

Impairment of the Plasmin Activation System in Primary Pulmonary Hypertension: Evidence for Gender Differences

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Keywords

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Summary

Primary pulmonary hypertension (PPH) is a rare disorder, with marked in-situ thrombosis of small pulmonary vessels occurring primarily in adult women. We investigated whether differences in the plasmin- and thrombin activation system are associated with the predominate affection of females. Plasma levels of plasminogen activator inhibitor type 1 (PAI-1), tissue-type plasminogen activator (t-PA), fibrinogen, thrombin-antithrombin (TAT) complexes, and prothrombin fragments (F1.2) were measured at baseline and after standardized venous occlusion (VO) in patients with PPH (24 female, 9 male). At baseline, females showed significant higher TAT levels ($p = 0.05$), higher t-PA antigen levels ($p = 0.01$) and higher fibrinogen levels ($p = 0.03$) with positive correlation to mean pulmonary artery pressure (mPAP), as well as nonsignificant lower t-PA activity, higher PAI-1 antigen and activity and F1.2 levels. After VO, females showed a significantly blunted increase in t-PA antigen ($p = 0.01$) and t-PA activity ($p = 0.001$), correlating with mPAP, as well as increased PAI-1 activity ($p = 0.05$). We hypothesize, that the observed presence of gender differences in the plasmin- and thrombin activation system in PPH leading to an antifibrinolytic/prothrombotic state might, in part, explain the female predominant incidence of this disease.

Introduction

PPH is a serious condition with still unknown etiology and poor prognosis (1-3). In one subgroup of PPH patients a history of appetite-suppressant drug abuse is present (4-6). The mechanism by which such drugs like aminorex increase the risk for the development of PPH is not known but some evidence suggests it may be caused by interactions with the serotonin transporters (7). Nevertheless, histopathologic studies of lung vessels in all PPH patients demonstrate marked vascular remodeling with formation of plexiform lesions and a high incidence of in-situ thrombosis (8-10). In accordance with this observed thrombotic lesions, alterations of plasma levels of the thrombin activation as well as plasmin activation system leading to a procoagulant-antifibrinolytic state have been reported (11-14). Furthermore, chronic anticoagulation

has been used in pulmonary hypertension based on observations from non-randomized studies suggesting increased survival in anticoagulated patients (15-17). These findings together point towards an important role of the coagulation system in the pathophysiologic mechanisms for maintaining or even initiating PPH (18). In addition, hypertension in general might be associated with a hypercoagulable state (19).

Epidemiological evidence shows that PPH occurs primarily in adult women (1, 2), but so far no data are available to explain this circumstance. Therefore, we investigated whether differences in plasma levels of the major components of the plasmin activation system (i.e. t-PA, the physiologic plasminogen activator within the vasculature, and PAI-1, the main inhibitor of the t-PA mediated proteolytic cascade), as well as markers of thrombin activation (TAT and F 1.2 complexes) are associated with the predominance of female PPH patients and the severity of the disease as compared to their male counterparts.

Patients and Methods

Patients. 33 consecutive patients with PPH (24 female and 9 male) and 33 control subjects (23 female, 10 male) were studied. All PPH patients underwent right heart catheterization prior to study enrollment. Other causes of pulmonary hypertension were excluded by medical history, pulmonary function testing, transthoracic echocardiography (TTE) and ventilation perfusion lung scans. All patients were on oral anticoagulation. Control subjects were normal volunteers and patients without organic heart disease, who underwent heart catheterization for exclusion of coronary heart disease due to chest pain. Mean PAP was estimated as 15 mmHg for controls. After obtaining informed consent venous occlusion tests were performed. The protocol was approved by the local ethic committee according to the Declaration of Helsinki.

Blood sampling and venous occlusion. To minimize diurnal alterations of fibrinolytic parameters, blood samples were collected into EDTA tubes (Vacutainer, Becton Dickinson; Rutherford NJ) between 8.00 a.m. and 10.00 a.m. after 12 h fasting (20, 21) and immediately centrifuged (4°C ; $3000 \times g$ for 15 min) to separate plasma. Blood samples for determination of TAT and F1.2 were collected into citrate tubes (Vacutainer) and processed the same way. Samples were stored at -70°C until further use. For determination of baseline values, blood was drawn from an antecubital vein without or with only minimal VO. For determination of post VO values, a standardized occlusion of 15 min duration was performed on the contralateral forearm (22, 23) followed by a second blood collection. Hematocrit (normal range male 42-52%, female 37-47%) and fibrinogen (according to Clauss, normal range 150-350 mg/dl) were determined using routine laboratory methods.

Determination of fibrinolytic parameters. t-PA antigen and t-PA activity were determined by a specific ELISA using monoclonal antibodies recognizing both free t-PA and t-PA/PAI-1 complexes (24) (Technoclone, Vienna, Austria). PAI-1 antigen was determined by specific ELISA using monoclonal antibodies (Technoclone). PAI-1 functional activity was determined using a modified functional titration assay (25) (Technoclone). Plasma concentrations of fibrinolytic parameters after VO were corrected for hemoconcentration by use of the

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correction factor (Ht_1 – hematocrit before, Ht_2 – hematocrit after VO): $f = ((Ht_1 (1-0.9 \times Ht_2)) / ((Ht_2 (1-0.9 \times Ht_1))) (26, 27)$.

Determination of coagulation activation markers. TAT and F1.2 plasma levels were determined by specific ELISA using polyclonal antibodies (Behring, Marburg, Germany).

Statistical analysis. Data are presented as mean ± SEM. After determination of the distribution pattern, statistical differences between groups were determined either by the Mann-Whitney U-test for fibrinolytic and coagulation activation markers, or by the unpaired t-test for demographic and hemodynamic parameters. Statistical differences before and after VO were determined by the Wilcoxon Signed Rank Test. Linear regression analysis was used to assess the relationship of PAP and pO_2 to the plasma values. Power calculations were performed for all comparisons.

Results

Demographic and hemodynamic data of study patients are presented in Table 1.

Within the PPH group 72.7% of the patients were female, without statistical differences in hemodynamic characteristics. PPH patients with a history of appetite-suppressant drug abuse were significantly older than those without (female 68 ± 7 vs. 46 ± 3 years, $p = 0.0001$; one male 71 vs. 55 ± 7 years). Apart from PAP and pO_2 no statistical significant differences between patients and control subjects could be observed.

PAI-1 Antigen and Activity Levels before and after VO

PAI-1 antigen levels showed no statistical significant differences between any group, neither at baseline nor after VO (after mathematical correction for hemoconcentration with the recommended correction factor 26, 27) (Table 2). Only female PPH patients showed moderately elevated baseline PAI-1 antigen levels compared to male patients or to female controls without reaching statistical significance. PAI-1 antigen showed no correlation to mPAP at baseline (female: $R = 0.3$, $p = 0.3$;

Table 2 PAI-1 and t-PA antigen at baseline, after VO and x-fold increase

	PPH			Control		
	Female	male	p	Female	male	p
Baseline						
PAI-1 (ng/ml)	103±30	73±40	n. s.	62±13	58±13	n. s.
t-PA (ng/ml)	8.1±0.9*	5.7±1.1	0.09	4.4±0.7	4.7±0.6	n. s.
After VO						
PAI-1 (ng/ml)	90±19	82±26	n. s.	67±14	85±23	n. s.
t-PA (ng/ml)	21.1±2.3	22.5±3.8	n. s.	22.1±3	21.5±2.1	n. s.
x-fold change						
PAI-1 (ng/ml)	1.3±0.4	1.6±0.3	n. s.	1.2±0.2	1.5±0.2	n. s.
t-PA (ng/ml)	3.1±0.4*	4.6±0.7	n. s.	6.2±1.2	5.5±0.6	n. s.

mean ± SEM; *p = 0.01 PPH versus control

male: $R = 0.4$, $p = 0.1$) or after VO (female: $R = 0.2$, $p = 0.6$; male: $R = 0.1$, $p = 0.6$).

Data on plasma levels of PAI-1 activity at baseline and after VO are presented in Fig. 1. At baseline, female PPH patients showed a trend for higher PAI-1 activity levels (7.5 ± 1.0 U/ml) compared to male patients (5.7 ± 1.5 U/ml), without reaching statistical significance ($p = 0.1$). After VO all groups showed a significant reduction in PAI-1 activity (Fig. 1, inset). Female PPH patients showed a significantly blunted decrease (2.9 ± 0.6 -fold PAI-1 activity decrease) compared to female controls (4.8 ± 1.2 -fold decrease, $p = 0.05$) or male PPH patients (3.6 ± 0.5 -fold decrease, $p = 0.07$). This results in significant higher PAI-1 activity after VO (4.0 ± 0.8 U/ml) in female PPH patients compared to male patients (1.1 ± 0.4 U/ml, $p = 0.04$) or female controls (1.0 ± 0.5 U/ml, $p = 0.01$). PAI-1 activity showed no correlation to mPAP, neither at baseline (female: $R = 0.05$, $p = 0.8$; male: $R = 0.1$, $P = 0.5$) nor after VO (female: $R = 0.2$, $p = 0.3$; male: $R = 0.3$, $p = 0.1$).

t-PA Antigen and Activity before and after VO

Data on plasma levels of t-PA antigen at baseline and after VO are presented in Table 2. At baseline, female PPH patients showed significantly higher t-PA antigen levels compared to controls. After VO, t-PA antigen significantly increased in all study groups. Due to a significantly blunted increase in female vs. male PPH patients, no significant differences were detectable between all groups after VO. Linear regression analysis of basal t-PA antigen levels to mPAP revealed a significant correlation only in female patients (Fig. 2).

Data on plasma levels of t-PA activity at baseline and after VO are presented in Fig. 3. At baseline, female PPH patients showed nonsignificant lower t-PA activity levels compared to male patients (0.11 ± 0.05 vs. 0.45 ± 0.2 U/ml, $p = 0.19$). After VO, t-PA activity was significantly lower in PPH patients compared to controls (female: PPH 1.9 ± 0.7 , controls 8.8 ± 1.6 U/ml, $p = 0.0007$; male: PPH 5.5 ± 2.2 , control 14.9 ± 3.2 U/ml, $p = 0.05$). Within the PPH group female patients showed significantly lower t-PA activity than males after VO ($p = 0.02$). T-PA activity after VO was inversely correlated to mPAP primarily in females (female: $R = 0.55$, $p = 0.001$; male: $R = 0.4$, $p = 0.09$), whereas t-PA activity at baseline was not correlated to mPAP (female: $R = 0.2$, $p = 0.2$; male: $R = 0.3$, $p = 0.1$).

TAT, F1.2 and Fibrinogen Levels at Baseline

Data on TAT, F1.2 and fibrinogen plasma levels are presented in Table 3. Female PPH patients showed significantly increased TAT

Table 1 Demographic and hemodynamic data

	PPH			Control		
	female	male	p	female	male	p
Number of patients	24	9		23	10	
Aminorex history	9	1		0	0	
Age (years)						
aminorex negativ	46±3	55±7	n. s.	45±3	47±3	n. s.
aminorex positiv	68±3	71		0	0	
Smokers	0	3		5	2	
Oral contraception	3	-		5	-	
Post-menopausal Status	11	-		10	-	
Body Mass Index (kg/m ²)	24.4±0.7	24.5±1.0	n. s.	23.6±0.9	25.1±0.5	n. s.
Systolic Aortic Pressure (mmHg)	123±4	126±6	n. s.	125±5	129±4	n. s.
Diastolic Aortic Pressure (mmHg)	77±2	80±3	n. s.	75±2	80±3	n. s.
Mean Aortic Pressure (mmHg)	92±2	95±3	n. s.	94±3	97±2	n. s.
Cardiac index (l/min/m ²)	2.1±0.7	2.2±0.4	n. s.	n. d.	n. d.	
Pulmonary Artery Pressure systolic (mmHg)	75±5	85±5	n. s.	n. d.	n. d.	
Pulmonary Artery Pressure diastolic (mmHg)	31±3	36±5	n. s.	n. d.	n. d.	
Pulmonary Artery Pressure Mean (mmHg)	48±4	53±5	n. s.	15	15	
Pulmonary Capillary Wedge Pressure (mmHg)	8±1	9±1	n. s.	n. d.	n. d.	
PO ₂ arterial (mmHg)	73±3	71±4	n. s.	92±5*	93±4*	n. s.

mean ± SEM; *p = 0.001 controls versus PPH

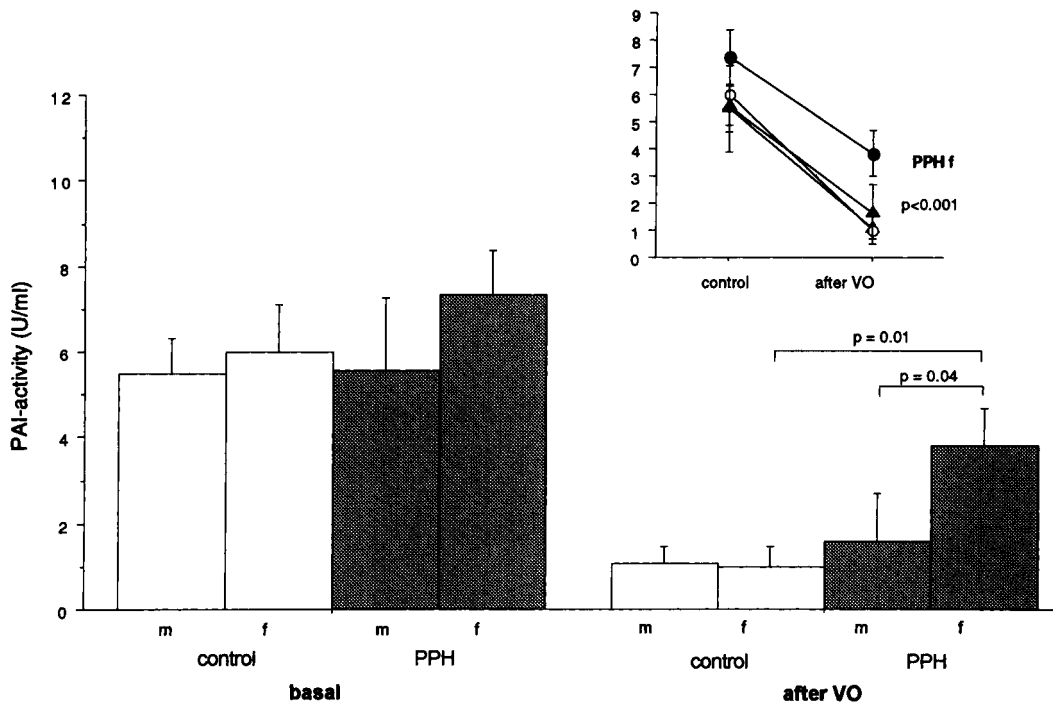


Fig. 1 Plasma levels of type 1 plasminogen activator inhibitor (PAI-1) activity at baseline (basal) and after venous occlusion (VO). At baseline no statistical significant differences are detectable. During VO PAI-1 activity decreases significantly in all groups [small inset: PPH: female: solid circles (●); male: solid triangles (▲); Controls: female: open circles (○); male: open triangles (△)], but remains significantly higher in female PPH patients after VO compared to male PPH patients or female controls

levels, as well as a trend for higher F1.2 levels compared to controls, without correlation to mPAP (TAT: female: $R = 0.2$, $p = 0.4$; male: $R = 0.3$, $P = 0.2$; F1.2: female: $R = 0.3$, $p = 0.2$; male: $R = 0.2$, $P = 0.5$).

Although mean fibrinogen levels were only slightly elevated (356 ± 13 mg/100 ml) in PPH, female patients showed significant higher levels than male patients or controls (Table 3) with positive correlation to mPAP (Fig. 4).

Hematocrit and pO_2 at Baseline

Although mean hematocrit was within the normal range in all groups at baseline, PPH patients showed significant higher values than controls (male: PPH $48 \pm 1.4\%$, controls $43 \pm 0.5\%$, $p = 0.0003$; female: PPH $45 \pm 1.2\%$, controls $39 \pm 0.8\%$, $p = 0.003$). Furthermore, the normal, sex related difference in hematocrit was absent in PPH patients (PPH: male vs. female $p = 0.19$; controls: male vs. female $p = 0.002$). Linear regression analysis revealed a highly significant positive correlation of hematocrit to mPAP in both groups (male: $R = 0.74$, $p < 0.0001$; female: $R = 0.54$, $p = 0.0005$). Data on pO_2 are presented in Table 1. No significant differences occurred within the PPH group or the control group, but pO_2 was significantly different between the two groups ($p = 0.001$). Linear regression analysis revealed a highly significant inverse correlation of pO_2 to hematocrit ($R = 0.73$, $p < 0.0001$) and mPAP ($R = 0.8$; $p = 0.0001$) in both groups.

Discussion

We could show that the previously observed prothrombotic state in PPH patients is mainly due to a female specific alteration of plasma levels of the major components of the plasmin activation system after VO (PAI-1 and t-PA), as well as a basal activation of coagulation as

shown by increased markers of the thrombin activation system (TAT, F1.2). This is schematically outlined in Fig. 5. At baseline, female PPH patients exhibit significant higher fibrinogen levels, but nonsignificant elevated PAI-1 antigen and activity levels, as well as lower t-PA activity levels. However, due to the small sample size, reflecting the rare incidence of PPH, there is insufficient statistical power to determine whether these moderate differences are significant. The "paradox"

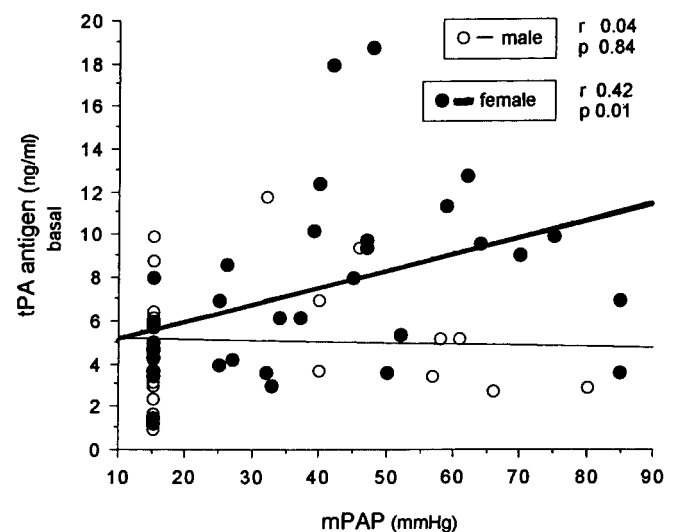


Fig. 2 Correlation of basal tissue type plasminogen activator (t-PA) to mean pulmonary artery pressure (mPAP). Baseline t-PA antigen levels show a statistical significant correlation to mPAP in females [solid circles (●), bold line (—)], in contrast to male individuals [open circles (○), thin line (—)]

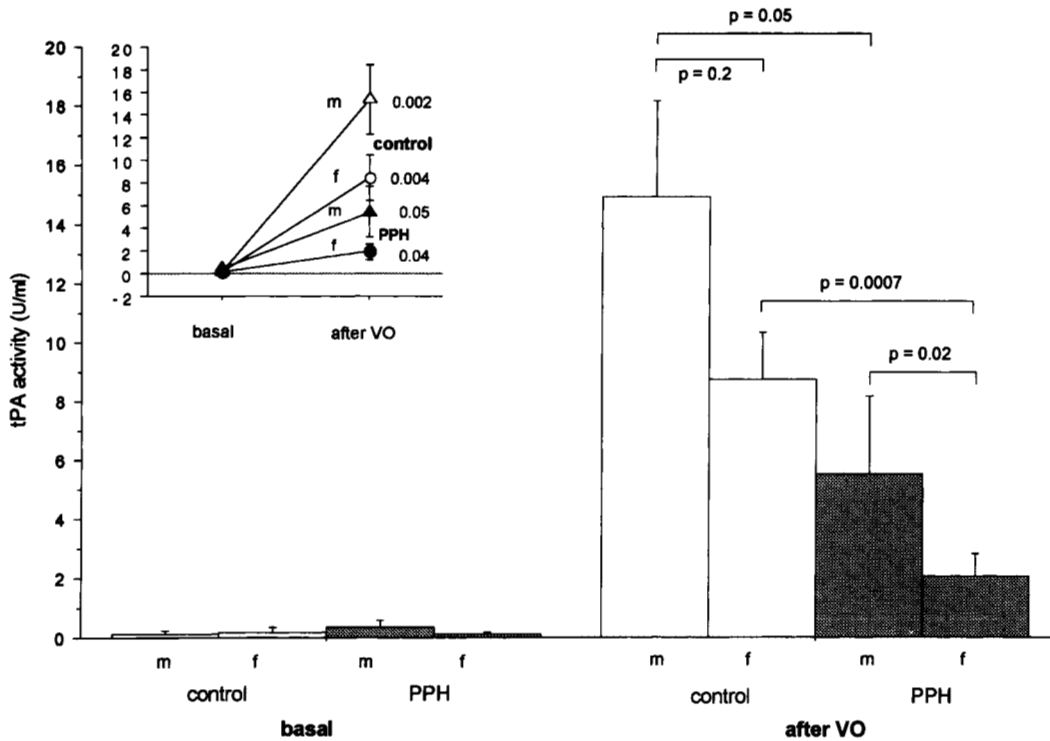


Fig. 3 Plasma levels of tissue type plasminogen activator (t-PA) activity at baseline (basal) and after venous occlusion (VO). At baseline no statistical significant differences are detectable. During VO t-PA activity increases significantly, but to a various degree, in all groups [small inset: PPH: female: solid circles (●); male: solid triangles (▲); Controls: female: open circles (○); male: open triangles (△)]. After VO t-PA activity is significantly lower in both PPH patient groups compared to controls and significantly lower in female PPH patients compared to male patients

higher t-PA antigen levels in our female PPH patients at baseline are presumably due to increased PAI-1/t-PA complex formation, as t-PA activity is not increased. After VO, female PPH patients show significant lower t-PA activity as well as higher PAI-1 activity levels, compared to male patients. Baseline fibrinogen levels and the blunted t-PA activity after VO correlates to the increase in mPAP only in females.

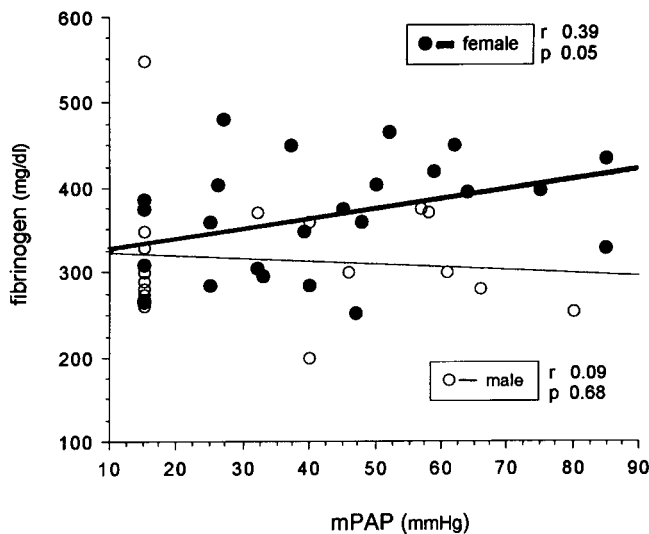


Fig. 4 Correlation of basal fibrinogen to mean pulmonary artery pressure (mPAP). Fibrinogen plasma levels show a positive correlation to mPAP in females [solid circles (●), bold line (—)], in contrast to male individuals [open circles (○), thin line (—)]

This leads, together with increased markers of coagulation activation (TAT, F1.2), to a prothrombotic-antifibrinolytic state primarily in female PPH patients with correlation to the severity of pulmonary hypertension.

The pathophysiologic mechanisms which lead to this observed gender differences in PPH remain speculative and seem to differ substantially from the mechanisms responsible for gender differences in the healthy population:

The physiologic fibrinolytic response in healthy men and women after VO results in higher fibrinolytic capacity and lower PAI-1 levels

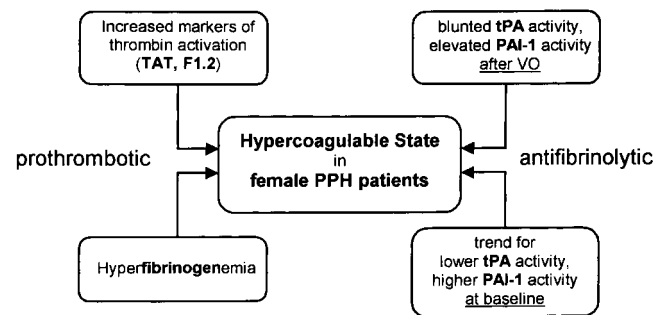


Fig. 5 Synopsis of the prothrombotic-antifibrinolytic state in female PPH patients. The hypercoagulability found primarily in female PPH patients is caused by a prothrombotic state – reflected by higher fibrinogen levels together with increased markers of thrombin activation (TAT, F1.2) at baseline, and an antifibrinolytic state – reflected by a blunted t-PA activity and higher PAI-1 activity after VO, as well as a trend for higher PAI-1 antigen and activity levels and lower t-PA activity levels at baseline

Table 3 TAT, F1.2 and fibrinogen plasma levels

	PPH			Control		
	Female	male	p	female	male	p
TAT ($\mu\text{g/L}$)	8.6 \pm 2.3*	4.1 \pm 1.2	0.05	2.0 \pm 0.5	2.7 \pm 0.7	n. s.
F 1.2 ($\mu\text{g/L}$)	1.8 \pm 0.3**	1.1 \pm 0.2	n. s.	0.7 \pm 0.2	1.0 \pm 0.2	n. s.
Fibrinogen (mg/dl)	375 \pm 15 ***	312 \pm 20	0.03	321 \pm 18	318 \pm 27	n. s.

mean \pm SEM; *p = 0.03; **p = 0.08, ***p = 0.05 PPH versus control

in females (28), which might be mediated by hormonal influences, especially by changes in estrogen levels, while TAT and F1.2 level remain unaffected (29, 30). Furthermore, recent evidence suggests that gender related differences in the plasmin activation system of healthy subjects may be explained by differences in obesity and accordingly in body mass index, which seem to correlate closer to plasma levels of PAI-1 and t-PA than genetic variances or age (31-33). The fact that women with PPH showed a different regulation of the plasmin activation system compared to healthy controls and the lack of differences in body mass index in our study population, largely excludes hormonal influences or obesity as potential explanation for the observed gender specific differences in the plasmin activation system in PPH patients.

PPH is associated with increased hematocrit values and impaired blood rheologic properties (34). We could demonstrate, that the slight increase in hematocrit is related to the severity of pulmonary hypertension and the extent of hypoxemia without any gender related differences. Therefore, chronic hypoxia, although being a potent regulator of the fibrinolytic system in vitro (35), itself is also no explanation for the observed female specific regulation of the plasmin and thrombin activation systems.

Based on former findings that anticoagulation might positively influence the course of patients with PPH (15-17), all our patients were on optimal chronic anticoagulation with coumadin. Chronic anticoagulation has shown controversial effects on circulating parameters of the thrombin and plasmin activation system: a modification of the hypercoagulable state in heart failure patients by a decrease of TAT and F1.2 plasma levels (36), no influence on F1.2, fibrinogen, PAI-1 and t-PA levels in post coronary bypass patients (37), as well as no influence on PAI-1 levels (38) or t-PA plasma levels but reduction of fibrinogen levels (39) in patients with atrial fibrillation. If at all, in or patients chronic anticoagulation would have influenced circulating levels of fibrinogen and parameters of the thrombin and plasmin activation system similarly and would therefore not distress our findings and their interpretation.

In contrast to our data Welsh et al. (12) described in PPH patients significantly elevated baseline PAI-1 levels with a positive correlation to mPAP, but no differences in TAT and F1.2 levels. Furthermore, in the hands of these authors fibrinogen levels were not elevated in PPH patients but in patients with secondary pulmonary hypertension. Possible explanations for these discrepancies are the different time point of blood collection (11 a.m. to 4 p.m. versus 8-10 a.m. in our hands), different age of study populations, the small number of patients investigated and different test systems used. Such differences in study design may also explain the elevated basal PAI-1 activity levels found by Eisenberg et al. (11), who also demonstrated elevated fibrinopeptide A levels, indicative for increased thrombin activation, in his series of patients with PPH.

Main limitation of our study is the relative small number of male PPH patients on which our gender hypothesis is tested. Unfortunately, due to the nature of the disease and its rare incidence, this small num-

ber simply reflects the existing gender ratio, which limits the inclusion of a higher amount of male PPH patients at our institution, as well as the statistical power to detect all potentially existing gender related differences.

To summarize, these demonstrated changes of thrombin and plasmin activation mechanisms may result in in-situ thrombosis and probably reflect a generalized diseased endothelium, which appears to be ineffective in maintaining patency within the pulmonary vessels mainly in female PPH patients. One could further speculate, that chronic anticoagulation, which has been shown to increase survival in non-randomized studies without evaluating gender related differences (15-17), might be beneficial primarily in female PPH patients. Whether these abnormalities represent the primary disturbance responsible for the occurrence of PPH mainly in female patients or the secondary response to a vascular injury of different origin remains unexplained. Nevertheless, these mechanisms, either responsible for initiation or perpetuation of pulmonary hypertension, seem to reflect a gender related phenomenon.

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