ORIGINAL

Decreased myo-inositol to chiro-inositol (M/C) ratios and increased M/C epimerase activity in PCOS theca cells demonstrate increased insulin sensitivity compared to controls

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Abstract. Previous studies from our and other labs have shown that insulin resistance is associated with an inositol imbalance of excess myo-inositol and deficient chiro-inositol together with a deficiency of myo-inositol to chiro-inositol epimerase *in vivo* and *in vitro*. In this report, we utilized well characterized theca cells from normal cycling women, with normal insulin sensitivity, and theca cells from women with polycystic ovary syndrome (PCOS), with increased insulin sensitivity to examine the myo-inositol to chiro-inisitol (M/C) ratio and the myo-inositol to chiro-inositol epimerase activity. PCOS theca cells with increased insulin sensitivity were specifically used to investigate whether the inositol imbalance and myo-inositol to chiro-inositol epimerase are regulated in a similar or the opposite direction than that observed in insulin resistant cells. The results of these studies are the first to demonstrate that in insulin sensitive PCOS theca cells the inositol imbalance goes in the opposite direction to that observed in insulin resistant cells, and there is a decreased M/C ratio and an increased myo-inositol to chiro-inositol epimerase activity. Further biochemical and genetic studies will probe the mechanisms involved.

Key words: Chiro-Inositol, Myo-Inositol, Epimerase, Polycystic ovary syndrome, Theca

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POLYCYSTIC ovary syndrome (PCOS) affects approximately 7-12% of women, and is the most common cause of infertility in reproductive aged women [1]. Reproductive endocrine abnormalities in PCOS include amenorrhea or oligomenorrhea, infertility, hirsutism, and acne resulting from increased ovarian androgen production [2-6]. Although there have been reports suggesting as high of 50% of women with PCOS have hyperinsulinemia and peripheral insulin resistance in adipose and skeletal muscle, ovarian theca and granulosa cells have been reported to be exquisitely sensitive to insulin and are not insulin resistant. Thus, a dichotomy is present with peripheral tissue insulin resistance and ovarian insulin sensitivity.

We have proposed an inositol imbalance consisting of excess myo-inositol and deficient chiro-inositol as a measure of insulin resistance [7]. This imbalance

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has been demonstrated in urine of type 2 diabetics [8], Rhesus monkeys progressing from normal to obese to diabetic [8], in muscle biopsies of type 2 diabetics during an insulin clamp [8], as well as in autopsy muscle specimens of type 2 diabetics [9]. In addition, deficiency of chiro-inositol in urine of Japanese subjects has been demonstrated to be linearly related to insulin resistance in type 2 diabetics, subjects with impaired glucose tolerance and normal subjects [10]. Further, lack of appearance of chiro-inositol glycan bioactivity (measured as PDH phosphatase (PDHP) activity) in blood of type 2 diabetic subjects during a glucose tolerance test, lack of chiro-inositol glycan bioactivity in women with PCOS during an insulin clamp, and lack of appearance of chiro-inositol glycan bioactivity release from placental membranes with insulin administration *in vitro* in women with preeclampsia have also been shown [11-13]. One glycan isolated from beef liver, named INS-2, has been identified, structure determined and chemically synthesized. Its structure is galactosamine-β-1,4 pinitol (3-O-methyl ether of D-Chiro-Inositol). It is insulin mimetic and insulin

sensitizing *in vivo* and *in vitro* [14].

Based on these studies of inositol imbalance; *i.e.* increased M/C ratio and of chiro-inositol glycan deficiency associated with insulin resistance, we hypothesized a defective epimerization of myo-inositol to chiro-inositol, an inversion of carbon 3 hydroxyl [7], as a cause of the inositol imbalance and chiro-inositol glycan deficiency. We next demonstrated *in vivo* in the GK type 2 diabetic rat that in the insulin sensitive tissues, muscle, liver and fat [^{3}H]myo-inositol conversion to [^{3}H]chiro-inositol was reduced from about 20–30% to under 5% [15]. We partially purified the myo-inositol to chiro-inositol epimerase from rat liver and demonstrated its absolute requirement for nucleotide, indicating that it acted *via* an oxido-reductive mechanism [16]. Analyses of tissue extracts from type 2 diabetic GK rat tissues compared to control Wistars demonstrated reduced epimerase enzyme activity [16].

As mentioned above, it is well established that ovarian cells from PCOS subjects are insulin sensitive compared to peripheral tissues, which are insulin resistant. In view of the above mentioned results demonstrating increased M/C ratios and decreased myo-inositol to chiro-inositol epimerase activity associated with insulin resistance in type 2 diabetes, GK type 2 diabetic rat and the lack or deficiency of chiro-inositol glycan in type 2 diabetes, PCOS and preeclampsia, we wished to determine whether ovarian theca cells from women with PCOS with increased ovarian insulin sensitivity demonstrated the opposite, *i.e.* decreased M/C ratios and increased epimerase activity. We now show this is in fact the case.

Research Design and Methods

Human theca interna tissue was obtained from follicles of women undergoing hysterectomy, following informed consent under a protocol approved by the Institutional Review Board of the Pennsylvania State University College of Medicine. Individual follicles were dissected away from ovarian stroma, dissected, and dispersed with 0.05% collagenase I, 0.05% collagenase IA, and 0.01% deoxyribonuclease, in medium containing 10% fetal bovine serum (FBS), as previously described [17]. The isolated follicles were size-selected for diameters ranging from 3-5 mm so that theca cells derived from follicles of similar size from normal and PCOS subjects could be compared. Theca cells were cultured on fibronectin coated dishes utiliz-

ing previously described growth medium (1:1 mixture of Dulbecco's Eagles Medium (DME) and Hams F-12 medium containing 5% FBS, 5% horse serum (HS), 2% UltroSer G, 20 nM insulin, 20 nM selenium, 1 μM vitamin E and antibiotics). The cells were grown in reduced oxygen tension (5% O_2, 90% N_2, and 5% CO_2) and given supplemental antioxidants (vitamin E and selenium) to prevent oxidative damage.

The theca cell cultures utilized in these studies were described and functionally characterized previously [18-20]. Experiments comparing PCOS and normal theca were performed utilizing 4th-passage (31-38 population doublings) theca cells isolated from size-matched follicles obtained from age-matched subjects. The use of fourth passage cells allowed us to perform multiple experiments from the same patient population, and were propagated from frozen stocks of second passage cells in the media described above. For all studies, theca cell cultures obtained from at least 5 independent normal and 5 independent PCOS patients were examined. The passage conditions and split ratios for all normal and PCOS cells were identical. For each of the experiments outlined in these studies fourth passage theca cells cells were grown to subconfluence and transferred into serum-free medium, containing DMEM/F12 1.0 mg/mL BSA, 100 μg/mL transferrin, 20 nM insulin, 20 nM selenium, 1.0 μM vitamin E and antibiotics, 48 h prior to being rinsed in PBS, flash frozen and processed for assay of myo-inositol to chiro-inositol epimerase.

The PCOS and normal ovarian tissue came from age-matched women, 28-40 years old. The diagnosis of PCOS was made according to established guidelines [21], including hyperandrogenemia, oligoovulation, and the exclusion of 21α-hydroxylase deficiency, Cushing's syndrome, and hyperprolactinemia. All of the PCOS theca cell preparations studied came from ovaries of women with fewer than six menses per year and elevated serum total testosterone or bioavailable testosterone levels, as previously described [18, 22, 23]. Each of the PCOS ovaries contained multiple subcortical follicles of less than 10 mm in diameter. The control (normal) theca cell preparations came from ovaries of fertile women with normal menstrual histories, menstrual cycles of 21-35 days, and no clinical signs of hyperandrogenism. Neither PCOS nor normal subjects were receiving hormonal medications at the time of surgery. Indications for surgery were dysfunctional uterine bleeding, endometrial cancer, and/or

pelvic pain.

Ovarian theca cells from women with polycystic ovarian syndrome and control normals were cultured, scraped, processed and analyzed for myo-inositol content, chiro-inositol content (plotted as a M/C ratio) and a myo-inositol to chiro-inositol epimerase assay was performed. Data obtained from these assays were plotted as scattergrams. The mean ± SE was determined and plotted as horizontal lines for normal and PCOS tissues. Unpaired *t*-tests were performed.

Myo-inositol to chiro-inositol epimerase

Plates were partially thawed at 4ºC. 500 µL of 10 mM HEPES pH 7 with protease inhibitor cocktail containing 1 mM AEBSF, 1:200 of 1.4 mg/mL protein stock aprotinin, 10 µM leupeptin, 10 µM pepstatin, 10 µM E64 and 1 mM mercaptoethanol was added (10 HPic). Cells were scraped, transferred to microcentrifuge tubes and homogenized by hand using a microcentrifuge tube homogenizer. Samples were centrifuged for 5 min at 4ºC. Assay tubes containing ± nucleotides (1 mM ea NAD^+, NADP, NADH and NADPH), 1 mM nicotinamide, 1 mM $MgCl_2$, 1 mM myo-inositol, 10 HPic were set up in total volume of 450 µL. Fifty µL of the cell supernatant from above centrifugation was added at timed intervals, vortexed, covered and placed in a 37°C incubator for 6 hours with gentle mixing. To stop the reaction 2 mL of ice-cold abs. ethanol was added, vortexed and incubated on ice for 20 min. Samples were dried *in vacuo*.

Samples were then processed as described below for chiro-inositol content. Total Units were calculated by subtracting the chiro-inositol content in the minus nucleotides tube from the chiro-inositol content in the plus nucleotides tube. Protein was analyzed by modified Bradford Protein Assay (Pierce® 660 nm Protein Assay kit). The Specific Activity (Units/µg protein) was calculated by dividing the Total Units by the Total Protein.

Myo-inositol and chiro-inositol content

Centrifugation filters were purchased from Fisher. An aliquot from the ovarian theca cell homogenate was transferred to a flame-seal ampoule, diluted to 6N HCl from 12N HCl, flame sealed and hydrolyzed at 100ºC for 48 hours. Samples were then transferred to centrifuge tubes, with the original vial washed three times with HPLC grade H_2O and dried *in vacuo*. Several 400 µL aliquots of H_2O were added to the centrifuge vial

and dried *in vacuo* in order to remove excess HCl.

Samples were reconstituted in 1 mL H_2O and then loaded onto a 3 mL bed volume mixed-bed ion exchange column and the pass-through collected. Column was washed with an additional aliquot of 19 mL H_2O. Samples were then dried *in vacuo* prior to analysis.

Samples reconstituted in 400 µL H_2O were centrifuged through a 0.2 µm filter and the filtrate injected onto Dionex MA-1 HPLC column. Myo-inositol and chiro-inositol were detected with an electrochemical detector consisting of a Au electrode and a Ag/AgCl reference electrode. The solvent was 100 mM NaOH running at 0.4 mL/min. The detected peaks were integrated and quantitated using Dionex PeakNet 6.4 software by comparing the area of the unknown to the area of the known standard (D-chiro-inositol was obtained from Cyvex, Inc. and myo-inositol from Sigma-Aldrich Chemical Company). The amounts of myo-inositol and chiro-inositol were expressed as nmoles and the ratio of myo-inositol to chiro-inositol (M/C) calculated.

For all the above experiments, Sera and growth factors were obtained from the following sources: FBS and DME/F12 (Irvine Scientific, Irvine, CA): horse serum (Life Technologies, Grand Island, NY); UltroSer G (Reactifs IBF, Villeneuve-la-Garenne, France): other compounds were purchased from Sigma (St. Louis, MO). All other chemicals were analytical grade or better and purchased from Cyvex Inc., Fisher Scientific and Sigma-Aldrich Chemical Company.

Statistical analysis

Unpaired *t*-test was performed using GraphPad Prism version 5.0f for Mac, GraphPad Software, San Diego California USA, www.graphpad.com.

Results

The myo-inositol to chiro-inositol epimerase activities and M/C ratios for both normal and PCOS ovarian theca cells are plotted separately as shown in Figs 1 and 2. Fig. 1 shows the epimerase values in a scattergram with mean ± SE as shown by horizontal lines. The myo-inositol to chiro-inositol epimerase specific activity (Units/µg protein) mean value for PCOS is 3 times as high as that for the normals (0.017 ± 0.003 (n=11) *vs*. 0.006 ± 0.002 (n=10), resp.). There is also more scatter for the PCOS ovarian theca cells values than for the normal ovarian theca cells (0.002 → 0.034
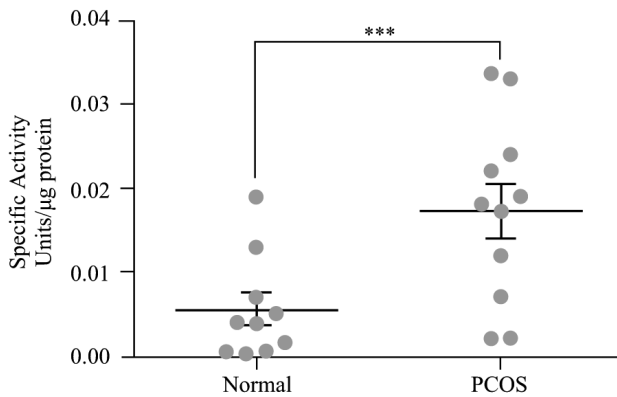
Fig. 1 Myo-inositol to chiro-inositol epimerase assay
    Data points for PCOS and normals shown in scatter plot
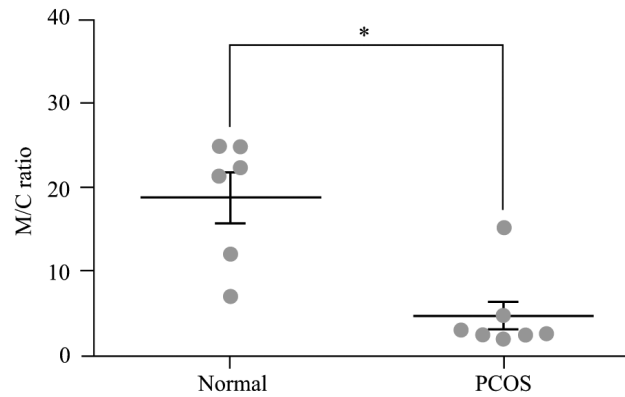   with mean ± SE shown as horizontal bars. *******, $p < 0.01$ *vs.*
    PCOS.

Fig. 2 Myo-inositol to chiro-inositol ratios
    Data points for PCOS and Normals shown in scatter plot
    with mean ± SE shown as horizontal bars. *****, $p < 0.002$ *vs.*
    normals.

vs. 0.0005 → 0.019, resp.).

Fig. 2 shows the M/C ratio in a scattergram with mean ± SE as shown by horizontal lines. The mean value for the M/C ratio of normals is about 4 times as high as that for PCOS (18 ± 3 (n=6) *vs.* 5 ± 2 (n=7), resp. and a range of (7 → 24 *vs.* 2 → 15, resp.). These results are in keeping what is seen with the epimerase results, and demonstrate that the PCOS ovary is insulin sensitive compared to control by these two parameters.

Two-tailed unpaired *t*-test ($p < 0.05$ was considered statistically significant) was performed resulting in $p < 0.01$ *vs.* PCOS for the epimerase and $p < 0.002$ *vs.* normals for the M/C ratios.

Discussion

Ovarian theca cells are recognized as one of the primary sources of excess androgen biosynthesis in the PCOS ovary [3, 24-26]. Using long-term cultures of normal and PCOS theca cells grown for successive population doublings in long-term culture, we have demonstrated that androgen production is elevated in theca cells isolated from the ovaries of women with PCOS, as compared to theca cells from the ovaries of normal cycling women [18, 27]. This increase in androgen production in PCOS theca cells results from increased mRNA accumulation of several steroidogenic enzymes, including cholesterol side chain cleavage (*CYP11A1*), 17α-hydroxylase (*CYP17A1*), and *HSD3B2* [18, 28]. Extensive examination of *CYP17A1* and *CYP11A1* gene expression in normal and PCOS theca cells has revealed that increased CYP17 and

CYP11 mRNA abundance results from both increased transactivation of the promoter and augmented mRNA stability in PCOS cells [20, 29] [23]. Moreover the 5' untranslated region of CYP17A1 and CYP11A1 mRNA have been shown to confer increased mRNA half life in PCOS theca cells as compared to normal theca cells, thus increasing *CYP17A1* and *CYP11A1* expression and androgen production in PCOS theca cells [23, 29].

Insulin acting through the insulin receptor stimulates androgen (*i.e.*, testosterone) production in theca cells, as does LH acting *via* cAMP [26]. Antibody blockade of the insulin receptor abolished insulin's stimulatory action, whereas effective antibody blockade of the insulin-like growth factor 1 receptor did not alter insulin's stimulation of theca cell testosterone biosynthesis [26]. Insulin's action on PCOS theca cells to produce testosterone is massively greater than on control theca cells [26], a measure of markedly increased insulin sensitivity. Nestler *et al.* have elegantly shown that chiro-inositol glycans are the signal transduction system for theca cell testosterone synthesis. A chiro-inositol containing glycan (INS-2) increased theca testosterone biosynthesis similarly to insulin [30]. Further, an INS-2, anti inositol glycan antibody, abolished insulin's stimulatory effect, but not that of hCG [30]. These findings suggest that inositol glycans serve as the signal transduction system for insulin's stimulation of human theca cell testosterone biosynthesis. Specifically, they demonstrated that external INS-2 activates theca cell testosterone production dose dependently as effectively as insulin.

Extracellular inositol glycan generation [31] as well as an ATP dependent inositol glycan transporter in liver have been demonstrated [32].

Our new data demonstrating increased M/C epimerase and decreased M/C ratios in PCOS theca cells provides a mechanistic explanation of how the PCOS ovary is more insulin sensitive. Increased M/C epimerase would provide increased chiro-inositol to be incorporated into precursor GPI-phospholipid and or precursor GPI-protein, which could then be cleaved into INS-2, thus enhancing insulin sensitivity by increasing glucose disposal [14]. INS-2 allosterically activates protein phosphatase PP2Cα to activate GS and mitochondrial PDHP to activate pyruvate dehydrogenase (PDH), both Mg^{2+} or Mn^{2+} requiring phosphatases (PPM family) leading to intracellular non-oxidative and oxidative glucose disposal and reduction of hyperglycemia [33]. This increased insulin sensitivity would also increase testosterone supply to induce peripheral insulin resistance. Mechanisms of testosterone inducing peripheral insulin resistance are not fully understood, but in an animal model, decreased GLUT4 and decreased glucose transport was observed [34]. In other studies, increased insulin sensitivity with administered testosterone has been observed [35, 36]. How the ovary is induced to increased insulin sensitivity *via* ovarian upstream mechanisms is unclear and requires further experimentation.

It is abundantly clear that there is marked peripheral insulin resistance *in vivo* in PCOS subjects [37]. However, when peripheral tissue cell lines from PCOS subjects are tested for the stability of the insulin resistance *in vitro*, variable results are seen [38, 39]. Thus at present there is not yet an agreed upon cell line that demonstrated stable insulin resistance in PCOS *in vitro*. For this reason we have not studied inositols and epimerase in peripheral cell lines from PCOS subjects.

In a paper entitled "The D-Chiro-Inositol Paradox in the Ovary" [40], the authors speculate that "PCOS patients with hyperinsulinemia likely present an enhanced MI to DCI epimerization in the ovary; this would result in an increased DCI/MI ratio (*i.e.* over-production of DCI), which would in turn would lead to a MI deficiency in the ovary."

The present data with theca cells from PCOS subjects and controls demonstrates that this is indeed the case. This is the first instance in which a cell with increased insulin sensitivity manifests an increased myo-inositol to chiro-inositol epimerase activity and a decreased myo-inositol to chiro-inositol ratio. In all previous papers, we have provided evidence for decreased epimerase activity and increased myo-inositol to chiro-inositol ratios in cases of insulin resistance [7-16].

Our experiments do not shed light on the continuing enigma of the primacy of increased testosterone versus the peripheral insulin resistance as the initiating event in PCOS. They do however provide evidence for the utility of both myo-inositol and chiro-inositol as effective agents in treatment. Certainly, a balance between the two inositols is required for normal physiological function and regulation of the myo-inositol to chiro-inositol epimerase opens a new avenue for future studies. Thus, IP3 [41], IP2 [42] and IP7 [43] as well as INS-2 [44] all inositol-containing molecules have been shown to act to allosterically control insulin signaling.

In this connection it is important to point out that the presence of the myo-inositol to chiro-inositol epimerase has been questioned in a recent paper by Lin, Gopalan and Ostlund entitled, "D-chiro-inositol is Absorbed but not Synthesized" [45]. The authors fed rats a chiro-inositol free diet for 10 or 12 weeks and then tested for chiro-inositol synthesis with heavy water or labeled myo-inositol. They conclude that there is no synthesis of chiro-inositol. Their failure in logic derives from the omission of the phrase "under our conditions"; *i.e.* the prolonged chiro-inositol free diet. The authors further fail to cite our *in vivo* epimerization of [³H]myo-inositol to [³H]chiro-inositol [15] and our partial purification, characterization with a demonstration of an absolute nucleotide requirement for a rat liver epimerase [16]. As stated above, in both *in vivo* and *in vitro* experiments, epimerase enzyme activity [16] was reduced *in vivo* in diabetic animals and in diabetic tissue extracts [15].

The present data on the decreased M/C ratios and the increased myo-inositol to chiro-inositol epimerase activity in PCOS ovarian theca cells further strengthens our argument that these two parameters are associated with insulin resistance and sensitivity. They open a new area of insight into this much-studied area.

Conflict of Interest

No conflict of interest for all authors of this manuscript.

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