ORIGINAL RESEARCH

Risk Factors for Nonplatelet Thromboxane Generation After Coronary Artery Bypass Graft Surgery

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***Background*—**Persistent thromboxane (TX) generation while receiving aspirin therapy is associated with an increased risk of cardiovascular events. The Reduction in Graft Occlusion Rates (RIGOR) study found that aspirin-insensitive TXA_2 generation, indicated by elevated urine 11-dehydro-TXB_2 ($UTXB_2$) 6 months after coronary artery bypass graft surgery, was a potent risk factor for vein graft thrombosis and originated predominantly from nonplatelet sources. Our goal was to identify risks factors for nonplatelet TXA_2 generation.

***Methods and Results*—**Multivariable modeling was performed by using clinical and laboratory variables obtained from 260 RIGOR subjects with verified aspirin-mediated inhibition of platelet TXA_2 generation. The strongest variable associated with $UTXB_2$ 6 months after surgery, accounting for 47.2% of the modeled effect, was urine 8-iso-prostaglandin (PG)$F_{2\alpha}$, an arachidonic acid metabolite generated nonenzymatically by oxidative stress (standardized coefficient 0.442, $P<0.001$). Age, sex, race, lipid therapy, creatinine, left ventricular ejection fraction, and aspirin dose were also significantly associated with $UTXB_2$ ($P<0.03$), although they accounted for only 4.8% to 10.2% of the modeled effect. Urine 8-iso-$PGF_{2\alpha}$ correlated with risk of vein graft occlusion (odds ratio 1.67, $P=0.001$) but was not independent of $UTXB_2$. In vitro studies revealed that endothelial cells generate TXA_2 in response to oxidative stress and direct exposure to 8-iso-$PGF_{2\alpha}$.

***Conclusions*—**Oxidative stress–induced formation of 8-iso-$PGF_{2\alpha}$ is strongly associated with nonplatelet thromboxane formation and early vein graft thrombosis after coronary artery bypass graft surgery. The endothelium is potentially an important source of oxidative stress–induced thromboxane generation. These findings suggest therapies that reduce oxidative stress could be useful in reducing cardiovascular risks associated with aspirin-insensitive thromboxane generation. **(*J Am Heart Assoc*. 2016;5:e002615 doi: 10.1161/JAHA.115.002615)**

Key Words: aspirin • isoprostane • oxidative stress • thrombosis • thromboxane

The cardioprotective property of aspirin derives principally from its antiplatelet effect resulting from the irreversible inhibition of the cyclooxygenase (COX)-1 enzyme and consequent suppression of platelet thromboxane A_2 (TXA_2) generation. In addition to mediating the activation of the platelet in which it is formed, TXA_2 is secreted and directly activates adjacent quiescent platelets and stimulates vasoconstriction via binding to surface thromboxane receptors.[1] Several clinical studies have shown that patients with persistent TXA_2 generation while taking aspirin therapy are at increased risk for atherothrombotic events, including death.[2,3]

Aspirin therapy is standard of care after coronary artery bypass graft surgery (CABG) in large part because of its ability to decrease the rate of vein graft thrombosis by half during the first postoperative year.[4] The Reductions in Graft Occlusion Rates (RIGOR) study investigated the hypothesis that failure of aspirin to adequately inhibit platelet activation would increase the incidence of early vein graft thrombosis after first-time CABG.[5] With the use of arachidonic acid platelet aggregometry, a specific indicator of platelet COX-1 activity, aspirin therapy was found to suppress platelet TXA_2 generation and inhibit aggregation in >95% and >99% of subjects at 3 days and 6 months after surgery, respectively. There was also no observable association between the rare failure to suppress platelet TXA_2 generation and vein graft occlusion assessed 6 months after surgery. Despite effective suppression of platelet-derived TXA_2 generation by the use of aspirin, measurement of urine levels of the stable TXA_2 metabolite 11-dehydroTXB_2 ($UTXB_2$) revealed persistent total-body TXA_2 generation in 73% and 31% of subjects at 3 days and

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6 months after surgery, respectively. Further, $UTXB_2$ ≥450 pg/mg creatinine measured 6 months after CABG was associated with a 2.6-fold increased risk of vein graft thrombosis compared with levels of <450 pg/mg creatinine.

These data indicate that a substantial percentage of patients taking aspirin continue to generate TXA_2 6 months after CABG that originates from predominantly nonplatelet pathways and is associated with an increased risk of early vein graft thrombosis. The source and stimuli for nonplatelet TXA_2 generation in patients with cardiovascular disease are largely unknown. The goal of this study was to use multivariable modeling to identify factors associated with nonplatelet TXA_2 generation.

Materials and Methods

Subjects

The Reduction in Graft Occlusion Rates (RIGOR) study was a multicenter observational study of 368 subjects undergoing first-time CABG between 2003 and 2006 that was designed to investigate the association between thrombotic risk factors and early saphenous vein graft occlusion. Patients were enrolled between October 2003 and October 2006 at 4 participating institutions: Johns Hopkins Hospital, Baltimore, MD; Christiana Hospital, Christiana, DE; Peninsula Regional Medical Center, Salisbury, MD; and Walter Reed Army Hospital, Washington, DC. Institutional human subject research review board approval was obtained at all participating sites, and all subjects provided written consent. A detailed description of the study design, patient characteristics, and principal findings has been previously published.[5–7] Patients ≥18 years of age undergoing first-time CABG with implantation of at least 1 saphenous vein graft were eligible for enrollment. Those with an anticipated requirement for postoperative oral anticoagulation or antiplatelet therapy other than aspirin were excluded, although those prescribed these agents for unforeseen postoperative conditions (eg, atrial fibrillation) continued in the study. All patients were administered aspirin (300–325 mg) within 24 hours of surgery. At hospital discharge, patients were given a supply of 325 mg enteric-coated aspirin and instructed to take 1 tablet daily for 6 months unless directed otherwise by their physician. Pill counts were performed at each postoperative encounter. Demographic, historical, procedural, clinical, and laboratory data were recorded for all patients.

Platelet Studies

Platelet-rich plasma was prepared from blood collected in 3.2% citrate by centrifugation at 100 rpm for 10 minutes, and the platelet count was adjusted to 180 000/mm^3 by the addition of platelet-poor plasma. Undiluted samples with a platelet count of <100 000/mm^3 were excluded from analysis. Impedance platelet aggregometry was performed by stimulation with arachidonic acid (0.5 mmol/L), ADP (20 μmol/L), epinephrine (50 μmol/L), and collagen (1 μg/mL) with use of a Chrono-Log Model 560CA aggregometer. The maximum aggregation response within 5 minutes was recorded in ohms. Subjects were considered to have aspirin-induced suppression of significant platelet COX-1 activity and TXA_2 generation if arachidonic acid–induced platelet aggregation was absent as indicated by a value of ≤1 Ω (normal range in our laboratory for aspirin-naïve subjects: 5–17 Ω) based on prior data demonstrating that suppression of platelet TXA_2 generation by >99% is required to suppress arachidonic acid–induced aggregation by 95%.[8] Shear-dependent platelet aggregation was measured by using the Platelet Function Analyzer-100® (PFA-100) device (Siemens Healthcare Diagnostics) as previously described[9] in whole blood collected in 3.8% citrate. Samples were tested with the collagen/ADP agonist cartridge, which assesses global platelet reactivity but is not affected by aspirin. Samples from subjects with a platelet count <50 000/m^3 were excluded from analysis. Samples with nonclosure were assigned a closure time (CT) value of 300 seconds, the maximum measurable by the device.

Measurement of Urine Prostanoids

11-Dehydro-thromboxane B_2 (TXB_2) was measured in urine ($UTXB_2$) with ELISA and expressed as a ratio to urine creatinine as previously described.[9] Aspirin responsiveness based on this assay was defined as $UTXB_2$ <400 pg/mg creatinine according to established criteria.[10]

Assessment of Saphenous Vein Graft Patency

Vein graft patency was assessed 6 months after CABG by the use of multidetector computed tomography coronary–angiography as previously described.[6] Data from clinically driven invasive coronary angiograms could be used for the primary end point analysis if performed within 6 weeks of the anticipated 6-month follow-up visit or if it was the only assessment of vein graft patency before an adverse clinical end point. Multisegmented grafts were statistically considered as separate vein grafts according to the Society of Thoracic Surgeons criteria. Reconstructed images were analyzed by 2 blinded reviewers and classified as patent (containing stenoses of 0–75%), significantly diseased (containing stenoses of 76–99%), or occluded (containing a 100% stenosis). There was 98% concordance in assessment of vein graft patency between reviewers. In cases of discordance, a third reviewer adjudicated all vein grafts in that patient.

In Vitro Prostanoid Generation

Human umbilical vein endothelial cells were maintained in EGM-2 medium (Lonza) at 37°C under 5% CO_2. Confluent cells in 10-cm plates were stimulated with hydrogen peroxide (Sigma-Aldrich) and 8-iso-prostaglandin (PG)$F_{2\alpha}$ (Cayman Chemical) for 1 hour at the indicated concentrations. Conditioned media were spiked with tetradeuterated 11-dehydro-TXB_2, TXB_2, and 8-iso-$PGF_{2\alpha}$ (Cayman Chemical) as internal standards before solid phase extraction by using 50-mg BondElut C18 reverse phase cartridges (Agilent Technologies) preconditioned with ethanol and water to concentrate eicosanoid species. Acidified samples (2% formic acid) were loaded and washed sequentially with water, 15% ethanol, and hexane and then eluted with ethyl acetate, dried, and resuspended in 15% acetonitrile. Calibrants were prepared in the same way over a 0.5- to 500-ng/mL concentration range. Liquid chromatography/mass spectrometry (MS)–MS was performed by using a Dionex UltiMate 3000 UHPLC system in line with a TSQ Quantiva triple quadruple mass spectrometer (Thermo Fisher Scientific). Chromatographic separation was performed with a Kinetex C18 (1.7 μm, 100 Å) 50×100-mm column maintained at 40°C. A multistep gradient with (A) water with 0.005% (v/v) acetic acid, pH 5.7, and (B) 5% methanol/95% acetonitrile with 0.005% acetic acid, at a flow rate of 0.6 mL/min was used. After a 15-μL injection, the gradient started at 15% B (0–0.6 minutes), increased to 40% B (0.6–2 minutes), increased to 95% B (2–4 minutes), was maintained at 95% B (4–4.5 minutes), decreased to 15% B (4.5–4.7 minutes), and was maintained at 15% B (4.7–8 minutes). Tandem MS was performed in negative ion mode with spray voltage set at 3.3 kV, ion transfer tube temperature at 356°C, and vaporizer temperature at 420°C. The sheath, auxillary, and sweep gases were set at 52, 16, and 2 AU, respectively. The following *m/z* transitions were monitored for quantification: *m/z* 353.2→193.1 (CE 26 eV) and 353.2→309.1 (CE 20 eV) for 8-iso-$PGF_{2\alpha}$; 357.2→197.1 (CE 26 eV) and 357.2→313.1 (CE 20 eV) for d4-iso-$PGF_{2\alpha}$, *m/z* 367.2→243.1 (CE 20 eV) and 367.2→305.1 (CE 16 eV) for 11-dehydro-TXB_2; *m/z* 371.2→247.1 (CE 20 eV) and 371.2→309.1 (CE 16 eV) for d4-11-dehydro-TXB_2; 369.2→169.1 (CE 18 eV) and 369.2→195.0 (CE 15 eV) for TXB_2; and 373.2→173.1 (CE 18 eV) and 373.2→199.0 (CE 15 eV) for d4-TXB_2. Area ratios of analyte to internal standard were calculated, and concentrations of the samples were determined from the standard curve. All data were processed and integrated in Xcalibur, version 3.0 (Thermo).

Statistical Analysis

$UTXB_2$ values were normalized by using the natural logarithmic transform. Univariate analyses were performed by using those variables deemed biologically plausible or supported by the literature. Colinearity of covariates was tested by using Fisher exact and Pearson correlation for categorical and continuous variables, eliminating highly collinear covariates based on clinical significance. All predictors with $P \leq 0.15$ on univariate analysis were included in a multivariable model that was optimized by using the corrected Akaike Information Criterion. To facilitate comparison of the contribution of independent variables in the multivariable model, the coefficient estimates are reported for independent variables standardized to a variance of 1 (β coefficients). The relative importance of the independent variables was further assessed by dominance analysis.[11] The regression was performed for all possible combinations of the identified predictors, the incremental contribution of each variable to the resulting models was averaged to obtain general dominance (additive decomposition), and conditional dominance evaluations were performed. The independent variables were ranked for their contribution to the multivariable model based on their dominance weights. For vein graft analysis, grafts classified as severely diseased were considered as patent. Univariate analyses were performed on a per-graft basis for the odds of occlusion versus patency for $UTXB_2$ and urinary (U)8-iso-$PGF_{2\alpha}$. Proportions were compared by using a χ^2 or Fisher's exact test, and comparisons among groups were made with ANOVA, McNemar, or Kruskal–Wallis testing, as appropriate. Analyses were performed by using Stata/MP 10.0 for Windows (StatCorp). Differences were considered significant when $P<0.05$.

Results

Study Population Characteristics

Of the 368 subjects undergoing first-time CABG enrolled in the RIGOR study, 299 had measurement of $UTXB_2$ and platelet reactivity at the time of assessment of vein graft patency 6 months after surgery. Thirty-nine subjects were excluded from the primary analyses: 2 because they had discontinued aspirin, 32 because they were taking additional nonaspirin antiplatelet agents, and 5 because of arachidonic acid–induced platelet aggregation ≥1 Ω despite aspirin therapy. Therefore, 260 subjects taking aspirin monotherapy with verified suppression of platelet COX-1 activity and TXA_2 generation by >99%[8] were used for the primary analyses. $UTXB_2$ in this study cohort was non–normally distributed (Figure 1) with a median of 328 pg/mg creatinine (IQR 232–451 pg/mg creatinine). Despite confirmed aspirin-induced suppression of arachidonic acid–induced platelet activation, 82 (31.5%) subjects had $UTXB_2$ ≥400 pg/mg creatinine, the accepted threshold with this assay for defining putative aspirin nonresponsiveness.[10] Table 1 shows the clinical characteristics of the study cohort as a whole, in subjects

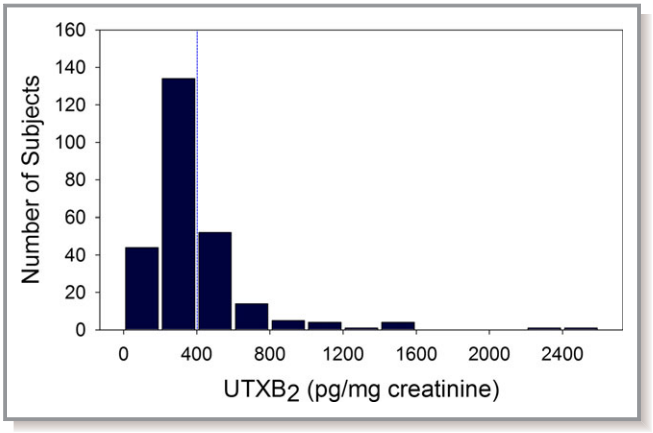

Figure 1. Distribution of urine 11-dehydroTXB$_2$ (UTXB$_2$) in the study cohort of 260 subjects. The blue dashed line indicates the putative threshold for defining aspirin responsiveness with this assay.[10]

stratified by UTXB$_2$, and in subjects excluded from the primary analyses.

Relationship Between Clinical and Laboratory Variables and UTXB$_2$

To identify potential stimuli and sources for nonplatelet thromboxane generation, univariate analyses were used to explore associations of a wide array of demographic, clinical, and laboratory variables to UTXB$_2$ expressed as a continuous variable (Tables 2 and 3). A multivariable model (fit statistic of 0.44, $P<0.0001$) was then constructed to identify independent predictors of UTXB$_2$ (Table 4). The strongest predictor of UTXB$_2$, accounting for nearly half of the modeled effect, was U8-iso-PGF$_{2\alpha}$, an isoprostane formed by nonenzymatic metabolism of arachidonic acid under conditions of oxidative stress.[12] Figure 2 shows the high degree of correlation between normalized values of U8-iso-PGF$_{2\alpha}$ and UTXB$_2$ in the study population. Age, race, and sex were also independently associated with UTXB$_2$ and accounted for ≈10% of the modeled effect, while lipid therapy (predominantly statins), renal function, left ventricular function, and aspirin dose contributed to lesser degrees.

Relationship of U8-Iso-PGF$_{2\alpha}$ to Early Vein Graft Failure

We previously found that aspirin-insensitive thromboxane generation, defined by elevated UTXB$_2$, was a novel and independent risk factor for vein graft thrombosis 6 months after CABG.[5] Given that U8-iso-PGF$_{2\alpha}$ is a major determinant of UTXB$_2$, we investigated whether there was a direct relationship of the former to early graft thrombosis. Stratification of RIGOR subjects, regardless of antiplatelet use or aspirin responsiveness, by tertile of U8-iso-PGF$_{2\alpha}$, revealed a proportional increase in the prevalence of vein graft occlusion (Figure 3), and normalized U8-iso-PGF$_{2\alpha}$ correlated with graft occlusion when considered on a per-graft basis (odds ratio 1.67, $P=0.001$). U8-iso-PGF$_{2\alpha}$ was not, however, an independent predictor of vein graft occlusion in multivariable modeling when UTXB$_2$ was included as a variable (data not shown), indicating the primacy of the latter.

Relationship Between Endothelial Thromboxane Generation and Oxidative Stress

The strong association between U8-iso-PGF$_{2\alpha}$ and UTXB$_2$ highly suggests, but does not in itself prove, a causal relationship between oxidative stress and nonplatelet TXA$_2$ generation. Because the endothelium is a potential major source of nonplatelet TXA$_2$ generation in vivo, we determined the effect of oxidative stress and direct stimulation with 8-iso-PGF$_{2\alpha}$ on endothelial TXA$_2$ generation. Exposure of human umbilical vein endothelial cells to hydrogen peroxide resulted in a dose-dependent increase in the concentration of 8-iso-PGF$_{2\alpha}$, TXB$_2$, and 11-dehydro-TXB$_2$ in the conditioned media, indicative of cellular TXA$_2$ generation (Figure 4A). Further, direct stimulation of human umbilical vein endothelial cells with 8-iso-PGF$_{2\alpha}$ results in endothelial TXA$_2$ production (Figure 4B), establishing a mechanistic link among oxidative stress, 8-iso-PGF$_{2\alpha}$ formation, and nonplatelet TXA$_2$ generation.

Discussion

The major findings of this study are that (1) oxidative stress–induced formation of 8-iso-PGF$_{2\alpha}$ is the strongest variable associated with nonplatelet TXA$_2$ generation in patients 6 months after CABG; (2) age, sex, race, lipid therapy, aspirin dose, and kidney and left ventricular function are also independently associated with nonplatelet TXA$_2$ generation, though to a much lesser degree; (3) U8-iso-PGF$_{2\alpha}$ directly correlates with incidence of early vein graft thrombosis, but its predictive power is not independent of UTXB$_2$; and (4) endothelial cells are capable of generating TXA$_2$ in response to both oxidative stress and direct stimulation with 8-iso-PGF$_{2\alpha}$, thus representing a potential source of nonplatelet TXA$_2$ generation in vivo.

Platelets are the predominant source of TXA$_2$ generation in healthy individuals, and measurement of stable TXA$_2$ metabolites in the urine has been used clinically as an indicator of the antiplatelet effects of aspirin. Substudies from the Heart Outcomes Prevention Evaluation (HOPE) and the Clopidogrel for High Atherothrombotic Risk and Ischemic Stabilization, Management and Avoidance (CHARISMA) trials found that in

Table 1. Baseline, Operative, and Postoperative Characteristics of the 260 Study Subjects Stratified by $UTXB_2$ and the 39 Excluded Subjects

Characteristic	<400 pg/mg Creatinine	≥400 pg/mg Creatinine	*P* Value	Total Included	Total Excluded	*P* Value
No. of patients	178	82		260	39	
Age, y	63 (55–69)	66 (57–73)	0.07	63 (56–71)	63 (59–72)	0.44
Male sex	153 (86%)	58 (71%)	0.006	211 (81%)	25 (64%)	0.02
White race	17 (10%)	18 (22%)	0.01	35 (13%)	5 (13%)	1.0
Body mass index, kg/m^2	29 (26–33)	28 (26–33)	0.87	29 (26–33)	26 (24–30)	<0.001
Medical history, n						
Hypertension	148 (83%)	65 (79%)	0.49	213 (82%)	32 (84%)	0.82
Dyslipidemia	150 (85%)	65 (79%)	0.29	215 (83%)	34 (89%)	0.48
Diabetes	56 (32%)	39 (48%)	0.018	95 (37%)	11 (29%)	0.47
Heart failure	16 (9%)	18 (22%)	0.006	34 (13%)	4 (10%)	0.80
Peripheral/cerebrovascular disease	28 (16%)	18 (22%)	0.226	46 (18%)	8 (21%)	0.66
Atrial fibrillation	5 (3%)	3 (4%)	0.71	8 (3%)	4 (10%)	0.06
Current tobacco use	33 (19%)	27 (33%)	0.017	60 (23%)	11 (28%)	0.55
Myocardial infarction	64 (36%)	39 (48%)	0.08	103 (40%)	18 (46%)	0.49
Prior PCI	40 (22%)	12 (15%)	0.18	52 (20%)	9 (23%)	0.67
Preoperative LVEF			0.78			0.75
≤30%	14 (8%)	8 (10%)		22 (8%)	3 (8%)	
30–50%	59 (33%)	29 (35%)		88 (34%)	11 (28%)	
>50%	105 (59%)	45 (55%)		150 (58%)	25 (64%)	
Urgent/emergent surgery	100 (56%)	57 (70%)	0.06	157 (60%)	30 (77%)	0.052
Euroscore	3 (1–5)	4 (3–6)	0.004	3 (2–5)	4 (2–5)	0.25
Arterial graft implanted	175 (98%)	79 (96%)	0.38	254 (98%)	36 (92%)	0.10
No. of SVGs per subject			0.2			0.03
1	48 (27%)	20 (24%)		68 (26%)	17 (44%)	
2	70 (39%)	41 (50%)		111 (43%)	12 (31%)	
3	44 (25%)	12 (15%)		56 (22%)	10 (26%)	
≥4	16 (9%)	9 (11%)		25 (10%)	0 (0%)	
Medications at the time of SVG patency assessment						
Aspirin	178 (100%)	82 (100%)	1.0	260 (100%)	37 (95%)	<0.001
Nonaspirin antiplatelet	0 (0%)	0 (0%)	1.0	0 (0%)	33 (85%)	<0.001
Aspirin low dose (<325 mg)	11 (6%)	10 (12%)	0.14	21 (8%)	12 (31%)	<0.001
Oral anticoagulation	6 (3%)	7 (9%)	0.12	13 (5%)	3 (8%)	0.49
β-Blocker	153 (86%)	61 (75%)	0.035	214 (82%)	35 (90%)	0.36
ACE inhibitor/ARB	113 (63%)	47 (57%)	0.4	160 (62%)	26 (67%)	0.60
Lipid-lowering agent	162 (91%)	65 (79%)	0.015	227 (87%)	37 (95%)	0.28

Values are median (IQR) or n (%). ACE indicates angiotensin-converting enzyme; ARB, angiotensin II receptor blocker; LVEF, left ventricular ejection fraction; PCI, percutaneous coronary intervention; SVG, saphenous vein graft; $UTXB_2$, urinary thromboxane B_2.

patients with either established or at high risk for cardiovascular disease who are receiving aspirin therapy, those with $UTXB_2$ in the highest quartile had a 1.66- to 1.80-fold increased risk of death, myocardial infarction, and stroke compared those in the lowest quartile.[2,3] An early interpretation of these results was that aspirin failed to adequately inhibit platelet COX-1 activity in a substantial number of subjects, leading to persistent TXA_2 generation, increased

Table 2. Univariate Analyses of the Associations of Subject Demographics, Past Medical History and Medication Use With $UTXB_2$ (Normalized by Natural Log Transformation of pg/mg Creatinine)

Characteristic	Standardized Coefficient*	*P* Value
Female sex	0.225	<0.001
Age, y	0.203	0.001
White race (versus nonwhite)	−0.188	0.006
Obesity (BMI ≥30 kg/m^2)	−0.101	0.093
Medical history		
Hypertension	−0.031	0.579
Dyslipidemia	−0.141	0.030
Diabetes	0.110	0.091
Current tobacco use	0.155	0.011
Former tobacco use	0.097	0.111
Myocardial infarction	0.052	0.394
Percutaneous coronary intervention	−0.054	0.272
Congestive heart failure	0.140	0.015
Cerebrovascular disease	0.044	0.524
Deep venous thrombosis/pulmonary embolus	−0.027	0.436
Peripheral vascular disease	0.167	0.010
Chronic obstructive pulmonary disease	0.139	0.016
Atrial fibrillation	0.038	0.581
Preoperative LVEF: <30% vs 30–50%	−0.110	0.360
Preoperative LVEF: <30% vs ≥50%	−0.148	0.211
Left ventricular ejection fraction (%)	−0.157	0.011
Euroscore: 0–2 vs 3–5	0.227	0.001
Euroscore: 0–2 vs ≥6	0.281	<0.001
CABG urgency: elective vs urgent or emergent	0.044	0.501
Medications		
Aspirin dose (81 mg vs higher)	−0.187	0.003
Oral anticoagulation	0.124	0.088
β-Blocker	−0.096	0.112
Angiotensin II receptor blocker	−0.046	0.488
Angiotensin-converting enzyme inhibitor	−0.029	0.636
Lipid therapy	−0.195	0.001
Diuretic	0.046	0.457
Insulin	0.020	0.726
Insulin sensitizer	−0.004	0.954
Insulin secretagogue	0.064	0.346

BMI indicates body mass index; CABG, coronary artery bypass graft surgery; LVEF, left ventricular ejection fraction; $UTXB_2$, urinary thromboxane B_2.
*Coefficients are standardized to 1 SD of the predictor. Huber–White sandwich estimates were used to produce robust estimates of variance.

Table 3. Univariate Analyses of the Associations of Laboratory Variables to $UTXB_2$ (Normalized by Natural Log Transformation of pg/mg Creatinine)

Characteristic	Standardized Coefficient*	*P* Value
Hematologic parameter		
Leukocyte count: 4.5–11×10^3 mm^{-3}	Reference	
Leukocyte count: ≤4.5×10^3 mm^{-3}	0.023	0.702
Leukocyte count: ≥11×10^3 mm^{-3}	0.078	<0.001
MCV 80–100 fL	Reference	
MCV <80 fL	0.067	0.354
MCV >100 fL	0.003	0.925
Hematocrit (%)	−0.089	0.183
Red cell distribution width (≤14.5 vs >14.5%)	0.130	0.042
RDW, %$^{-3}$	0.141	0.024
Platelet count (<150 vs ≥150×10^3 mm^{-3})	−0.032	0.582
Reticulocyte (ln %)	−0.083	0.273
Mean platelet volume (%$^{-1}$)	−0.050	0.401
Immature platelet fraction (ln %)	0.055	0.390
Blood group: O vs other	−0.015	0.805
Rh positivity	0.071	0.341
Creatinine (−[mg/dL]$^{-½}$)	−0.166	0.002
C-reactive protein (<5 vs ≥5 mg/L)	0.139	0.027
Fibrinogen <390 vs ≥390 mg/dL	0.124	0.049
vonWillebrand factor (>150% vs ≤150%)	0.192	0.002
Urine 8-iso-$PGF_{2\alpha}$ (ln pg/mg creatinine)	0.500	<0.0001
Urine 8-iso-$PGF_{2\alpha}$ (<1061 vs ≥1061 pg/mg creatinine)	0.353	<0.001
Fasting serum insulin (ln μU/mL)	−0.008	0.91
Impedance platelet aggregation in ohms to		
ADP (20 μmol/L)	0.041	0.452
Collagen (1 μg/mL)	−0.006	0.910
Epinephrine (50 μmol/L)	0.078	0.188
PFA-100 collagen/ADP (closure time in s)	−0.014	0.811
PFA-100 collagen-epinephrine (closure time in s)	−0.097	0.147

MCV indicates ; PG, prostaglandin; RDW, ; $UTXB_2$, urinary thromboxane B_2.
*Coefficients are standardized to 1 SD of the predictor. Huber–White sandwich estimates were used to produce robust estimates of variance.

platelet reactivity, and elevated cardiovascular risk. The RIGOR study also found that elevated $UTXB_2$ was associated with increased cardiovascular risk, being a potent and independent risk factor for early vein graft thrombosis.[5] Unlike the HOPE and CHARISMA data, which did not measure

Table 4. Independent Risk Factors for $UTXB_2$* After Adjustment of Other Variables by Multivariable Regression Analysis

Characteristic	Standardized Coefficient	*P* Value	Dominance Weight	Dominance Ranking
Urine 8-iso-$PGF_{2\alpha}$ (ln pg/mg creatinine)	0.442	<0.001	0.472	1
Age, y	0.239	<0.001	0.102	2
Female sex	0.129	0.015	0.093	3
White race (versus nonwhite)	−0.172	0.009	0.085	4
Lipid therapy	−0.161	0.004	0.077	5
Creatinine (−[mg/dL]$^{-½}$)	−0.152	0.002	0.072	6
Aspirin dose (81 mg vs higher)	−0.145	0.004	0.052	7
Left ventricular ejection fraction (%)	−0.113	0.032	0.048	8

PG indicates prostaglandin; $UTXB_2$, urinary 11-dehydro thromboxane B_2 (pg/mg creatinine).
*Normalized using natural log transform.

platelet-specific TXA_2 generation, the RIGOR data revealed that failure of aspirin to inhibit platelet COX-1 and TXA_2 generation was in fact rare, occurring in <1% of subjects 6 months after surgery. This provided compelling evidence that aspirin-insensitive TXA_2 generation in patients with cardiovascular disease, unlike in healthy individuals, predominantly originates from nonplatelet sources.

The current analysis extends our previous results and identifies oxidative stress as a potentially major stimulus for nonplatelet TXA_2 generation by showing a strong correlation with 8-iso-$PGF_{2\alpha}$, an arachidonic acid metabolite formed nonenzymatically in a variety of cell types by free radical oxidation.[12] While frequently used as a marker of oxidative stress, 8-iso-$PGF_{2\alpha}$ is also a biologically active prostanoid that can bind to and activate cellular thromboxane receptors (see reviews13 and 14). Although it is not as potent a platelet agonist as TXA_2, 8-iso-$PGF_{2\alpha}$ can potentiate platelet activation in response to collagen and ADP as well as directly stimulate vasoconstriction. In 2 small prior studies involving patients with unstable angina and diabetes who were taking aspirin, a correlation between $UTXB_2$ and 8-iso-$PGF_{2\alpha}$ was identified, though in neither was the source of TXA_2 generation specifically evaluated.[15,16] Our analysis not only establishes a strong association between 8-iso-$PGF_{2\alpha}$ and aspirin-insensitive TXA_2 generation in a larger study cohort but also conclusively demonstrates that the latter originates predominantly from nonplatelet sources.

Mounting evidence suggests that oxidative stress—induced generation of 8-iso-$PGF_{2\alpha}$ and nonplatelet TXA_2 is more than just linearly associated but is causally linked. Treatment with the antioxidant vitamin E has been shown to reduce both U8-iso-$PGF_{2\alpha}$ and $UTXB_2$ levels in aspirin-naïve smokers.[17] More definitive are the findings that fetal porcine cerebral and retinal microvessels generate TXA_2 when incubated with 8-iso-$PGF_{2\alpha}$, an effect that is blocked by indomethacin.[18,19] Our data not only confirm this finding in macrovascular endothelial cells but also reveal that oxidative stress itself can be a primary stimulus for endothelial TXA_2 generation. Whether this effect is mediated by the endothelial

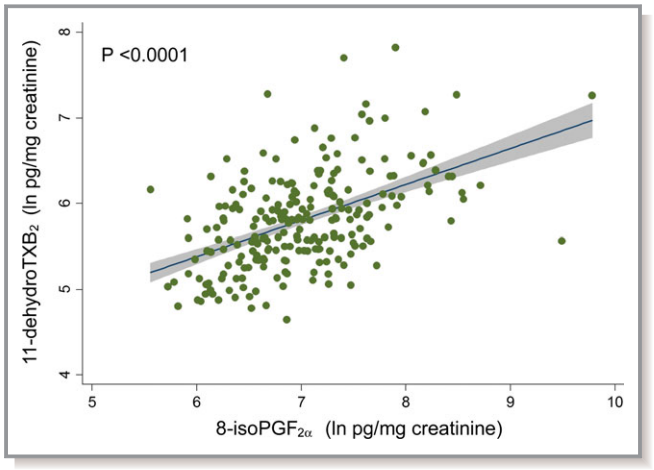

Figure 2. Linear regression of normalized levels of urine 11-dehydro-thromboxane (TX)B_2 to 8-iso-prostglandin (PG)$F_{2\alpha}$ in 228 subjects.

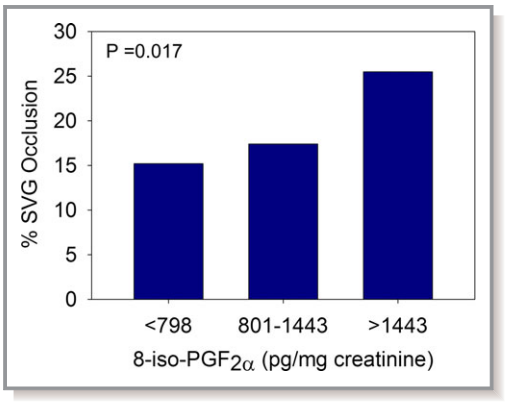

Figure 3. Prevalence of saphenous vein graft (SVG) occlusion in 225 subjects stratified by tertile of urine 8-iso-prostaglandin (PG)$F_{2\alpha}$.

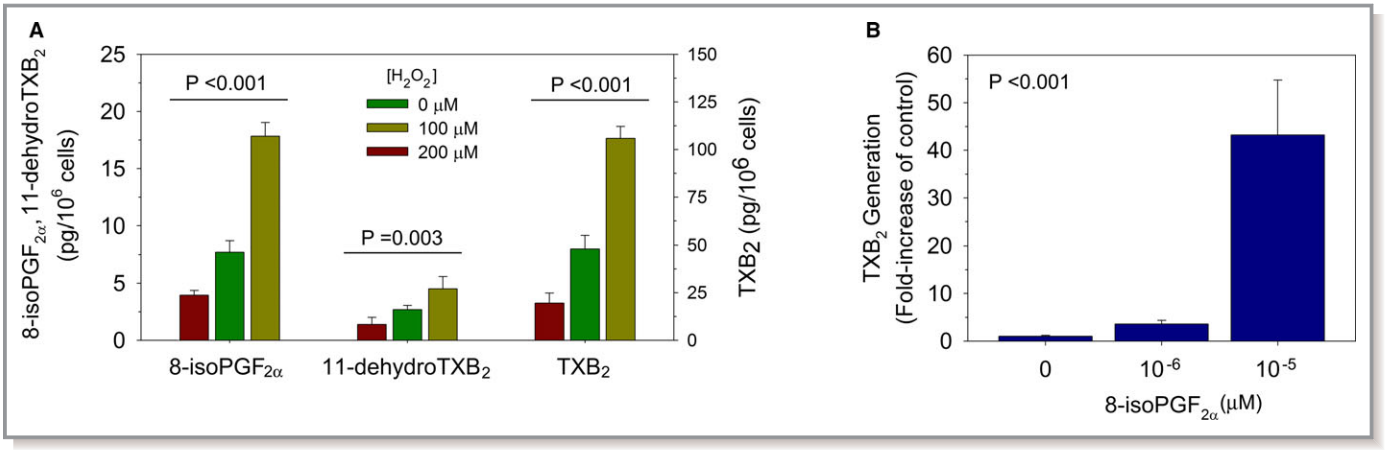

Figure 4. A, Human umbilical vein endothelial cells (HUVECs) under oxidative stress by exposure to hydrogen peroxide (H_2O_2) for 1 hour generate thromboxane (TX) and isoprostanes in a dose-dependent manner. B, HUVECs exposed to 8-iso-prostaglandin (PG)$F_{2\alpha}$ for 1 hour generate thromboxane in a dose-dependent manner. Values shown are the mean of n=3 ±SEM.

generation of 8-so-$PGF_{2\alpha}$ and autocrine stimulation of cellular thromboxane receptors or involves additional intracellular pathways is an area of active investigation.

Because 8-iso-$PGF_{2\alpha}$ is a stable prostanoid that freely circulates, its biological effects can be widespread and distinct from sources of origin. Inflammatory cells are capable of directly generating TXA_2 and 8-iso-$PGF_{2\alpha}$, and patients undergoing cardiac surgery with cardiopulmonary bypass have a marked inflammatory response with an observable increase in U8-iso-$PGF_{2\alpha}$ in the early postoperative period.[20–22] In the RIGOR study, $UTXB_2$ was significantly higher 3 days after CABG than at 6 months, though only the latter correlated with risk of vein graft occlusion.[5] While we did find significant correlations of $UTXB_2$ with white blood cell count, C-reactive protein, or fibrinogen on univariate analyses, none of these variables was independently associated with $UTXB_2$, suggesting that inflammation was not a major stimulus for nonplatelet TXA_2 generation 6 months after CABG. It is conceivable that the vein grafts themselves contribute to TXA_2 generation given that surgical preparation and pressure-induced distention are known to increase oxidative stress in vein segments.[23,24] However, we did not observe any correlation between the number of vein graft segments implanted at the time of surgery and either $UTXB_2$ or U8-iso-$PGF_{2\alpha}$ 6 months later (data not shown). The elevated $UTXB_2$ and U8-iso-$PGF_{2\alpha}$ observed in a substantial percentage of the RIGOR study cohort are, therefore, likely due predominantly to underlying cardiovascular disease or its risk factors, rather than effects of the CABG per se. $UTXB_2$ has previously been shown to be elevated in diabetics compared with nondiabetics.[16] While this was also true in our analysis, diabetes was not found to be an independent predictor of $UTXB_2$ when 8-iso-$PGF_{2\alpha}$ was considered as a variable, suggesting that they are different manifestations of the same pathologic process.

The mechanism by which nonplatelet TXA_2 generation could adversely affect cardiovascular risk is currently unknown. TXA_2 necessarily acts locally because of a short half-life (≈30 seconds) due to degradation to biologically inert TXB_2. Although aspirin-inhibited platelets cannot generate appreciable amounts of TXA_2, they could potentially still aggregate in response to locally generated nonplatelet TXA_2 to cause thrombosis. However, several pieces of evidence argue against platelet hyperreactivity being a major mediator of cardiovascular risk by nonplatelet TXA_2 generation. First, the addition of clopidogrel did not reduce cardiovascular risk in CHARISMA subjects with elevated $UTXB_2$.[3] Second, $UTXB_2$ in the RIGOR study cohort was independent of multiple parameters of platelet reactivity, including shear-dependent platelet aggregation and whole blood aggregation performed in response to multiple different agonists (Table 3). Third, we found no correlation between U8-iso-$PGF_{2\alpha}$ and platelet reactivity, suggesting that circulating 8-iso-$PGF_{2\alpha}$ did not significantly "prime" or potentiate platelet aggregation in response to more physiologic agonists (data not shown). Rather than potentiating platelet reactivity, local TXA_2 generation could predispose to thrombus formation by altering endothelial thromboresistance. Consistent with this concept are the recent findings that thromboxane receptor activation stimulates tissue factor expression in both endothelial cells and monocytes.[25,26]

A significant finding of our analysis was that U8-iso-$PGF_{2\alpha}$ correlated directly with the incidence of early vein graft thrombosis. This suggests that therapies aimed at reducing oxidative stress might be a viable strategy to reduce nonplatelet TXA_2 generation and improve outcomes after cardiac surgery. Antioxidants have been shown to be efficacious at reducing the incidence of postoperative atrial fibrillation.[27,28] While they have not been evaluated for

efficacy at reducing graft failure, this is an eminently testable hypothesis.

Our study has several potential limitations. Although we examined a wide array of clinical and laboratory variables associated with $UTXB_2$ generation in subjects 6 months after CABG, presumably at a time when the effects of surgery have subsided, it is possible that the relative contributions of oxidative stress and other identified factors differ in populations with various forms or severity of cardiovascular disease. Although the RIGOR cohort was extensively phenotyped and we evaluated numerous potential variables, the 8 variables identified in the multivariable modeling account for only slightly less than half of the modeled effect on $UTXB_2$. Thus, additional risk factors for nonplatelet TXA_2 generation remain to be identified.

In summary, we identified several risk factors for nonplatelet TXA_2 generation in subjects 6 months after CABG. Not only was oxidative stress-induced formation of 8-iso-$PGF_{2\alpha}$ the strongest identified risk factor, but it also directly correlated with risk of early vein graft thrombosis. In vitro studies revealed that macrovascular endothelial cells are capable of generating TXA_2 under conditions of oxidative stress and with direct stimulation with 8-iso-$PGF_{2\alpha}$, not only establishing a mechanistic link between oxidative stress and nonplatelet TXA_2 generation but pointing to dysfunctional endothelial cells as a potentially major source. These findings provide valuable insights into the pathobiology of nonplatelet TXA_2 generation and identify potential therapeutic strategies for its suppression.

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Disclosures

None.

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