Increased Levels of Prostaglandin D₂ Suggest Macrophage Activation in Patients With Primary Pulmonary Hypertension*

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**Study objective:** TXA₂ (thromboxane A₂) is a lipid mediator believed to be produced primarily by platelets in normal subjects, although macrophages are capable of synthesis. There is increased production of TXA₂ in patients with primary pulmonary hypertension (PPH), which may reflect augmented production by macrophages. The objective of this study was to determine if macrophages are activated in PPH and whether they contribute to the increased production of TXA₂.

**Study type:** Case control.

**Setting:** University hospital.

**Methods:** We measured the urinary metabolites of three mediators that predominantly derive from different cell types in vivo: (1) TX-M (platelets and macrophages), a TXA₂ metabolite; (2) prostaglandin D₂ (PGD₂) metabolite (PGD-M); and (3) N-methylhistamine (mast cells), a histamine metabolite, in 12 patients with PPH and 11 normal subjects.

**Results:** The mean (± SEM) excretion of both TX-M and PGD-M at baseline was increased in PPH patients, compared to normal subjects (460 ± 50 pg/mg creatinine vs 236 ± 16 pg/mg creatinine [p = 0.0006], and 1,390 ± 221 pg/mg creatinine vs 637 ± 65 pg/mg creatinine [p = 0.005], respectively). N-methylhistamine excretion was not increased compared to normal subjects. There was a poor correlation between excretion of TX-M and PGD-M (r = 0.36) and between excretion of PGD-M and methylhistamine (r = 0.09) in individual patients.

**Conclusion:** In patients with PPH, increased levels of PGD-M, without increased synthesis of N-methylhistamine, suggest that macrophages are activated. The lack of correlation between urinary metabolite levels of TXA₂ and PGD₂ implies that macrophages do not contribute substantially to elevated TXA₂ production in patients with PPH. They may, however, have a role in the pathogenesis and/or maintenance of PPH, which warrants further investigation.

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**Key words:** macrophages; platelets; primary pulmonary hypertension; prostaglandin D₂; thromboxane

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Although the etiology of primary pulmonary hypertension (PPH) remains unknown, persistent vasoconstriction, in situ thrombosis, and proliferation of both intimal and medial cell wall compartments contribute to the loss of pulmonary vascular surface area. The arachidonic acid metabolite, TXA₂ (thromboxane)
A), a vasoconstrictor, a mitogen for smooth-muscle cell growth, and an agonist for platelet aggregation, may contribute to many of these changes.\(^3,4\) Previously, we have shown that the major urinary metabolite of \(\text{TXA}_2\), 11-dehydro-TxB\(_2\) (TX-M), is elevated in patients with PPH compared to normal subjects, suggesting that thromboxane plays an important role in the pathogenesis of PPH.\(^5\)

Studies\(^6-10\) in normal subjects showing a substantial decrease in TXA\(_2\) metabolite excretion using low-dose aspirin to inhibit platelet cyclooxygenase activity indicate that the majority of TXA\(_2\) synthesis occurs in platelets. However, it is well established\(^8-10\) that stimulated macrophages are capable of enhanced production of TXA\(_2\). Several studies lend support to the presence of activated macrophages in patients with PPH. Both Galiè et al.\(^11\) and Humbert et al.\(^12\) have demonstrated elevated levels of macrophage cytokine products when compared to normal subjects. Another study\(^13\) reported increased amounts of the proinflammatory transcription factor, nuclear factor-\(\kappa\)B, in alveolar macrophages obtained from PPH patients. The contribution of macrophages to enhanced excretion of urinary TX-M in patients with PPH is unknown.

We hypothesized that macrophages are activated in patients with PPH, contributing to the increased production of TXA\(_2\). To investigate this, we measured the urinary excretion of TXA\(_2\) and prostaglandin D\(_2\) (PGD\(_2\)) metabolite (PGD-M) ([\(9\alpha,11\beta\)-dihydroxy-15-oxo-2,3,18,19-tetranorep-5-ene-1,20-dioic acid] [the major urinary metabolite of PGD\(_2\)], and prostanoids derived primarily from platelets/macrophages and macrophages/mast cells, respectively).\(^9,10,14,15\) We also measured urinary excretion of N-methylhistamine, the major metabolite of histamine, a product of activated mast cells but not of macrophages, to determine more specifically the source of PGD-M.

**Materials and Methods**

**Clinical**

Patients with PPH were sequentially enrolled without selection bias. Patients were in New York Heart Association (NYHA) class III or class IV and were being considered for long-term epoprostenol therapy. PPH was defined using the criteria from the National Institutes of Health registry of PPH.\(^16\) Healthy, non-smoking adult volunteers were used as control subjects. The study was approved by the institutional review board, and informed consent was obtained from all subjects for the collection of urine and blood specimens. To avoid the potentially confounding effects of physical activity and diet inherent in 24-h urine collections, first-voided morning urine samples were collected from 12 PPH patients and 11 normal subjects. Treatment with nonsteroidal anti-inflammatory medications, including aspirin and cyclooxygenase-2 inhibitors, was discontinued at least 1 week prior to sample collection. Samples were kept on ice until centrifuged and aliquoted into 1.5-mL vials. The samples were then frozen at \(-80^\circ\text{C}\) until analysis was performed. All measurements were normalized to urinary creatinine to control for dilutional differences in urinary flow among patients.

**Hemodynamic Data**

Diagnostic right-heart catheterization, as part of routine patient evaluation, was performed in nine patients at the time urine samples were obtained. Three patients did not undergo right-heart catheterization at the time of sample collection but had documented severe pulmonary hypertension without evidence of secondary causes. Right atrial pressure, pulmonary artery pressure (PAP), and pulmonary wedge pressure were measured directly, and cardiac output was determined either by thermodilution method or Fick calculation (with measured oxygen consumption) and adjusted for body surface area to determine cardiac index (CI). Pulmonary vascular resistance index (PVRI) was calculated using the standard formula: (mean PAP – pulmonary wedge pressure)/CI.

**Eicosanoid Analysis**

TX-M or PGD-M were quantified using stable isotope dilution techniques in conjunction with gas chromatography-mass spectrometry (GC/MS), as previously described.\(^15\) Samples were spiked with 1 to 2 ng of the respective internal standards, subjected to solid-phase extraction on preconditioned octadecyl-silyl cartridges, and after derivatization of appropriate functional groups (depending on the analyte) with pentfluorobenzyl bromide, bis(trimethylsilyl)trifluoroacetamide, and methoxamine hydrochloride to enhance volatility for gas chromatography analysis, purified by thin-layer chromatography. Samples underwent gas chromatography using a 15-m DB-1701 column followed by negative-ion chemical ionization mass spectrometry. Comparison of peak areas between the exogenously introduced internal standard and that generated by the endogenous compound allowed for rigorous quantification.

**Urinary Methylhistamine Analysis**

Methylhistamine concentrations, normalized to urinary creatinine values, were determined by GC/MS using methods of Morrow and coworkers\(^17\) as previously described.

**Statistical Analysis**

All values are presented as mean \(\pm\) SD. Fisher’s Exact \(t\) Test was used for comparison between PPH patients and normal subjects. Significant differences are reported at a value of \(p < 0.05\).

**Results**

All PPH patients were significantly limited by impaired right-heart function: nine patients were in NYHA class III, and three patients were in NYHA class IV. Three patients were receiving long-term therapy with calcium-channel blockers, five patients were receiving digoxin, and five patients were receiving supplemental oxygen either part-time or full-time. Five patients were receiving long-term antico-
agulation with warfarin, and one patient was treated with low-dose aspirin (which was discontinued > 1 week prior to obtaining samples). No patient was receiving epoprostenol at the time baseline measurements were obtained.

Seven PPH patients were female, five PPH patients were male, and the average (± SD) age was 24 ± 17 years (range, 2 to 60 years). The mean age of normal subjects was 37 ± 9 years, and the female/male ratio was 6/5. The mean PAP was 68 ± 6 mm Hg, CI was 2.1 ± 0.3 L/min/m², and PVRI was 30.6 ± 2.1 U × m².

Urinary PGD-M levels were significantly increased in patients with PPH. Mean (± SEM) excretion of PGD-M was 1,390 ± 221 pg/mg creatinine in PPH patients compared to 637 ± 65 pg/mg creatinine in normal subjects (p = 0.005; Fig 1). The crude odds ratio for PPH associated with a median urinary PGD-M value of > 761 pg/mg creatinine was 8.75. Urinary TX-M levels were elevated in patients with PPH as well. The average urinary excretion of TX-M was 460 ± 48 pg/mg creatinine in PPH patients, compared to 236 ± 16 pg/mg creatinine in normal subjects (p = 0.0006; Fig 2). This difference confirms our earlier observation using radioimmunoassay techniques.

Figure 3 demonstrates the relationship between excretion of TX-M and PGD-M for individual patients. There is a poor correlation between excretion of TX-M and PGD-M (r = 0.36), suggesting that the elevated levels of these metabolites reflect synthesis by more than one cell type. There was no correlation between excretion of TX-M or PGD-M and PAP, CI, or PVRI. However, there was a significant negative correlation between TX-M (but not PGD-M) and mixed venous oxygen saturation (r = −0.73; p < 0.05).

Average methylhistamine value for PPH patients was 135 ± 14 ng/mg creatinine, not significantly different from the value obtained in normal subjects (163 ± 19 ng/mg creatinine), implying that mast cells are not activated in patients with PPH. The relationship between excretion of PGD-M and methylhistamine is shown in Figure 4, demonstrating that there is no significant correlation in PPH patients (r = 0.1; p = 0.7).

**DISCUSSION**

In this study, we observed increased PGD-M excretion in patients with PPH compared to normal control subjects. Immunocytochemical studies have demonstrated that a variety of monocytic cells account for the majority of PGD₂ synthesis in rats. In humans, a number of investigators have shown that the predominant source of PGD₂ is macrophages and mast cells. Mast cells are also capable of releasing significant amounts of histamine. However, although mast cells have been shown to selectively release histamine in response to activation by neuropeptides, they have not been found to release PGD₂ without concomitant release of histamine. Therefore, the finding of normal levels of urinary methylhistamine argues against mast cell activation in patients with PPH. Furthermore, the lack of correlation between excretion of metabolites of PGD₂ and histamine suggests that macrophages are the source of, or at the very least contribute substantially to, elevated PGD-M.

Platelets normally produce little PGD₂, although they are capable of synthesizing it when TXA₂ synthesis is inhibited. TXA₂ synthesis is increased,
not inhibited, in PPH, making it unlikely that platelets are the source of PGD2. The lack of correlation between excretion of PGD-M and TX-M, primarily a platelet-derived eicosanoid, in individual patients lends further support to PGD2 production by another cell type.

Other cells, including eosinophils, endothelial cells, and a variety of antigen-presenting cells in addition to macrophages, have the capacity for PGD2 synthesis in vitro.18,24–26 For example, small amounts of PGD2 are produced from activated eosinophils in asthmatic patients after stimulation with platelet-activating factor.24 There is no evidence of eosinophilia or of eosinophil activation in PPH making them an unlikely source for PGD2 in our patients. In another study, cultured human umbilical vein endothelial cells demonstrated increased extracellular production of PGD2 after stimulation with interleukin (IL)-1, which was believed to be the result of increased intracellular production of PGH2, the precursor of PGD2.25 Stimulated umbilical vein endothelial cells behave quite differently from pulmonary arterial endothelial cells, and coupled with the fact that the pulmonary endothelium is clearly abnormal in patients with PPH, makes an endothelial source of PGD2 unlikely as well. While we cannot completely exclude the contribution of alternative cell types to the production of PGD2 in patients with PPH, there is no evidence to support activation of cells other than platelets or macrophages.

A growing body of literature bolsters the theory that macrophages are in an activated state in patients with PPH, and suggests the possibility that alveolar macrophages, since they reside in close proximity to the distal pulmonary arteries and arterioles, may contribute substantially to increased PGD2 synthesis. Fartoukh and colleagues27 reported a fourfold increase in the steady-state levels of macrophage inflammatory protein-1α in patients with severe PPH. Galiè et al11 have noted a fourfold increase in plasma levels of tumor necrosis factor-α, and Humbert and coworkers12 have observed elevated levels of IL-1 and IL-6, all of which are cytokine products of macrophages. More recently, direct evaluation of alveolar macrophages from patients with PPH by Raychaudhuri et al13 has demonstrated increased production of nuclear factor-κB, a proinflammatory cytokine product of activated macrophages. In the absence of mast cell activation, measurement of PGD-M reflects in vivo macrophage activity. Our finding of elevated PGD-M adds to the existing evidence that macrophages are activated in PPH.

Using GC/MS, a rigorously quantitative method for determination of mediator concentrations in biological fluids, we also confirmed the results of our earlier study5 demonstrating increased TX-M excretion in patients with PPH. The major source of urinary TX-M in normal subjects is believed to be platelets.6,7 This conclusion is based on significant suppression of urinary TX-M after administration of low doses of aspirin, an irreversible inhibitor of platelet cyclooxygenase. Urinary TX-M is significantly increased in patients with PPH, and it is possible that extrapolation of such findings in normal subjects may not be valid. In addition, we have found that in patients undergoing bone marrow transplantation, who have >95% reduction in circulating platelet counts, excretion of TX-M is not reduced indicating substantial capability for thromboxane synthesis in
other cell types (B.W. Christman, MD; unpublished data; May 1999). Although our findings suggest that macrophages do not appear to be the source of augmented TXA2 production in patients with PPH, additional studies, looking at specific macrophage populations such as tissue or alveolar macrophages or peripheral blood monocytes, are needed to determine if there is differential induction of specific cyclooxygenase products. Alternatively, other cell types, such as fibroblasts or even transformed endothelial or smooth-muscle cells, may contribute to increased TXA2 synthesis.

A shortcoming of our studies is the lack of direct measurement of macrophage products in patients with PPH. In vivo studies must by necessity be inferential. Direct measurement of specific cell products ex vivo, while providing more definitive data regarding mediator production by specific cell types, may or may not reflect in vivo conditions. The combination of both types of studies will provide more definitive data on the role of macrophages in PPH, and these studies are underway.

In summary, we have found that PGD2 is produced in increased amounts in patients with PPH, suggesting macrophage activation. We have also confirmed, using physicochemical techniques, that total body synthesis of TXA2 is increased in patients with PPH. The lack of correlation between the production of these two eicosanoids suggests that macrophages do not contribute substantially to TXA2 production in patients with PPH. However, macrophages may contribute to the pathogenesis of PPH via other mechanisms. In conjunction with previous findings, our study supports a more central role for macrophages in the pathogenesis and/or maintenance of the pulmonary arteriopathy in patients with PPH, and underscores the need for additional studies examining the role of macrophages in this disease.

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