm (cephalad limit) and the top of the greater trochanter (caudal limit) (Taylor *et al.* 1998).

Analytic methods

MNC isolation and culture were performed on a 20 ml fasting blood sample drawn before ingestion of the

Table 1 Subject characteristics

	Control		PCOS	
	Lean (n=6)	Obese (n=6)	Lean (n=6)	Obese (n=6)
Age (years)	33 $\pm$ 2	30 $\pm$ 3	26 $\pm$ 2	28 $\pm$ 2
Height (cm)	165.6 $\pm$ 1.0	163.1 $\pm$ 3.6	165.4 $\pm$ 3.9	165.7 $\pm$ 2.4
Body weight (kg)	59.1 $\pm$ 2.2	91.9 $\pm$ 4.7 ^{a,b}	63.9 $\pm$ 3.5	96.8 $\pm$ 6.0 ^{c,d}
Body-mass index (kg/m ²)	21.6 $\pm$ 0.8	34.5 $\pm$ 1.2 ^a	23.3 $\pm$ 0.5	35.2 $\pm$ 1.6 ^{c,d}
Total body fat (%)	27.1 $\pm$ 1.8	43.8 $\pm$ 0.4 ^{a,b}	30.7 $\pm$ 1.7	44.2 $\pm$ 1.0 ^{c,d}
Truncal fat (%)	22.8 $\pm$ 2.4	43.2 $\pm$ 0.6 ^{a,b}	29.7 $\pm$ 2.6 ^e	45.2 $\pm$ 0.9 ^{c,d}
Waist circumference (cm)	71.3 $\pm$ 2.0	101.1 $\pm$ 3.4 ^{a,b}	78.0 $\pm$ 2.7	96.2 $\pm$ 8.0 ^{c,d}

Values are expressed as means $\pm$ S.E. ^aObese controls vs lean controls, $P < 0.0003$; ^bObese controls vs lean PCOS, $P < 0.002$; ^cObese PCOS vs lean controls, $P < 0.002$; ^dObese PCOS vs lean PCOS, $P < 0.01$; ^eLean PCOS vs lean controls, $P < 0.02$.

glucose beverage during the OGTT. The cells were isolated by Histopaque-1077 density gradient centrifugation (Boyam 1968), washed two times in pyrogen-free saline, re-suspended in RPMI (0.3 mg/ml L-glutamine, 100 U/ml penicillin and 100 μ g/ml streptomycin) with serum substitute TCH, and seeded in coated culture plates (2.510⁶ cells/ml). The culture medium was supplemented with D-glucose at varying concentrations to mimic a euglycemic (5 mM) or hyperglycemic (10 or 15 mM) environment. The cells were incubated (humidified, 5% CO₂, 37° C) for 24 h. Cell supernatants were subsequently collected (10 000 g for 2 min) and stored at -70 °C until analysis. Plasma glucose concentrations were measured by the glucose oxidase method (YSI, Yellow Springs, OH, USA), while plasma insulin concentrations were measured by double-antibody RIA (Linco Research, St Charles, MO, USA). Plasma CRP concentrations were measured by high-sensitivity ELISA (Alpha Diagnostics International, San Antonio, TX, USA). TNF α concentrations were also measured by ELISA (BioSource International, Camarillo, CA, USA). All samples from each subject were measured in duplicate in the same assay. The interassay and intra-assay coefficients of variation for all assays were 7% and 12% respectively.

Statistics

The StatView statistical package (SAS Institute, Cary, NC, USA) was used for data analysis. The difference in values under either hyperglycemic condition (10 or 15 mM) and the euglycemic baseline (5 mM) for primary dependent variables, such as TNF α release from MNC, was calculated to represent the incremental change. Descriptive data and the incremental change of variables were compared between groups by unpaired Student's *t*-test or ANOVA for multiple-group comparisons followed by post hoc analysis. Differences between the incremental change of variables within groups were analyzed by paired Student's *t*-test. Alterations in TNF α release from MNC

within groups were expressed as the percent change between either hyperglycemic condition (10 or 15 mM) and the euglycemic condition (5 mM, 100% baseline). Differences in the MNC-derived TNF α response among the different glycemic conditions within groups were analyzed by repeated-measures ANOVA. Regression analyses used the Pearson (*r*) correlation for parametric data and the Spearman rank order (ρ) correlation for nonparametric data. All values are expressed as means $\pm$ S.E.M. An α -level of 0.05 was used to determine statistical significance.

Results

Age and height were similar among groups (Table 1). Weight, body-mass index (BMI), percent total body fat and waist circumference were significantly ($P < 0.01$) greater in obese subjects than in those who were lean whether or not they had PCOS, but were similar when women with PCOS were compared with weight-matched controls. The obese also exhibited significantly ($P < 0.01$) greater percent truncal fat than lean subjects whether or not they had PCOS. However, percent truncal fat was significantly ($P < 0.02$) greater in lean women with PCOS than in lean controls.

Circulating levels of LH, testosterone and androstenedione were significantly ($P < 0.05$) higher in women with PCOS than in control subjects independently of body weight (Table 2). Circulating DHEA-S levels were modestly higher in women with PCOS than in weight-matched controls. Levels of glucose while fasting and 2 h after glucose ingestion were similar in women with PCOS to those of controls independently of body mass. All 12 control subjects had a normal glucose response during the OGTT with 2-h glucose levels of 62–138 mg/dl. Glucose values at 2 h were consistent with impaired glucose tolerance in two lean women with PCOS (141 and 148 mg/dl) and one obese woman with PCOS (192 mg/dl). Fasting insulin levels

Table 2 Plasma hormone, glucose, insulin and C-reactive protein (CRP) levels

	Control		PCOS	
	Lean (n=6)	Obese (n=6)	Lean (n=6)	Obese (n=6)
LH (mIU/ml)	3.3 ± 0.4	2.6 ± 0.3	14.4 ± 1.7 ^{a,b,c}	7.6 ± 1.2
Testosterone (ng/dl)	42.8 ± 5.8	32.7 ± 6.2	72.2 ± 6.7 ^{a,b}	67.7 ± 7.4 ^{d,e}
Androstendione (ng/ml)	1.8 ± 0.2	2.0 ± 0.1 ^b	3.2 ± 0.4 ^{a,b}	2.9 ± 0.3 ^{d,e}
DHEA-S (μg/dl)	167 ± 20	205 ± 29	319 ± 76	322 ± 67
Fasting glucose (mg/dl)	85.3 ± 2.0	84.3 ± 3.2	83.3 ± 3.2	87.8 ± 1.9
2-h glucose (mg/dl)	99.0 ± 11.8	121.8 ± 6.0	112.5 ± 11.2	113.5 ± 16.4
Insulin (μIU/ml)	8.5 ± 1.2	13.7 ± 2.5	11.5 ± 2.1	18.6 ± 3.5
CRP (ng/ml)	219 ± 92	6935 ± 139 ^f	1314 ± 412 ^{b,c}	6988 ± 1711 ^e
IS _{HOMA}	32.1 ± 4.5	52.8 ± 10.7	43.1 ± 8.6 ^e	73.7 ± 14.8 ^e

Values are expressed as means ± s.e. ^aLean PCOS vs lean controls, *P*<0.005; ^bLean PCOS vs obese controls, *P*<0.003; ^cLean PCOS vs obese PCOS, *P*<0.05; ^dObese PCOS vs obese controls, *P*<0.02; ^eObese PCOS vs lean controls, *P*<0.05; ^fObese controls vs lean controls, *P*<0.0005.

were modestly higher, and plasma CRP levels were significantly higher (*P*<0.05) in the obese whether or not they had PCOS (Table 2). CRP levels were also modestly elevated in lean women with PCOS compared with lean controls.

IS_{HOMA} was significantly (*P*<0.05) higher in obese women with PCOS than in lean controls and lean women with PCOS (Table 2). Lean women with PCOS exhibited an IS_{HOMA} that was similar to that of obese controls and lean controls. IS_{HOMA} was positively correlated with BMI (*r*=0.58, *P*=0.004), percent body fat (*r*=0.49, *P*<0.02) and percent truncal fat (*r*=0.57, *P*<0.004) for the combined groups (data not shown).

TNFα release from MNC under euglycemic conditions (5 mM) was similar in women with PCOS compared with controls (Table 3). After exposure of MNC to hyperglycemia at the 10 mM glucose concentration, the incremental change in TNFα release of women with PCOS increased significantly (*P*<0.03) compared with that of controls, which slightly declined (7.0 ± 3.3 vs −2.9 ± 2.2). When subjects were grouped by body mass, the incremental change in TNFα release from MNC exposed to 10 mM glucose increased significantly in obese women with PCOS compared with either lean controls (*P*<0.005)

or obese controls (*P*<0.05). In contrast, the incremental change in TNFα release from MNC of lean women with PCOS was similar to lean controls after the 10 mM glucose exposure. There were no significant differences in the TNFα response of women with PCOS compared with control subjects regardless of body weight after exposure of MNC to 15 mM glucose.

The percent change in TNFα release from MNC in control subjects remained unchanged under hyperglycemic conditions (10 mM, 103 ± 20%; 15 mM, 136 ± 33%) compared with the euglycemic condition (5 mM, 100%). In contrast, the women with PCOS exhibited a significant (*P*<0.02) progressive increase in the percent change in TNFα release from MNC under hyperglycemic conditions compared with the euglycemic condition. The MNC-derived TNFα response to hyperglycemia was significantly greater in women with PCOS (10 mM, 410 ± 124%, *P*<0.03; 15 mM, 541 ± 200%, *P*<0.05) than in controls. When subjects were grouped by body mass, lean controls exhibited no difference in the percent change in TNFα release from MNC under hyperglycemic conditions compared with the euglycemic condition (Fig. 1). Obese controls and lean women with PCOS exhibited a modest increase in the percent change in

Table 3 TNFα release from mononuclear cells (MNC) under euglycemic and hyperglycemic conditions *in vitro*

	5 mM	10 mM	Δ	15 mM	Δ
MNC TNFα (pg/ml)					
Lean controls (n=6)	9.4 ± 4.1	3.6 ± 0.8	−5.8 ± 4.0	6.4 ± 1.8	−3.0 ± 2.7
Obese controls (n=6)	4.5 ± 1.4	4.5 ± 1.5	0.02 ± 1.6	8.5 ± 5.3	8.5 ± 5.3
Lean PCOS (n=6)	3.0 ± 0.4	5.4 ± 1.8	2.4 ± 1.6	7.9 ± 4.5	4.9 ± 4.5
Obese PCOS (n=6)	3.0 ± 0.7	14.5 ± 6.1	11.6 ± 6.0 ^{a,b}	21.9 ± 13.6	19.0 ± 13.3

Values are expressed as means ± s.e. Δ: calculated differences between TNFα concentrations under either hyperglycemic condition (10 or 15 mM) and euglycemic baseline (5 mM). ^aObese PCOS vs lean controls, *P*<0.005; ^bObese PCOS vs obese controls, *P*<0.05.

Figure 1 Percent change in TNF α release from MNC under hyperglycemic conditions (10 and 15 mM) compared with the euglycemic condition (5 mM, 100% baseline). □: lean controls; ■: obese controls; ▨: lean women with PCOS; ■: obese women with PCOS. *TNF α response from MNC exposed to 10 mM glucose was significantly higher in obese women with PCOS than in either control group ($P<0.003$). †TNF α response from MNC exposed to 15 mM glucose was significantly higher in obese women with PCOS than in either control group ($P<0.04$).

TNF α release from MNC in response to hyperglycemia compared with lean controls. However, there was a significant increase in the MNC-derived TNF α response to hyperglycemia in obese women with PCOS (10 mM, $P<0.003$; 15 mM, $P<0.04$) compared with either control group.

After exposure of MNC to hyperglycemia at the 10 mM glucose concentration, the percent change in TNF α release from MNC was positively correlated with BMI, percent truncal fat and IS_{HOMA} for the combined groups, and with BMI for women with PCOS (Table 4). The percent change in TNF α release from MNC was also positively correlated with androstenedione for the combined groups after exposure to both 10 and 15 mM glucose, and for women with PCOS after exposure to 15 mM glucose. The percent change in TNF α release from MNC and testosterone were positively correlated for women with PCOS after exposure to 15 mM glucose, and negatively correlated for controls after exposure to both 10 and 15 mM glucose.

Discussion

Our data clearly show that *in vitro* exposure to hyperglycemia causes an increase in TNF α release from MNC of obese women with PCOS. The *in vitro* conditions demonstrate that the enhanced TNF α response is directly related to hyperglycemia, and occurs at a glucose concentration similar to the postprandial state (10 mM) or higher (15 mM). The TNF α response is directly related to the degree of insulin resistance and to the levels of androgens. Since TNF α is a proinflammatory cytokine, these findings provide further support for the role of inflammation in the development of insulin resistance and hyperandrogenism in PCOS. TNF α , in particular, may contribute to the decline in insulin action in obese women with PCOS. Furthermore, the association of TNF α with BMI, percent total body fat and percent truncal fat suggests that the combination of PCOS and increased adiposity may be a key determinant of the MNC-derived TNF α response.

Table 4 Spearman rank correlation analysis examining the relationship between the percent change in TNFα release from mononuclear cells and BMI, percent total body fat, percent truncal fat, IS_{HOMA}, testosterone and androstenedione

		BMI (kg/m ²)	Total body fat (%)	Truncal fat (%)	IS _{HOMA}	Testosterone (ng/dl)	Androstenedione (ng/ml)
(A) 10 mM glucose concentration							
Combined groups (n=24)	ρ	0.425	0.379	0.448	0.397	0.271	0.645
	P	0.041*	0.069	0.032*	0.049*	0.193	0.002*
PCOS (n=12)	ρ	0.601	0.455	0.510	0.392	0.182	0.587
	P	0.046	0.132	0.090	0.194	0.547	0.048*
Controls (n=12)	ρ	0.315	0.441	0.427	0.336	−0.755	0.098
	P	0.297	0.144	0.157	0.266	0.012*	0.745
(B) 15 mM glucose concentration							
Combined groups (n=24)	ρ	0.337	0.326	0.346	0.257	0.290	0.525
	P	0.107*	0.118	0.097*	0.217*	0.164	0.012*
PCOS (n=12)	ρ	0.322	0.126	0.140	0.175	0.594	0.245
	P	0.286*	0.676	0.643	0.562	0.048*	0.417
Controls (n=12)	ρ	0.357	0.497	0.448	0.364	−0.601	0.427
	P	0.237	0.100	0.138	0.228	0.046*	0.157

The percent change in TNFα release was determined by comparing the (A) 10 mM or (B) 15 mM hyperglycemic condition with the 5 mM euglycemic condition. *P<0.05.

observed in obese women with PCOS, and its potential role in inducing insulin resistance in these individuals.

Hyperglycemia within the physiologic range does not result in increased TNFα release from MNC in normal circumstances. Lean controls in the present study exhibited no change in MNC-derived TNFα release in response to either hyperglycemic culture condition. This is in contrast to a previous report of increases in TNFα release from MNC of normal individuals exposed to hyperglycemia *in vitro* (Morohoshi *et al.* 1996). In this study, however, the glucose concentration required to achieve this response was above the physiologic range. Since TNFα is a known mediator of insulin resistance (Hotamisligil *et al.* 1994, 1995, Del Aguila *et al.* 1999), the lack of increase in TNFα release from MNC may be a physiologic benefit in the presence of hyperglycemia. Thus, facilitation of glucose disposal in lean controls may be due to the control of TNFα release to optimize insulin signaling in the postprandial state.

In contrast, the MNC of obese women with PCOS have increased sensitivity to hyperglycemic conditions in the physiologic range. Obese women with PCOS are in a proinflammatory state, as shown by the elevations of plasma CRP observed in this group that are consistent with previous reports (Kelly *et al.* 1996, Bastard *et al.* 1999, Yudkin *et al.* 1999). MNC-derived TNFα release increased in response to either hyperglycemic culture condition in obese women with PCOS compared with obese controls. Oral intake of glucose, lipid and protein has been noted to elicit similar proinflammatory responses *in vivo* (Mohanty *et al.* 2000, 2002, Aljada *et al.* 2004). It is possible that in obese women with PCOS, feeding results in increased TNFα release from MNC in the postprandial state to promote the insulin resistance observed in

these individuals. This concept is supported by the positive correlation between the TNFα response and IS_{HOMA}. Previous reports of a reduction in oxidative stress and inflammatory mediators after caloric restriction in the obese, and after a 2-day fast in normal subjects provide further corroboration (Dandona *et al.* 1998, 2001a, 2001b).

Our data suggest a link between adiposity and MNC-derived TNFα release in PCOS. There was a direct relationship between the change in TNFα release from MNC under hyperglycemic culture conditions and abdominal adiposity. It is possible that the inflamed adipose tissue in the abdominal region of obese women with PCOS perpetuates the increased sensitivity of MNC to hyperglycemia manifested by the increased TNFα release observed in culture. Our data also demonstrate a direct relationship between the degree of insulin resistance by IS_{HOMA} and abdominal adiposity. These findings are consistent with previous observations in young adults demonstrating that changes in insulin sensitivity are a function of abdominal adiposity (Kriktetos *et al.* 2004, Linne 2004). Thus, increased TNFα release from MNC may promote the insulin resistance observed in obese women with PCOS.

The MNC of obese controls and lean women with PCOS exhibited only modest increases in TNFα release in response to hyperglycemia. Both of these groups are in a proinflammatory state, as evidenced by the elevated CRP concentrations in accordance with previous observations (Kelly *et al.* 1996, Boulman *et al.* 2004). The lack of statistical significance in the increases in TNFα release in either of these groups and the elevated CRP concentrations in lean women with PCOS may be due to the small sample size. Nevertheless, there is a stepwise increasing trend in the TNFα response, with progressively higher

hyperglycemic conditions when study subjects are grouped by body weight. It is possible that the increased abdominal adiposity observed in lean women with PCOS also perpetuates increases in hyperglycemia-induced TNF α release from MNC to promote insulin resistance in this group. Nevertheless, the presence of PCOS in combination with a greater amount of adiposity may explain the higher TNF α response and greater degree of insulin resistance evident in obese women with PCOS.

In PCOS, TNF α release from MNC in response to hyperglycemia may be capable of directly stimulating hyperandrogenism. This is suggested by the direct correlation of the TNF α response with plasma levels of testosterone and androstenedione in women with PCOS. We have also demonstrated direct correlations of ROS generation (González *et al.* 2006) and activated NF κ B (unpublished data) with these androgen levels. Infiltration of the ovary by MNC-derived macrophages has been previously reported (Best *et al.* 1996). Ovarian steroidogenic enzymes responsible for androgen production are stimulated by oxidative stress and inhibited by antioxidants, such as statins, *in vitro* (Piotrowski *et al.* 2005, Rzepczynska *et al.* 2005). Circulating androgen levels decline in women with PCOS in response to statin therapy *in vivo* (Banaszewska *et al.* 2005). TNF α stimulates *in vitro* proliferation of androgen-producing theca cells (Spazynsky *et al.* 1999). Thus, it is attractive to consider that increased TNF α release from glucose-activated MNC recruited into the polycystic ovary may be the result of a local inflammatory response that stimulates ovarian androgen production in women with PCOS.

In conclusion, MNC of obese women with PCOS exhibit increased TNF α response when directly exposed to hyperglycemic conditions *in vitro* in the physiologic range. Our findings suggest that the increased abdominal adiposity in women with PCOS promotes a proinflammatory state, especially in those who are obese. The associations of the TNF α response with measures of adiposity and androgen levels suggest that MNC-derived TNF α release contributes to insulin resistance and hyperandrogenism, particularly when the combination of PCOS and increased adiposity is present.

Acknowledgement

We thank the nursing staff of the General Clinical Research Center for supporting the implementation of the study and assisting with data collection.

Funding

This research was supported by National Institutes of Health Grants HD-01273 (Women's Reproductive Health Research Program) to the Department of

Obstetrics and Gynecology at MetroHealth Medical Center and MO1 RR-000080 to the General Clinical Research Center. The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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Received 17 November 2005

Accepted 25 November 2005