Effects of Unfractionated Heparin and Glycoprotein IIb/IIIa Antagonists Versus Bivalirudin on Myeloperoxidase Release From Neutrophils

Guohong Li, Alison C. Keenan, Justin C. Young, Margaret J. Hall, Zehra Pamuklar, E. Magnus Ohman, Steven R. Steinhubl, Susan S. Smyth

Objectives—The objective of this study was to determine whether adjunctive therapy during percutaneous coronary intervention (PCI) affects markers of systemic inflammation or platelet activation. Despite different mechanisms of action, direct-thrombin inhibition with bivalirudin during PCI provided similar protection from periprocedural and chronic ischemic complications as compared with unfractionated heparin (UFH) plus planned use of GPIIb/IIIa antagonists in the REPLACE-2 and ACUITY trials.

Methods and Results—Patients undergoing nonurgent PCI of a native coronary artery were randomized to receive adjunctive therapy with bivalirudin or UFH+epifibatide. Interleukin (IL)-6 and C-reactive protein (CRP) transiently increased in both groups after PCI. In the UFH+epifibatide, but not the bivalirudin group, myeloperoxidase (MPO) levels were elevated 2.3-fold above baseline (P=0.004) immediately after PCI. In an in vitro assay, heparin and to a lesser extent enoxaparin, but not bivalirudin or epifibatide, stimulated MPO release from and binding to neutrophils and neutrophil activation. A mouse model of endoluminal femoral artery denudation was used to investigate further the importance of MPO in the context of arterial injury.

Conclusions—Adjunct therapy during PCI may have undesired effects on neutrophil activation, MPO release, and systemic inflammation. (Arterioscler Thromb Vasc Biol. 2007;27:0-0.)

Key Words: platelets ■ neutrophils ■ myeloperoxidase ■ percutaneous coronary intervention ■ adjunctive therapy

Aggressive antithrombotic therapy, in particular antplatelet therapy with glycoprotein platelet glycoprotein [GP] IIb/IIIa antagonists, thienopyridines, and aspirin used in conjunction with unfractionated heparin, have consistently been shown to decrease the risk of periprocedural thrombotic complications associated with percutaneous coronary interventions (PCI).1,2 Bivalirudin, a direct thrombin inhibitor, was approved for use in PCI as an alternative to heparin before to widespread use of GPIIb/IIIa antagonists. Recently, the Randomized Evaluation in PCI Linking Angiomax to Reduced Clinical Events (REPLACE)-2 and the Acute Catheterization and Urgent Intervention Triage strategY (ACUITY) trials demonstrated that bivalirudin used with GPIIb/IIIa antagonists on a provisional basis provided similar protection from periprocedural ischemic and hemorrhagic complications compared with heparin plus planned use of GPIIb/IIIa antagonists.3,4 In the REPLACE-2 trial of low- to moderate-risk patients undergoing PCI, the primary composite end point at 30 days (incidence of death, myocardial infarction, urgent repeat revascularization, or in-hospital major bleeding) occurred in 9.2% of patients in the bivalirudin group versus 10.0% of patients in the unfractionated heparin (UFH) plus GPIIb/IIIa antagonist group.5 At 1 year, a nonsignificant trend toward lower mortality with bivalirudin was observed (1.9% in bivalirudin group and 2.5% in heparin plus GPIIb/IIIa antagonist group).3 The results from the ACUITY trial also suggest that in patients with acute coronary syndromes undergoing PCI, routine use of bivalirudin is associated with similar ischemic outcomes as UFH or low-molecular weight heparin+GPIIb/IIIa antagonists.4

Given their distinct modes of action, it is not readily apparent why bivalirudin would provide similar efficacy as compared with heparin plus GPIIb/IIIa antagonists. GPIIb/IIIa antagonists are potent inhibitors of platelet aggregation.5 However, GPIIb/IIIa antagonists do not block platelet activation and may theoretically act as partial platelet agonists.6 In addition, heparin can also activate platelets7,8 and may have deleterious effects on other vascular cells. Bivalirudin, in
contrast, does not directly prevent platelet aggregation, but through direct thrombin inhibition, may reduce platelet and vascular cell activation and thereby lower local and systemic inflammatory responses.

To gain insight into the efficacy of bivalirudin in comparison to GPIIb/IIIa antagonists plus UFH, we performed serial analysis of markers of platelet activation and systemic inflammation in patients undergoing PCI for stable angina randomized to receive either bivalirudin or UFH + eptifibatide. The results indicated that PCI is associated with an acute inflammatory reaction regardless of adjunct therapy and that immediately after the procedure levels of myeloperoxidase (MPO), a hemoprotein released by activated neutrophils and to a lesser extent monocytes, were elevated in the UFH + eptifibatide group. Because elevated levels of MPO have been associated with poor outcomes in patients presenting with chest pain and acute coronary syndromes, we investigated the effects of adjunct therapy on MPO release by isolated neutrophils in the absence and presence of activated platelets. In addition, we examined the role of MPO in the response to arterial injury in a mouse model. Our results suggest that heparin may have undesired effects during PCI by enhancing neutrophil function and MPO release.

Methods

Patient Population

All procedures were performed in accordance with guidelines of the Institutional Review Boards of the University of North Carolina. Patients were eligible for study if they were over 21 years of age and undergoing a percutaneous coronary intervention involving no more than 2 stent implantations. Patients were excluded if the intervention was being performed in the setting of acute myocardial infarction or troponin-positive acute coronary syndrome. Other exclusion criteria included heparin administration in the last 24 hours, long-term warfarin anticoagulation, acute inflammatory condition (eg, cancer, autoimmune disorder), thrombocytopenia (platelet count < 100,000), bleeding diathesis, and renal failure requiring dialysis.

Study Protocol

A total of 24 patients undergoing nonurgent PCI were randomized to receive bivalirudin alone or UFH + the GPIIb/IIIa antagonist eptifibatide. All patients received oral clopidogrel (300 mg) and were on aspirin before the intervention. Bivalirudin was administered as a bolus (0.75 mg/kg i.v.) before the procedure and then infused at 1.75 mg/kg/h for the duration of the procedure. Patients randomized to UFH + eptifibatide received a bolus of heparin (50 to 70 U/kg) before the procedure and 2 boluses of eptifibatide (180 μg/kg i.v.) 10 minutes apart before the procedure followed by an infusion of 3 μg/kg/min for 18 hours. Blood was collected before the intervention, and then immediately, 18 hours, 72 to 96 hours, and 7 days after PCI.

In Vitro Assays

Methodological details of the in vitro assays can be found in an online supplemental materials (available online at http://atvb.ahajournals.org).

Statistics

For the clinical study, inflammatory and platelet markers were compared over time to baseline levels with Wilcoxon rank sum test with continuity correction using a 2-sided test. Differences between the 2 groups at a given time point were analyzed by \( t \) test. Spearman analysis was used to assess the correlation of parameters over time. SAS software version 8 (SAS Institute) was used for analysis. Results from in vitro assays are presented as mean ± SEM and are derived from at least 3 separate experiments run in duplicate or triplicate and were analyzed by \( t \) test or ANOVA, where appropriate. Significance was defined as \( P = 0.05 \).

Results

Patient Characteristics

The demographics of patients undergoing planned PCI with stent implantation and randomized to receive either bivalirudin (n = 12) or UFH + eptifibatide (n = 12) are listed in Table 1. There were no statistically significant differences in mean age, weight, gender, or percentage of patients with diabetes mellitus, hypertension, hyperlipidemia, history of myocardial infarction, or previous revascularization between the 2 treatment groups. In addition, the angiographic data for the 2 groups was similar in terms of number of vessels treated, total lesion length, and average number of stents deployed (Table 2).

Markers of Platelet Activation

There were no differences in either P-selectin exposure or binding of the GPIIb/IIIa activation-dependent antibody PAC-1, as measured by mean fluorescent intensity and percentage of positive platelets, in unstimulated blood or after PCI in either treatment group (supplemental Figure IA and IB). In the 7 days after PCI, there was a steady reduction in P-selectin exposure in TRAP-treated blood in both treatment groups, which may reflect the fact that all patients were started on daily clopidogrel at the time of their intervention (supplemental Figure IC). As has been previ-

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**TABLE 1.** Demographic Data

<table>
<thead>
<tr>
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<th>Eptifibatide Group (n = 12)</th>
<th>Bivalirudin Group (n = 12)</th>
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</thead>
<tbody>
<tr>
<td>Age, y*</td>
<td>63 ± 4</td>
<td>62 ± 4</td>
</tr>
<tr>
<td>Gender, M</td>
<td>33%</td>
<td>54%</td>
</tr>
<tr>
<td>Active smokers</td>
<td>17%</td>
<td>9%</td>
</tr>
<tr>
<td>Former smokers</td>
<td>33%</td>
<td>36%</td>
</tr>
<tr>
<td>Hypertension</td>
<td>75%</td>
<td>91%</td>
</tr>
<tr>
<td>Hyperlipidemia</td>
<td>58%</td>
<td>83%</td>
</tr>
<tr>
<td>Diabetes</td>
<td>25%</td>
<td>54%</td>
</tr>
<tr>
<td>History of MI</td>
<td>33%</td>
<td>36%</td>
</tr>
<tr>
<td>Previous revascularization</td>
<td>33%</td>
<td>27%</td>
</tr>
<tr>
<td>Weight, kg*</td>
<td>85.8 ± 6</td>
<td>96.8 ± 5</td>
</tr>
</tbody>
</table>

*Mean ± SEM.

**TABLE 2.** Angiographic Data

<table>
<thead>
<tr>
<th></th>
<th>Eptifibatide Group</th>
<th>Bivalirudin Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vessels treated, n*</td>
<td>1.2 ± 0.3</td>
<td>1.2 ± 0.1</td>
</tr>
<tr>
<td>Total lesion length</td>
<td>22 ± 5</td>
<td>36 ± 9</td>
</tr>
<tr>
<td>Average stent, n*</td>
<td>1.3 ± 0.3</td>
<td>1.6 ± 0.4</td>
</tr>
<tr>
<td>% Taxus</td>
<td>19%</td>
<td>17%</td>
</tr>
<tr>
<td>% Cypher</td>
<td>44%</td>
<td>28%</td>
</tr>
</tbody>
</table>

*Mean ± SEM.
ously reported, immediately after PCI there were lower sCD40L levels in patients randomized to UFH+eptifibatide (P=0.024; supplemental Figure 1D). A correlation between sCD40L levels and binding of PAC-1, a monoclonal antibody which recognizes the activated form of GPIIb/IIIa (integrin αIIbβ3), was observed immediately after PCI (r=0.597; P=0.007). By 18 hours, sCD40L returned to baseline in both groups. No difference in plasma RANTES levels, a chemokine released by activated platelets, was observed between the treatment groups (data not shown). Likewise, no differences in circulating platelet-leukocyte aggregates in whole blood were observed after PCI in the 2 groups (data not shown).

Inflammatory Markers
PCI was associated with an acute inflammatory response, as judged by approximately 6-fold and 2-fold elevations in IL-6 and CRP levels, respectively, at 18 hours after the procedure in both treatment groups (P<0.05 compared with baseline; Figure 1A and 1B). Circulating levels of tissue factor and soluble intercellular adhesion molecule-1 (ICAM-1), a cell adhesive protein shed from activated endothelium and leukocytes, were similar in both treatment groups (data not shown). Surprisingly, immediately after the procedure, myeloperoxidase (MPO) levels were elevated 2.3-fold above baseline in the UFH+eptifibatide (7.5±0.7 versus 3.2±0.6 ng/mL; P=0.002; Figure 2C), but not in the bivalirudin group (2.9±0.6 versus 2.6±0.8 ng/mL). There was an inverse correlation between MPO levels and sCD40L immediately after PCI (r=-0.452; P=0.0047).

Effect of Adjunctive Therapy on MPO Release From Neutrophils
To understand the potential effects of adjunctive therapy on white blood cell function, we sought to determine whether these drugs influenced MPO release from isolated neutrophils. When coincubated with isolated neutrophils, neither bivalirudin nor eptifibatide altered baseline MPO release from isolated neutrophils. When coincubated with isolated neutrophils, neither bivalirudin nor eptifibatide altered baseline MPO release from isolated neutrophils.

Interestingly, the addition of activated, but not resting, platelets to isolated neutrophils reduced the levels of MPO in the supernatant (supplementary Figure IIA). Pretreatment of platelets with either eptifibatide or antibodies to CD40L restored levels of MPO in the supernatant by 20% (P=0.031; supplemental Figure IIA). Pretreatment of platelets with either eptifibatide or antibodies to CD40L restored levels of MPO in the supernatant (supplementary Figure IIB). Bivalirudin had no effect on MPO release from neutrophils in the presence of platelets.

MPO and Arterial Injury in Mice
Elevated levels of MPO have been associated with adverse outcomes in patients presenting with chest pain,12,13 but have not been extensively studied in the context of PCI. To study the heparin–MPO nexus in the context of arterial injury, we used a well-characterized model in which an angioplasty guide wire is used to denude the endothelium and trigger endoluminal arterial injury.14 We have previously reported that within hours neutrophils are recruited to the site of injury by adherent platelets in this model. Immunohistochemical staining of the vessels at 6 hours after injury confirmed the presence of MPO (Figure 3A). Chlorotryptosine protein adduct formation, produced by reaction with MPO-generated hypochlorous acid, was also detected along injured vessels (Figure 3A). Pretreatment of mice with heparin (200 U, i.p.) 30 minutes before surgery increased MPO levels in injured vessels (Figure 3B). Fondaparinux (200 µg, i.p.) did not affect MPO levels, consistent with observations in the in vitro assay. Finally, to determine whether MPO contributes to the development of intimal hyperplasia in this model, injury was performed in wild-type mice and mice deficient in MPO (MPO−/−). At 28 days after femoral wire injury, neointimal area was 37% smaller in MPO−/− mice as compared with wild-type control group (8600±2,200 µm² versus 5400±1,500 µm²; n=10 arteries per genotype; Figure 3C), but the difference was not statistically significant (P=0.245). Likewise, no statistically significant differences were ob-
served in lumen or media areas or intima/media ratios (lumen 20 500±6500 μm² versus 27 900±8500, P=0.498; media 12 400±2000 μm² versus 13 200±3000, P=0.827).

**Discussion**

A major finding of our study was that the use of UFH+eptifibatide was associated with a rapid rise in plasma MPO levels immediately after percutaneous coronary revascularization that appears to be attributable to a direct effect of heparin on neutrophils. MPO is a hemoprotein released by neutrophils, and to a lesser extent macrophages, that serves as a prominent pathway for leukocyte-mediated oxidation of lipids and nucleic acids. MPO-derived modifications have been observed in atherosclerotic plaque, and MPO levels are higher in patients with angiographically-documented coronary artery disease. In patients presenting with chest pain, plasma MPO levels have been shown to be an independent predictor of early myocardial infarction and major adverse
cardiac outcomes at 30 days and 6 months.12,13 Although our sample size was small, our results are in agreement with 2 other recent publications that indicate that heparin can increase MPO levels. Keating et al18 reported higher levels of MPO in blood obtained from the coronary ostium after administration of UFH/epitifibatide, and Baldus et al19 found elevated MPO levels in patients receiving UFH alone. The latter study demonstrated that heparin could release MPO from endothelial cells but did not examine effects on neutrophils.

Our studies with isolated neutrophils indicate that heparin and enoxaparin, but not bivalirudin, fondaparinux, or epitifibatide, stimulate MPO release. Thus, the use of heparin as adjunct therapy during PCI may account for higher levels of circulating MPO. Additionally, we observed that heparin promotes the binding of endogenously-released MPO to neutrophils. Recently, MPO binding to integrin αMβ2 (CD11b/CD18) was proposed to function in an autocrine feedback loop that activates neutrophils independently of catalytic activity of the enzyme.11 We observed that both heparin and MPO stimulated MMP-9 release and respiratory burst in neutrophils, suggesting that heparin may modulate the activation state of neutrophils via the cytokine-like effects of MPO.

The fact that the addition of activated platelets decreased MPO levels suggests that MPO may bind to platelet-stimulated neutrophils, platelet-neutrophil aggregates, or activated but not resting platelets. Pretreatment with either antibodies to CD40L, or epitifibatide blunted this effect, suggesting that the effect may be mediated by surface...
expression of CD40L on activated platelets and/or the activated conformation of GPIIb/IIIa.

The significance of transient elevations of plasma MPO levels after PCI is unclear, but there is reason to believe that MPO may be a mediator of cardiovascular disease. For example, MPO has recently been shown to contribute to adverse left ventricular remodeling after myocardial infarction in an animal model. A major unfavorable outcome of stent implantation is the development of intimal hyperplasia and clinical restenosis. In a recent report, brief treatment of rat carotid arteries with MPO and H₂O₂ elicited intimal hyperplasia through the production of hypochlorous acid. Our results indicate that both MPO and chlorotyrosine-adduct formation accumulate along arteries after endoluminal injury in a mouse model. Heparin increased levels of vessel-associated MPO. However, we failed to observe a statistically significant difference in intimal area in mice deficient in MPO. Although these results may reflect the lack of a role for MPO in the injury response process, they need to be interpreted cautiously, as our studies may have been underpowered to detect a small difference in vessel areas in normal and MPO-deficient animals. Additionally, murine neutrophils contain only a fraction of the amount of MPO found in their human counterparts. In a mouse model of atherosclerosis, lack of MPO did not attenuate lesion development; yet, transgenic mice expressing human MPO in macrophages have exaggerated atherosclerosis. Thus, differences in vascular cells and the injury response in mice may limit the extrapolation of our observations to humans.

We did not observe differences in platelet activation, as measured by platelet P-selectin expression or monoclonal antibody PAC-1 binding, in samples of peripherally-collected whole blood up to 1 week after PCI in patients who received UFH + eptifibatide as compared bivalirudin. These results are similar to those reported by Keating et al., although in their study, UFH + eptifibatide therapy was associated with elevated platelet surface expression of P-selectin and platelet-leukocyte aggregates in blood sampled from the coronary ostium before PCI. It is possible that the use of clopidogrel at the time of intervention blunts any effect of adjunctive therapy on markers of platelet activation, and it is likely that the gradual decline in agonist-induced activation of platelets that we observed over the 7 days after PCI was related to the administration of clopidogrel. In patients receiving UFH + eptifibatide, levels of soluble CD40L were lower immediately after the procedure, which would be consistent with the observation that eptifibatide blocks CD40L liberation from platelets.

As has been shown by others, our results indicate that PCI is associated with an acute inflammatory response in patients with stable coronary artery disease, as manifest by a rise in IL-6 and C-reactive protein (CRP) levels in the first 24 hours after the procedure. In other studies, use of the GPIIb/IIIa antagonists abciximab and eptifibatide has been associated with a postprocedural reduction in IL-6 and CRP values as compared with placebo, and a direct comparison of the two agents indicated an equivalent effect on postprocedural inflammatory markers. Our results in a small population of stable patients suggests that there may not be a substantial difference in the change in IL-6 and CRP levels postprocedure in patients receiving bivalirudin or UFH + eptifibatide as adjunctive antithrombotic therapy.

In conclusion, our results suggest that the use of bivalirudin or UFH + eptifibatide in patients with stable coronary artery disease undergoing PCI is associated with similar effects on general inflammatory and platelet markers. Use of UFH + eptifibatide may be associated with transient eleva-

Figure 3. MPO deposition and the development of intimal hyperplasia after arterial injury. Mice were subjected to endothelial denudation injury as described under Materials and Methods. A, Immunohistochemical staining with secondary antibody alone (control) or with antibodies to MPO or chlorotyrosine 6 hours after injury. B, Vessel-associated MPO detected as described in Materials and Methods by ELISA in samples from mice (n=4 per condition) treated without (control) or with heparin (200 U i.p.) or fondaparinux (200 µg, i.p.) administered 30 minutes before surgery. C, Representative CME-stained sections of vessels taken at 4 weeks injury from a wild-type and MPO-null mouse.
tions in MPO levels, perhaps as a direct consequence of actions of heparin on white blood cells. Larger clinical studies are required to understand the prognostic significance of transient elevations in MPO levels in this setting.

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Disclosures

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References


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**Supplemental Text and Figures for article by Li et al “Effects of Unfractionated Heparin and Glycoprotein IIb/IIIa Antagonists versus Bivalirudin on Myeloperoxidase Release from Neutrophils”**

**Supplemental Methods**

Flow cytometry.

For platelet analysis, blood was collected into 3.8% citrate and processed within 15 minutes of collection. P-selectin expression and activated GPIIb/IIIa, detected by binding of the activation-dependent monoclonal antibody PAC-1, were measured in blood without and with thrombin-receptor activating peptide (TRAP) stimulation. For these measurements, 450 μl of whole blood was incubated with vehicle or TRAP (15 μM) for 5 minutes prior to the addition of FITC-anti-P-selectin, FITC-PAC1, or FITC-isotype matched control (BD Biosciences, Mountain View, CA). Blood was incubated with antibodies for 30 min and then fixed with 1% paraformaldehyde. All samples were analyzed on a FacsCalibur flow cytometer (Becton Dickinson; Franklin Lakes, NJ) using CellQuest software. For P-selectin expression and PAC1 binding, 10,000 platelet events were acquired and the mean specific fluorescent intensity and the percentage of positive platelets were reported after subtracting non-specific binding defined by using isotype-matched control antibodies. As might be expected, at each time point, the mean platelet fluorescent intensity for individual activation markers significantly correlated with the percentage of platelets expressing the activation marker.

Platelet-leukocyte aggregates were detected by incubation of 5 μl of whole blood with 10 μg/ml FITC-anti-CD42a and 10 μg/ml of PE-anti-CD45 in a total volume of 100 μl for 30 minutes at room temperature at which time blood was fixed with 1% paraformaldehyde. Two-color flow cytometric analysis for platelet-leukocyte aggregates was performed at previously described 11 and values reported as percent of leukocytes with attached platelets (as detected by coincident green fluorescence).

Soluble markers.

Whole blood was collected into vacutainer clotted tubes, and serum prepared by centrifugation (3000g for 10 min). Serum was prepared within 10 min of blood collection and stored in aliquots at -80°C until used in ELISAs for tissue factor (TF; American Diagnostica), MPO (American Laboratory
Product Company), ICAM-1 (R&D systems), IL-6 (R&D Systems), and C-reactive protein (CRP) following manufacturer’s instructions using Luminex technology.

RANTES and sCD40L assays were performed on serum prepared from platelet-poor plasma prepared by centrifugation of citrate-anticoagulated whole blood. The plasma was additionally spun at 10,000g for 10 min to remove in residual platelets. The resulting platelet-poor-plasma was adjusted to 10 mM CaCl$_2$ and incubated with 5 units of thrombin (Enzyme research Labs, South Bend, IN) for 15 min at room temperature. The samples were centrifuged, and the resulting serum used in RANTES (Biosource International Inc.; Camarillo, CA) and sCD40L (Alexis Biochemicals; San Diego, CA) ELISAs according to manufacturer’s directions.

**Platelet-Neutrophil Co-Aggregate Assay**

Blood was collected into 1/10 volume of 3.8% citrate from healthy donors who had abstained from any anti-platelet drugs for one week in accordance with guidelines of the Institutional Review Boards of the University of North Carolina and the University of Kentucky. The blood was centrifuged (150 g x 20 min) to prepare platelet-rich plasma (PRP). Platelets were isolated from the PRP by readjusting it with ACD and centrifuging at 150g for 20 min. The resulting platelet pellet was resuspended in CGS (120 mM NaCl, 30 mM trisodium citrate, and 30 mM dextrose, pH 7.0) containing 134 nM PGI$_2$ and washed twice by centrifugation (1800 g x 10 min). After the last centrifugation, the washed platelets were resuspended in Tyrodes buffer (138mM NaCl, 2.7mM KCl, 0.4mM NaH$_2$PO$_4$, 12mM NaHCO$_3$, 10mM Heps 0.1% glucose pH 7.4) containing 0.35% bovine serum albumin. Neutrophils were isolated from the remaining blood by Dextran sedimentation followed by Ficoll-Hypaque gradient and hypotonic lysis of erythrocytes using sterile technique. The neutrophils were washed and resuspended in cold Tyrode’s buffer. Immediately before use, MgCl$_2$ and CaCl$_2$ were added to the suspension of neutrophils to bring the concentration of both divalent ions to 1 mM.

Neutrophils (2 x 10$^7$/ml) alone or with soluble P-selectin (R&D Systems, Minneapolis, MN) or with platelets (2 x 10$^8$/ml) were incubated in a final volume of 400 µL in siliconized glass tubes placed in an aggregometer (Platelet Ionized Calcium Aggregometer, PICA, ChronoLog, Haverton, PA) at 37°C and stirred at 1,000 revolutions per minute. Although the shear rate produced by this stirring speed will
vary throughout the cuvette, it should be approximately 250 seconds⁻¹. After five minutes, the suspension containing the neutrophils was transferred to an eppendorf tube and spun at 11,000 g for 1 min at 4°C. The supernatant was removed, and MPO in the supernatant was measured by ELISA (Assay Designs, Ann Arbor, MI). A standard curve of purified MPO was used to convert absorbance readings to values in ng/ml.

**MPO Binding to and Activation of Neutrophils**

To detect MPO binding, neutrophils were suspended in PBS containing 0.5 mM MgCl₂, 1 mM CaCl₂, and 1 mg/ml glucose and stimulated with 10 IU/ml UFH or 100 nM phorbol ester (as a positive control) for 45 min at 37°C in a 5% CO₂ incubator. 10⁶ cells were incubated (30 min at 4°C) with PE-conjugated anti-MPO (a gift kindly provided by Dr. Lau from University Hospital Hamburg-Eppendorf, Germany); and FITC-conjugated anti-CD66b antibody (BD Pharmingen). Isotype-matched mouse IgG served as control. Two-color flow cytometric analysis on 10,000 events was performed by using a FACSCalibur flow cytometer with Cellquest software as previously described. The percentage of CD66b positive cells was assessed after correction for the percentage of cells that were reactive by using an isotype-matched control.

Neutrophils (5x10⁵ cells in 400 μl) were stimulated with indicated reagents for 45 min at room temperature. The cells were centrifuged and supernatants collected and stored at -70°C until analysis. MMP9 and elastase was measure by ELISA (R&D Systems and AXXORA, LLC, respectively) following the manufacturers’ directions. PMN respiratory burst was measured as previously described by flow cytometry using dihydrorhodamine 123 (DHR 123). Briefly, isolated neutrophils were suspended in Hanks Balanced Salt Solution (HBSS) containing 0.25% BSA. Aliquots of PMNs (2x10⁶ cells in 200 μl) were loaded with 1 μM DHR123 (Molecular Probes) for 5 min at 37°C, and then stimulated with 1.0 μg/ml MPO (Calbiochem) or 100 nM PMA (Sigma Chemical Co.) for 30 min at 37°C. Reagent blank tubes were prepared by adding only vehicle HBSS to the cell suspension. Samples were analyzed immediately by flow cytometry.

**Mouse Model of Arterial Injury**

Wild-type C57BL/6 and MPO-deficient (MPO-/-) mice were the generous gift of Dr. Charles
Jennette (Department of Pathology, the University of North Carolina) and bred in the Department of Laboratory Animal Medicine facility at the University of North Carolina. Mice were weaned at 14 days, maintained on a 12 h light and 12 h dark cycle, and fed water and standard rodent chow (Isopro 300; Purina Mills, Richmond, IN) *ad libitum*. All procedures conformed to the recommendations of "Guide for the Care and Use of Laboratory Animals" (Department of Health, Education, and Welfare publication number NIH 78-23, 1996) and were approved by the University of North Carolina and the University of Kentucky’s Institutional Animal Care and Use Committees.

Endothelial denudation of mouse femoral arteries was performed as previously described. In brief, mice were anesthetized with inhaled isoflurane (~ 1 – 3% mixed with oxygen) and viewed under a Zeiss OPMI 6S surgical dissecting scope. The femoral artery was exposed, and a 0.014” (0.36mm) diameter angioplasty guide wire (Hi torque CROSS-IT 200XT guide wire; Guidant; Indianapolis, IN) was introduced into the arterial lumen through an arteriotomy made in the distal portion of the vessel. The guide wire was advanced to the level of the aortic bifurcation and pulled back three times. Animals were euthanized at various times by perfusion with 60 ml of 4% paraformaldehyde in PBS at 220ml/hour administered with a syringe pump via a cannula in the left ventricle. Hindlimbs were excised en bloc, fixed, and decalcified. The blood vessel was processed for histology by embedding in paraffin a 5 mm segment of the limb containing the femoral artery from the inguinal ligament to its division into the epigastric artery. Multiple serial sections were taken at 400 μm intervals and stained with combined Masson’s elastin stain or processed for immunohistochemistry using antibodies to MPO (Dako) or chlorotyrosine (Cell Science, Inc). The following measurements were made from digitized images using Image J software: lumen area, area beneath the internal elastic lamina (IEL), and area beneath external elastic lamina (EEL). Intimal area was derived from IEL – lumen areas and medial area from EEL – IEL areas. For each vessel, average areas on five serial sections spanning 2 mm of the injured vessel was calculated.

To measure MPO activity, mice were perfused with PBS/0.2% BSA at 37°C. After removing fat and connective tissues, the arteries were weighed and homogenized in lysis buffer (200 mM NaCl, 2 mM EDTA, 10 mM Tris, pH 7.4, 10% glycine) containing protease inhibitors (Roche Applied Science).
The homogenate was centrifuged (1500 g for 15 min) to remove cell debris, and MPO activity in the supernatant was measured by ELISA (Hycult Biotechnology). Data were normalized to wet weight of arterial sample.
Supplemental Figure I. Platelet activation markers in peripherally-collected whole blood in patients before and at times up to one week after elective PCI with stent implantation.

Monoclonal antibody PAC-1 binding and P-selectin expression were detected by flow cytometry and soluble CD40L levels were determined by ELISA assay as described in Materials and Methods.

Spearman correlation coefficients indicated that P-selectin expression as detected by mean fluorescence intensity (MFI) and percent of positive platelets were highly correlated (p = <0.0001); thus, only MFI results are reported. A similar correlation was also observed with PAC-1 binding as detected by MFI and percent of positive platelets (p<0.0001). Soluble CD40L (sCD40L) levels were significantly lower in the UFH + eptifibatide group immediately after the procedure (p = 0.024).
Supplemental Figure II. Effect of isolated platelets on myeloperoxidase (MPO) release from isolated neutrophils. (A) Isolated neutrophils were co-incubated with buffer, resting platelets (PGI₂-treated; dark bars) or activated platelets (TRAP-treated; light bars) and soluble MPO detected by ELISA as described in Materials and Methods. Bivalirudin (2.5 μg/ml) or eptifibatide (1 – 60 μg/ml) was included in the reaction, as indicated. (B) Isolated neutrophils were incubated with buffer (dark bars) or activated platelets (TRAP-treated; light bars) in the presence of vehicle, isotype-matched control antibody, or anti-CD40L antibody (20 μg/ml) and soluble MPO detected by ELISA. Combined results from three separate experiments are graphed as mean ± SD.