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A study of aspirin and clopidogrel in idiopathic pulmonary arterial hypertension

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ABSTRACT: Idiopathic pulmonary arterial hypertension (IPAH) is characterised by *in situ* thrombosis and increased thromboxane (Tx) A_2 synthesis; however, there are no studies of antiplatelet therapy in IPAH. The aim of the current study was to determine the biochemical effects of aspirin (ASA) and clopidogrel on platelet function and eicosanoid metabolism in patients with IPAH.

A randomised, double-blind, placebo-controlled crossover study of ASA 81 mg once daily and clopidogrel 75 mg once daily was performed. Plasma P-selectin levels and aggregometry were measured after exposure to adenosine diphosphate, arachidonic acid and collagen. Serum levels of TxB_2 and urinary metabolites of TxA_2 and prostaglandin I_2 (Tx-M and PGI-M, respectively) were assessed.

A total of 19 IPAH patients were enrolled, of whom nine were being treated with continuous intravenous epoprostenol. ASA and clopidogrel significantly reduced platelet aggregation to arachidonic acid and adenosine diphosphate, respectively. ASA significantly decreased serum TxB_2, urinary Tx-M levels and the Tx-M/PGI-M ratio, whereas clopidogrel had no effect on eicosanoid levels. Neither drug significantly lowered plasma P-selectin levels. Epoprostenol use did not affect the results.

In conclusion, aspirin and clopidogrel inhibited platelet aggregation, and aspirin reduced thromboxane metabolite production without affecting prostaglandin I_2 metabolite synthesis. Further clinical trials of aspirin in patients with idiopathic pulmonary arterial hypertension should be performed.

KEYWORDS: Aspirin, clinical trial, hypertension, platelets, pulmonary

Idiopathic pulmonary arterial hypertension (IPAH) is characterised by sustained elevation in pulmonary artery pressure and pulmonary vascular resistance, which ultimately leads to right heart failure and death. The pathological findings include medial hypertrophy, intimal fibrosis and *in situ* thrombosis in the small muscular pulmonary arteries [1]. Platelet aggregation, a prerequisite for thrombus formation, is increased in patients with IPAH, and the degree of platelet activity is associated with functional class and survival [2, 3]. Soluble P-selectin levels are increased in IPAH, reflecting platelet activation [4].

IPAH is also characterised by abnormal arachidonic acid (AA) metabolism, resulting in alterations in the synthesis of thromboxane (Tx)A_2 and prostaglandin I_2 (PGI_2) [5]. TxA_2 is a potent vasoconstrictor and smooth muscle mitogen and promotes platelet aggregation [6]. Previously, it has been demonstrated that the major urinary metabolite of TxA_2 (11-dehydro-TxB_2 (Tx-M)) is elevated in patients with IPAH compared with healthy volunteers [5]. Although platelets are the predominant source of TxA_2 in healthy volunteers [7, 8], the source in IPAH is unknown. Due to the known adverse effects of TxA_2 on the pulmonary vasculature, investigators have previously attempted to treat IPAH with Tx synthase inhibitors, however, side effects and lack of efficacy limited these studies [9, 10]. The failure of Tx synthase inhibitors in IPAH parallels their ineffectiveness in other cardiovascular diseases characterised by increased TxA_2 and platelet aggregation, which are, on the contrary, effectively treated with antiplatelet agents.

It has also been shown that the major urinary metabolite (2,3-dinor-6-keto-prostaglandin $F_{1\alpha}$ (PGI-M)) of PGI_2, a vasodilator that inhibits smooth muscle proliferation and platelet aggregation, is decreased in patients with IPAH [5]. Administration of synthetic PGI_2 analogues has

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proven clinically effective, confirming that eicosanoid imbalance contributes to the risks of morbidity and mortality in IPAH [11–13]. An oral agent with a favourable safety profile that reduces TxA_2 production and platelet aggregation without decreasing PGI_2 levels could, therefore, provide significant benefit to patients with IPAH.

Aspirin (ASA) inhibits platelet aggregation and inactivates cyclooxygenase (COX), which catalyses the first step of TxA_2 and PGI_2 formation. This irreversible inhibition of COX normally eliminates further platelet Tx production (as platelets are unable to generate new COX). Therefore, low-dose ASA reduces Tx-M to <20% of baseline values and serum TxB_2 to <5% of baseline values with minimal effects on systemic PGI_2 generation [7, 8]. Clopidogrel inhibits platelet aggregation by preventing platelet binding of adenosine diphosphate (ADP). While clopidogrel could decrease TxA_2 levels by inhibition of ADP-induced platelet activation and secondary AA release and metabolism, it does not directly affect COX-1 or eicosanoid production. Although platelet inhibition is efficacious in systemic vascular disease [14–16], specific antiplatelet therapy with either ASA or clopidogrel has not been evaluated in IPAH.

The aim of this study was to determine the biochemical effects of treatment with ASA and clopidogrel in patients with IPAH. The current authors hypothesised that both ASA and clopidogrel would inhibit *ex vivo* platelet aggregation and decrease plasma P-selectin levels. It was also hypothesised that platelets are the primary source of TxA_2 in patients with IPAH and that low-dose ASA would, therefore, reduce TxA_2 production without affecting PGI_2 synthesis.

METHODS AND MATERIALS

Study subjects

Patients were recruited from the Pulmonary Hypertension Centers at Vanderbilt University Medical Center (Nashville, TN, USA) and New York Presbyterian Hospital (New York, NY, USA). Inclusion criteria were: 1) diagnosis of IPAH; 2) ⩾18 yrs of age; 3) New York Heart Association (NYHA) functional class I, II or III; and 4) clinical stability (*i.e.* without change in pulmonary arterial hypertension medical regimen within 1 month prior to enrolment). Patients who had any of the following were excluded: 1) other forms of pulmonary arterial hypertension; 2) a contraindication to ASA or clopidogrel; 3) thrombocytopenia (defined as platelet count $\leqslant 75{,}000 \cdot \mu L^{-1}$); 4) history of intracranial haemorrhage or chronic thromboembolic disease; 5) renal failure; or 6) inability or unwillingness to avoid nonsteroidal anti-inflammatory, ASA, or warfarin use for the duration of the trial. The study protocol was approved by the local Institutional Review Board of each institution (Vanderbilt University Medical Center, Nashville, TN; Columbia University, New York, NY, USA).

Study design

This study was a double-blind, placebo-controlled, three-period crossover study comparing ASA 81 mg once daily with placebo and clopidogrel 75 mg once daily with placebo. After informed consent, patients were instructed to stop warfarin 2 weeks before the baseline evaluation and to refrain from nonsteroidal medications and cigarette use for the duration of the trial. The research pharmacy assembled numbered packages of three bottles (labelled with period 1, 2 or 3) containing placebo, ASA, or clopidogrel in capsules, which were identical in colour and taste. Sequence allocation was balanced with respect to treatment with continuous intravenous epoprostenol and centre. All investigators, subjects and laboratory personnel were blinded to the treatment sequence.

Patients were evaluated at baseline and after a 2-week treatment period with each drug; a 2-week washout period followed each treatment period. Patients were assessed in the early morning (~ 08:00 h) and were fasting overnight. Patients maintained a diary of study medication use and recorded any new medications taken during the study period. At each visit, a pill count was performed and the diary reviewed to assess compliance. Haemodynamic data were collected from the most recent right heart catheterisation before enrolment in the study.

The primary outcomes were as follows: 1) platelet aggregometry, 2) serum TxB_2 level, 2) urinary Tx-M concentration, 3) urinary PGI-M concentration, 4) Tx-M/PGI-M ratio, and 5) plasma P-selectin level. Secondary outcomes included symptoms and side effects.

Methods

Ex vivo platelet aggregation was assessed at baseline and after each drug period (2, 6 and 10 weeks) in response to collagen ($4\ \mu g \cdot mL^{-1}$), ADP (3.3, 5 and $10\ \mu g \cdot mL^{-1}$), and AA (4, 6, 8 and $10\ \mu g \cdot mL^{-1}$). Aggregation was assessed in platelet-rich plasma with an aggregometer. For each agonist, the percentage of maximal light transmission achieved within 6 min of the addition of the agonist was recorded. Maximal platelet aggregation after addition of an agonist allows most light to be transmitted (*e.g.* 80%), whereas inhibition of platelet aggregation blocks light from being transmitted (*e.g.* 20%).

Blood from study participants was collected in tubes containing sodium EDTA and centrifuged at $1{,}000 \times g$ for 10 min, within 30 min of collection, to separate plasma. The Human P-Selectin immunoassay kit (R&D Systems Inc., Minneapolis, MN, USA) was used with protocols provided by the manufacturer. Serum TxB_2 and urinary levels of Tx-M and PGI-M were analysed using stable isotope dilution methodology with gas chromatography/mass spectrometry, as previously described [9].

Analysis

The data are expressed as mean±SD, median (interquartile range) or frequency (95% confidence interval), except where otherwise noted. t-Tests, rank-sum tests and Fisher's exact tests were used, as appropriate. Linear mixed-effects modelling was used to assess differences between ASA, clopidogrel and placebo. The models included fixed effects for drug, period, sequence, centre, epoprostenol use and the drug–epoprostenol interaction. Subject number was included as a random effect. Pairwise comparisons between ASA, clopdiogrel and placebo were performed only if there was a significant difference overall between treatment groups. Raw baseline data and least squares means and standard errors from the fully adjusted models are reported. Log transformation was used for the Tx-M/PGI-M ratio, which was highly skewed; back-transformed results are reported from the multivariate model.

With $\alpha=0.05$ and $\beta=0.20$, eight subjects were required to detect a 1 SD difference in end-points. To assess drug effects stratified by continuous intravenous epoprostenol use, the sample size was increased to 16. The number of subjects was further increased to 20 to allow for a 20% drop-out rate. The trial was closed after enrolling 19 patients due to prolonged recruitment.

Primary analyses were performed by intention-to-treat including all patients; secondary analyses included completers only. A p-value <0.05 was considered significant.

RESULTS

Nineteen patients were enrolled from April 2002 until December 2003 (table 1). Nine (47%) patients were treated with intravenous epoprostenol at a median (range) dose of 20 (16–35) ng·kg^{-1}·min^{-1}, and nine (47%) were treated with oral bosentan. The median time from the most recent right heart catheterisation to study entry was 248 (125–369) days. Patients receiving epoprostenol had higher mean right atrial and pulmonary artery pressures than those who were receiving oral therapy. Baseline Tx-M levels were significantly elevated compared with previously reported controls in the present authors' laboratory [17]. Baseline P-selectin levels were similar to control values published by the manufacturer of the assay.

TABLE 1 Baseline characteristics

Characteristic	All	Epo	No epo	p-value#
Subjects n	19	9	10	
Age yrs	43±15	45±14	40±16	0.52
Female	14 (74)	6 (67)	8 (80)	0.63
Ethnicity/race				0.63
Hispanic/Caucasian	2 (11)		2 (20)	
Non-Hispanic/ African-American	4 (21)	2 (22)	2 (20)	
Non-Hispanic/Caucasian	13 (68)	7 (78)	6 (60)	
NYHA functional class				0.57
I	3 (16)	2 (22)	1 (10)	
II	9 (47)	3 (33)	6 (60)	
III	7 (37)	4 (45)	3 (30)	
Haemodynamics				
RAPm mmHg	5±4	8±5	3±2	0.009
PAPm mmHg	46±18	54±19	39±15	0.07
CI L·min^{-1}·m^{-2}	2.9±1.4	2.4±1.3	3.3±1.4	0.15
PVR Wood units	9.8±7.9	12.6±9.4	7.2±5.6	0.14
PCWPm mmHg	9±4	10±5	8±3	0.22
Biochemical assays				
Serum TxB_2 ng·mL^{-1}	64±49	47±44	80±50	0.15
Urine Tx-M ng·mg Cr^{-1}	0.40±0.19	0.45±0.22	0.35±0.15	0.27
Urine PGI-M† ng·mg Cr^{-1}	2.5±3.4	5.1±3.4	0.2±0.3	0.0002
Urine Tx-M/PGI-M ratio¶	2.4±2.9	0.15±0.11	4.5±4.6	0.0003
Plasma P-selectin ng·mL^{-1}+	36±7	34±7	37±7	0.47

Data are presented as mean±SD or n (%). Epo: chronic IV epoprostenol; NYHA: New York Heart Association; RAPm: mean right atrial pressure; PAPm: mean pulmonary artery pressure; CI: cardiac index; PVR: pulmonary vascular resistance; PCWPm: mean pulmonary capillary wedge pressure; TxB_2: thromboxane B_2; Tx-M: 11-dehydro-thromboxane B_2; Cr: creatinine; PGI-M: 2,3-dinor-6-keto-prostaglandin $F_{1\alpha}$. #: Epo *versus* no epo. ¶: n=18, +: n=17.

All patients received the study treatments in the assigned order. Compliance with the avoidance of nonsteroidal anti-inflammatory drug guidelines was 100%. Four patients dropped out of the trial, three due to worsening symptoms of pulmonary arterial hypertension and one due to a transient ischaemic attack. Patients who dropped out were not significantly different from those who completed the study in terms of demographic, haemodynamic, platelet or eicosanoid variables at baseline (data not shown).

There was significant suppression of *ex vivo* platelet aggregation with ASA and with clopidogrel. The results from ADP 3.3 and 10 μg·mL^{-1}, AA 6 and 10 μg·mL^{-1}, and collagen are shown in figure 1; other agonist doses produced similar results. Clopidogrel reduced ADP-induced platelet aggregation compared with ASA and placebo. Both ASA and clopidogrel reduced AA-induced platelet aggregation; however, ASA had a significantly greater effect than clopidogrel ($p<0.001$). Only ASA reduced collagen-induced platelet aggregation. Drug effects were similar in patients with or without intravenous epoprostenol therapy (data not shown).

ASA reduced serum TxB_2, urine Tx-M and the urine Tx-M/PGI-M ratio compared with clopidogrel and placebo (table 2). Overall, ASA reduced TxB_2 by almost 80% ($p<0.001$), although there was significant variation among patients. ASA also lowered urine Tx-M by 78% compared with baseline values ($p<0.001$; fig. 2a), but had no effect on urine PGI-M. Clopidogrel had no effect on eicosanoid metabolites. ASA and clopidogrel modestly decreased plasma P-selectin levels, although this did not reach statistical significance, and there was a possible sequence effect ($p< 0.01$). There were no significant differences in ASA or clopidogrel effects between patients receiving epoprostenol and those who were not (fig. 2).

There were several minor protocol deviations in the trial. Three patients missed a total of seven doses of study drug; no patient missed more than two doses in any single period. One patient had a colonic polypectomy during the washout between periods 2 and 3. This patient's washout period was extended by 1 week. One patient did not save her first morning urine sample, and a second, fasting, morning sample was collected for analysis. Five patient assessments for ADP, six patient assessments (for three patients) for AA, and two patient assessments for collagen were missing or technically inadequate. There was one missing PGI-M value.

There were no significant differences between the effects of the study drugs on NYHA functional class or adverse events. Two patients complained of bruising, one while receiving placebo and one while receiving clopidogrel. One patient complained of haemorrhoidal bleeding and one reported nausea while taking clopidogrel. There were no significant period or carry-over effects, other than for P-selectin, as noted. When only patients who completed the entire study were analysed (15 out of the 19 who enrolled), results were consistent with the intention-to-treat analysis (data not shown).

DISCUSSION

This is the first randomised, double-blind, placebo-controlled clinical trial to evaluate the biochemical effects of antiplatelet

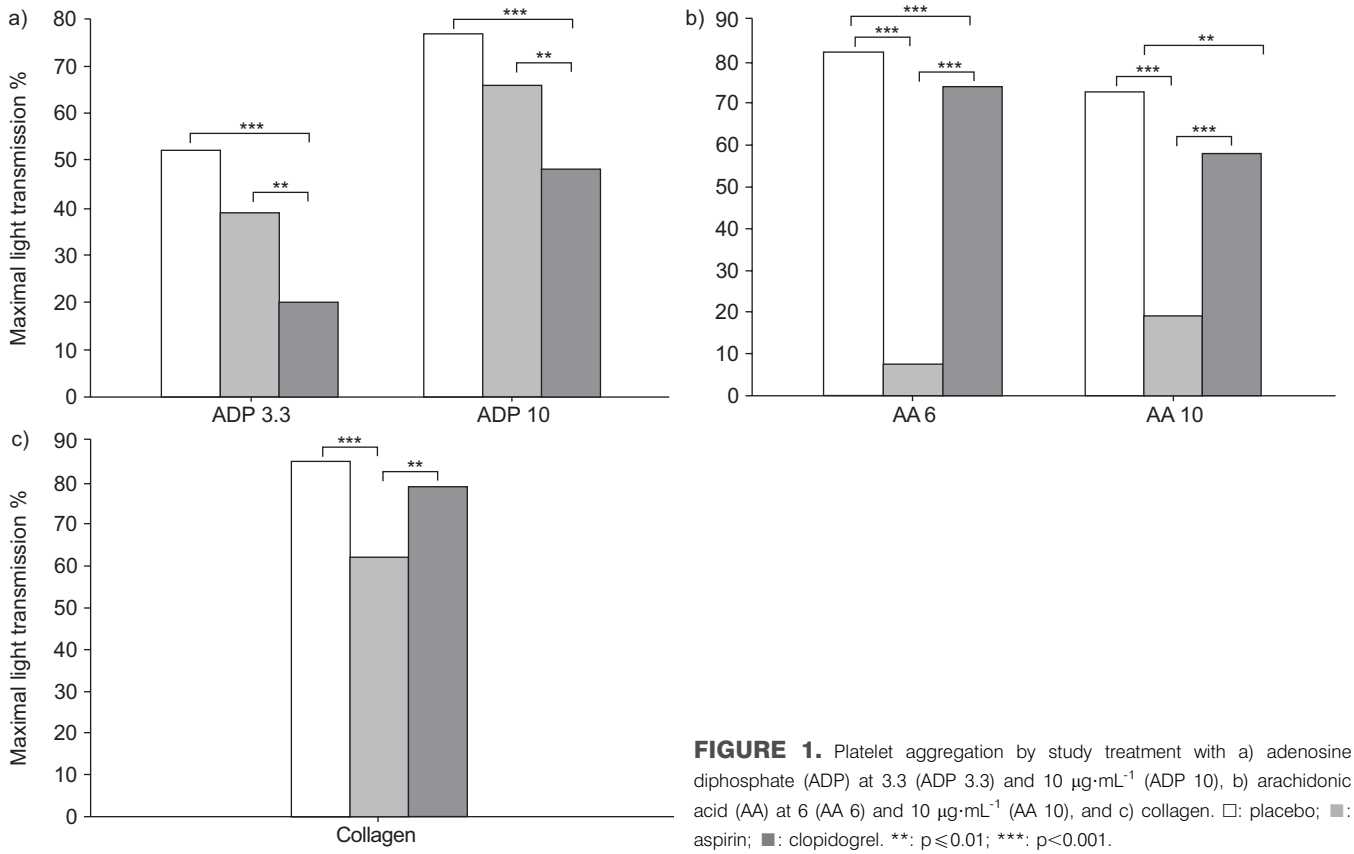

FIGURE 1. Platelet aggregation by study treatment with a) adenosine diphosphate (ADP) at 3.3 (ADP 3.3) and 10 μg·mL^{-1} (ADP 10), b) arachidonic acid (AA) at 6 (AA 6) and 10 μg·mL^{-1} (AA 10), and c) collagen. □: placebo; ■: aspirin; ■: clopidogrel. **: p≤0.01; ***: p<0.001.

therapy in patients with IPAH. Both low-dose ASA and clopidogrel reduced *ex vivo* platelet aggregation. ASA also decreased serum TxB_2 levels and urine Tx-M, but not urine PGI-M, therefore significantly improving the Tx-M/PGI-M ratio. Neither ASA nor clopidogrel significantly decreased P-selectin levels, which were normal at baseline.

ASA almost completely inhibited AA-induced platelet aggregation whereas clopidogrel had less of an effect. Conversely, clopidogrel had a much greater effect on ADP-induced aggregation than did ASA. Collagen-induced platelet aggregation, a major stimulus for *in vivo* aggregation, was inhibited with ASA only. While the extent to which *ex vivo* studies reflect *in vivo* conditions remains uncertain, these data confirm a normal inhibitory response of platelets to these agents in patients with IPAH.

ASA was consistent in reducing serum and urine Tx metabolites in all patients in the study. ASA reduced the urinary excretion of Tx-M by almost 80% of the value on placebo, similar to the reduction seen in both healthy volunteers and in patients with coronary artery disease [7, 8]. Platelets are the major source of TxA_2 in these groups, whereas the contributions of vascular, megakaryocytic and other sources to TxA_2 production in IPAH are currently unknown. While similar decreases were seen in serum TxB_2 levels, previous studies of healthy volunteers reported 94–100% suppression of serum TxB_2 with ASA [8, 18–20]. As serum

TABLE 2 Eicosanoid metabolites and plasma P-selectin after drug administration

Variable	Placebo	ASA	Clopidogrel	p-value		
				ASA *versus* placebo	Clopidogrel *versus* placebo	ASA *versus* clopidogrel
Serum TxB_2 ng·mL^{-1}	63±9	13±9	59±9	<0.0001	0.75	0.0003
Urine Tx-M ng·mg Cr^{-1}	0.33±1.1	0.09±1.1	0.32±1.1	<0.0001	0.44	<0.0001
Urine PGI-M ng·mg Cr^{-1}	2.6±0.6	2.4±0.5	3.2±0.6	0.68	0.45	0.24
Urine Tx-M/PGI-M	0.46±1.2	0.16±1.2	0.42±1.2	<0.0001	0.68	<0.0001
Plasma P-selectin ng·mL^{-1}	35±2	31±2	31±2	0.05	0.09	0.85

Data are presented as least squares mean±SE after adjustment for period, sequence, centre, epoprostenol use and the drug by epoprostenol interaction. ASA: aspirin; TxB_2: thromboxane B_2; Tx-M: 11-dehydro-thromboxane B_2; Cr: creatinine; PGI-M: 2,3-dinor-6-keto-prostaglandin $F_{1\alpha}$.

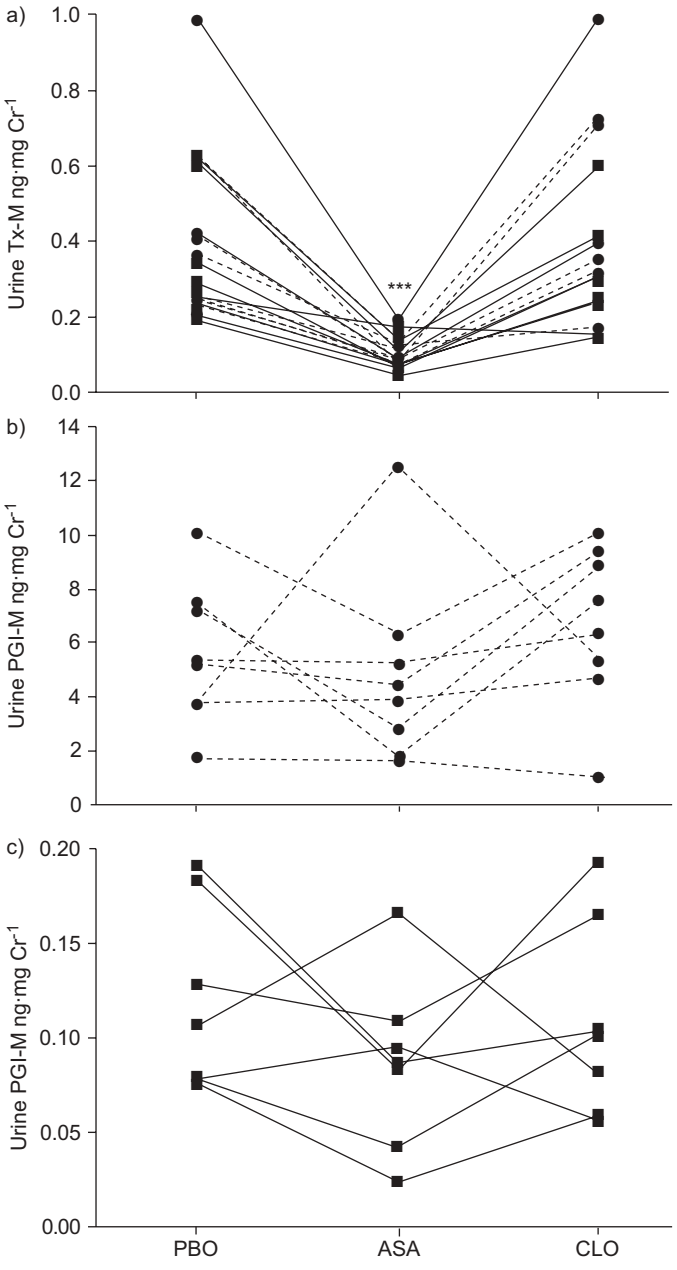

FIGURE 2. a) Urine 11-dehydro-thromboxane B_2 metabolite (Tx-M), and b) and c) urine 2,3-dinor-6-keto-prostaglandin $F_{1\alpha}$ (PGI-M) after drug administration stratified by treatment with epoprostenol (●) or without epoprostenol (■) for subjects with complete data. Cr: creatinine. ***: $p \leqslant 0.001$ for aspirin (ASA) *versus* placebo (PBO) and for ASA *versus* clopidogrel (CLO). All other comparisons were nonsignificant.

TxB_2 reflects maximal platelet production, platelet ASA resistance seems the most likely explanation. Alternatively, other cellular sources could account for these findings.

Biochemical ASA resistance is a phenomenon of persistent platelet activation despite ASA administration, measured by *in vivo* (TxB_2) or *ex vivo* (platelet aggregometry) testing. Smaller (but still clinically and statistically significant) reductions were found in TxB_2 than were observed with ASA in other patient populations and in normal subjects; however, there was a preserved effect on platelet aggregometry. Insufficient ASA dosing does not explain this finding, as similar doses of ASA (60–80 mg) result in almost complete TxB_2 suppression in other reports [8, 19, 21], and 81 mg is within the recommended dosing range for the majority of cardiovascular indications.

The present authors' previous studies of abnormal eicosanoid imbalance in IPAH suggest an alternative explanation [17]. Nucleated cellular sources of TxA_2, such as endothelial cells, macrophages and monocytes, can regenerate COX after ASA administration and possibly account for the residual TxB_2 production. This would explain the incomplete TxB_2 suppression in the face of robust ASA-induced platelet antagonism, as demonstrated *ex vivo*. Other potential causes of ASA resistance include increased formation of isoprostanes (nonenzymatic products of lipid peroxidation reflective of oxidant stress) and elevated norepinephrine levels [22–24]. Augmented production of both has been reported in IPAH [25, 26]. Lastly, the increased variability inherent in TxB_2 measurement may account for less suppression compared with Tx-M. Regardless, ASA suppressed the majority of Tx production. As TxA_2 is a potent pulmonary vasoconstrictor and smooth muscle mitogen, long-term suppression of this eicosanoid with ASA could prevent progression of disease in IPAH as it does in other cardiovascular diseases.

Clopidogrel had little effect on Tx-M despite substantial inhibition of ADP-dependent *ex vivo* aggregation. A recent study in healthy volunteers evaluating the effect of therapeutic doses of clopidogrel on *ex vivo* TxA_2 production showed a significant decrease in TxA_2; the relevance of this to *in vivo* effects is unclear [27]. The failure of clopidogrel to decrease TxA_2 metabolites in this study suggests that inhibition of ADP-induced aggregation is insufficient to alter platelet AA metabolism. Furthermore, the lack of impact of clopidogrel on collagen-mediated aggregation is consistent with differential effects of antiplatelet therapies on platelet function.

Treatment with continuous intravenous epoprostenol was not randomly assigned and it is, therefore, difficult to draw conclusions about the effect of this therapy on platelet function or other biomarkers in IPAH from these data. Epoprostenol therapy was determined by clinical indication and the availability of other therapies. Not surprisingly, patients receiving epoprostenol tended to be sicker, potentially biasing comparisons between patients receiving epoprostenol and patients who were not. However, certain conclusions may be drawn. Tx-M levels were elevated despite treatment with epoprostenol, consistent with a recent study in which the present authors found that chronic epoprostenol therapy had no effect on urinary Tx-M in IPAH [26]. The results also indicate that the effects of ASA and clopidogrel are similar, regardless of treatment with or without epoprostenol. Importantly, low-dose ASA had a substantial effect on platelet aggregometry and Tx-M levels compared with both placebo and clopidogrel, irrespective of therapy for IPAH.

Plasma P-selectin levels were not elevated in this study population, in contrast with previous studies of patients with PAH [4, 28]. However, the less severe haemodynamic derangements, previous long-term treatment for IPAH and

clinical stability of the selected study population preclude comparisons with other cohorts. An apparent sequence effect (one sequence of drugs had a higher mean P-selectin level than the others) also weakens the conclusions that may be drawn from this analysis.

There are a number of limitations of this study. First, the crossover study design requires the assumption of disease stability over the time of the trial. Therefore, unstable and NYHA class IV patients were excluded from the study. Even so, several patients had medication dose adjustments and four dropped out of the study, testifying to the fluctuating nature of IPAH, even in apparently stable patients. However, systematic changes in biomarkers over the time of the study and drug-by-period interactions were not found, making significant bias less likely. Analysis of ''completers only'' resulted in the same findings as the intent-to-treat approach. Secondly, the number of patients enrolled was small, however, the power calculations were conservative and the estimates robust. Thirdly, other markers of platelet activation, *e.g.* β-thromboglobulin or platelet factor 4, were not evaluated, which may have provided additional information regarding the role of platelets in IPAH. Fourthly, this study did not address the effect of epoprostenol on platelet function, which would require a different study design. Finally, concurrent healthy controls were not studied, so comparisons of the effects of ASA and clopidogrel in IPAH must be made with the results of prior research, which has established the effects of these drugs in other populations.

Conclusions

Aspirin and clopidogrel inhibited platelet aggregation in patients with idiopathic pulmonary arterial hypertension. However, only aspirin significantly inhibited thromboxane A_2 synthesis, although not to the full extent seen in previous studies. These results suggest that most circulating thromboxane A_2 in patients with idiopathic pulmonary arterial hypertension is produced by platelets, although additional cell types, *e.g.* macrophages or monocytes, may contribute. The present study has shown that aspirin produces significant antiplatelet effects and restores the normal eicosanoid balance in patients with idiopathic pulmonary arterial hypertension. Future studies should focus on the potential clinical benefit and safety of long-term aspirin therapy in pulmonary arterial hypertension.

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