Transgenic overexpression of plasminogen activator inhibitor-1 promotes the development of polycystic ovarian changes in female mice

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Abstract

Reproductive age women (5–10\%) are affected by the polycystic ovarian syndrome (PCOS), a diagnosis which confers lifelong cardiovascular and reproductive health implications. Plasminogen activator inhibitor-1 (PAI-1), the main physiological inhibitor of plasminogen activation, is consistently elevated in women with PCOS, regardless of metabolic status. Interestingly, the plasminogen system has long been implicated in proteolytic processes within the dynamic ovary. A non-physiologic elevation in PAI-1 may thus contribute systemically to endothelial dysfunction and locally to abnormal ovarian phenotype and function. We herein characterize the phenotypic alterations in ovaries from transgenic mice, which constitute a stable form of human PAI-1 and determine the plasma testosterone level in these mice as opposed to their unaffected counterparts. Over half of the ovaries from transgenic mice were found to contain large cystic structures, in contrast to wild-type controls of the same genetic background (53\% (N=17) vs 5\% (N=22); P=0.001). Plasma testosterone was nearly twofold elevated in transgenic female mice versus wild-type females (0.312 ng/ml \( \pm \) 0.154 (N=10) vs 0.181 ng/ml \( \pm \) 0.083 (N=8); P=0.014). An elevation in PAI-1 therefore appears to predispose mice to the development of this abnormal architecture, which in turn is associated with an increase in plasma testosterone. Therefore, we propose that an inappropriate elevation in PAI-1 contributes to the development of polycystic structures; these findings may thus reorient the efforts aimed at the development of therapeutic agents for the treatment of this increasingly common syndrome.

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Introduction

Approximately 1 in 15 women in the United States is affected by the polycystic ovarian syndrome (PCOS), thus giving it the distinction as the most common endocrinopathy in this age group (Adams et al. 2004, Apridonidze et al. 2005). The cardinal features of this syndrome include hyperandrogenism, chronic anovulation, and polycystic ovaries; however, of most concern is the observation that these young women demonstrate a greater propensity towards the development of metabolic disease as well as infertility and recurrent miscarriage (Chang & Katz 1999). Nearly, 40\% of women with PCOS exhibit insulin resistance, a condition that is only aggravated by the increased prevalence of obesity in women with this syndrome (Legro et al. 2004).

Dahlgren et al. (1992) predict that women with PCOS are at a sevenfold increased risk for myocardial infarction using a risk factor profile. Indeed, vascular endothelial dysfunction, a surrogate marker for cardiovascular disease, has been well described in this population (Kravariti et al. 2005). Despite the increased prevalence of cardiovascular risk factors described in women with PCOS, an increased prevalence of cardiovascular events has yet to be clearly established (Pierpoint et al. 1998, Wild et al. 2000, Lo et al. 2006). This paradoxical observation continues to drive investigation into the pathophysiology of PCOS and the clinical implications of its diagnosis.

There is ample amount of observational data within the literature to support an association between an elevation in plasminogen activator inhibitor-1 (PAI-1) and PCOS (Sampson et al. 1996, Atiomo et al. 1998, Orio et al. 2004). PAI-1 is a member of the superfamily of serine protease inhibitors and prevents plasminogen activation via its specific and rapid inhibition of plasminogen activators. Tissue plasminogen activator (t-PA) mediates fibrinolysis, while urokinase plasminogen activator (u-PA) plays a critical role in cell-surface plasminogen activation; both serine proteases are vital to cell migration and tissue remodeling. PAI-1 is synthesized in the liver, adipose tissue, and vasculature.
The transcription of PAI-1 is regulated by a number of hormonal, metabolic, and inflammatory stimuli, including glucose, very low density lipoprotein (VLDL), insulin, growth factors such as transforming growth factor-β (TGF-β), as well as the inflammatory cytokines tumor necrosis factor (TNF-α) and IL-6. Recent epidemiologic studies have reported that an elevation in plasma PAI-1 predicts the future development of diabetes mellitus (Festa et al. 2002, 2006, DeTaeye et al. 2005, LiJnen 2005, Vaughan 2005). Thus, it is not surprising that PAI-1 is emerging as an informative biomarker predicting metabolic and cardiovascular risk.

In addition to physiological factors that regulate PAI-1 production, plasma PAI-1 levels are also determined by genetic factors. A common diallelic polymorphism located at −675 bases from the transcription start site of PAI-1 is the best characterized genetic determinant. Individuals that carry the 4G allele have increased plasma levels of PAI-1. The 4G allele has been reported to exist at a higher frequency in women with PCOS when compared with age and BMI-matched controls (Diamanti-Kandarakis et al. 2004). This finding suggests a mechanistic link between PCOS and PAI-1. PAI-1 has additionally been implicated as the only measurable vascular risk factor associated with both obese and non-obese PCOS in the setting of normal as well as high insulin levels (Sills et al. 2003, Tarkun et al. 2004). Sampson et al. (1996) determined that PAI-1 was higher in women with PCOS characterized by menstrual abnormalities than those by regular menses. Glueck et al. (2006) demonstrated that an elevated PAI-1 is an independent risk factor for the development of early first trimester miscarriage in these women; improvement in this risk profile accompanies a reduction in PAI-1 achieved with 6 months of metformin therapy. Systemically, an elevation in PAI-1 may thus not only contribute to the increased risk of metabolic disease in this population, but at the level of the ovary, may also explain impaired ovulation and thus infertility.

A novel line of transgenic mice that express a stable form of active human PAI-1 were developed and characterized in our laboratory (Eren et al. 2002). Specifically, the transgenic mice constitutively express a stable variant of active human PAI-1. This variant is formed by the substitution of specific amino acids, which prolong active PAI-1’s half-life to > 145 h. These mutations prolong stability yet do not have any effect on other functional domains within PAI-1 (Berkenpas et al. 1995). The transgene is under control of the 5.9 kb fragment of the murine proendothelin-1 promoter (mPPET-1), a system previously established to target gene expression to endothelial cell-specific elements (Harats et al. 1995). Physiologic expression of mouse PAI-1 remains unaltered. Mice of the identical B6D2 background and of matched age served as control animals and are herein referred to as ‘wild-type mice.’ All mice received identical feed and were housed in groups with mice of the same strain. There were no differences in external genitalia between wild-type and transgenic mice. For purposes of histological analyses, animals were euthanized by cervical dislocation following anesthesia with isoflurane inhalation. Ovaries were harvested, formalin fixed, and embedded within paraffin blocks. Five micron tissue sections from each ovary were prepared for analyses. All animal protocols were approved by the Vanderbilt University Institutional Animal Care and Use Committee.

Materials and methods

Experimental animals

Transgenic mice were generated as previously described (Eren et al. 2002). Specifically, the transgenic mice constitutively express a stable variant of active human PAI-1. This variant is formed by the substitution of specific amino acids, which prolong active PAI-1’s half-life to > 145 h. These mutations prolong stability yet do not have any effect on other functional domains within PAI-1 (Berkenpas et al. 1995). The transgene is under control of the 5.9 kb fragment of the murine proendothelin-1 promoter (mPPET-1), a system previously established to target gene expression to endothelial cell-specific elements (Harats et al. 1995). Physiologic expression of mouse PAI-1 remains unaltered. Mice of the identical B6D2 background and of matched age served as control animals and are herein referred to as ‘wild-type mice.’ All mice received identical feed and were housed in groups with mice of the same strain. There were no differences in external genitalia between wild-type and transgenic mice. For purposes of histological analyses, animals were euthanized by cervical dislocation following anesthesia with isoflurane inhalation. Ovaries were harvested, formalin fixed, and embedded within paraffin blocks. Five micron tissue sections from each ovary were prepared for analyses. All animal protocols were approved by the Vanderbilt University Institutional Animal Care and Use Committee.

Human specimens

All ovary specimens were identified through a tissue archive maintained in the Department of Pathology and were retrieved following approval by Vanderbilt...
University's Institutional Review Board. Due to the retrospective nature of the study, informed consent was not obtained. Five specimens were obtained, which satisfied both of the following criteria: 1) gross and histologic diagnosis of 'polycystic ovaries' and 2) a clinical diagnosis of PCOS provided at the time of ovariectomy. Five unaffected ovarian specimens were also obtained. Additional information collected at the time of specimen retrieval was age at the time of ovariectomy. Five micron paraffin-embedded tissue sections were cut from each block for histological analyses.

**Histopathology**

Ovarian sections were stained with Masson's trichrome, Movat pentachrome, and hematoxylin and eosin stains. Expression of human PAI-1 in mouse ovarian tissues was assessed by immunohistochemistry specific for human PAI-1 antigen; the primary antibody does not exhibit cross-reactivity with mouse PAI-1 antigen. Primary mouse monoclonal antibody to human PAI-1 primary antigen (Molecular Innovations, Southfield, Michigan; dilution 1:25) and anti-rabbit IgG biotinylated goat secondary antibody with the immunohistochemistry (IHC) Mouse Link (Innogenex, San Ramon, CA, USA) were used for detection of stable human PAI-1 antigen. Streptavidin–horseradish peroxidase (HRP) conjugate and 3-amino-9-ethyl-carbazole (Chromogen Stable Solution; Biogenex, San Ramon, CA, USA) as substrate were used for visualization. Similarly, localization of human PAI-1 in human ovarian tissues was undertaken with rabbit anti-human PAI-1 immunoglobulin (IgG) fraction, biotin-labeled primary antibody (Molecular Innovations; dilution 1:500). These sections were counterstained with hematoxylin in order to provide contrast. All sections presented were stained within the same session in order to minimize variability secondary to staining technique. All sections were photographed under 4×20× magnification using an Olympus BX40 microscope with an Optronics Magnafire digital camera (Optronics, Goleta, CA, USA). Digital image analysis and processing of each photomicrograph was performed with ImagePro Plus (MediaCybernetics, Silver Spring, MD, USA). All images were processed equally. Sections were examined and characterized by a single, blinded investigator.

**Clinical chemistry**

Mice were anesthetized by isoflurane inhalation and blood samples were taken by retro-orbital bleed. All blood samples were centrifuged at 750 g for 15 min at 4 °C, and plasma fractions were immediately frozen and stored at −80 °C. Human PAI-1 antigen levels were determined using the Immulyse PAI-1 kit (Biopool, Ventura, CA, USA), which measures the active, complexed, and latent forms of human PAI-1. The within-assay variability is 5%; all comparisons were made within the same assay. The least detectable concentration is 0.9 ng/ml; the midrange of the assay is 25 ng/ml.

Plasma levels of testosterone were determined using the Testosterone RIA DSL-4100 kit (Diagnostic Systems Laboratories Inc., Webster, TX, USA). The within-assay variability is 6-4% and the between assay variability is 11%. The sensitivity of the assay is 0.05 ng/ml. The percentage cross-reactivity of the testosterone antiserum is 100% with testosterone, 6-6% with 5α-dihydrotestosterone, and 0-4% with 17β-estradiol; estrone, estriol, corticosterone, dehydroepiandrosterone (DHEA), and progesterone are non-detectable.

**Statistical analysis**

Discrete variables were analyzed using the Fisher’s exact test to accommodate the small sample size. Continuous variables were analyzed using the Mann–Whitney U test to accommodate non-parametric data. Data are reported as the mean ± s.d. for continuous variables and as the absolute number and percent prevalence in the population for binomial variables. All tests are two-tailed and a P<0.05 was taken to be significant. All data analyses were performed using SPSS v. 13 (SPSS Inc., Chicago, IL, USA).

**Results**

**Mouse populations**

Ovaries were obtained from 17 transgenic mice with a mean age of 9 months ± 1 and 22 wild-type mice with a mean age of 9 months ± 1. Plasma was obtained from eight wild-type mice with a mean age of 8 months ± 3, and ten transgenic mice with a mean age of 9 months ± 1±0. The difference in age between the groups was not statistically significant in either the histological (P=0.172) or the plasma (P=0.388) analyses.

**Histological findings in transgenic versus wild-type ovaries**

Histological characteristics were systematically evaluated. In contrast to wild-type mice, ovaries from transgenic mice demonstrated little evidence of ovulation: corpora lutea were rarely identified and ovaries lacked representation of the various stages of follicle development. Stromal volume was increased; and upon higher magnification, the hypertrophied theca (interstitium) was composed of large lipid-laden
vacuoles. Antral follicles were characterized by an uneven disorganized layer of granulosa cells. Many of the ovaries were encased within a thickened tunica (Fig. 2A). The area of ovaries from transgenic mice \((N=8, 4.95 \text{ mm}^2 \pm 1.87)\) was not significantly larger than that observed in ovaries from wild-type mice \((N=14, 4.09 \text{ mm}^2 \pm 2.22; P=0.365)\). The number of preantral follicles were counted and expressed as a percentage of the total number of follicles in that histological section. There did not appear to be an excess of preantral follicles \((51\% \pm 30 \text{ of } 22 \pm 18 \text{ total number of follicles})\) in transgenic mice \((N=8)\), when compared with wild-type animals \((N=14; 42\% \pm 24 \text{ of } 27 \pm 17 \text{ total number of follicles}; P=0.330)\).

Cysts were identified and counted under the 10× objective and defined as an open cavity, with or without follicular fluid, of at least 50 μm in diameter, and lined by not more than one layer of non-luteinized granulosa cells. The ovaries from transgenic mice (9 out of 17, 53%) were more likely to harbor cystic structures than their wild-type counterparts (1 out of 22, 5%; \(P=0.001)\).

Biochemical analysis of transgenic versus wild-type plasma

The Rotterdam Consensus defines PCOS, after the exclusion of related disorders, by two of the following three features: 1) oligo or anovulation, 2) clinical and/or biochemical signs of hyperandrogenism, or 3) polycystic ovaries. We therefore hypothesized, given this characteristic ovarian phenotype, that the transgenic females would have significantly higher levels of testosterone when compared with their wild-type counterparts. We found that plasma testosterone was elevated nearly twofold in transgenic females \((N=10, 0.312 \text{ ng/ml} \pm 0.154)\) versus wild-type animals \((N=8, 0.181 \text{ ng/ml} \pm 0.083; P=0.014; \text{Fig. 3})\).

As expected, plasma levels of human PAI-1 were increased in the transgenic animals, with a mean value of 32.1 ng/ml \pm 14.5, while human PAI-1 was undetectable in plasma samples from wild-type mice. Transgenic mice which exhibited cystic structures, however, did not have a significantly higher level of PAI-1 \((N=5, 35.4 \text{ ng/ml} \pm 24.7)\) than those transgenic mice which were unaffected \((N=4, 28.7 \text{ ng/ml} \pm 13.2; P=0.724)\).

Localization of human PAI-1 antigen in murine ovaries

We then investigated the localization of human PAI-1 in the ovaries of mice employing immunohistochemistry. We found that human PAI-1 is abundant throughout

![Figure 2](image1)

**Figure 2** Ovaries from mice that overexpress PAI-1 exhibit structural changes, which approximate ovaries from humans with PCOS. (A) Ovaries from transgenic mice exhibit readily apparent cysts (Cy) as well as hyperplasia of the theca (interstitium; Ti) and only rare corpora lutea (CL), in contrast to their wild-type counterparts. (B) Masson Trichrome stain, 10× magnification, scale 100 μm. These features are similar to those found in the human polycystic ovary, when compared with an unaffected human ovary. Movat pentachrome stain, 4× magnification, scale 500 μm.

![Figure 3](image2)

**Figure 3** Plasma testosterone was elevated nearly twofold in transgenic females \((N=10, 0.312 \text{ ng/ml} \pm 0.154)\) versus wild-type animals \((N=8, 0.181 \text{ ng/ml} \pm 0.083; P=0.014)\).
the ovaries of transgenic animals. At low power, PAI-1 expression was readily noted in the granulosa cells of the developing follicles, thickened tunica, and cyst lining. At higher magnification, human PAI-1 expression was visible throughout the hypertrophied theca (interstitium) as well. Conversely, human PAI-1 cannot be identified within the ovaries of wild-type animals (Fig. 4A).

**Localization of human PAI-1 antigen within human ovaries**

Based on the findings in transgenic mice, we then determined the extent and location of PAI-1 accumulation in human ovary specimens. Sections of ovarian tissue was examined from five women with polycystic ovarian morphology (age of women 30.2 years ± 4.1) and compared with five unaffected human ovary specimens (age of women 33.8 years ± 4.3). Specifically, the five ovary specimens with polycystic morphology exhibited characteristics consistent with such a histopathological diagnosis, specifically, stromal hypertecosis, a thickened tunica, cystic structures, atretic follicles, and a distinct lack of corpora lutea (Fig. 2B; Hughesdon 1982, Takahashi et al. 1994). Interestingly, PAI-1 accumulation was evident within the polycystic ovary specimens and localized to the granulosa cells lining cystic structures as well as atretic follicles; PAI-1 was not detected within the thecal cell layer, stroma, corpora lutea, or corpora albicantia. These findings contrast to the scant amount of PAI-1 detected within unaffected ovary specimens (Fig. 4B).

**Discussion**

Transgenic mice that constitutively express a stable variant of human PAI-1 develop ovaries with cystic structures. Furthermore, these transgenic mice exhibit androgen levels that are nearly twofold higher than their unaffected counterpart. These novel findings suggest that the well-recognized clinical association of elevated plasma PAI-1 in patients with PCOS may provide a pathophysiological insight into the development of this ovarian morphology. The detection of abundant human PAI-1 antigen within atretic follicles and cystic structures of human polycystic ovary specimens lends strength to this hypothesis.

The plasminogen activator system has long been recognized as integral to normal ovarian physiology (Peng et al. 1993). The hormone-controlled expression of PAs (t-PA and u-PA) and their primary inhibitor, PAI-1, is critical to follicular development and atresia, the breakdown of the follicular wall during ovulation, and the maintenance and regression of the corpus luteum. A spatial expression of the plasminogen system within the ovary exists and is under gonadotropin control, primarily luteinizing hormone (LH). The majority of PAI-1 is produced within the theca (interstitium), whereas the expression of the plasminogen activators is specific to the granulosa cells (Peng et al. 1993, Hagglund et al. 1996, Liu et al. 1996, 1997, 2004, Liu 2004).

Animal models of gonadotropin-induced ovulation have defined the temporal expression of the plasminogen system in the processes of ovulation and the
maintenance and regression of the corpus luteum. These processes have been defined in animal models of gonadotropin-induced ovulation. PAI-1 is highest immediately prior to ovulation particularly in the center of the ovary, where the follicles which are not going to be selected for ovulation reside. Levels of t-PA peak within the granulosa cells of the preovulatory follicle just prior to ovulation, whereas PAI-1 levels reach a nadir. Preovulatory follicles nearest to the surface of the ovary express less of the inhibitor, thus leading to their selective rupture. The coordinated expression of the plasminogen system, under the influence of gonadotropin secretion, therefore allows for a window of t-PA excess, which results in the activation of plasminogen to plasmin within the follicular fluid, and ultimately results in controlled proteolytic breakdown of the follicular wall (Peng et al. 1993, Liu et al. 1996, Liu 2004). The formation and maintenance of the corpus luteum requires rapid angiogenesis and tissue remodeling under the control of the plasminogen system. If fertilization does not take place, PAI-1 levels regress leaving an excess of plasminogen activators. The structural and functional regression of luteal tissues then ensues (Liu et al. 1996, 1997).

We propose that a disordered proteolytic program is present in our transgenic mouse model. The human PAI-1 gene product is under the control of the mPPET-1, which targets transgene expression to the vasculature and epithelial surfaces (Harats et al. 1995). A constitutive elevation in intra-ovarian PAI-1 leaves little opportunity for an excess of plasminogen activator, thereby functionally inhibiting proteolytic breakdown of the follicular wall. In this model, follicular growth is excessive and unchecked by the usual postovulatory stimuli, which in turn leads to cyst formation. Finally, a thickened tunica develops secondary to impaired plasmin-mediated tissue remodeling. The development of this abnormal architecture in turn may then further impair physiologic LH-mediated ovulation.

The hyperandrogenemia which is characteristic of PCOS leads to excessive early follicular growth, which then impairs follicular development as well as the selection and ovulation of a dominant follicle; we theorize that an imbalanced plasminogen system further aggravates this situation (Jonard & Dewailly 2004, Diamanti-Kandarakis & Piperi 2005). These changes contribute to the loss of the dynamic architecture of the ovary and to the regression, or atresia, of follicles. The resulting hypertrophied theca (interstitium) prompts a deleterious feedback loop, which then perpetuates testosterone production (Kyei-Mensah et al. 1998).

A chronic elevation in LH alone has previously proven sufficient for the development of cystic architecture similar to that seen in our animal model. A transgenic mouse model, which expresses a chimeric LHβ subunit in gonadotropes, has been described. In this model, chronic overexpression of LH leads to infertility, polycystic and markedly enlarged ovaries, hydronephropathy, pyelonephritis and granulosa cells tumors (Risma et al. 1995). LHβ transgenic mice are also more obese than their wild-type counterparts (Kero et al. 2003). Our transgenic mouse model shares only the characteristic of cyst formation. Additionally, the thickened tunica observed in our mice as well as in ovaries from women with PCOS is absent in this LH transgene model, suggesting that an inappropriate elevation in LH alone is insufficient to produce morphology identical to that seen in PCOS. Levels of LH were not assessed in our animals due to inadequate amounts of plasma as well as challenges inherent to the measurement of this hormone, including its short half-life and pulsatile nature. Although it would not be surprising to observe a mild elevation in LH in our animal model, it is unlikely that an LH excess in mice transgenic for human PAI-1 is solely driving the ovarian phenotype observed.

It has previously been proposed that immunohistochemical localization of intra-ovarian PAI-1 can be sensitive to the stage of development of the ovary samples or phase of the menstrual cycle (Liu 2004). Nevertheless, all five of our human polycystic ovary specimens uniformly stained strongly for the presence of PAI-1 within the granulosa cells lining the cystic structures. None of the ovaries from women without PCOS demonstrated significant PAI-1 expression. These findings lend strength to our hypothesis that a constitutively high level of PAI-1 expression is, at least in part, responsible for the development of polycystic ovarian morphology.

Elevated plasma levels of circulating PAI-1 in women with PCOS may be explained by a variety of hormonal, metabolic, and genetic mechanisms inherent to this disorder, including an increase in visceral adiposity, hyperinsulinemia, or an aberrant polymorphism within the PAI-1 promoter. While it does not appear that PAI-1 excess alone is sufficient for the development of polycystic structures, the hypothesis that an excess in PAI-1 contributes to the development of the syndrome deserves further exploration. Furthermore, a ‘second-hit hypothesis’ may also merit consideration. In this model, a human female may possess an underlying genetic predisposition to the development of PCOS and then acquire a second mechanism, which may then serve to elevate PAI-1 to the extent that ovarian function is ultimately disturbed and endothelial, metabolic, and reproductive dysfunction ensues.

Whereas the clinical significance of PAI-1 excess in women with PCOS appears well established in the areas of insulin resistance and infertility, the significance of the increased prevalence of cardiovascular risk markers remains poorly understood. Whether an elevation in PAI-1 predisposes these women to an increased risk of cardiovascular disease remains to be established. While
there have been an abundance of studies predicting increased cardiovascular disease in this population based upon risk factors, there is as of yet few if any studies demonstrating harder endpoints. The reasons for this may be several fold: PCOS is a syndrome of young women that has only recently been recognized and thus we may not yet be appreciating cardiovascular sequelae, which will surface as the population ages, the true prevalence of PCOS is likely underestimated, and finally we likely still do not fully understand all of the factors that play a role in the etiology of coronary artery disease in women, including the impact of race and ethnicity (Pierpoint et al., 1998, Wild et al., 2000, Lo et al., 2006).

Based on our findings, we anticipate that the contribution of PAI-1 to the development of PCOS deserves further investigation. Additional studies are currently underway in our laboratory to further investigate the specific contribution of each functional domain within PAI-1 to the development of this phenotype. Specifically, we are evaluating the histological architecture of ovaries from mice deficient in PAI-1 as well as transgenic mice expressing a human PAI-1 variant that is unable to effectively inhibit all serine proteases. Ultimately, the investigation into new pharmacological agents that are capable of directly antagonizing PAI-1 will have the potential to restore proteolytic balance in tissues such as the ovary, and may embody a multi-faceted treatment for the prevention of PCOS and its complications.

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