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Inflammatory cytokines and chemokines, skeletal muscle and polycystic ovary syndrome: Effects of pioglitazone and metformin treatment

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ABSTRACT

Objective. Chronic low-grade inflammation is a common feature of insulin resistant states, including obesity and type 2 diabetes. Less is known about inflammation in Polycystic Ovary Syndrome (PCOS). Thus we evaluated the impact of PCOS on circulating cytokine levels and the effects of anti-diabetic therapies on insulin action, cytokine and chemokine levels and inflammatory signaling in skeletal muscle.

Methods. Twenty subjects with PCOS and 12 healthy normal cycling (NC) subjects of similar body mass index were studied. PCOS subjects received oral placebo or pioglitazone, 45 mg/d, for 6 months. All PCOS subjects then had metformin, 2 g/day, added to their treatment. Circulating levels of cytokines, chemokines, and adiponectin, skeletal muscle markers of inflammation and phosphorylation of signaling proteins, insulin action evaluated by the hyperinsulinemic/euglycemic clamp procedure and Homeostasis Model Assessment of Insulin Resistance were measured.

Results. Circulating levels of a number of cytokines and chemokines were generally similar between PCOS and NC subjects. Levels in PCOS subjects were not altered by pioglitazone or metformin treatment, even though whole body insulin action and adiponectin levels increased with pioglitazone. In spite of the lack of change in levels of cytokines and chemokines, several markers of inflammation in skeletal muscle were improved with Pio treatment.

Conclusions. PCOS may represent a state of elevated sensitivity of inflammatory cells in skeletal muscle to cytokines and chemokines, a property that could be reversed by pioglitazone treatment together with improved insulin action.

Abbreviations: ANCOVA, analysis of covariance; BMI, body mass index; CV, coefficient of variation; DHEA, dehydroepiandrosterone; DHEA-S, dehydroepiandrosterone sulfate; FG, Ferman-Galloway score; GDR, glucose disposal rate; GROα, growth-related oncogene alpha; HOMA-IR, homeostasis model assessment of insulin resistance; IFNγ, interferon gamma; IGT, impaired glucose tolerance; IKK, Ikappa B kinase; IL, interlukin; JNK, c-jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MCP-1, monocyte chemoattractant protein-1; MIP-1β, macrophage inflammatory protein-1beta; NC, normal cycling subject; NF-kB, nuclear factor kappa-light-chain-enhancer of activated B cells; PCOS, polycystic ovary syndrome; SHBG, sex hormone binding globulin; T, testosterone; T2D, type 2 diabetes; TNFα, tumor necrosis factor alpha; VEGF, vascular endothelial growth factor; WHR, waist–hip ratio.

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1. Introduction

Low-grade chronic inflammation has been identified as a common feature of insulin resistant conditions such as obesity and T2D (reviewed in [1]) Such inflammation is characterized by elevated levels of inflammatory cytokines (eg, IL-6, MCP-1, TNFα) in the circulation and in adipose tissue. These cytokines are produced both by macrophages and other cells recruited to adipose tissue and in a cell-autonomous manner by adipocytes (reviewed in [2]). Such cytokines, whatever the source, act through the p44/42 MAPK p38 MAPK, JNK, and IKK/NFκB pathways to both further induce the production of inflammatory cytokines and to stimulate kinases that, through serine phosphorylation of Insulin Receptor Substrate-1, contribute to the development of insulin resistance (reviewed in [3]). Recent evidence has demonstrated positive effects of anti-inflammatory treatments to reduce insulin resistance [1]. The involvement of inflammation in skeletal muscle, the predominant insulin target tissue, in whole body insulin resistance has not been studied to the same extent as that of adipose tissue, though it has been reported that the macrophage content of skeletal muscle is elevated in obese, insulin-resistant individuals [4].

Polycystic ovary syndrome, while defined as a disease of reproduction, has a considerable impact on metabolism (reviewed in [5,6]). A significant proportion of women with PCOS display glucose intolerance or overt insulin resistance that is exacerbated by obesity [7,8]. The features of insulin resistance in PCOS differ in multiple ways from insulin resistance in T2D [7,9]. Evidence for the presence of chronic low-grade inflammation in PCOS is mixed, with reports of either normal [10] or elevated [11] circulating levels of inflammatory cytokines. In many instances it appears that obesity is the major determinant of circulating cytokine levels in these individuals, not necessarily the absence or presence of PCOS [10,12,13]. Reports on the effects of anti-diabetic therapies on circulating levels of inflammatory cytokines in subjects with PCOS range from no changes to significant reductions [14,15].

The current study was designed to address two questions. First, to determine if there is a relationship between inflammation and insulin resistance in PCOS and if any such relationship might differ from what has been well established in obesity and diabetes [1], we investigated similarly overweight to obese NC and PCOS subjects. Second, as the antidiabetic agents metformin and pioglitazone have been shown to have anti-inflammatory actions in obesity and diabetes [1] and are also frequently used to treat PCOS (reviewed in [16]), we evaluated if there would be similar effects of these treatments on insulin action and cytokine and chemokine levels in PCOS subjects. Inflammatory responses in skeletal muscle were assessed by monitoring markers of infiltration by neutrophils (CD15) and macrophages (CD68), as well as signaling through the p44/42 MAPK, p38 MAPK, JNK, and IKK/NFκB pathways (IkBα content).

2. Methods

2.1. Subjects

Normal cycling (control) non-diabetic, non-hirsute female subjects were screened by medical history, physical examination and laboratory evaluation. Non-diabetic PCOS subjects were recruited. The diagnosis of PCOS was based on criteria recommended by the 1990 National Institutes of Health Conference on PCOS [17]: 1. evidence of chronic anovulation or oligoamenorrhea, 2. clinical or biochemical evidence of hyperandrogenism, 3. exclusion of pregnancy, thyroid disease, prolactinoma, Cushing's syndrome, and late-onset nonclassic congenital hyperplasia. None of the subjects had taken: oral contraceptives, glucocorticoids, antiandrogens, ovulation inducing agents or antidiabetic or antiobesity medications in the 2 months before screening. None of the subjects had a family history of type 2 diabetes. All procedures were performed in the early or mid-follicular phase (d 2–8) of the subjects' menstrual cycle, except in those subjects who did not have regular menses. The experimental protocol was approved by the Human Research Protection Program of the University of California, San Diego. Informed written consent was obtained from all subjects after explanation of the protocol.

2.2. Materials

Antibodies against these proteins were obtained from the following sources: CD15 (Pierce, Rockford, IL), CD68 and CXCR2 (R&D Systems, Minneapolis, MN), IkBα, phospho-p44/42 MAPK, p44/42, phospho-p38 MAPK, p38, phospho-JNK, and JNK (Cell Signaling Technology, Beverly, MA). Horseradish peroxidase conjugated anti-mouse and anti-rabbit IgGs were from Santa Cruz Biotechnology (Santa Cruz, CA), SuperSignal enchanced chemiluminesence substrate was from Pierce (Rockford, IL). Electrophoresis reagents were from Bio-Rad (Richmond, CA) or Invitrogen (Carlsbad, CA).

2.3. Study design

Following the pretreatment testing described below, including muscle biopsy, subjects with PCOS were randomly assigned to receive either oral placebo or pioglitazone, 45 mg/d, for 6 months (Phase 1). Clinical characteristics were similar between subjects assigned to the two groups. At the end of the first treatment period each subject underwent the same tests as in the pretreatment phase. Besides the 75 gm OGTT and clamp procedures, this testing included dual energy absorptiometry for measurement of total body fat and a leuprolide stimulation test [18]. All subjects then had metformin, 2 gm/day, added to continuing pioglitazone or placebo treatment (Phase 2). All testing was repeated after an additional 3 months.

2.4. In vivo evaluation of insulin action

All studies were performed after a 12–14 hour overnight fast. Blood was collected and serum isolated and immediately stored at −80 °C. Insulin action in the fasting state was determined in all subjects by calculating the HOMA-IR [19]. All of the PCOS subjects and a subset of the NC subjects (n = 7) underwent both a 75 gm oral glucose tolerance test and had maximal insulin action measured by a 3-h hyperinsulinemic (300 mU m^{-2} min^{-1}) euglycemic (5.0–5.5 mmol/L) clamp; the GDR was measured during the last 30 min of the clamp [20]. Percutaneous needle biopsies of vastus lateralis muscle were performed prior to insulin infusion [20] and muscle tissue was immediately frozen in liquid nitrogen.

2.5. Assay for circulating factors

Serum insulin levels were determined by RIA (Linco Research Inc.) with intraassay CV less than 7%. Circulating serum total adiponectin was measured using a commercially available RIA kit (Millipore, St. Louis, MO). The lower limit of detection with this assay was 2 μg/mL; the inter- and intra-assay CVs were 7% and 11% respectively. Cytokines and chemokines were analyzed with a MILLIPLEX MAP kit (Millipore Corp, Billerica, MA) using a BioPlex 200 instrument (Bio-Rad Corp, Hercules, CA). Sensitivities (in pg/mL), inter- and intra-assay CVs for each analyate are as follows: IL-1β (0.4, 7%, 6%), IL-6 (0.3, 12, 8), IL-8 (0.2, 12, 7), IL-10 (0.3, 9, 5), IL-15 (0.4, 10, 7), GROα (10.1, 12, 5), TNFα (0.1, 16, 10), IFNγ (0.1, 6, 5), MCP-1 (0.9, 12, 6), MIP-1β (4.5, 11, 5), VEGF (5.8, 8, 6).

2.6. Protein extraction

Fifty–100 mg of muscle tissue was homogenized using a Polytron at half maximum speed for 1 min on ice in 500 μl of 1× solubilization buffer [21]. Tissue lysates were solubilized for 30 min at 4 °C with vortexing and centrifuged for 10 min at 14,000*g*. The supernatants were stored at −80 °C. Protein concentration was determined by the Bradford method (Bio-Rad).

2.7. Electrophoresis and Western blotting

Procedures for the electrophoresis, transfer and Western blotting of proteins are similar to standard methods [21]. Detection was by enhanced chemiluminescence, followed by densitometric analysis. Quantification of band intensity was performed using Alpha Innotech ChemiImager 4000 v.4.04 software (San Leandro, CA).

2.8. Statistical analysis

Statistical analysis was performed using GraphPad Prism 5.0 (GraphPad, San Diego, CA) and the Statistical Package for Social Sciences v.19 (SPSS, Chicago, IL). Within group comparisons (treatment effects) were evaluated by paired *t* test. Between group comparisons were evaluated by independent group *t* test if data was normally distributed and with a Mann–Whitney test for non-normally distributed data, and by ANCOVA after adjusting for age and BMI. The Pearson correlation test was used for univariate correlation analysis. For results that were not normally distributed, data was log-transformed for statistical analysis and then back-transformed and reported in original units as mean ± SEM. Statistical significance was accepted as $p < 0.05$. The number of individual determinations for each measurement is indicated in brackets in the tables or figure legends.

3. Results

3.1. Research subjects

Clinical characteristics relating to the metabolic and reproductive phenotypes of the NC and PCOS subjects are presented in Table 1. The PCOS subjects are a subset of individuals included in an earlier report on the effects of pioglitazone treatment on reproductive and metabolic features of PCOS [18]. PCOS subjects were younger than the normal cycling control subjects. The two groups were equally obese. As expected, PCOS subjects were more hirsute and had elevated total T and DHEA-S levels; differences in SHBG levels did not attain significance ($p = 0.135$). PCOS subjects had similar HbA1c values compared to NC subjects, and were euglycemic ($p = 0.53$ vs NC), a status that was maintained under the influence of modestly elevated insulin levels ($p = 0.055$). Results of the oral glucose tolerance test revealed that greater than 50% of the PCOS group (13 of 20) met the criteria for IGT. Only two of the NC subjects could be classified as having IGT. The PCOS subjects displayed insulin resistance in both the fasting state (HOMA-IR) and of more than 30% ($p < 0.01$) in the ability of insulin to stimulate whole body glucose disposal (GDR), primarily into skeletal muscle (Table 1).

3.2. Circulating cytokines, chemokines, and adiponectin

Serum levels of a number of inflammation-related cytokines and chemokines were measured in the fasting state (Fig. 1). There were no significant differences between the NC and PCOS groups, though there was a tendency ($p = 0.062$) for MIP-1β levels to be lower in PCOS subjects. Further comparisons after adjusting for age and BMI as covariates also did not reveal any differences between groups in cytokine and chemokine levels. Potential influence of the hyperandrogenic environment characteristic of PCOS on cytokine and chemokine levels was evaluated by looking for associations between levels of these factors in PCOS subjects and sex steroid hormones. Statistically significant associations were seen between MCP-1, TNFα and MIP-1β levels with DHEA and DHEA-S (Table 2). Of the other factors presented in Fig. 1, no significant correlations were found between levels of any of them and free T, androstenedione, DHEA, DHEA-S or SHBG (Table 2). None of these associations

Table 1 – Subject characteristics.

	NC	PCOS
Age (y)	32 ± 1 [12]	28 ± 1* [20]
BMI (kg/m^2)	39.4 ± 2.2 [12]	35.6 ± 1.5 [20]
WHR	0.88 ± 0.02 [11]	0.90 ± 0.01 [19]
Fasting [glucose] (mM)	4.87 ± 0.21 [12]	5.27 ± 0.10 [20]
2 h [glucose] (mM)	7.08 ± 0.30 [7]	8.89 ± 0.47* [20]
Fasting [Insulin] (pM)	107 ± 16 [12]	168 ± 20 [20]
HbA1c (%)	5.33 ± 0.13 [7]	5.30 ± 0.10 [19]
GDR (mg kg^{-1} min^{-1})	8.55 ± 0.69 [7]	5.76 ± 0.35† [19]
HOMA-IR	2.07 ± 0.25 [12]	3.46 ± 0.34* [20]
Serum [adiponectin] (μg/mL)	12.2 ± 2.1 [10]	9.7 ± 1.5 [16]
F-G score	3.3 ± 0.9 [7]	14.2 ± 1.8† [20]
Total T (ng/mL)	0.68 ± 0.10 [7]	1.44 ± 0.16 † [20]
DHEA-S (ng/mL)	115 ± 13 [7]	248 ± 22† [20]
SHBG (nM)	23.8 ± 5.8 [7]	16.6 ± 1.9 [20]

F-G score – Ferman-Galloway score, WHR – waist–hip ratio, HbA1c – hemoglobin A1c, DHEA-S -DHEA sulfate.
Ave ± SEM.
*$p < 0.05$ vs NC, †$p < 0.01$ vs NC, by unpaired *t*-test.

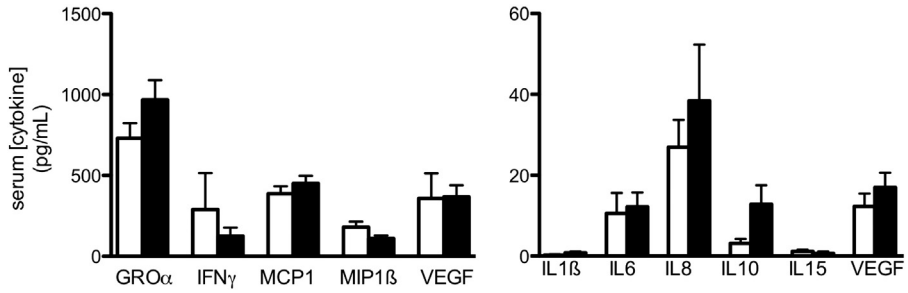

Fig. 1 – Circulating cytokine and chemokine levels in normal cycling (open bars) and PCOS (solid bars) subjects. Samples collected in the fasting state. Results are average + SEM. Numbers for individual determinations for each factor are (NC, PCOS): GROα (12, 18), IFNγ (5, 13), MCP1 (12, 18), MIP-1β (7, 18), VEGF (11, 13), IL-1β (8, 13), IL-6 (11, 17), IL-8 (12, 18), IL-10 (12, 18), IL-15 (10, 13), TNFα (12, 18).

were observed in NC subjects. Indeed, some of the correlations differed significantly between groups (Table 2). For the PCOS subjects there was a positive association between circulating SHBG levels and maximally stimulated insulin action (GDR, r = 0.543, p = 0.020), but not in the fasting state (HOMA-IR). Statistically significant relationships were not observed between either measure of insulin action and either free T, androstenedione, DHEA or DHEA-S (not shown). Insulin action in NC subjects was also not significantly associated with free T, androstenedione, DHEA or DHEA-S, or SHBG (not shown).

There were limited associations between cytokine and chemokine levels and measures of adipose tissue content and distribution in our sample of PCOS subjects (Table 3). No such relationships were observed in NC subjects (Table 3). However, a number of significant correlations were found between individual cytokines and chemokines and markers of glucose tolerance and insulin action in the PCOS subjects (Table 4). Many of these associations were lost upon performing partial correlational analysis while controlling for BMI, though significant correlations remained between MIP-1β and VEGF for HOMA-IR and GDR and for VEGF alone with 2 hour glucose. Somewhat similar results were obtained after controlling for WHR, with significant correlations remaining between MIP-1β and VEGF for HOMA-IR and GDR, as well as IL-6 and MIP-1β with fasting insulin. In addition, the association between GROα and GDR attained significance after controlling for WHR. Only one of these associations was shared with NC subjects, between IFNγ and fasting insulin (Table 4); in a number of instances the relationships between cytokine and chemokine levels and markers of glucose tolerance and insulin action differed significantly between the NC and PCOS subjects (Table 4).

Circulating levels of the insulin sensitizing adipokine, adiponectin, were not significantly different between the NC and PCOS subjects (p = 0.313, Table 1). In PCOS subjects, total adiponectin levels were strongly positively correlated with insulin action (GDR, r = 0.66, p = 0.04 and HOMA-IR, r = −0.65, p = 0.04) but not with reproductive hormones (free T, androstenedione, DHEA, DHEA-S or SHBG, not shown). The same was true in NC subjects except for a significant correlation of adiponectin with DHEA-S (r = −0.832, p = 0.042). A significant association between adiponectin and cytokine or chemokine levels was observed only with VEGF (r = −0.855, p = 0.014) in PCOS subjects and with MCP-1 in NC subjects (r = 0.926, p = 0.024).

3.3. *Treatment effects on insulin action and circulating cytokines and chemokines*

After 6 months of placebo treatment of PCOS subjects there was no change in insulin stimulated whole body glucose

Table 2 – Relationships (Pearson's r) between circulating cytokine and chemokine levels and sex steroid hormones in normal cycling (NC) and PCOS subjects (baseline).

	Total testosterone		DHEA		DHEA-S		SHBG	
	NC	PCOS	NC	PCOS	NC	PCOS	NC	PCOS
Il-1β								
IL-6	−0.452	0.179	−0.408	−0.523*	−0.029	−0.508	−0.179	0.370
IL-8	0.336	−0.118	−0.487	0.278	0.042	0.098	−0.582	−0.364
IL-10	−0.263	0.201	−0.221	−0.531	−0.724†	−0.212	−0.673	0.181
IFNγ	−0.922*	−0.183†	−0.046	−0.141	−0.696	−0.410	0.887*	−0.139†
GROα	−0.592	0.112	0.548	−0.176	0.467	0.237	−0.618	−0.172
MCP-1	−0.091	−0.271	−0.258	0.701*	−0.823*	−0.440	−0.026	−0.089
MIP-1β	0.411	−0.030	−0.861*	−0.491	0.220	−0.516*	0.461	0.014
TNFα	0.217	0.208	−0.214	0.655*	−0.213	−0.474	−0.222	0.349
VEGF	0.431	0.0132	−0.126	−0.267	−0.879*	−0.102	0.884*	−0.050†

*p < 0.05, † p < 0.05 vs NC.

Table 3 – Relationships (Pearson's r) between circulating cytokine and chemokine levels and anthropomorphic features in normal cycling (NC) and PCOS subjects (baseline).

	BMI		Waist circumference		Waist–hip ratio	
	NC	PCOS	NC	PCOS	NC	PCOS
GROα		0.102	−0.824	−0.149	−0.095	−0.338
IL-1β	0.332	−0.159	−0.325	0.114	−0.463	0.232
IL-6	−0.504	0.416†	−0.752	−0.370	−0.110	−0.352
IL-8	−0.055	0.208	−0.055	0.579*	−0.463	−0.810*
IL-10		0.545*		0.357		−0.036
IFNγ	0.797	0.527	0.587	0.200	−0.428	−0.170
MCP-1	0.362	0.12	0.688	0.191	0.124	0.024
MIP-1β	0.118	0.313	0.088	−0.049	−0.181	−0.289
TNFα	−0.562	0.051	−0.921*	−0.069†	−0.096	−0.010
VEGF	0.118	0.183	0.510	0.144	−0.050	0.059

* $p < 0.05$, † $p < 0.05$ vs NC.

disposal, while there was a strong tendency ($p = 0.0502$) toward decay in insulin action in the fasting state (HOMA-IR) (Fig. 2A). Adding metformin for 3 more months did not have any effect on either GDR ($p = 0.49$ vs placebo, $p = 0.80$ vs baseline) or HOMA-IR ($p = 0.33$ vs placebo, $p = 0.36$ vs baseline). Meanwhile, 6 months of treatment with pioglitazone resulted in a significant elevation of GDR that was unaltered by subsequent metformin addition ($p = 0.47$ vs Pio alone). Improvements in HOMA-IR were also seen. Serum total adiponectin levels responded in a similar manner, with a sizable increase after pioglitazone treatment and no effect of treatment with metformin alone (Fig. 2B). Adding metformin to pioglitazone treatment actually led to a reduction in adiponectin levels compared to the initial period of pioglitazone monotherapy ($p = 0.046$), though levels were still elevated compared to baseline. The magnitude of increases in GDR and adiponectin levels with pioglitazone treatment were associated ($r = 0.61$, $p = 0.045$).

Pioglitazone-induced increases in insulin action and adiponectin levels were not accompanied by net changes in circulating cytokine and chemokine levels (Fig. 3). None of the treatments had a consistent effect on levels of any of the factors of interest. However, some relationships were apparent between individual changes in cytokine and chemokine levels and metabolic responses to pioglitazone treatment. Treatment-related reductions in IL-6, IL-10, TNFα and MIP-1β levels were significantly correlated with falls in fasting insulin (not shown). Interestingly, all of these cytokines and chemokines were also associated with fasting insulin levels in the baseline state (Table 4), though the initial association with IL-10 was not statistically significant ($p = 0.07$). In addition, the drop in fasting glucose levels with pioglitazone treatment was related to reductions in IL-8 levels ($r = 0.73$, $p = 0.04$).

3.4. *Treatment effects on inflammation in skeletal muscle*

While pioglitazone treatment had no consistent effect on circulating cytokine and chemokine levels, the effects of treatment on inflammation in skeletal muscle, the major site of pioglitazone -augmented insulin-stimulated glucose disposal, were evaluated by looking at muscle content of markers for the presence of neutrophils (CD15) [22] and macrophages (CD68) [23]. Pioglitazone treatment resulted in reductions in the content of both markers in muscle, significantly so for CD15 (Fig. 4), and with a strong tendency ($p = 0.052$) for CD68. Neither change was associated with individual changes in circulating cytokine and chemokine levels. In addition, with pioglitazone treatment there was a tendency ($p = 0.08$, $n = 9$) for a reduction, to 75 ± 13% of baseline, in the protein expression in skeletal muscle tissue of CXCR2, the receptor mediating neutrophil recruitment by GROα and IL-8 [24]. A major process in inflammatory signaling pathways is the phosphorylation and ultimate degradation of IkBα (2). Pioglitazone treatment led to an increase in the expression of IkBα protein in muscle (Fig. 4), indicative of reduced inflammation. Metformin treatment had no effect on either CD68 or IkBα content in muscle (Fig. 4).

3.5. *Inflammatory signaling in skeletal muscle*

Inflammatory signaling is mediated through activation of a number of pathways (reviewed in [25]), including p44/42

Table 4 – Relationships (Pearson's r) between circulating cytokine and chemokine levels and glucose tolerance and insulin action in normal cycling (NC) and PCOS subjects (baseline).

	Fasting glucose		Fasting insulin		HOMA-IR		2 hr glucose		GDR	
	NC	PCOS	NC	PCOS	NC	PCOS	NC	PCOS	NC	PCOS
GROα	0.704*	0.168†	−0.389	0.332	−0.377	0.544*†	−0.010	−0.177	0.271	−0.437
IL-1β	−0.156	−0.050	−0.156	−0.382	−0.210	−0.538	0.929	−0.158†	0.696	0.102
IL-6	0.633	0.405	−0.257	0.644*†	−0.386	0.358	−0.095	0.421	0.617	−0.447†
IL-8	0.764*	0.271†	0.115	0.341	0.095	0.203	0.648	0.403	0.310	−0.332
IL-10		0.559*		0.479		0.669*		0.110		−0.600*
IFNγ	0.715	0.300	0.997*	0.652*	0.739	0.157	0.058	0.221	−0.415	−0.33
MCP-1	0.229	−0.171	−0.648*	0.338†	−0.410	0.450†	−0.556	−0.004	−0.547	−0.296
MIP-1β	−0.002	0.533*	−0.342	0.764*	0.298	0.626*	0.006	0.511*	0.299	0.637*
TNFα	−0.921*	0.480†	−0.260	0.641*	−0.390	0.131	−0.199	0.278	0.489	−0.256
VEGF	0.508	0.285	−0.356	0.557†	−0.287	0.626*†	0.076	0.590*	−0.288	−0.682*

*$p < 0.05$, †$p < 0.05$ vs NC.

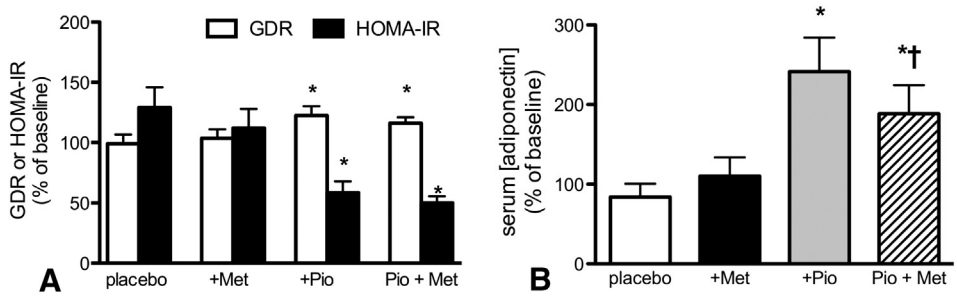

Fig. 2 – Effects of treatment on insulin action and circulating adiponectin levels in PCOS subjects. A. Treatment induced changes in HOMA-IR (n = 6-9) and maximally insulin-stimulated glucose disposal rate (GDR, n = 6). B. Changes in serum adiponectin levels. n = 10 for + Pio, n = 7 for placebo, +Met and Pio + Met. Results presented as % of the paired baseline (untreated) value for each individual, average + SEM. *p < 0.05 vs paired control, †p < 0.05 vs +Pio by paired t-test.

MAPK, p38 MAPK and JNK. The expression and activity of these kinases was evaluated in muscle biopsies obtained in the fasting state. While there was considerable heterogeneity within groups, the protein expression of p44/42 MAPK was significantly lower in muscle from PCOS subjects compared to NC subjects, that of p38 MAPK did not differ between groups and there was a tendency (p = 0.094) for JNK expression to be increased in PCOS muscle (Fig. 5A). Six months of pioglitazone treatment had no effect on expression of any of the kinases (Fig. 6A). Serine phosphorylation of each of these proteins is an accepted surrogate for kinase activity. Phosphorylation of p44/42 MAPK was elevated in muscle from untreated PCOS subjects compared to NC subjects (Fig. 5B). However, there were no significant effects of pioglitazone treatment on phosphorylation of p44/42, p38 or JNK (Fig. 6B). Metformin treatment was also without effect on either kinase protein expression or phosphorylation.

4. Discussion

PCOS is the most common endocrinopathy confronting reproductive-aged women. Beyond the hyperandrogenemia and reproductive impacts, metabolic dysfunction is a common feature of PCOS. A considerable proportion of women with PCOS display obesity and/or insulin resistance [5], with the former exacerbating the latter. Evidence has accumulated implicating chronic low-grade inflammation in the pathogenesis of obesity-related insulin resistance (1). Such inflammation is characterized by elevated circulating levels of cytokines and increased infiltration of adipose tissue and skeletal muscle by inflammatory cells, such as neutrophils and macrophages.

In our sample of similarly overweight-to-obese NC and PCOS subjects we found no significant differences between groups for any of the cytokines and chemokines measured. This was true even though the PCOS subjects, while having an average BMI equal to that of the NC subjects, displayed greater insulin resistance. Our findings would be consistent with a

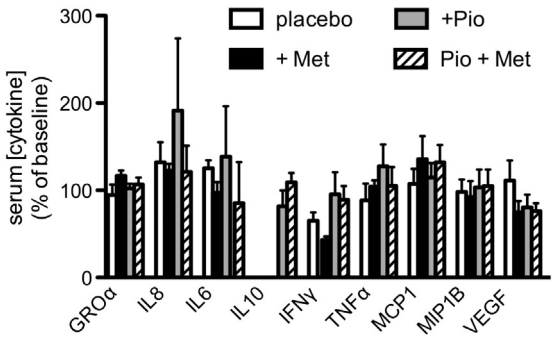

Fig. 3 – Effects of treatment of PCOS subjects on serum cytokine and chemokine levels. Results presented as % of the paired baseline (untreated) value for each individual, average + SEM, number of individual determinations: n = 12 for + Pio, 10 for Pio + Met, 7 for placebo, and 5 for + Met for all factors except VEGF, where n = 7, 7, 5 and 5, respectively.

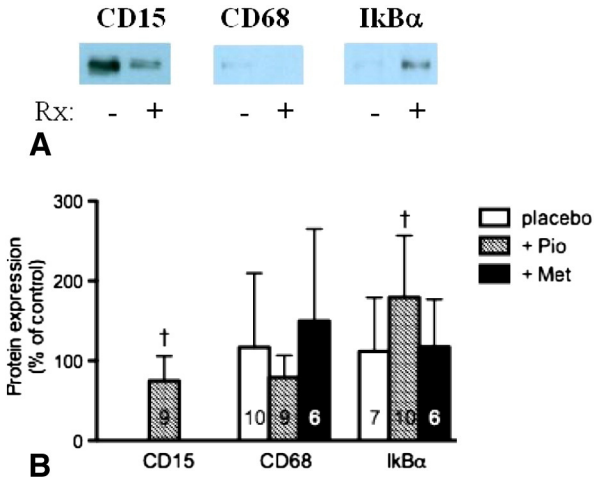

Fig. 4 – Effects of treatment on markers of inflammation in skeletal muscle from PCOS subjects. Muscle biopsies were obtained in the fasting state from PCOS subjects after the indicated treatments. A. Representative western blot showing changes in markers of neutrophil (CD15) and macrophage (CD68) content, as well as inflammatory signaling (IkBα) after treatment. B. Quantification of blots, results expressed relative to paired baseline/control value. Results are average + SEM, n = 7–10 for placebo, n = 6 for Met and n = 9–10 for pioglitazone. †p < 0.05 vs paired control.

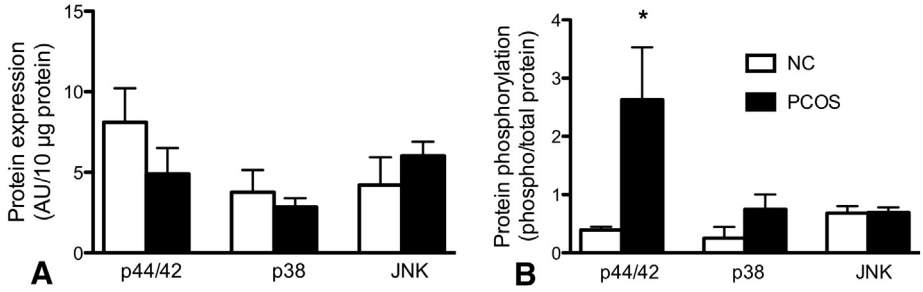

Fig. 5 – Effects of PCOS on markers of inflammatory signaling in skeletal muscle. Muscle biopsies were obtained in the fasting state from normal cycling (open bars) and PCOS subjects (solid bars). A. Kinase protein expression in NC (n = 5–6) and PCOS (n = 14–18) subjects, normalized against total tissue protein. B. Protein phosphorylation in NC (n = 5–6) and PCOS (n = 14–17) subjects, presented as the ratio of phosphorylated protein over total specific protein. *p < 0.05 vs NC by unpaired t-test.

recent meta-analysis of the literature on this topic [26]. Overall, the literature regarding cytokine and chemokine levels in PCOS subjects appears evenly split between reports of no differences between NC and PCOS subjects when matched for weight [10,26,27] and elevated levels with PCOS [27]. In our subjects cytokine and chemokine levels tracked indicators of fasting insulin and appeared to be independent of the reproductive hormones characteristic of PCOS, except for the cases of MCP-1 and TNFα with DHEA and MIP-1β with DHEAS. These results are in general agreement with other reports of a lack of association between cytokine and androgen levels in PCOS [10,12,27]. Interestingly, one difference between NC and PCOS subjects was that NC subjects failed to display any of the associations between sex hormones and cytokine and chemokine levels.

We and others have reported previously that the insulin resistance in individuals with PCOS differs in multiple ways from the insulin resistance characteristic of T2D [9,28]. The current findings reveal several other differences between PCOS and T2D. One is the dissociation between circulating cytokine and chemokine levels and whole body insulin action in PCOS, where such levels are essentially normal, when compared to NC women of similar BMIs, even in the presence of greater glucose intolerance and overt insulin resistance. Second, unlike the situation in obesity and T2D [1], but for IL-8 and IL-10, there were no associations between cytokine and chemokine levels and measures of adipose tissue content and distribution. Third is the failure of pioglitazone and/or metformin treatment to alter circulating cytokine and chemokine levels in our group of PCOS subjects, which contrasts with the responses in other populations of insulin resistant individuals, such as those with IGT or T2D. In those groups thiazolidinedione treatment has been shown to reduce circulating levels of TNFα and IL-6 [29–33]. The limited data available concerning effects of antidiabetic drugs on cytokine and chemokine levels in PCOS is mixed, with metformin being shown to reduce hsCRP [14,34,35] and IL-6 [15] levels in some instances and without effect in others [10,36] as well as pioglitazone treatment lowering hsCRP [14,35], yet having no effect on IL-6 [14], MCP-1 or MIP-1α [15] levels. The latter reports would agree with the current findings. With regard to treatment effects on insulin action in PCOS, direct comparisons of metformin and thiazolidinediones have found thiazolidinediones to be superior [37–39], inferior [40] or equivalent [41] to metformin. Two studies evaluated rosiglitazone and metformin combination therapy and found no further benefit over either metformin alone [40] or rosiglitazone alone [37]. Our results would be consistent with the later report. Markers of inflammation were not followed in these studies. Such variability in responses to treatment, both metabolic and inflammatory, could be due in part to differences in time and dosing of treatment [42], but also

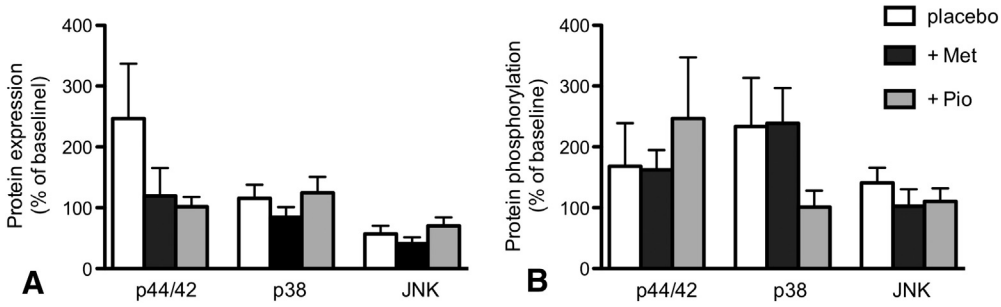

Fig. 6 – Effects of treatment on markers of inflammatory signaling in skeletal muscle from PCOS subjects. Muscle biopsies were obtained in the fasting state from PCOS subjects before and after treatment with placebo (open bars, n = 6), Met (shaded bars, n = 6) or Pio (solid bars, n = 9–10). A. Regulation of kinase protein expression in PCOS subjects after treatment. B. Regulation of kinase phosphorylation. Results expressed relative to paired baseline/control value. Results are average + SEM. †p < 0.05 vs paired control.

reinforces the considerable heterogeneity of the reproductive [43] metabolic [6,8] and, potentially, inflammatory phenotype(s) displayed by individuals with PCOS.

The lack of association between individual pioglitazone-induced changes in circulating cytokine and chemokine levels and markers of neutrophil and macrophage content in skeletal muscle of PCOS subjects suggests that the treatment effect on inflammation in muscle occurs either at the level of cytokine and chemokine content within muscle itself or in the responsiveness/sensitivity of these inflammatory cells to cytokines, which itself may be altered in PCOS. Indeed, there is evidence in support of the latter concept, as TNFα production in response to lipopolysaccharide stimulation was higher in monocytes isolated from insulin resistant PCOS subjects than in controls [44].

Activation of many of the kinases involved in inflammatory signaling such as p44/42 MAPK, p38 MAPK and JNK, has been demonstrated in circulating cells in obesity and T2D (reviewed in [45]). Information about the behavior of the same kinases in skeletal muscle tissue is less abundant. Both the activity and protein expression of p44/42 were found to be normal in muscle from T2D subjects [46]. Protein expression of p38 is also normal [47–49] in T2D muscle while there is opposing data regarding phosphorylation, either normal [48] or elevated [47,50]. JNK protein expression is also normal in diabetic muscle [48] while phosphorylation is elevated [51]. Taken together, the literature suggests that the insulin resistance of obesity and T2D may be associated with activation of p38 MAPK and JNK in muscle. Even less is known about the behavior of inflammatory kinases in muscle in PCOS. We are aware of a single report in skeletal muscle cells cultured from individuals with PCOS where p38 activity was found to be normal, as was p44/42 protein expression, while p44/42 activity was elevated [52]. Those observations regarding p44/42 contrast in part with the current results in muscle tissue (Fig. 5), though p44/42 activity/phosphorylation was higher in both skeletal muscle tissue and myocytes from PCOS subjects when compared to NC subjects. One possible explanation for such differences is that we studied muscle tissue, where measures would include the contributions of multiple cell types, including skeletal muscle resident macrophages, while Corbould et al [52] evaluated a homogenous population of myocytes. Meanwhile, one possible explanation for elevated p44/42 MAPK phosphorylation in PCOS muscle in the presence of normal inflammatory cytokine levels could be increased sensitivity of p44/42 to cytokines. Either way, the available evidence indicates that inflammatory signaling in skeletal muscle is yet another feature that differs between individuals with PCOS and those with T2D.

Mention has been made of the considerable variability regarding both the impact of PCOS on circulating cytokine levels and responses to pharmacologic interventions, at least with regard to IL-6, MCP-1, MIP-1α and IL-18, the most commonly studied such factors in PCOS [26,53]. Given such variability, a limitation of the present report is the modest number of subjects included in each treatment group. However, for MCP-1, VEGF, IL-1β, IL-8, and TNFα our results would be consistent with multiple reports showing no differences between PCOS subjects and age and BMI-matched normal cycling controls [26] and little or no response to metformin or thiazolidinedione treatment [15]. Strengths of the current report are that, as far as we can determine, the current report presents new information regarding; circulating levels of GROα, IFNγ, MIP-1β, IL-10 and IL-15 in PCOS, relationships between those levels and androgen levels and insulin action in the same subjects, and, responses to metformin and thiazolidinedione treatment. Furthermore, while several groups have compared metformin and thiazolidinedione mono- and combination therapy of individuals with PCOS [37,40], effects on cytokine and chemokine levels were not reported. Treatment effects on several markers of inflammation in skeletal muscle in PCOS, as well as the protein expression and phosphorylation of key kinases involved in inflammatory signaling also represent new information. With regard to translation of our findings, in this population of overweight-to-obese PCOS subjects, the results suggest that pioglitazone is superior to metformin in improving both insulin action and inflammation in skeletal muscle.

In conclusion, the current results suggest that the relationship between inflammation in skeletal muscle and insulin resistance in PCOS differs from that in other insulin resistant states, such as obesity and T2D. As circulating levels of selected cytokines and chemokines are not routinely altered in PCOS, PCOS may represent a state of elevated sensitivity of inflammatory cells to cytokines and chemokines. This in turn could result in increased neutrophil and macrophage infiltration into skeletal muscle, contributing to insulin resistance. A further contributor to the existence of a pro-inflammatory state in PCOS in spite of normal cytokine levels could be the reported presence of polymorphisms in genes involved with the TNFα and IL-6 signaling systems that might influence the activity of those pathways [54]. The beneficial effects of pioglitazone treatment could involve both improving insulin sensitivity of muscle and reversing the augmented sensitivity of inflammatory cells.

Author contributions

TPC designed and conducted the in vitro portions of the study, collecting, and analyzing data and wrote the manuscript. VA designed and conducted the in vivo primary study, participated in data interpretation and review of the manuscript. SRM participated in in vivo study design and writing of the manuscript. RRH participated in study design, data interpretation and writing of the manuscript.

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Conflict of interest

TPC, VA and SRM have nothing to declare. RRH has previously consulted for and received grant support (7/02-6/05) from Takeda Pharmaceuticals North America.

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