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Insulin resistance in polycystic ovary syndrome is associated with defective regulation of ERK1/2 by insulin in skeletal muscle in vivo.

By

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ABBREVIATIONS: polycystic ovary syndrome (PCOS), insulin receptor substrate-1 (IRS1), protein kinase-B (PKB), extracellular regulated protein kinase (ERK1/ERK2), phosphatidyl inositol (PI), body mass index (BMI)

Abstract

Insulin resistance is a recognised feature of polycystic ovary syndrome (PCOS). However, the molecular reason(s) underlying this reduced cellular insulin sensitivity is not clear. This study compares the major insulin signalling pathways in skeletal muscle isolated from PCOS and controls. We measured whole body insulin sensitivity and insulin signalling in skeletal muscle biopsies taken before and after acute exposure to hyperinsulinaemia in nine women diagnosed with PCOS and seven controls. We examined the expression, basal activity and response to *in vivo* insulin stimulation of three signalling molecules within these human muscle samples, namely, insulin receptor substrate-1 (IRS1), protein kinase-B (PKB) and extracellular regulated protein kinase (ERK1/ERK2). There was no significant difference in the expression, basal activity, or activation of IRS1 or PKB between PCOS and control subjects. However, there was a severe attenuation of insulin stimulation of the ERK pathway in muscle from all but two of the PCOS women (the two most obese), and an accompanying trend towards higher basal phosphorylation of ERK1/2 in PCOS. These data are striking in that the metabolic actions of insulin are widely believed to require the IRS1-PKB pathway rather than ERK, and the former has been reported as defective in some previous PCOS studies. Most importantly, the molecular defect identified was independent of adiposity. The altered response of ERK to insulin in PCOS was the most obvious signalling defect associated with insulin resistance in muscle from these patients.

Introduction

Polycystic Ovary Syndrome (PCOS) is a common endocrine disorder affecting up to 10% of women of reproductive age. The central features of this heterogeneous disorder are hyperandrogenism, oligomenorrhoea and infertility, with obesity in up to 60% of patients [1, 2]. Women with PCOS exhibit peripheral insulin resistance similar in magnitude to that seen in type 2 diabetes (T2DM) [3, 4]; this is reported to be independent of body mass index (BMI), but is clearly exacerbated by associated obesity. Forty percent of obese PCOS women develop impaired glucose tolerance with up to 10% developing T2DM [5-10]. Insulin resistance is believed to be critical to the pathophysiology of the disorder with the use of insulin-sensitising agents like metformin, thiazolidinediones, and diazoxide, widely recognised to confer therapeutic benefits [11]. The high risk of progression from insulin resistance to T2DM associated with PCOS may indicate that the two diseases share a common molecular defect [12, 13]; however, the molecular reason(s) for impaired insulin action in either disease remains unclear.

The two best characterised insulin signalling pathways are the Ras-ERK pathway and the IRS-PKB pathway. The actions of these two pathways are diverse, with the Ras-ERK pathway primarily mediating mitogenic effects, and the metabolic actions of insulin primarily requiring the IRS-PKB pathway. Activation of the oncogene Ras, which ultimately leads to stimulation of ERK1/2 (for review see [14]), promotes expression of immediate early genes involved in growth and proliferation. This pathway also regulates a diverse range of transcription factors and signalling molecules that are likely to be involved in more widespread cellular processes. Activation of the IRS-PKB pathway is initiated by the phosphorylation of Insulin Receptor Substrate (IRS) proteins by the insulin receptor tyrosine kinase [15]. This leads to activation of phosphatidylinositol (PI) 3-kinase and downstream targets including protein kinase B (PKB, often called Akt) [16-18]. Activated PKB is involved in many actions of insulin such as regulation of glucose transport [19, 20], gluconeogenesis [21], and glycogen synthesis [22], but this pathway also controls aspects of growth and apoptosis [23].

In T2DM, there is evidence that insulin signalling is defective at the level of IRS1, due to negative regulation of this protein by high fatty acid and triglyceride levels [24-28]. The negative regulation involves increased Ser/Thr phosphorylation of IRS1 at a number of residues [29-31], resulting in ubiquitinylation and subsequent degradation of the IRS1 protein [26]. Indeed, some studies report defective IRS signalling in muscle of insulin resistant volunteers [32], while disruption of IRS2 in mice results in diabetes [33]. Molecular signalling has been studied in cultured skin fibroblasts, adipocytes, myotubes, theca cells and skeletal muscle biopsies from PCOS patients [34-46]. The data are somewhat conflicting, but overall suggest impaired metabolic signalling (IRS-PKB) and enhanced, or intact, mitogenic signalling (Ras-ERK). Indeed, the evidence suggests that distinct signalling problems may exist in different insulin responsive tissues [47].

The aim of this study was to investigate the activation state of these two major insulin signalling pathways in skeletal muscle biopsies in response to experimental hyperinsulinaemia *in vivo*, in women with and without PCOS. We hypothesised that a clear molecular defect underlying resistance to insulin-mediated glucose uptake would be detectable in muscle from women with PCOS.

Experimental

Materials

Antibodies to phospho-PKB (Ser473), phospho-ERK1/2 (Thr202/Tyr204), and total ERK1/2 were from Cell Signaling Technology (Beverly, MA, U.S.A.). Those for IRS1 and total PKB were from Upstate Biotechnologies (Lake Placid, NY, U.S.A.), and the anti- β -Actin was purchased from Sigma-Aldrich, Inc. (St Louis, MO, U.S.A.).

Methods

Local Ethical Committee approval was obtained for the study and written informed consent was obtained from all participants.

Subjects: Female volunteers with PCOS (Rotterdam Criteria [48]) without a history of diabetes or impaired glucose tolerance attending the combined Gynaecology/Endocrinology Infertility Clinic of Ninewells Hospital, a tertiary referral centre, were invited to participate. Ten women with PCOS were recruited, though on further assessment one subject was not included in the study as she did not fulfil the diagnostic criteria. The criteria used for the diagnosis of Polycystic Ovary syndrome included, oligo/ amenorrhoea (6 or fewer menstrual cycles in the previous year), elevation of serum total testosterone and/ or free androgen index 2SD greater than the reference range established in the local laboratory, and polycystic ovarian morphology on transvaginal ultrasound scan [49]. Women attending the Gynaecology clinic for sterilisation were invited to participate as control subjects, their numbers being supplemented by newspaper advertisement. Eight controls were recruited to the study. One control subject experienced approximately five minutes of asymptomatic hypoglycaemia (< 2.0 mmol/L) due to loss and re-establishment of venous access during her second muscle biopsy and was not included in the study. Seven controls were therefore included in the data analysis. Six of the control subjects had regular menstrual cycles, no evidence of hirsutism, and normal ovarian morphology on transvaginal ultrasound scan. The seventh control subject was amenorrhoeic as she was using Levonorgestrel intra uterine system for contraception, but had a history of regular menstrual cycles, no hirsutism, and normal ovarian morphology on scan. Besides this subject, none of the controls or PCOS subjects were on any sex steroid hormone treatment. The subjects were not on any medications or diet known to alter carbohydrate metabolism for at least three months prior to the study.

Assays: LH, FSH, oestradiol, progesterone, testosterone were measured by chemiluminometric immunoassays using a Roche Modular SWA (E170 unit) in conjunction with Roche calibrators and reagents. SHBG and insulin were measured by chemiluminometric immunoassays using a Siemens IMMULITE 2000 analyzer in conjunction with Siemens calibrators and reagents. Free androgen index was calculated from testosterone and SHBG ($(\text{testosterone}/\text{SHBG}) \times 100$).

Total cholesterol, HDL-cholesterol, triglycerides and glucose were measured by photometric assays using a Roche Modular SWA (P800 unit) in conjunction with Roche calibrators and reagents. LDL-cholesterol was calculated from total cholesterol, HDL-cholesterol and triglycerides by means of the Friedewald formula; it was not applied to samples with serum triglycerides greater than 4.0 mmol/L.

Assessment of insulin sensitivity: All subjects underwent an insulin tolerance test (ITT) following an overnight fast, as previously described [50, 51]. The parameter K_{itt} , derived from the slope of plasma glucose values in response to a weight-adjusted insulin bolus during the linear decline of plasma glucose ($t = 3$ -15 mins), has been shown to correlate highly with the M-value derived from the “gold standard” method for insulin sensitivity measurement, the hyperinsulinaemic, euglycaemic clamp (counter regulatory hormone responses occur at $t = 15$ -20 mins) [52].

Intravenous cannulae were inserted into right and left antecubital veins, one for blood sampling and the other for administration of insulin and glucose. Venous blood was collected every three minutes during the study period for bedside assessment of plasma glucose (Yellow Springs YSI 2300, Ohio, USA) with concomitant sampling for later assay of plasma insulin. After baseline blood sampling, Actrapid insulin 0.1 unit/kg body weight (NovoNordisk, Bagsvaerd, Denmark) was administered at time $t = 0$ mins. A slow saline infusion was maintained through that line for infusion of 10% Dextrose solution at the end of the study, or earlier according to predefined “rescue” hypoglycaemia plasma glucose criteria.

Calculation of insulin sensitivity: K_{itt} insulin sensitivity was calculated from the slope of least square analysis of the plasma glucose analysis between $t = 3$ and $t = 15$ mins (expressed as % fall/minute)[50].

Human skeletal muscle extraction: Forceps biopsies were taken according to established procedures from the right vastus lateralis muscle prior to insulin exposure (basal) [53]. A second biopsy was taken from a different incision on the same leg, at $t = 15-20$ mins (insulin stimulated). Muscle biopsies were snap frozen in liquid nitrogen.

Preparation of Protein Extracts for Western Blotting: Protein extracts were obtained as detailed before [53]. In brief, muscle biopsies were thawed on ice and homogenized (Dounce, 10-15 strokes) in 0.5 ml ice-cold lysis buffer (25 mM Tris-HCl (pH 7.4), 50 mM NaF, 100 mM NaCl, 1 mM sodium vanadate, 5 mM EGTA, 1 mM EDTA, 1% (v/v) Triton X-100, 10 mM sodium pyrophosphate, 0.27 M sucrose, Complete Protease inhibitor tablets (1 tablet/10 ml), and 0.1% (v/v) 2-mercaptoethanol). Lysates were obtained from the supernatant fraction after 10 min centrifugation at 13,000 r.p.m., and then pre-cleared for 1 h at 4°C with Protein G-Sepharose in PBS 50% (v/v). This process removed contaminating antibodies present due to variable amounts of blood in the muscle samples. Potential contamination of muscle protein lysate with adipose protein was measured by the ratio of troponin C to fatty acid binding protein (FABP4) in every lysate, and in no case was there significant contamination (data not shown).

Western Blot Analysis: Protein extracts (30-40 μ g) were separated on Novex SDS 4-12% polyacrylamide gels. Following transfer to nitrocellulose, blots were blocked with 5% (w/v) non-fat milk in TBST (Tris-buffered saline containing 0.1% (v/v) Tween 20) for 1 h, then incubated with primary antibodies at 4 °C overnight, prior to incubation for 1 h at room temperature with secondary antibody and development using an enhanced chemiluminescence (ECL) (Amersham Biosciences, Inc.). Insulin treatment of cells induces the phosphorylation of ERK1/2 at Tyr-187 and Thr-185 (numbers relate to human ERK2 sequence) [54], and this dual phosphorylation activates the enzyme. Hence a phosphospecific antibody to this motif (P-ERK) provides a measure of the activity status of ERK1/2 [45]. PKB is activated by phosphorylation on two residues, namely Thr-308 and Ser-473 (see [16-18] for review). Phosphorylation of both residues is required for PKB activation, therefore, an increase in Ser-473 phosphorylation is often used as a surrogate marker of activation. Phosphorylation status was assessed by the ratio of phosphorylated to total protein for ERK1/2 and PKB. Protein loading was assessed by β -actin measurement, and IRS1 levels corrected for loading.

Quantification: Protein bands were scanned and quantified by densitometry using AIDA Image Analyzer software.

Statistical Methods: SPSS 9v 15 and MINITAB® Statistical Software (Release 15) was used to perform most of the data handling and data analysis. Results are reported as mean response and the standard error of the mean (SEM). For some of the quantities measured, variation increased markedly with central location; these quantities were analysed on a logarithmic scale to help

stabilise variation. The principal procedure used to test the hypothesis of no difference between the mean responses in the two populations, (i.e. the control and the PCOS populations), was the two independent sample Student's t-test using an adjustment to the degrees of freedom to allow for unequal variances. For the results of the test procedure to be meaningfully interpreted and applied generally, the sampling procedure used to select the subjects must be assumed to introduce no biases.

Results

Demographic characteristics of the nine women with PCOS and seven healthy control subjects (all Caucasian) are shown in Table 1. Thyroid function tests, prolactin and 17 hydroxyprogesterone (17-OHP) levels were within normal limits for all participants. None of the women had evidence of impaired fasting glucose, although one control had a first degree relative with Type 2 Diabetes Mellitus (T2DM).

There were no statistically significant differences in weight or adiposity between the healthy and PCOS groups, although control subjects were not fully matched to PCOS women for obesity neither group was clinically obese (BMI 25.4 vs 29.8 kg/m², $p=0.072$). Neither the fasting blood glucose (4.5 v 4.4 mmol/l, $p=0.73$), nor the insulin levels (6.7 v 18 mU/L, $p=0.055$) were significantly different between the groups. However, the PCOS patients were more insulin resistant with a lower K_{itt} (4.8 v 3.2 %/min, $p=0.024$). The insulin area under curve during the insulin tolerance test was similar in two groups (table 1).

The mean time interval between insulin bolus and second biopsy in control subjects was 15.6 (1.2) min and that for PCOS was 18.3 (1.1) min ($p=0.117$). The mean insulin measurement at time of second biopsy in controls was 91 (13.3) μ U/l and that for PCOS was 109.8 (16.3) μ U/l ($p=0.393$). The regulation of the key signalling molecules, PKB, ERK and IRS1 by insulin was assessed by immunoblot (Fig.1). We attempted to measure other facets of signalling, however the signal to noise ratio was too weak to provide conclusive results, either due to the time point being studied, or the quality of available reagents. There was a trend towards higher basal ERK phosphorylation in the PCOS samples (Fig.2A versus 2B), however this was not statistically significant (Fig.2C). We found that insulin increased the ratio of phospho-ERK to total ERK in 5 out of the 7 control volunteers, whereas ERK phosphorylation was actually reduced in most of the 9 PCOS volunteers (Fig.2A and B). Most strikingly, there was a significant difference in the average level of induction of ERK by insulin between controls and PCOS subjects (2.04 v 0.71, $p<0.05$, Fig.2C).

Ser-473 phosphorylation was higher in the post-insulin biopsy in all but two volunteers (Fig.3A and 3B). The average induction of Ser-473 phosphorylation for both groups was around 4-fold (Fig.3C). One healthy volunteer, and one PCOS volunteer, had comparatively high basal Ser-473 phosphorylation, and in both cases subsequent exposure to insulin did not stimulate Ser-473 phosphorylation. There was no significant difference in Ser-473 phosphorylation between the two groups either before or after insulin injection, or a significant difference in the degree of induction following insulin between groups (Fig.3C).

IRS1 protein levels are reported to be reduced in muscle from insulin resistant people with T2DM, and we have previously observed a 2-3 fold induction of IRS1 protein levels following

exposure of healthy muscle to insulin *in vivo* [53]. In both healthy (3.1 fold, Fig.4A), and PCOS (5.3 fold, Fig.4B) groups, there was a significant induction of IRS1 following exposure to insulin. Indeed, all but one of the volunteers had increased IRS1 in the post-insulin biopsy. There was a great deal of variation in basal expression of IRS1 between individuals (Fig.1 and Fig.4), however there was no significant difference in expression of IRS1 between control and PCOS muscle, either in the fasted or post-insulin states (Fig.4C). Similarly, average induction of IRS1 by insulin was not significantly different between groups (Fig.4D).

There was no correlation between BMI and the defective regulation of ERK in the PCOS group (Fig.5A). Indeed, the only two PCOS volunteers that showed some ability to activate ERK in response to insulin had two of the highest BMI values of the group (Fig.5A). Nevertheless, we performed a sub-analysis where the three PCOS subjects with the highest BMI were omitted, resulting in a drop in the average BMI of the group to 27.6 ($p=0.37$ when compared to the control group). Despite the smaller sample size the insulin regulation of ERK in this leaner PCOS group remained significantly different from the control group (0.589 ± 0.27 , $p=0.034$ compared to control). In order to investigate the effect of BMI further, we regrouped all of the volunteers (control and PCOS) into low (≤ 27 kg/m²) or high BMI (>27 kg/m²) cohorts. Insulin activation of ERK was lower in the high BMI group; although this did not reach statistical significance (Fig.5B), this simple analysis argues against BMI as the factor driving the differences in ERK activation observed in the PCOS group. Importantly, while the high BMI group activated ERK relatively weakly in response to insulin, the PCOS group generally exhibited reduced ERK phosphorylation after insulin administration (Fig.2).

Discussion

Discovering the molecular defect underlying insulin resistance in specific tissues in distinct insulin resistant states should allow a more accurate diagnosis and directed targeting of interventions. Our results are novel in that they clearly implicate the ERK pathway in insulin resistance in intact muscle from women with PCOS for the first time. In particular, we observed reduced ERK activity in skeletal muscle following exposure to insulin with almost all PCOS subjects, contrasting with the physiological *increase* in ERK activity expected in response to insulin. It remains to be determined whether the defect in the ERK pathway we have observed in intact muscle tissue is conserved in other insulin sensitive tissues such as adipose and liver, or in the insulin sensitive cells of the ovary. Loss of insulin regulation of ERK has only been reported previously in tumour cells lacking PTEN (which opposes the action of PI 3-kinase), and in these cells the effect on the ERK pathway was much more dramatic than the effect on the PI 3-kinase-PKB pathway [55].

Much of the early work on insulin signalling in PCOS was performed in cultured skin fibroblasts [34-38]. The results suggested increased phosphorylation of serine residues of the insulin receptor in cultured fibroblasts isolated from PCOS skin. This was accompanied by reduced tyrosine phosphorylation of the receptor, indicative of reduced receptor signalling [34]. Despite this, downstream activation of PKB was normal in the fibroblasts from PCOS [37]. There are no reports on the regulation of ERK in these cells.

In contrast, phosphorylation and expression of the insulin receptor in more “classical” insulin target tissues (adipose and muscle) appeared comparatively normal [39, 40]. However, in cultured adipocytes from PCOS subjects, tyrosine phosphorylation *and* expression of IRS1 was

reduced [41]. Interestingly, reduced GLUT4 expression was also reported in adipocytes from PCOS, and this (like the study presented here) was independent of obesity [43].

We have focused our work on insulin regulation of muscle, as this is the major tissue involved in insulin-stimulated glucose disposal. Previous work in cultured human myotubes provided evidence for slightly higher IRS1 levels in cells isolated from PCOS muscle, compared to control cells, as well as a two fold induction of phosphorylation of Ser-312 of IRS1 [44]. Interestingly, IRS1 associated PI 3-kinase activity was reduced in the PCOS cells when a correction was made for the increased IRS1 level [44]. However PKB and ERK activities were not measured.

Signalling analysis in cells cultured from isolated muscle is not consistent with *in vivo* analysis of signalling in human PCOS muscle. Two previous studies have analysed insulin action in intact PCOS muscle [40, 45]. Dunaif and colleagues found increased IRS2, but not IRS1 expression in intact muscle [40], suggestive of a difference in the PCOS associated defect between intact muscle, isolated myoblasts and adipose (where IRS2 appeared normal [42]). However, in both cultured muscle cells and intact muscle, IRS1 signalling was reduced (measured by PI 3-kinase associated activity) with PCOS, but PKB activation was not assessed. Most recently, Hojilund *et al* reported small reductions in IRS1-PKB responses to insulin in intact muscle, and the reduced signalling could be reversed by treatment with insulin sensitising agents [46]. We could not detect abnormalities in PKB activation by insulin, suggesting a relatively normal IRS1-PI 3-kinase response in PCOS muscle, at least at the insulin concentration and time point studied here. The study protocols do differ slightly in that we have used an ITT to assess insulin sensitivity and initiate signalling events, as opposed to a hyperinsulinemic-euglycemic clamp. In addition, the mean BMI of the PCOS subjects in our study was much lower (29.8).

Basal phosphorylation of ERK has been previously reported to be significantly higher in isolated PCOS muscle compared to control [45], but the insulin response was not reported. These concord with our observation of a trend towards increased basal ERK phosphorylation in PCOS muscle (Fig.2C). Although insulin regulation of ERK has not been previously analysed in PCOS *in vivo*, a high basal *and* insulin stimulated activity of the pathway, downstream of Ras, was observed in cultured myotubes from PCOS compared to controls [45]. Abnormally high ERK activity has also been seen in theca cells from PCOS subjects [56], although this was linked to abnormal androgen production rather than response to insulin. Therefore, the most striking difference to the previous analysis of ERK in PCOS muscle cells is our observation that insulin *reduces* ERK activity *in vivo*, rather than inducing it to even higher levels. The reasons for the discrepancy may be related to our *in vivo* analysis as compared to the model of cultured myotubes (see earlier). A similar discrepancy has been reported for insulin stimulated glucose uptake and GLUT4 expression. These appear normal in cultured myotubes from PCOS muscle [44], whereas PCOS muscle exhibits reduced glucose uptake in response to insulin *in vivo* [40]. Interestingly, ERK activation may influence regulation of glucose uptake in muscle [57].

Finally, although extreme BMI has been linked to defective signalling in non-PCOS populations, multiple analyses of our data indicate that PCOS has a BMI-independent effect on insulin regulation of ERK. Our PCOS group were not overtly obese (average of 29.8 BMI), and although they were generally heavier than the controls there was no significant difference in BMI between groups. Importantly there was no significant difference in ERK activation by insulin

between groups when subjects were reclassified according to BMI rather than PCOS status (Fig.5). Moreover, the two most obese patients with PCOS were the only ones in whom insulin was able to activate ERK to any extent; in contrast, ERK was markedly upregulated in the majority of control subjects (Fig 2A vs 2B). If we omitted three of the PCOS subjects to match the BMI of the groups more closely, the defective ERK signalling was still apparent in the leaner PCOS group. Therefore, although it remains likely that high BMI could worsen some molecular signalling defects, our data strongly suggest that the loss of ERK regulation in this population is due to PCOS and not BMI

In summary, we have found compelling evidence of an abnormal insulin regulation of the Ras-ERK pathway in intact muscle from PCOS women. This is of particular interest, as it has previously been considered that a defect in the IRS-PKB pathway was more likely to underlie the clinical development of insulin resistance. The IRS-PKB pathway is known to be vital in the regulation of glycogen synthesis, gluconeogenesis and glucose transport by insulin and defects in this pathway have been found in animal models of T2DM as well as in some studies on human T2DM and PCOS. IRS1 levels are reduced in T2DM, but in muscle from PCOS they are normal (Fig.4 and [40]), while in isolated muscle cells IRS1 is actually increased in PCOS [44, 46]. This may suggest distinct signalling problems in these insulin resistant states, and it will be of interest to determine whether defects in the ERK pathway in a healthy population correlate with insulin resistance and indicate an increased risk of developing PCOS rather than T2DM.

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Figure Legends:

Table 1: Clinical and Biochemical data of PCOS and control women (A), and individual parameters used as part of the diagnosis (B).

Figure 1: Representative immunoblots for analysis of IRS1, PKB and ERK. 30 mg of protein lysate prepared from muscle biopsies isolated from control and PCOS volunteers, after an overnight fast (-), or from the same individual after a 20 min exposure to insulin (+), were subjected to SDS-PAGE, transferred to nitrocellulose and analysed using specific antibodies as indicated. Reactive species were visualised by ECL.

Figure 2: Assessment of ERK activity. Immunoblots of 7 control and 9 PCOS muscle biopsies, before and after exposure to insulin for 20 mins, were quantified. The ratio of phospho-ERK to total ERK was determined for every biopsy and that ratio is shown for the control (A) and PCOS (B) volunteers, before (basal) and after insulin for each individual. In addition, the average \pm SEM fasted and post-insulin ratios for each group are given (C), along with the average \pm SEM fold change in ERK phosphorylation (C).

Figure 3: Analysis of PKB activity. Immunoblots of 7 control and 9 PCOS muscle biopsies, before and after exposure to insulin for 20 mins, were quantified. The ratio of phospho-473-PKB to total PKB was determined for every biopsy and that ratio is shown for the control (A) and PCOS (B) volunteers, before (basal) and after insulin for each individual. In addition, the average \pm SEM fasted and post-insulin ratios for each group are given (C), along with the average \pm SEM fold change in PKB phosphorylation (C).

Figure 4: Analysis of IRS1 expression. Immunoblots of 7 control and 9 PCOS muscle biopsies, before and after exposure to insulin for 20 mins, were quantified. The ratio of IRS1 to β -actin was determined for every biopsy and that ratio is shown for the control (A) and PCOS (B) volunteers, before (basal) and after insulin for each individual. In addition, the average \pm SEM fasted and post-insulin ratios for each group are given (C), along with the average \pm SEM fold change in IRS1 levels (D).

Figure 5: There is an obesity independent effect of PCOS on insulin regulation of ERK. (A) The insulin regulation of ERK in the PCOS group is presented relative to the BMI of the volunteers. (B) The volunteers were divided into two groups dependent on BMI, low (≤ 27 , n=6) and high (>27 , n=9). The average fold induction of ERK in each group \pm SEM is shown.

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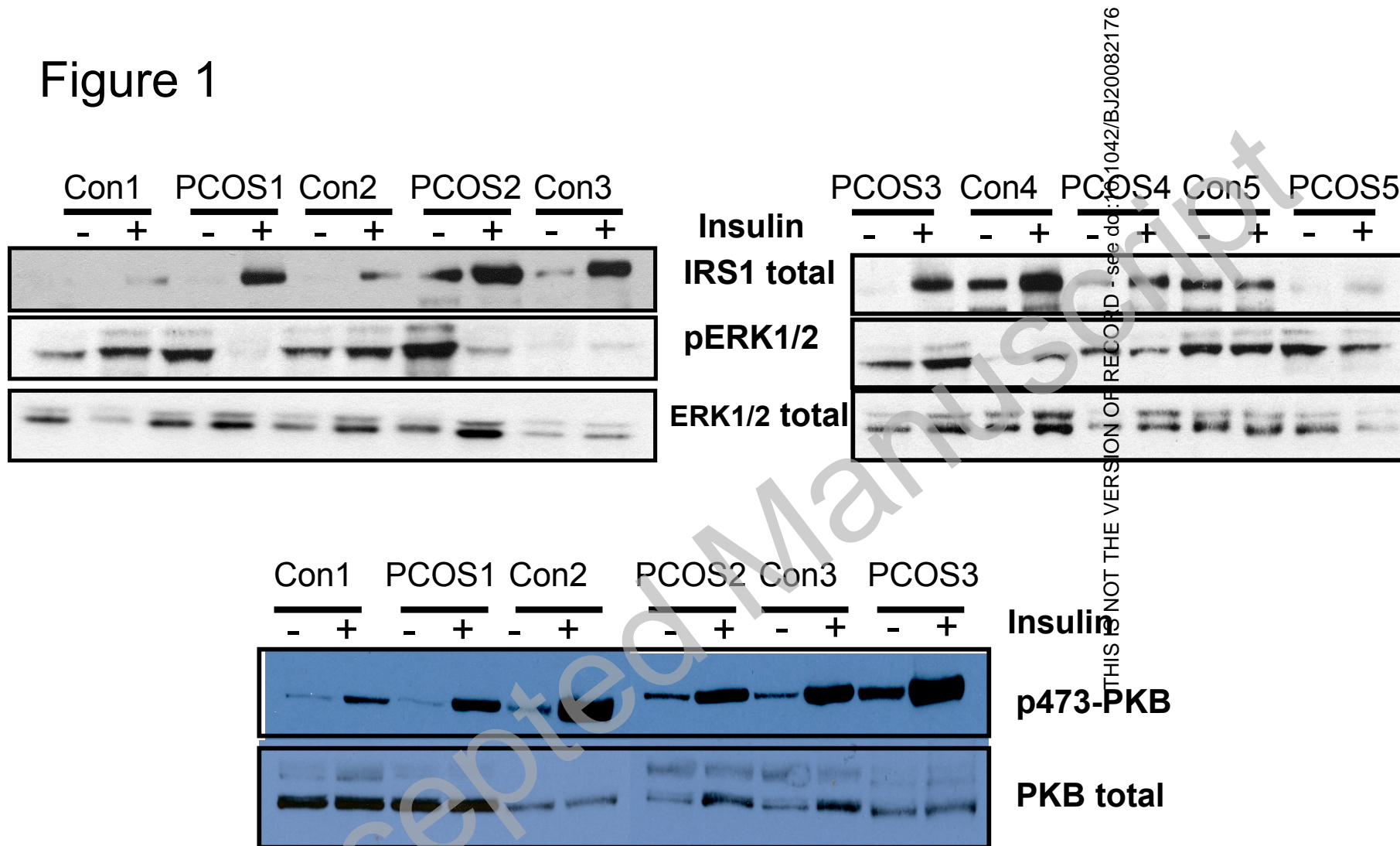
A

	Controls (mean +/- SEM) (n=7)	PCOS (mean +/- SEM) (n=9)	p value (Con vs PCOS) (2 sample t-test)
Age (yrs)	33.3 (1.0)	27.1 (1.4)	0.003
BMI (kg/m ²)	25.4 (1.6)	29.8(1.5)	0.07
Waist: hip ratio	0.83(0.03)	0.87(0.02)	0.38
Serum testosterone (nmol/L)	1.6(0.22)	3.3(0.13)	<0.001
SHBG (nmol/L)	55.5(10)	27.5(3.2)	0.012
Free Androgen Index	3.43(1.02)	10.74(2.63)	0.008
Serum FSH U/l	5.14(1.35)	4.99(0.58)	0.912
Serum LH U/l	3.09(0.63)	8.14(1.6)	0.004
Serum progesterone nmol/l	2.38 (0.17)	2.83 (0.3)	0.267
Fasting Glucose mmol/l	4.46(0.17)	4.35(0.25)	0.73
Fasting Insulin mU/l	6.71(1.95)	18(5.88)	0.055
Fasting serum triglyceride (mmol/L)	0.74(0.15)	1.09(0.09)	0.083
Total cholesterol (mmol/L)	4.02(0.3)	4.69(0.29)	0.136
HDL Cholesterol (mmol/L)	1.504(0.061)	1.544(0.099)	0.74
Insulin sensitivity K _{itt} (% / min)	4.76(0.55)	3.24(0.23)	0.024
Insulin area under curve mU/l	4136(645) (n=6)	5014(587) (n=8)	0.336

B

PCOS subjects	Age years	BMI	Serum Testosterone nmol/L	Insulin Sensitivity K _{itt}
1	23	20.3	2.9	3.28
2	30	27.7	3.2	2.42
3	22	34.1	3.8	3.64
4	31	33.7	2.7	3.38
5	31	27	3.6	2.66
6	25	29	3.5	3.56
8	26	29.2	3.1	4.61
9	33	32.5	3.1	2.52
Controls				
1	34	27.2	1.7	5.17
2	31	23.2	0.9	5.37
3	38	20	1.0	3.74
4	34	23.8	2.5	7.29
5	30	22.7	2.0	5.15
6	34	32.9	1.3	3.68
7	32	28.3	1.8	2.92

Figure 1



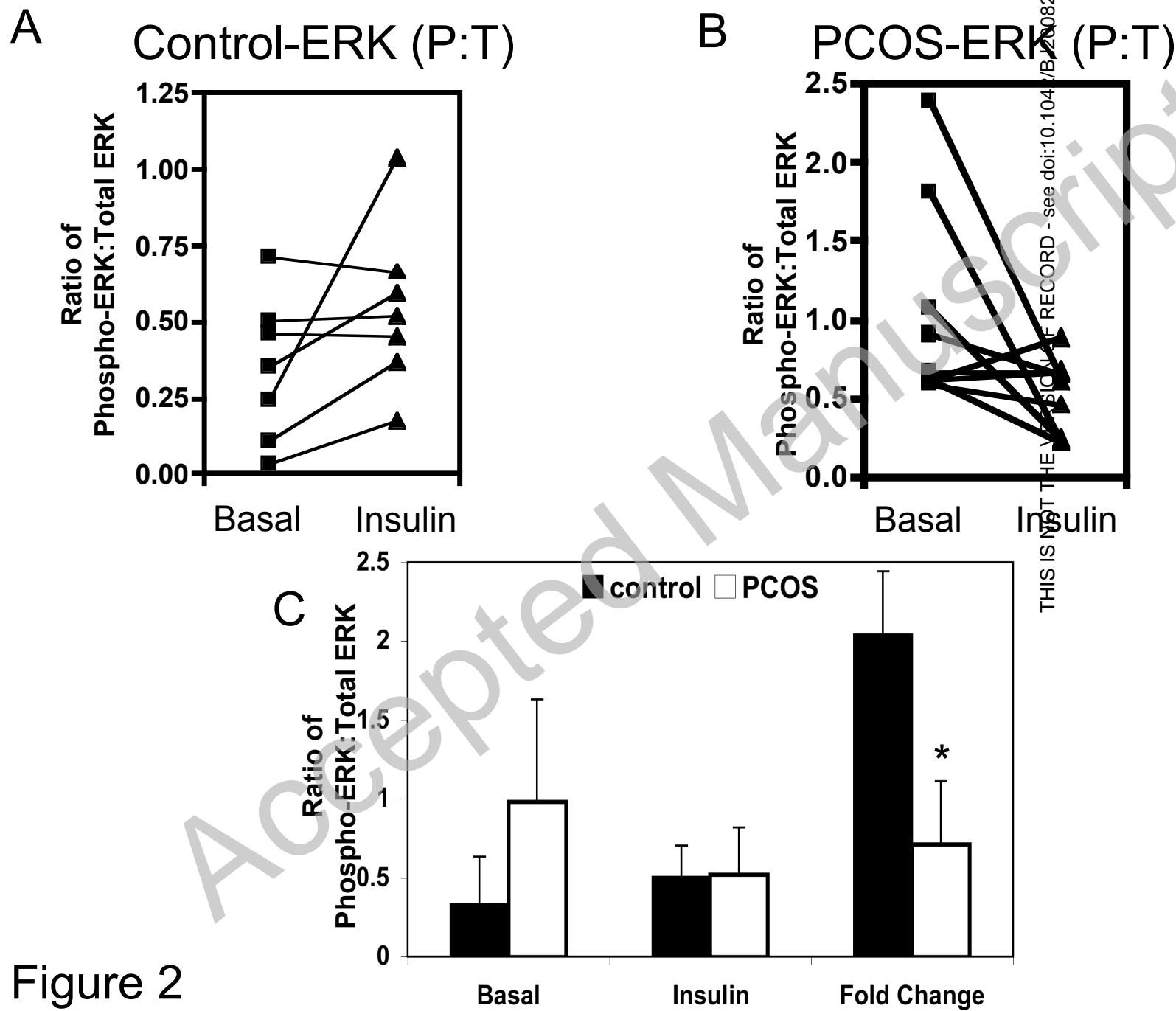


Figure 2

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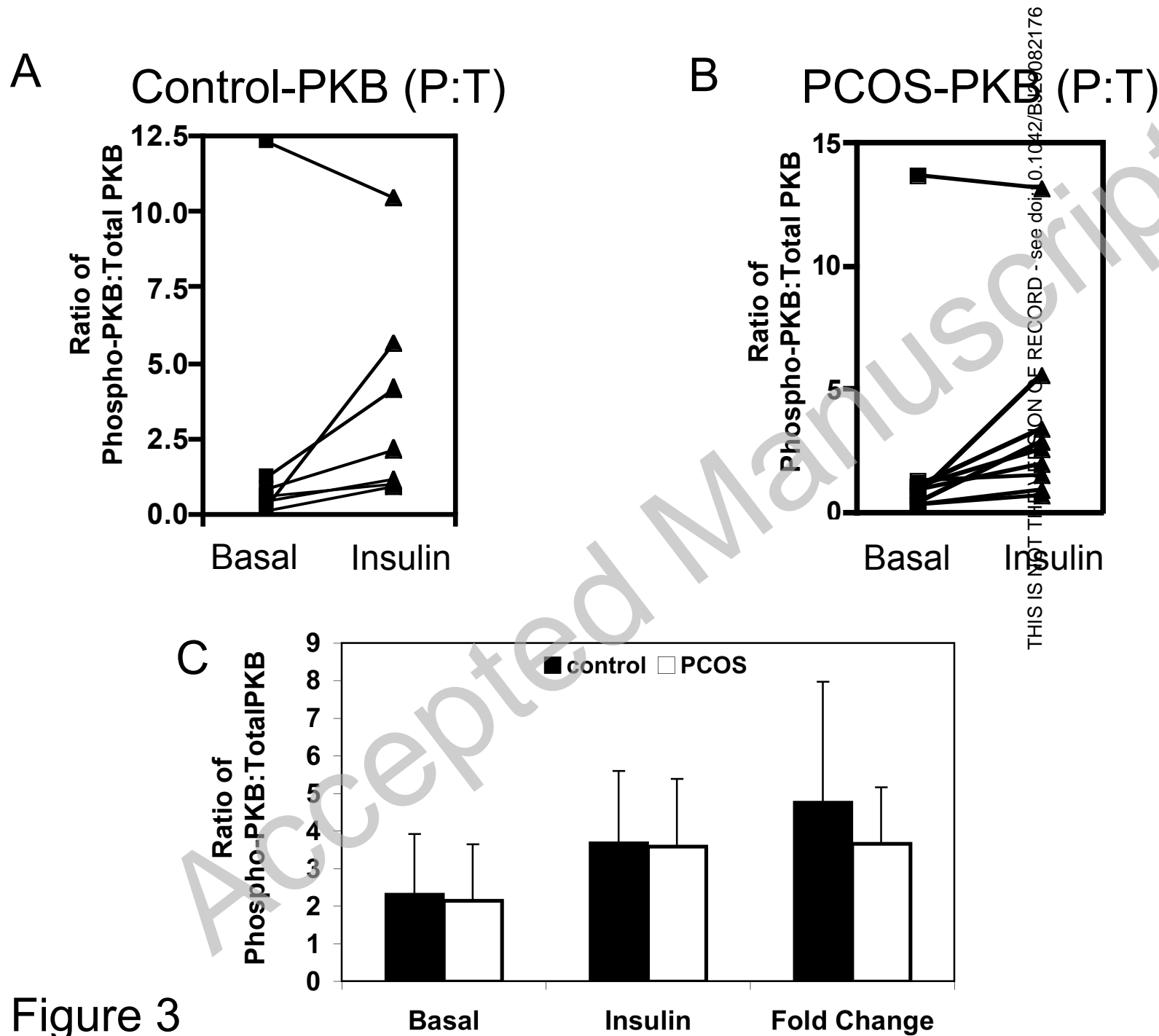


Figure 3

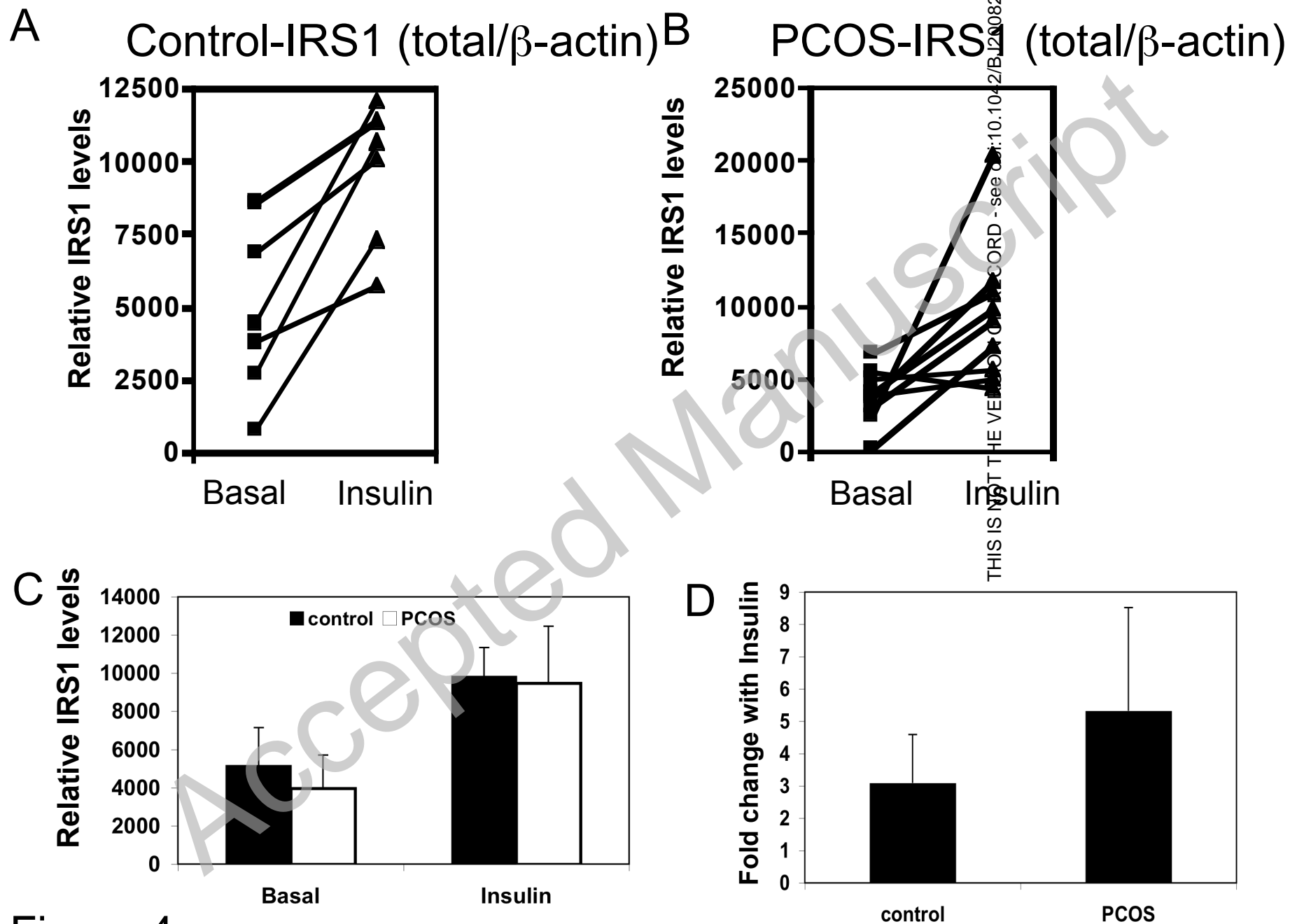
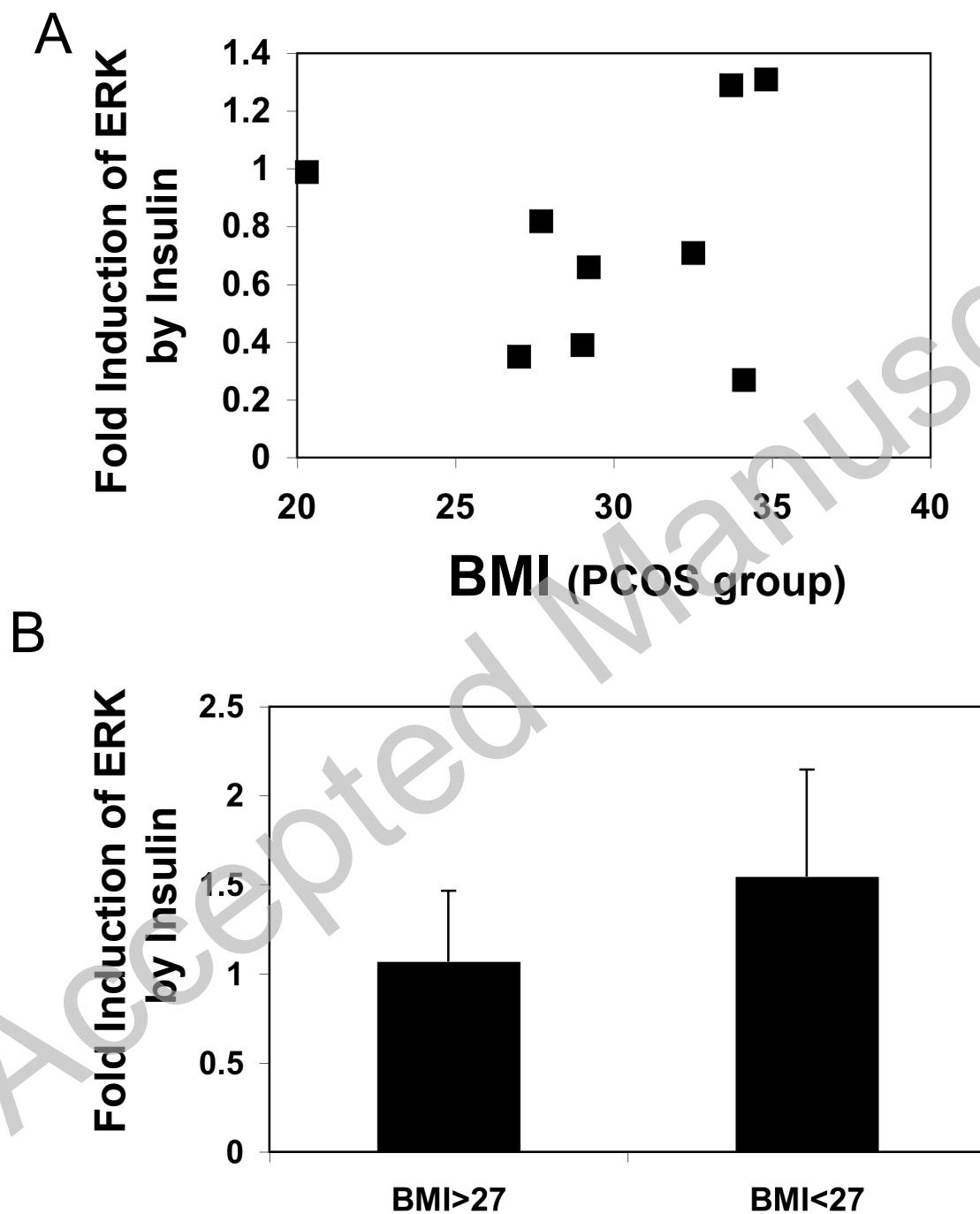


Figure 4

Figure 5



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