

# EXPRESSION OF ANDROGEN RECEPTOR IN HUMAN PLACENTAS FROM NORMAL AND PREECLAMPTIC PREGNANCIES

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## SUMMARY

**Objective:** To elucidate the expression of androgen receptor (AR) in human placenta from normal and preeclamptic (PE) pregnancies.

**Materials and Methods:** This study was performed at the Division of Obstetrics and Gynecology, Kaohsiung Chang Gung Memorial Hospital, Academic Medical Center. Forty normal pregnant women at  $37.0 \pm 0.89$  weeks' gestation and 40 women with preeclampsia at  $35.9 \pm 1.33$  weeks' gestation were included in this study. Serum testosterone was measured before delivery. Immunochemical methodology was employed in formalin-fixed, paraffin-embedded sections from the placentas of all subjects. AR expression was evaluated in placentas collected from 15 normal pregnant women and 12 PE women.

**Results:** Women with preeclampsia had an elevated serum testosterone level ( $0.52 \pm 0.13$  vs.  $0.34 \pm 0.11$  ng/mL;  $p < 0.01$ ). The expression of AR in both placentas was confirmed using immunohistochemistry and was primarily present in the nucleus and cytoplasm of syncytiotrophoblasts and stromal cells. There was a significant increase in AR mRNA expression in PE placentas compared with normal placentas.

**Conclusion:** These data suggest that the increased AR expression may alter AR-mediated function on syncytiotrophoblasts and stromal cells in PE placentas, and be a possible mechanism for its association with preeclampsia. [Taiwan J Obstet Gynecol 2009;48(3):262–267]

**Key Words:** androgen receptors, placenta, preeclampsia

## Introduction

Preeclampsia is one of the major causes of mortality in pregnant women. Excluding ethnic factors, the incidence of preeclampsia is about 5% globally. From 1974 to 1985, the incidence of preeclampsia in Taiwan was 2.06%, but preeclampsia has been implicated as the cause of 23.7% perinatal maternal deaths [1]. Unfortunately, the cause of preeclampsia remains unclear to

date. Preeclampsia is associated with abnormal placentation, reduced placental perfusion, and systemic vasospasm. Vascular and hemostatic hyperreactivities involving the renin–angiotensin system, eicosanoids, and platelets have all been implicated.

Several reports have demonstrated, through human and animal studies, the association of androgens, especially testosterone, with hypertension. Baker et al pointed out that women with polycystic ovary disease, a disease associated with hyperandrogenemia, are at increased risk of pregnancy-induced hypertension [2]. Recent studies have shown that androgens have important effects on vascular reactivity, the renin–angiotensin system, eicosanoids, and platelets.

Androgens, i.e. testosterone and androstenedione, are produced in the ovarian thecal cells. In addition, a



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local factor, inhibin, within the follicles was recently shown to increase androgen production by thecal cells, which in turn increased circulating androgen levels in women. The serum concentrations of inhibin A and the potent androgen testosterone were higher in patients with preeclampsia than in matched control subjects with normal pregnancies [3,4]. It is possible that the effects of increased serum inhibin in preeclampsia may be manifested through increased circulating androgen levels.

Women with prior preeclampsia have elevated serum-free testosterone levels and free androgen index [5]. Acromite et al [6] reported that levels of the potent androgen testosterone were significantly higher in primigravid women with preeclampsia than in normotensive women at similar gestational and maternal ages. Serin et al [7] found that testosterone and free testosterone levels were found to be significantly higher in preeclamptic (PE) women in the third trimester compared with the values in normotensive controls. They concluded that higher blood androgen levels measured in PE patients may be implicated in the pathogenesis of preeclampsia.

Several studies have shown that androgen can cause physiologic changes strikingly similar to those seen in preeclampsia. Androgens also decrease prostacyclin production *in vitro* and increase production of other eicosanoids (including thromboxane), resulting in a thromboxane/prostacyclin ratio that favors vascular constriction and coagulation in a way similar to that seen in preeclampsia [8]. As normal pregnancy progresses and the placenta grows, androgens tend to decline and estrogens tend to increase as a result of increased placental enzyme activity. Hahnel et al [9] showed that pregnancies complicated by hypertension have a deficiency in placental aromatization enzymes, which may lead to high levels of androgens, including testosterone.

It is possible that androgens may play a mediating or amplifying role in the pathophysiology of preeclampsia. However, the relationship between androgen and the placental androgen receptor (AR) in PE pregnancy is unclear. The purpose of our study was to compare the expression of placental AR between normal and PE pregnancies.

## Materials and Methods

In this prospective, controlled, cross-sectional study, we evaluated 80 women with singleton pregnancies who received perinatal care from August 2001 to January 2002 at the Division of Obstetrics and Gynecology, Kaohsiung Chang Gung Memorial Hospital, a tertiary

clinical care center. All women included in this study gave informed consent. Blood samples were obtained from peripheral blood taken from 40 pregnant women with preeclampsia and 40 healthy, gestational age-matched pregnant women. Samples were centrifuged at 3,000 rpm for 15 minutes, and serum was stored in microtubes at  $-80^{\circ}\text{C}$  until assayed.

Preeclampsia was defined as new onset hypertension after 20 weeks' gestation with systolic blood pressure  $>140$  mmHg, diastolic pressure  $>90$  mmHg, or both, measured on two occasions  $>6$  hours apart, and significant proteinuria (300 mg per 24 hours). No subject had a history of hypertension or renal disease. Control subjects were apparently healthy and without hypertension and proteinuria. Immunohistochemical staining was performed on the placentas from both groups. Twelve women were selected randomly from among the 40 PE women, and 15 gestational age-matched subjects were then selected randomly from the 40 control subjects. We performed real-time quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) analysis on placentas from 12 PE patients and 15 normal pregnant women.

### Androgen assay

Non-fasting serum samples were drawn at a non-standardized time of the day, centrifuged at room temperature and stored at  $-80^{\circ}\text{C}$  until assay. Total testosterone was measured by means of a commercially available radioimmunoassay, using a sensitive and specific rabbit anti-human testosterone antibody (Diagnostic Products Corp., Webster, TX, USA). Mean intra-assay coefficients of variation (CV) of the assays were 7.7, 8.1 and 6.7% at 0.52, 5.05 and 13.77 ng/mL, respectively ( $n=14$ ). Mean interassay CV were 10.5, 5.7 and 8.1% at 0.57, 6.00 and 13.62 ng/mL, respectively ( $n=11$ ).

### Immunohistochemical stain

Placental specimens were fixed with paraformaldehyde and embedded in paraffin, and immunohistochemistry was performed on 4- $\mu\text{m}$  sections using the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA, USA). Tissue sections were deparaffinized in xylene, dehydrated in a graded series of ethanol. Following a phosphate buffered saline (PBS) rinse, the endogenous peroxidases were quenched on incubation for 15 minutes with 3%  $\text{H}_2\text{O}_2$  in absolute ethanol followed by a 10-minute rehydration in PBS. The tissue sections for AR determination were heated in a microwave for 14 minutes in antigen retrieval solution (10 mM citrate buffer) before incubation with primary antibody. After initial incubation with blocking serum for 30 minutes at room temperature, rabbit polyclonal antibody raised against AR (N-20, sc-816; Santa Cruz Biotechnology, Inc., Santa

Cruz, CA, USA) was applied at a concentration of 4 µg/mL and 1:200. Finally, sections were counterstained with hematoxylin, dehydrated in a graded series of ethanol, cleared with xylene, mounted in Permount, and coverslips placed over for evaluation by light microscopy.

#### **Placental tissue collection, RNA extraction, and cDNA preparation**

Specimens (0.25 g) of human placentas were collected from PE pregnant women ( $n=12$ ) and normotensive pregnant women ( $n=15$ ). They were washed with PBS solution, and resuspended in guanidine isothiocyanate buffer with  $\beta$ -mercaptoethanol then stored at  $-80^{\circ}\text{C}$ . Total RNA was extracted from placental tissue by Trizol reagent (Invitrogen, Grand Island, NY, USA). Briefly, 1 mL of Trizol was added to the placental tissue. The mixture was pipetted to agitate and allowed to sit for 5 minutes at room temperature. Chloroform (0.2 mL) was added, mixed, and allowed to incubate at room temperature for 3 minutes. The mixture was centrifuged at 12,000g for 15 minutes, and the supernatant was transferred to a fresh tube. Isopropanol (0.5 mL) was added, mixed, and incubated for 10 minutes at room temperature. The solution was processed by centrifuge at 12,000g for 10 minutes, and the RNA was purified. The pellet was washed once with 70% ethanol, resuspended in water, and stored at  $-80^{\circ}\text{C}$ . The quality of the RNA samples was determined by electrophoresis through agarose gels and staining with ethidium bromide.

Total RNA (2 µg) from the sample preparation was reverse transcribed in 25 µL as follows: 0.1 µg of random hexamer primers (Amersham Pharmacia Biotech, Inc., Buckinghamshire, UK) were denatured for 10 minutes at  $70^{\circ}\text{C}$  in a gradient cycler (DNA Engine; MJ Research, Watertown, MA, USA). The subsequent reverse transcription was done at  $42^{\circ}\text{C}$  for 1 hour by adding 5X reverse transcriptase buffer (500 mM each deoxynucleotide triphosphate [dNTP], 3 mM  $\text{MgCl}_2$ , 2.75 mM KCl, and 50 mM Tris-HCl [pH 8.3]), 1 mM dNTP (Promega, Madison, WI, USA), 10 IU of ribonuclease inhibitor (Promega), and 100 U of Moloney murine leukemia virus reverse transcriptase (Promega). The reverse transcriptase was inactivated by heating at  $95^{\circ}\text{C}$  for 5 minutes and cooling at  $4^{\circ}\text{C}$  for 5 minutes.

#### **Real-time quantitative RT-PCR analysis**

##### *Preparation of real-time PCR mix*

All PCR reactions were performed with an ABI Prism 7900 Sequence Detection System (Perkin Elmer–Applied Biosystems, Courtaboeuf, France). PCR was performed using the SYBR Green PCR Core Reagents Kit (Perkin Elmer–Applied Biosystems). The amplification reactions were performed in a 50-µL final volume containing

10X SYBR Green buffer, 3 mM  $\text{MgCl}_2$ , 2.5 mM dNTP, 1.25 U of Taq-Gold, 0.25 U of reverse transcriptase, 0.4 U ribonuclease inhibitor, 10 µL RNA. Final AR (forward primer sequence: CCT ggC TTC CgC AAC TTA CAC; reverse primer sequence: ggA CTT gTg CAT gCg gTA CTC A) and  $\beta$ -actin (forward primer sequence: TCA CCC ACA CTg TgC CCA TCT ACg A; reverse primer sequence: CAg Cgg AAC CgC TCA TTg CCA ATg g) forward and reverse primer concentrations were 0.4 µM. To reduce the variability between replicates, PCR premixes which contained all the reagents expected to be used for the cDNA were prepared and aliquoted into 1.5-mL microfuge tubes. Experiments were performed with duplicates for each data point.

##### *Real-time RT-PCR time and temperature profiles*

The real-time RT-PCR protocol used for AR was as follows: denatured program ( $60^{\circ}\text{C}$  for 10 minutes,  $95^{\circ}\text{C}$  for 10 minutes), amplification and quantification program repeated 40 times ( $95^{\circ}\text{C}$  for 20 seconds,  $65^{\circ}\text{C}$  for 30 seconds,  $72^{\circ}\text{C}$  for 30 seconds), and melting curve program ( $95^{\circ}\text{C}$  for 15 seconds,  $60^{\circ}\text{C}$  to  $95^{\circ}\text{C}$  with a heating rate of  $0.2^{\circ}\text{C}$  per second).

##### *Calibration curves*

All samples with threshold cycle ( $C_t$ ) CV values higher than 1% were retested. The quantity of AR mRNA was calculated using the  $C_t$  method. Briefly, the fluorescence signal threshold was defined such that each PCR reaction was in the exponential phase. The  $C_t$  value was the cycle at which the fluorescence signal of the reaction exceeded this value.  $C_t$  values were proportional to the starting copy number (using the Perkin Elmer–Applied Biosystems analysis software), according to the manufacturer's manual.

$C_t$  values of the AR target gene were normalized to the  $C_t$  value of a housekeeping gene,  $\beta$ -actin. The precise amount of total RNA added to each reaction (based on absorbance) and its quality (i.e. lack of extensive degradation) were both difficult to assess. We, therefore, also quantified transcripts of the gene  $\beta$ -actin as the endogenous RNA control, and each sample was normalized on the basis of its  $\beta$ -actin content. Results are presented as:  $2^{\Delta C_t}$ , where  $\Delta C_t = C_{t\beta\text{-actin}} - C_{t\text{target gene}}$ .

##### *Statistical analysis*

The SigmaStat 9.0 statistical package (Jandel Corporation, San Rafael, CA, USA) was used for data analysis. All  $p$  values were two-sided, and a  $p$  value of  $<0.05$  was considered statistically significant. The nonparametric Mann-Whitney  $U$  test (StatView 1996; Abacus Concepts, Inc., Berkeley, CA, USA) was used to evaluate the variation in expression between human placental tissues.

## Results

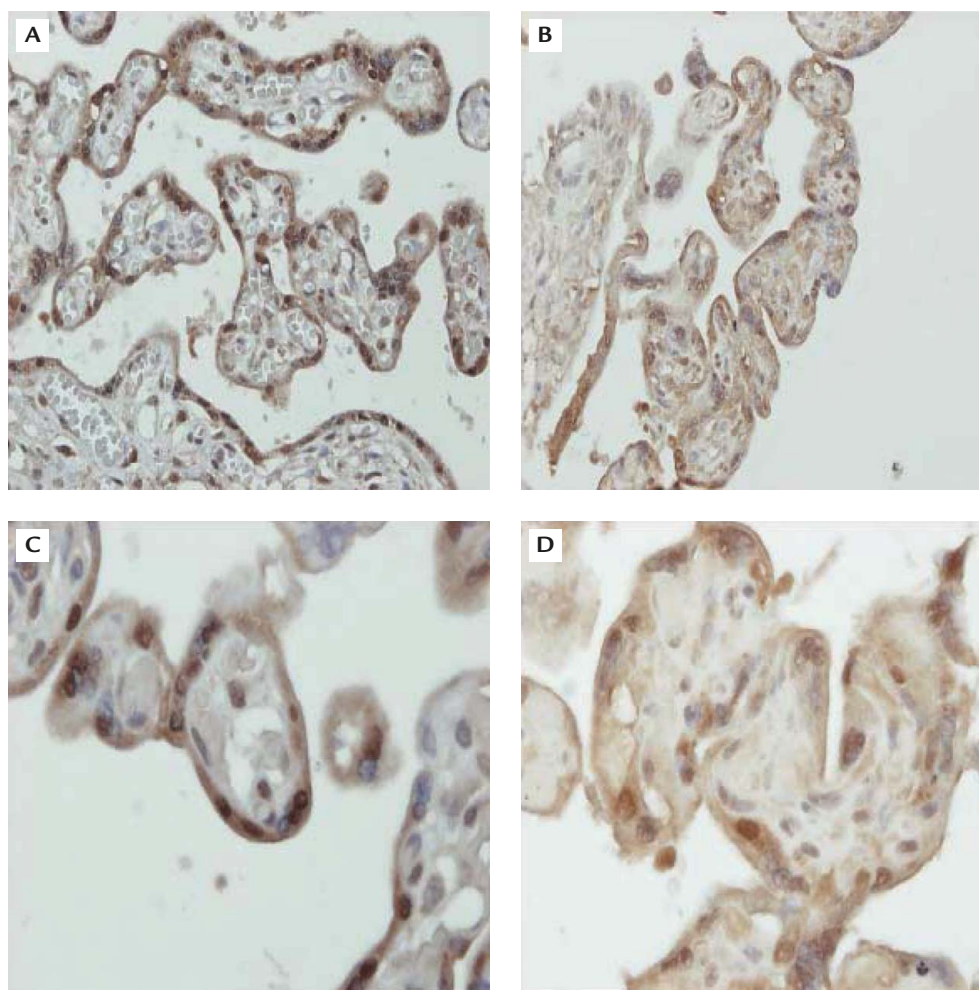
The clinical characteristics of the PE and normal pregnancy women were comparable and matched by maternal age and gestational age. The birth weight was greater in the normal pregnancy group. The systolic and diastolic blood pressures were higher in the PE pregnancy group. PE women were characterized by elevated serum

testosterone levels ( $p < 0.01$ ; Table 1). The microscopic examination of PE placentas showed small-sized villi with many syncytial knots and focal perivillous and intervillous fibrinoids. Focal calcification and necrosis were noted at the edge of the placenta. Syncytiotrophoblasts and stromal cells in human placentas were positive for the AR with nuclear and cytoplasmic staining (Figure). The AR mRNA levels were presented as

**Table 1.** Patient characteristics\*

	Preeclamptic women ( $n = 40$ )	Normal pregnant women ( $n = 40$ )	$p$
Gestational age (yr)	$29.6 \pm 5.0$	$28.7 \pm 4.4$	NS
Gestational week (wk)	$35.9 \pm 1.33$	$37.0 \pm 0.89$	NS
Body weight (g)	$1,822.0 \pm 726.1$	$3,268.2 \pm 368.8$	$< 0.01$
Systolic blood pressure (mmHg)	$179.4 \pm 18.7$	$123.0 \pm 8.5$	$< 0.01$
Diastolic blood pressure (mmHg)	$110.5 \pm 11.5$	$77.6 \pm 8.0$	$< 0.01$
Serum testosterone (ng/mL)	$0.52 \pm 0.13$	$0.34 \pm 0.11$	$< 0.01$

\*Data are expressed as mean  $\pm$  standard deviation. NS = not significant.



**Figure.** Syncytiotrophoblasts and stromal cells are positive for androgen receptor with nuclear and cytoplasmic staining. Androgen receptor distribution is illustrated for (A, C) normal specimens and for (B, D) preeclampsia specimens. (A, B) Original magnification  $\times 200$ ; (C, D) original magnification  $\times 400$ .



**Table 2.** Androgen receptor (AR) expression levels are displayed as a ratio between the target gene and a reference gene ( $\beta$ -actin) to correct for variation in the amounts of RNA

	Normal ( <i>n</i> = 15)	Preeclampsia ( <i>n</i> = 12)	<i>p</i> *
AR: $\beta$ -actin	3.7 $\pm$ 1.8	36.8 $\pm$ 19.0	<0.001

\*Mann-Whitney U test.

a ratio between the target gene and a reference gene ( $\beta$ -actin) to correct for variation in the amounts of RNA. There was significantly increased mRNA expression of AR in PE placentas compared with that in normal placentas (Table 2).

## Discussion

Androgens are synthesized and secreted into the bloodstream for circulation largely as a form of testosterone, which enters its target cells before it can exert its specific function. Patients with polycystic ovarian disease, a syndrome with hyperandrogenism, have a higher risk of developing preeclampsia. The association of preeclampsia and polycystic ovarian disease supports a relationship with androgens. Several studies demonstrated that the levels of androgen were significantly higher in women with preeclampsia than in normotensive women with similar body mass index, gestational age, and chronologic age [7]. Two *in vitro* studies showed reduced conversion of androgens to estrogens in placental tissue from PE pregnancies. Accordingly, several studies have consistently supported the important role of androgens in the development of arterial hypertension. Our results support previous studies which found elevated serum testosterone levels in PE women.

The AR is a member of the steroid hormone receptor family, which is found in a variety of tissues, and changes throughout development, aging, and malignant transformation. A concomitant increase in both serum androgens and elevations in endometrial ARs in women with polycystic ovarian syndrome has been reported [10]. ARs could be demonstrated in the deciduas and trophoblastic cells from three first-trimester human samples using immunohistochemistry [11]. However, the study of Chan et al [12] showed the absence of ARs in all samples from human first-trimester placenta and deciduas. These observations indicated that there is little AR expression in early human placenta and deciduas. Our study showed that the positive staining of ARs was observed primarily in the nucleus and cytoplasm of syncytiotrophoblast and stromal cells

in human placentas. To our knowledge, for the first time, we demonstrated that the AR is expressed in normal and PE placentas at near-term pregnancy. Several previous reports have shown that AR levels in Sertoli cells are more sensitive to androgen regulation in adult as compared with those in immature animals. According to our findings, we hypothesize that the greater the gestational age, the higher is the expression of AR in human placentas.

Here, we also found that AR expression was significantly increased in PE placentas compared with normal placentas. Recently, several reports pointed out that androgens induce a variety of effects on blood vessels. Testosterone induces endothelium-independent vasodilation [13]; conversely, it can act via the endothelium to oppose relaxation and produce vasoconstriction [14]. The latter action varies according to gender, being more marked in men, a fact that may contribute to the increased risk of vascular events in men [15]. Increasing evidence suggests possible adverse effects of androgens on the vasculature. *In vitro* studies suggest that androgens may directly accelerate atherosclerosis by stimulating the proliferation of vascular smooth muscle cells [16]. Ling et al [17] showed that testosterone, but not dehydroepiandrosterone or estradiol, at physiologic concentrations, enhances apoptosis-related damage in human vascular endothelial cells after serum deprivation. It has been reported in the past that androgens can cause physiologic changes strikingly similar to those seen in preeclampsia. Androgens also decrease prostacyclin production *in vitro* and increase production of other eicosanoids (including thromboxane), resulting in a thromboxane to prostacyclin ratio which favors vascular constriction and coagulation in a way similar to that seen in preeclampsia.

At the cellular level, increased androgen bioactivity may result from elevated circulating androgen concentrations and/or increased AR transactivational activity which are influenced by the AR gene. Only limited information is available regarding the possible involvement of arterial ARs in atherogenesis processes. Hanke et al [18] first showed an upregulation of the expression of arterial AR mRNA with testosterone; they hypothesized that vascular ARs might play an important role in signaling of the testosterone effects at the level of the arterial vessel wall. This "total androgen bioactivity" might contribute to the risk of preeclampsia. In the future, further evaluation of their biologic actions in PE placentas will undoubtedly contribute to our understanding of androgen effects in preeclampsia.

In conclusion, this study demonstrated that the AR is expressed in normal and PE placentas in near-term and term pregnancy. In addition, there is a significant

increase in AR mRNA expression in PE placentas compared with that in normal placentas. Further study of the AR gene and its influence is important in the future.

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