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RESEARCH ARTICLE

Molecular Characteristics of polycystic ovary syndrome by Real Time PCR.

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Abstract

The study carried out to evaluate the effect of some genes that play role in the polycystic syndrome.

Martials and methods: Three ovarian normal women and Three ovarian tissue PCOS women through laparoscopic surgery in Hospital of Mansoura University, extraction total RNA from ovarian tissue (Extraction of total RNA by The IQeasy plus CTB RNA Extraction Mini Kit intron biotechnology, Korea). In addition, total RNA to cDNA by ((Synthesis cDNA by Maxima RT PreMix Kit, intron biotechnology, Korea.). Finally, preparation of reagent of the Real MOD Real time PCR Core Kit and Master Mix provides a system intron biotechnology, Korea. Reading by Real-time PCR performed with ABI Prism 7900 (Applied Biosystems).

Results: Pyruvate dehydrogenase kinase 4 (PDK4) and Serine transcription (SET) downregulated gene in normal women while upregulated in polycystic ovary syndrome. *While* NR4A1 (nuclear receptor subfamily 4, group A, member 1) and HIP-55 (src homology 3 domain-containing protein are upregulated gene in normal women while downregulated in polycystic ovary syndrome.

Conclusion: There are some genes play important role in polycystic ovary syndrome as PDK4, NR4A1, SET and HIP-55 genes.

Introduction:-

The first definition was proposed by the National Institute of Health (NIH) in 1990 in which clinical and biochemical signs of hyperandrogenism or hyperandrogenemia and clinical symptoms of ovulation disorder as amenorrhea, oligomenorrhea or infertility in the absence of non-classical adrenal hyperplasia are the diagnostic criteria of the disease (***Naderi et al., 2011***, ***Mehrabian et al., 2011***).

The second definition (Rotterdam) was presented by Fertility and Embryology Association of Europe and America Fertility Society in Rotterdam conference in 2003 and has considered two criteria from the following three criteria as criteria for diagnosis of PCOS : Oligoovulation: menstrual period more than 40 days or anovulation less than 9 cycles per year. Clinical hyperandrogenism: (acne, hirsutism, and androgenic alopecia) or biochemical hyperandrogenism (elevated serum androgen levels). The presence of polycystic ovaries on pelvic ultrasound: (more than 12 follicles measuring 2 to 9 mm and ovarian volume greater than 10 mm) (***Akbari. 2010, Rahmanpour et al, 2008***). Whoever. **Sayera *et al*. (2012)** reported that, The PCOS is a heterogeneous condition, which defined by the presence of two out of the following three criteria: Oligo- and/or anovulation; hyperandrogenism (clinically or biochemically); and polycystic ovary, with exclusion of other etiology.

Palomba*et al.* (2013) reported that, several criteria proposed to define PCOS. The Rotterdam consensus workshop concluded that PCOS is a syndrome of ovarian dysfunction and its diagnosis is confirmed by the presence of two of the following three disorders: oligomenorrhea or amenorrhea, hyperandrogenism (e.g., hirsutism, acne, alopecia) or hyperandrogenemia (e.g., elevated levels of total or free testosterone), and polycystic ovaries on ultrasonography**.** Moreover, polycystic ovarian syndrome (PCOS) is a complex and multifactorial disorder believed to be the

consequence of a complex interaction between genetic, immunological, and environmental factors**. (Praveen*et al.* 2016)**

Fei-Yet al. (2004) concluded that, we selected four genes (NR4A1, PDK4, HIP-55 andSET) for semiquantitative real-time PCR analysis. These genes are differentially expressed in PCOSovaries and involved in various biologic functions that might have a role in the pathogenesis of PCOS. The sequences of primers used and relative changes in expression levels of these genes as determined by real-time RT-PCR.A paired *t*-test showed that the differences in the levels of expression of these genes between normal subjects and PCOSpatients are statistically significant. The patterns of these gene expressions obtained fromcDNA microarray and real-time RT-PCR analyses were similar, although the absolute ratios were different due to the potential difference in assay sensitivity and dynamics of these two assays.

Pyruvate dehydrogenase complex (PDHC) regulates the oxidative metabolism of glucose, which can be inhibited by isoforms of PDK. Recently, increased PDK4 activity has been implicated in the pathogenesis of insulin resistance and non-insulin-dependent diabetes mellitus (NIDDM). In the muscle cell of NIDDM patients, PDK4 mRNA expression correlates negatively with glucose uptake rate, and decreases following the improvement of insulin sensitivity and the reduction of weight (**Muller *et al.* 1998, Rosa *et al.* 2003**).

(Zhao *et al.* 2012) showed there are the relationship between ALAT and PDK4. Alanine transferred to the circulation mainly by skeletal muscle. There are two main pathways of alanine production: directly from protein degradation, and via the transamination of pyruvate by alanine aminotransferase (ALAT). Women with PCOS have been implicated to have higher levels of ALAT in the serum, which could accelerate the transamination of pyruvate to alanine. Additionally.

NR4A1 is a steroid/thyroid hormone-responsive orphan nuclear receptor that contains three key functional domains: a steroid hormone receptor ligand-independent transactivation domain (AF-1), a nuclear hormone receptor zinc finger domain (ZnF_C4) and a hormone receptor ligand-binding domain (HOLI) (Figure). No natural ligand for NR4A1 has yet been identified. NR4A1 thought to play a role in transcriptional regulation through binding of the ZnF_C4 domain to hormone response elements in DNA (**Moehren *et al.*, 2004**). This domain contains multiple finger-like structures and interacts with several target molecules, including DNA, RNA, proteins, and/or lipid substrates (**Laity *et al.*, 2001**). The range of interactions implies that the ZnF_C4 domain has multiple functions in different molecular processes. The HOLI domain influences NR4A1 nuclear translocation and its association with DNA (**Bledsoe *et al.*, 2004**).

ExpressionNR4A1 has been detected at varying levels in different human tissues, with particularly high levels in the adrenal cortex, lungs, prostate, ovaries, testes, heart, muscle, thyroid, trachea, olfactory bulb and adrenal gland ,LocalisationNR4A1 is a nuclear hormone receptor that activated by association with its ligand to move into the nucleus. For example, in response to n-Butylidenephthalide induced cell death signals, NR4A1 translocates into mitochondria to enhance apoptosis ((**Su *et al.*, 2004**)

FunctionNR4A1 is involved in multiple molecular processes, including signal transmission, transcriptional regulation, mediation of cell growth, induction of apoptosis, and cell cycle control (**Mohan *et al.*, 2012**). NR4A1 acts as a hormone receptor and is stimulated by ligand binding to move into the nucleus and associate with DNA to regulate transcription of multiple genes (**Wu *et al.*,2002**). NR4A1 is also involved in several complex pathways that mediate cell survival and apoptosis. Furthermore, NR4A1 dysfunction has been associated with inflammation and carcinogenesis. In terms of post-translational modifications, NR4A1 is phosphorylated by protein kinase B at Serine 350 and its acetylation is modulated by p300 and HDAC1 (**Li *et al.*, 2006**).

Martials and methods:-

Three ovarian normal women and three ovarian tissue PCOS women through laparoscopic surgery in Hospital of Mansoura University, extraction total RNA from ovarian tissue (Extraction of total RNA by The IQeasy plus CTB RNA Extraction Mini Kit intron biotechnology, Korea). In addition, total RNA to cDNA by ((Synthesis cDNA by Maxima RT PreMix Kit, intron biotechnology, Korea.). Finally, preparation of reagent of the Real MOD Real time PCR Core Kit and Master Mix provides a system intron biotechnology, Korea. Reading by Real-time PCR performed with ABI Prism 7900 (Applied Biosystems).show table (1&2). Results analyzed using the relative Ct

method. The Ct value, which is inversely proportional to the initial template copy number, is the calculated cycle number where the fluorescence signal emitted is significantly above background levels.

Comparative or ΔΔCt method for relative quantitation:-

ΔCT value: is the difference between the CT value of the target gene and the CT value of the corresponding endogenous reference gene, such as a housekeeping gene,

ΔCT = CT (target gene) – CT (endogenous reference gene):-

First, the difference between the Ct values (ΔCt) of the gene of interest and the housekeeping gene calculated for each experimental sample. Then, the difference in the ΔCt values between the experimental and control samples ΔΔCt is calculated. **ΔΔCT = average ΔCT (sample of interest) – average ΔCT (reference sample or calibrator sample)**.The fold-change in expression of the gene of interest between the two samples is then equal to **2^ (-ΔΔCt).**

Table (1): Show the preparation of reagent for RT-PCR reading.

[Reagents]	**1 test /50 scale**
2x RealMOD Green Real-time PCR Master mix Solution	25ul
PCR Forward Primer PCR	0.45 ul
PCR Reverse Primer	0.45 ul
Template	3ul
DW	Up to 50ul

Table (2): Show the arrangement of four primers forward and reverse (**NR4A1, PDK4, HIP-55 and SET)**

No.	Gene name	Primer (5^{-} - 3^{-})		tm	size
1	NR4A1	F	CATGGTGAAGGAAGTTGTC	55 °C	289
		R	AAAGCCAGGGATCTTCTC		
2	PDK4	F	CCAGACCAACCAATTCACATC	55 °C	285
		R	ACCAGCCAAAGGAGCATTC		
3	HIP-55	F	ATGTGACCATCAACGCAC	55 °C	207
		R	CCCAGAAGCTGTCTTTACC		
4	SET	F	CGAGCTACCAATGAAGGC	55 °C	199
		R	AAGCCTGGAAGTTCCGATAC		

Pyruvate dehydrogenase kinase 4 mRNA (PDK4), Nuclear receptor subfamily 4, group A, member 1(NR4A1), hedgehog interacting protein (HIP-55), Serine translocation (SET)

Result:-

Cycle Threshold (CT) genes that play role of polycystic ovary syndrome (PCOS) women and controls:-

Data resulted in table (3) and illustrated in figure (1) showed highly significant decrease ($p<0.01$) Pyruvate dehydrogenase kinase 4 mRNA (PDK4) (25.7 ± 1.33) at the comparison with control group (28.9 ± 0.99). Obtained data in table (3) showed highly significant in increase ($p<0.01$) Nuclear receptor subfamily 4, group A, member 1(NR4A1) (29.0 ± 1.33) at the comparison with control group (25.9 ± 0.7). . Obtained data in table (3) and illustrated in figures (1) showed high significant increase the SET translocation (myeloid leukemia-associated) showed high significant decrease ($p<0.01$) (24.5 ± 1.08) at the comparison with control group (26.8 ± 0.91). The sac homology 3 domain-containing protein HIP-55 showed high significant increase ($p<0.01$) (024.5 ± 1.08) at the comparison with control group (26.8 ± 0.91). The B acting showed non-significant ($p<0.01$) (22.31 ± 0.61) at the comparison with control group (22.41 ± 0.46).

The difference between the CT value of the target gene and the CT value of the corresponding endogenous reference gene, (ΔCT) genes that play role of polycystic ovary syndrome (PCOS) in women and controls.

Data resulted in table (4) and illustrated in figures (2) showed highly significant decrease ($p<0.01$ Pyruvate dehydrogenase kinase 4 mRNA (PDK4) (3.39 ± 1.59) at the comparison with control group (6.48 ± 1.17). Obtained data in table (4) showed highly significant in increase ($p<0.01$) Nuclear receptor subfamily 4, group A, member 1(NR4A1) (6.69 ± 1.61) at the comparison with control group (3.48 ± 0.84). . Obtained data in table (4) and illustrated in figures (2) showed high significant increase ($p<0.01$) in the SET translocation (myeloid leukemia-associated) showed high significant decrease ($p<0.01$) (2.19 ± 1.10) at the comparison with control group

(4.38 ± 1.088). The src homology 3 domain-containing protein HIP-55 showed high significant increase (p<0.01) (4.69 ± 1.13) at the comparison with control group (2.48 ± 0.84).

Relative quantities displaying ΔΔCT and Fold induction genes that play role of polycystic ovary syndrome (PCOS) women and controls:-

Data resulted in table (5) and illustrated in figures (3 A) showed ΔΔCT increase Pyruvate dehydrogenase kinase 4 mRNA (PDK4) (3.09) and SET translocation (myeloid leukemia-associated) (2.19). ΔΔCT decrease Nuclear receptor subfamily 4, group A, member 1(NR4A1) (-3.21) and the src homology 3 domain-containing protein HIP-55 (-2.21).

Data resulted in table (5) and illustrated in figures (3 B) showed Fold induction decrease Pyruvate dehydrogenase kinase 4 mRNA (PDK4) (-8.51) and SET translocation (myeloid leukemia-associated) (-4.56).Fold induction increase Nuclear receptor subfamily 4, group A, member 1(NR4A1) (9.25) and the src homology 3 domain-containing protein HIP-55 (4.62).

Table (3): CT *genes that play role of polycystic ovary syndrome (PCOS) women and controls.*

Cases parameter	Normal	PCOS	P-value
PDK4	**28.9 ± 0.99**	**25.7 ± 1.33*** **	**0.000**
NR4A1	**25.9 ± 0.7**	**29.0 ± 1.33*** **	**0.000**
HIP-55	**24.9 ± 0.87**	**27.0 ± 1.54*** **	**0.000**
SET	**26.8 ± 0.91**	**24.5 ± 1.08** **	**0.001**
B actin	**22.41 ± 0.46**	**22.31 ± 0.61^{ns}**	**0.675**

Mean with dissimilar superscript letter are significantly different at (P<0.05)
(p<0.05) =* (p<0.01) =** (p<0.001) =***
Pyruvate dehydrogenase kinase 4 mRNA (PDK4), Nuclear receptor subfamily 4, group A, member 1(NR4A1), HIP-55 (hedgehog interacting protein -55) SET translocation (Serine translocation) and beta actin (B actin).

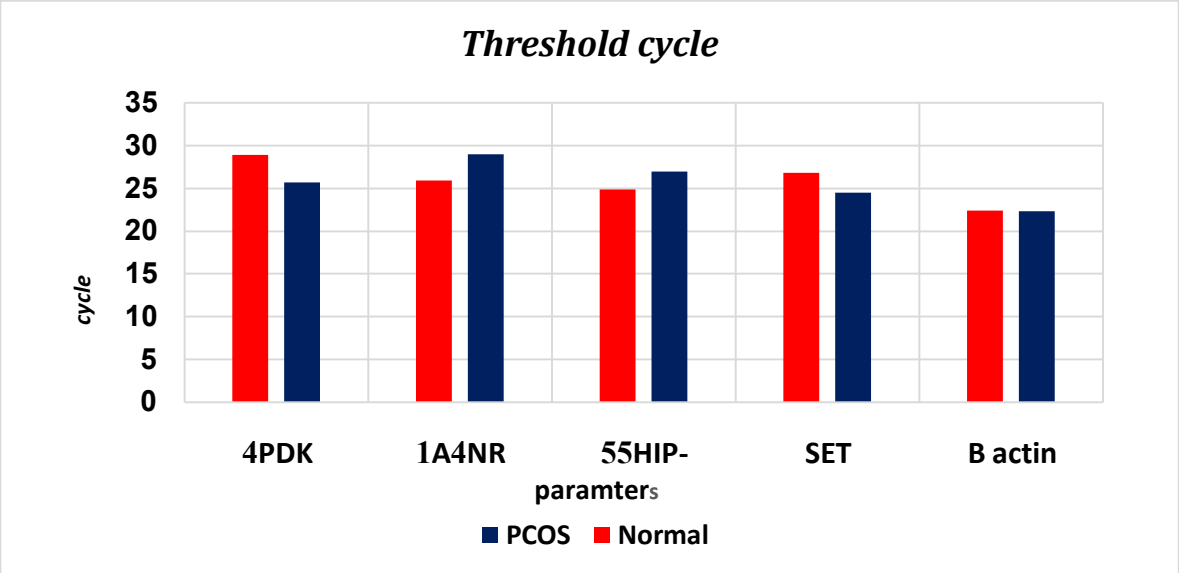


Figure (1): CycleThreshold (CT) *genes that play role of polycystic ovary syndrome (PCOS) women and controls.*

Table (4): ΔCT *genes that play role of polycystic ovary syndrome (PCOS) woman and controls.*

Cases parameter	Normal	PCOS	P-value
PDK4	**6.48 ± 1.17**	**3.39 ± 1.59** **	**0.001**
NR4A1	**3.48 ± 0.84**	**6.69 ± 1.61*** **	**0.000**
HIP-55	**2.48 ± 0.84**	**4.69 ± 1.13*** **	**0.000**
SET	**4.38 ± 1.088**	**2.19 ± 1.10** **	**0.006**

Mean with dissimilar superscript letter are significantly different at (P<0.05)
(p<0.05) =* (p<0.01) =** (p<0.001) =***

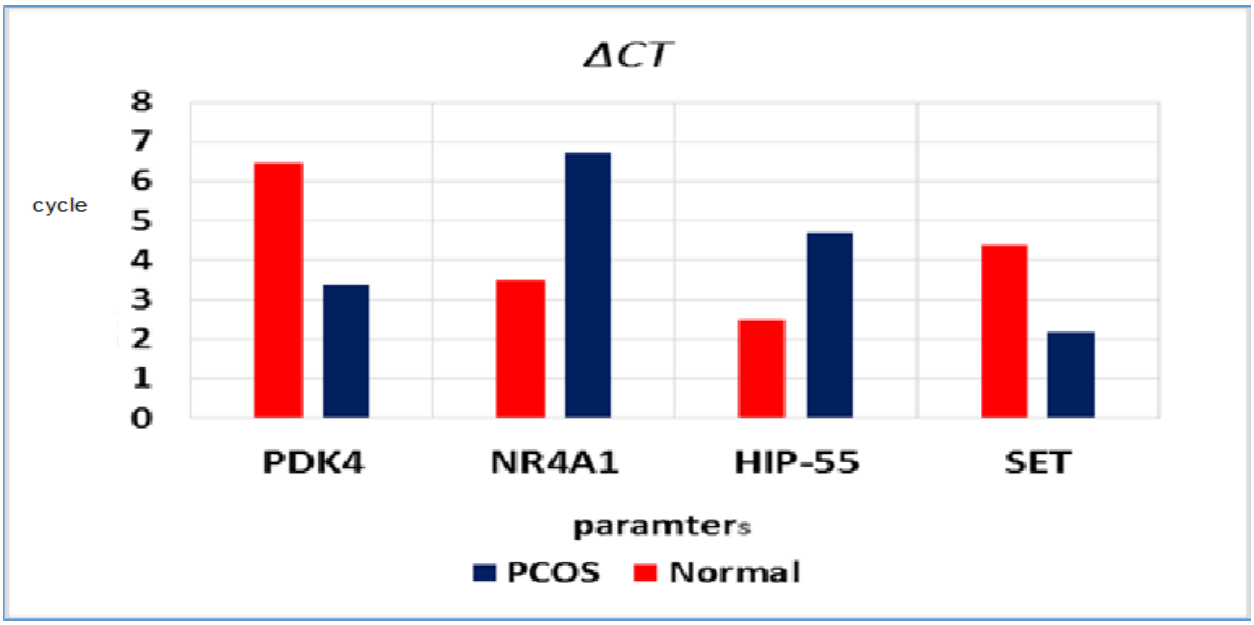
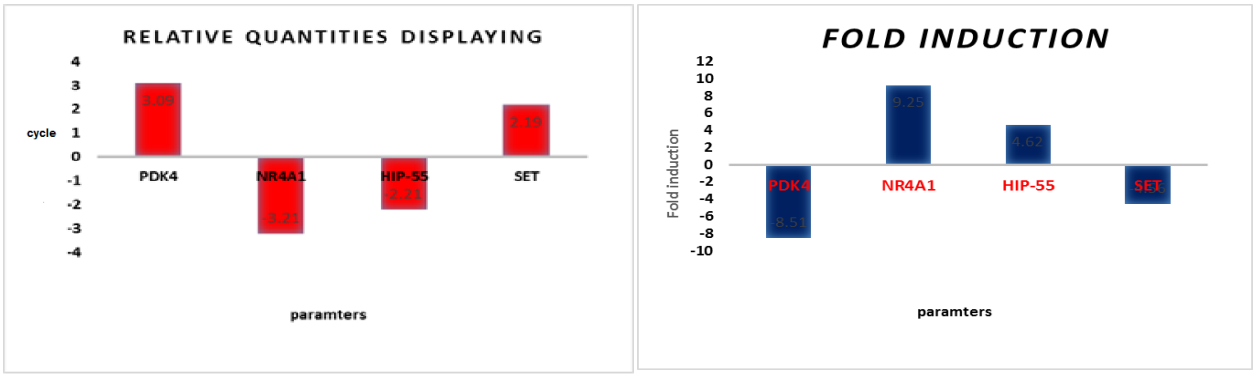


Figure (2): show some ΔCT genes that play role of polycystic ovary syndrome (PCOS) women and controls.

Table (5): *Relative quantities displaying (*ΔΔCT*) and* Fold induction *genes that play role of polycystic ovary syndrome (PCOS) women and controls.*

Cases parameter	ΔΔCT	$2^{-\Delta\Delta CT}$ Fold induction
PDK4	**3.09**	**-8.51**
NR4A1	**-3.21**	**9.25**
HIP-55	**-2.21**	**4.62**
SET	**2.19**	**-4.56**



(A) **(B)**

Figure (3): (A) ΔΔCT *genes show upregulating of NR4A1and HIP-55 genes while PDK4 and SET genes area upregulating in PCOS.(B):* Fold induction *(Normalized target gene expression level) that play role of polycystic ovary syndrome (PCOS) women and controls.*

Discussion:-

In this study, we used recently developed real time PCR technology to examine the differential gene expression patterns between normal and PCOS ovaries, and were able to identify several genes expressed at changed levels in PCOS patients compared with normal subjects. These genes are involved in a wide range of biologic functions, including gene/protein expression, cell signaling/cell communication and metabolism.. Many genes identified in this study were found to be associated with hormone production, metabolism and apoptosis in other tissues, but their roles in human ovaries and PCOS have not been documented before.

PDK4 (pyruvate dehydrogenase kinase 4):-

In the present study show, pyruvate dehydrogenase kinase 4 is down regulated gene in normal women while upregulated in polycystic ovary syndrome this result agree with ***Fei-Yet al. (2004)***that concluded, pyruvate dehydrogenase kinase 4 is down regulated gene in normal women while upregulated in polycystic ovary syndrome. Due to increased expression of pyruvate dehydrogenase kinase 4 (PDK4) mRNA in PCOS patients can enhance the peripheral concentration of this enzyme and subsequently promote the conversion of pyruvate to lactate, supporting the higher lactate concentration and glycolytic rate in our results. In addition, all control subjects have normal weight and insulin sensitivity, and we need samples from control women with obesity or insulin resistance for comparison to further analyze the effect of obesity and insulin resistance on the metabolic changes in PCOS.(**Zhao *et al.* 2012**)

Insulin has numerous biologic functions in target tissues, such as glycogen synthesis, steroidogenesis,DNA synthesis and lipogenesis. There is increasing evidence that PCOS is associated with hyperinsulinemia, insulin-resistance and dyslipidemia.Insulin resistance has been observed in cultured skin fibroblasts derived from PCOS patients (**Book&Dunaif 1999**). However, peripheral insulin resistance cannot fully explain the abnormal insulin action inPCOS ovary. In our study, we found that some genes involved in insulin functions changed their expression levels in PCOS ovary, as PDK4 pyruvate dehydrogenase kinase 4) (**Rosa *et al.* 2003,Sugden*et al.* 2003**) Pyruvate dehydrogenase complex (PDHC) regulates the oxidative metabolism of glucose, which can be inhibited by isoforms of PDK. Recently, increased PDK4activity has been implicated in the pathogenesis of insulin resistance and non-insulin -dependent diabetes mellitus (NIDDM). In the muscle cell of NIDDM patients, PDK4 mRNA expression correlates negatively with glucose uptake rate, and decreases following the improvement of insulin sensitivity and the reduction of weight (**Muller *et al* ,1998 .Rosa *et al.* 2003**). We found that PDK4mRNA was upregulated in PCOS ovary, although we need to investigate whether this increased expression of PDK4 mRNA means the existence of insulin resistance in PCOS ovary.

Moreover,(**Sara *et al.*, 2016**) resulted that, the pyruvate dehydrogenase complex (PDC) catalyzes the conversion of pyruvate to acetyl-CoA in mitochondria and is a key regulatory enzyme in the oxidation of glucose to acetyl-CoA. Phosphorylation of PDC by the pyruvate dehydrogenase kinases (PDK) inhibits its activity. The expression of the pyruvate dehydrogenase kinase 4 (PDK4) gene increased in fasting and other conditions associated with the switch from the utilization of glucose to fatty acids as an energy source. Transcription of the PDK4 gene is elevated by glucocorticoids and inhibited by insulin. In this study, we have investigated the factors involved in the regulation of the PDK4 gene by these hormones. Glucocorticoids stimulate PDK4 through two glucocorticoid receptor (GR) binding sites located more than 6000 base pairs upstream of the transcriptional start site. Insulin inhibits the glucocorticoid induction in part by causing dissociation of the GR from the promoter. Previously, we found that the estrogen related receptor alpha (ERRα) stimulates the expression of PDK4. Here, we determined that one of the ERRα binding sites contributes to the insulin inhibition of PDK4. A binding site for the fork head transcription factor (FoxO1) is adjacent to the ERRα binding sites. FoxO1 participates in the glucocorticoid induction of PDK4 and the regulation of this gene by insulin. Our data demonstrate that glucocorticoids and insulin each modulate PDK4 gene expression through complex hormone response units that contain multiple factors.

NR4A1 (nuclear receptor subfamily 4, group A, member 1) and HIP-55 (hedgehog interacting protein):-

In the present study show, NR4A1 (nuclear receptor subfamily 4, group A, member 1) and HIP-55 (src homology 3 domain-containing protein are upregulated gene in normal women while downregulated in polycystic ovary syndrome this result agree with Fei-Yet al. (2004 Our study indicates that there is a group of genes up- or downregulated in PCOS ovary that might result in reduced apoptosis. These genes include NR4A1 (nuclear receptor subfamily 4, group A, member 1) and HIP-55 (src homology 3 domain-containing protein). They regulate apoptosis via different pathways such as the c-Jun N-terminal kinase signaling cascade (JNK)/NF- kB, p53/BCL2/BAX and p73/c-myc. Most of them finally influence apoptosis through the mitochondrial death-signaling pathway, resulting in the defect in the release of cytochrome C**.**due to data suggest that the overexpression of survival or ant apoptotic factors and downregulation of apoptosis inducers led to the blocking of follicle apoptosis and atresia in PCOS ovary.We hypothesize that the blocking of apoptosis and atresia affects follicle development at both gonadotropin-independent and -dependent stages,and contributes to the excessive recruitment ofpreantral follicles and accumulation of multiple small antral follicles as well as hyperproliferation oftheca-interstitial cells.The most interesting gene among this group isNR4A1, which is dramatically upregulated innormal adult ovary compared with fetal ovary (adult/fetal=7·1), but downregulated in PCOS ovary. NR4A1, also known as TR3, Nur77 or NGFI-B, has beenconfirmed as an inducer of apoptosis. It causesmitochondria to release cytochrome C (**Li *et al.* 2000**).

Considering that the major cell typesexpressing NR4A1 are thecal cells of follicles indifferent sizes, we suggest that the downregulationof NR4A1 might contribute to the hyperproliferation of theca cells from small antral follicles inPCOS ovary (**Park *et al.* 2001**). As a nuclear receptor, NR4A1 mRNA expresses rapidly andtransiently in granulosa cells of preovulatoryfollicles after the preovulatory luteinizing hormone(LH) surge in adult cycling rats (**Park *et al.* 2001**)suggesting that NR4A1 may play a role inovulation by initiating a cascade of expression of ovulation-specific genes in ovulatory follicles inresponse to LH surge. Considering that NR4A1might play a protective role in atherogenesis, which is one of the long-term sequelae of PCOS, the expression pattern of NR4A1 in the PCOSpatient's circulation system needs further investigations (**Arkenbout *et al.* 2002**). These findings suggest that the downregulation of NR4A1 may affect multiple signal pathways and contribute to the development of various abnormalities in PCOSovary simultaneously.

The most interesting gene among this group is NR4A1, which is dramatically upregulated in normal adult ovary compared with fetal ovary (adult/fetal=7·1, but downregulated in PCOS ovary. NR4A1, also known as TR3, Nur77 or NGFI-B, has been confirmed as an inducer of apoptosis. It causes mitochondria to release cytochrome C (**Li *et al.* 2000**). Considering that the major cell types different sizes, we suggest that the downregulation of NR4A1 might contribute to the hyperproliferation of theca cells from small antral follicles in PCOS ovary (**Park *et al.* 2001**).

SET (Serine translocation):-

In the present study show, SET (Serine translocation) is downregulated gene in normal women while upregulated in polycystic ovary syndrome this result agree with ***Fei-Yet al. (2004)*** that concluded, SET (Serine translocation) is down regulated gene in normal women while upregulated in polycystic ovary syndrome.

Our cDNA microarray analysis also discovered gene named SET (SET translocation) that can regulate androgen production by P450c17. Cytochrome P450c17 catalyzes 17α-hydroxylation during cortisol synthesis and 17, 20-lyase activity duringsex steroid production. 17, 20-lyase activity, but not17α-hydroxylation activity, can be inhibited byPP2A (protein phosphatase 2A). The serinephosphoprotein SET inhibits PP2A specificallyand fosters 17, 20-lyase activity (**Pandey & Synthia 2003**). Since SET and PP2A are the posttranslational regulators of androgen biosynthesis,changes in their expression might contribute to the development of hyperandrogenism in PCOS.

Moreover, SET was originally identified as a translocated gene in acute undifferentiated leukemia it is a 39-kDa phosphoprotein widely expressed in various tissues, especially in steroidogenic cells within the central nervous system, adrenal gland, and gonad. As a transcriptional regulatingfactor, SET not only exerts function by binding to the transcriptional coactivators CBP/p300 (**Karetsou*et al.* 2005**), but also acts directly as a transcriptional factor of P450c17. All these indicated that SET regulated androgen synthesis in steroidogenic cells by regulation of both the transcriptional and posttranslational levels of P450c17 and CYP17. In mouse eggs PP2A was needed for both continued metaphase arrest and successful exit from meiosis (**Chang *et al.*2005**), which suggested that SET may participate in oocyte maturation. However, the function of SET protein in human ovary in regulating androgen production and oocyte development should be further studied. Hyperandrogenism is the central defect in PCOS patients (**Azzi*et al.*2006**) which related to the increased expressions of steroido-genic enzymes and the increased androgen biosynthesis.

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