Comparison of Platelet Function and Morphology in Patients Undergoing Percutaneous Coronary Intervention Receiving Bivalirudin Versus Unfractionated Heparin Versus Clopidogrel Pretreatment and Bivalirudin

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We hypothesized that direct thrombin inhibition could attenuate platelet activation and release of soluble CD40 ligand (sCD40L), a marker of inflammation, during percutaneous coronary intervention (PCI). To assess platelet function under flow conditions with bivalirudin versus unfractionated heparin (UFH), we employed the cone and plate(let) analyzer (CPA) assay in drug-spiked blood samples from volunteers (n = 3) in vitro, and then in PCI patients who received bivalirudin alone (n = 20), UFH alone (n = 15), and clopidogrel pretreatment plus bivalirudin (n = 15). Scanning electron microscopy was employed to image bivalirudin or UFH-treated platelets to determine whether platelet function observations had a morphologic explanation. Enzyme immunoassay was used to measure sCD40L levels in PCI patients. In vitro, bivalirudin decreased platelet surface coverage; UFH increased platelet surface coverage. In PCI patients, bivalirudin alone decreased platelet surface coverage, UFH alone increased platelet surface coverage, and clopidogrel pretreatment plus bivalirudin additively reduced platelet surface coverage. Unlike UFH, bivalirudin did not activate platelets in SEM studies. Bivalirudin alone or coupled with clopidogrel significantly reduced plasma sCD40L in PCI patients. In conclusion, our findings suggest that under flow conditions, bivalirudin alone or coupled with clopidogrel may have an antiplatelet effect versus UFH alone during PCI. These data suggest that bivalirudin and UFH may confer an anti-inflammatory effect by reducing sCD40L during PCI. © 2007 Elsevier Inc. All rights reserved. (Am J Cardiol 2007;100:417–424)

Methods

Patient population: The institutional review board of Mount Sinai Hospital (New York, New York) approved all study protocols. All participating patients undergoing cardiac catheterization or staged PCI and volunteers provided informed consent.

Exclusion criteria included acute myocardial infarction within 24 hours, active internal bleeding, recent (within 6 weeks) gastrointestinal or genitourinary bleeding of clinical significance, bleeding diathesis, history of cerebrovascular accident within 1 year or with a residual neurologic deficit, recent (within 6 weeks) major surgery or trauma, intracranial neoplasm, arteriovenous malformation or aneurysm, severe uncontrolled hypertension >180/100 mm Hg or severe hypotension on enrollment to the study. Administration of a thrombolytic, dextran, oral adenosine diphosphate receptor inhibitor, or oral anticoagulant within 24 hours, hypersensitivity to aspirin or the study drugs or their compo-
nents, baseline prothrombin time >1.2 times control, or <100,000 platelets/μl excluded potential patients. Patients treated with UFH or low-molecular-weight heparin were excluded. Patients with a baseline activated clotting time ≥160 seconds were excluded because an activated clotting time above this level was likely due to a residual anticoagulant effect that could confound analysis of the effect of bivalirudin on platelet function and sCD40L. Patients after coronary artery bypass surgery who underwent diagnostic angiography and required heparin were excluded. Because bivalirudin is excreted predominantly renally, patients on dialysis and/or with a creatinine level ≥3.0 mg/dl were excluded.

Fifty patients were enrolled in this prospective, nonrandomized observational study. Of the 50, 35 PCI patients received an intravenous 0.75 mg/kg bolus followed by a 1.75 mg/kg/hour infusion of bivalirudin. Of the 35 patients in the bivalirudin cohort, 15 received clopidogrel before treatment (300 mg) ≥3 hours before bivalirudin administration, leaving 20 patients who received bivalirudin alone. The UFH cohort consisted of the remaining 15 PCI patients who received UFH 40- to 50-U/kg bolus.

Adjunct glycoprotein (GP) IIb/IIIa inhibitor was administered at the operator’s discretion to all patients receiving UFH and 9 of 20 patients receiving bivalirudin alone after all study blood sampling was completed. Those patients receiving clopidogrel before treatment and bivalirudin were not administered any GP IIb/IIIa therapy, at the operator’s discretion.

All patients who underwent PCI received aspirin 325 mg before the procedure. In those patients receiving bivalirudin or UFH alone, the clopidogrel 300-mg oral loading dose was given after the second blood sample was collected.

**Blood samples:** In all blood sample collections, the initial 2 ml of blood was discarded and gently aspirated to limit artifactual activation of platelets. Two blood samples were collected from the arterial access sheath in sodium citrate Vacutainers (Becton Dickinson, Franklin Lakes, New Jersey) at baseline and 5 minutes after administration of bivalirudin or UFH. Patients receiving periprocedural clopidogrel plus bivalirudin had a baseline blood sample collected before clopidogrel administration by venipuncture of the antecubital vein and then immediately after arterial sheath insertion (≥3 hours after clopidogrel administration) but before bivalirudin administration. Another blood sample was then collected 5 minutes after administration of bivalirudin. Blood samples were centrifuged at 2,000 g for 15 minutes at 22°C and platelet-poor plasma was stored at −70°C. Whole blood for the in vivo cone and plate(let) analyzer (CPA) assay and plasma sCD40L quantification were obtained from the same PCI patients.

**Platelet function assessment:** The dose-response study blood samples were procured from 3 volunteers (2 men and 1 woman, mean age 25 ± 1 years). Blood (4 ml) was collected into citrate tubes by venipuncture from each medication-free volunteer. Bivalirudin 5 mg/ml was diluted in saline and added to aliquots of whole blood, resulting in final concentrations of 1,000, 12, 0.022, and 0.0067 μg/ml. These concentrations demonstrated platelet function with the therapeutic steady-state concentration (12 μg/ml) achieved during PCI compared with extremely high and low bivalirudin concentrations. Using the same volunteers’ blood, UFH 5,000 U/ml was diluted in saline and added to aliquots of whole blood, resulting in final concentrations of 10, 1, 0.5, and 0.1 U/ml.

To extend our in vitro platelet function observations into the clinical domain, we employed the CPA assay in PCI patients. Citrated whole blood (200 μl) was aliquoted into plates consisting of 4 separate polystyrene wells (Nalge Nunc International, Roskilde, Denmark) and subjected to a shear rate of 1,875 s⁻¹ with a rotating Teflon cone for 2 minutes. Each well was washed 4 times with 2 ml of distilled water, stained with diluted May-Grünwald stain (2:1 v/v in distilled water) for 1 minute, and washed again with ~1 ml of distilled water.

Computerized planimetric measurements of each well plate were performed at 10-fold magnification by an investigator blinded to treatment assignments. Images were digitized with a Sony DKC-5000 camera (Tokyo, Japan) using Adobe Photoshop 4.0 (San Jose, California) on a Power Macintosh 8500 computer (Cupertino, California). Platelet surface coverage, expressed as percent platelet surface coverage, represents the duality of platelet function: platelet adhesion and aggregation. Platelet surface coverage was assessed in the 4 quadrants of each well (4 wells per plate = 16 quadrants per plate), allowing for a minimum of 4 and maximum of 16 measurements per treatment, using NIH Image 1.6 software (National Institutes of Health, Bethesda, Maryland). The mean of these measurements determined the platelet surface coverage for each treatment. Results were expressed as percent platelet surface coverage.

**Electron microscopic analysis of platelet morphologic change:** Scanning electron microscopy was employed to determine whether bivalirudin or UFH could induce platelet morphologic changes. Whole blood from healthy volunteers was mixed with acid citrate-dextrose solution (6:1, vol/vol, pH 5.1) containing 1 mM theophylline and 1 μg/ml prostaglandin E₁. Platelets were isolated by differential centrifugation at 210g for 15 minutes. These platelets were washed 1 time in calcium and albumin-free Tyrode solution (pH 6.2) containing 1 U/ml apyrase. Washed platelets were resuspended in albumin-free Tyrode solution containing 2 mM calcium chloride and 1 mM magnesium chloride (pH 7.2) using polystyrene tubes (Becton Dickinson). Resuspended platelets (0.9 ml at a concentration of 250,000 platelets/μl) were then incubated with final anticoagulant concentrations of 12 μg/ml bivalirudin or 0.1, 0.5, and 1.0 U/ml UFH. After a 30-minute room temperature incubation, samples were fixed with 3% glutaraldehyde. A drop of the fixed platelet suspension was placed on a glass coverslip coated with Sta-On (Surgipath Medical Industries, Inc., Richmond, Illinois). Samples were then placed in 0.2 M sodium cacodylate (pH 7.3) to prevent specimen drying. These fixed samples were washed with sodium cacodylate for 10 minutes and then washed with 1% osmium tetroxide for 1 hour. Samples were then sputter-coated with platinum and observed.
under a Hitachi S530 scanning electron (Tokyo, Japan) microscope.

**Detection of sCD40L:** Quantification of sCD40L in plasma from PCI patients was performed to evaluate whether bivalirudin conferred an anti-inflammatory effect compared with UFH. Duplicate experiments were performed with an enzyme immunoassay (R&D Systems, Minneapolis, Minnesota) according to the manufacturer’s instructions.

**Statistical analysis:** All subjects and patients served as their own control (before vs after treatment). Percent change from baseline in platelet surface coverage, in vitro and by treatment cohort, was first tested for normal distribution and then analyzed using paired t tests. Plasma sCD40L was tested for normal distribution and compared between before and after treatment within each treatment cohort using the Wilcoxon signed-rank test. Patient demographics were compared across the 3 treatment cohorts. Continuous variables were analyzed using the analysis of variance model with treatment cohort as the factor. Categorical data were analyzed using chi-square test. A p value <0.05 was considered statistically significant. Data are presented as mean ± SEM.

**Results**

**Study population:** Fifty patients undergoing coronary angiography and/or PCI were enrolled in the study. Study population demographics are listed in Table 1.

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**Table 1**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Bivalirudin Alone (n = 20)</th>
<th>UFH Alone (n = 15)</th>
<th>Clopidogrel + Bivalirudin (n = 15)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yrs)</td>
<td>61 ± 13</td>
<td>62 ± 9</td>
<td>69 ± 14</td>
<td>NS</td>
</tr>
<tr>
<td>Men</td>
<td>12 (60%)</td>
<td>10 (67%)</td>
<td>8 (53%)</td>
<td>NS</td>
</tr>
<tr>
<td>Statins</td>
<td>10 (50%)</td>
<td>8 (53%)</td>
<td>10 (67%)</td>
<td>NS</td>
</tr>
<tr>
<td>Angiotensin II type 1 receptor blocker</td>
<td>1 (5%)</td>
<td>4 (27%)</td>
<td>3 (20%)</td>
<td>NS</td>
</tr>
<tr>
<td>Angiotensin-converting enzyme inhibitor</td>
<td>6 (30%)</td>
<td>6 (30%)</td>
<td>8 (53%)</td>
<td>NS</td>
</tr>
<tr>
<td>Platelets (1,000/U/L)</td>
<td>259</td>
<td>219</td>
<td>256</td>
<td>NS</td>
</tr>
<tr>
<td>Hypertension*</td>
<td>16 (80%)</td>
<td>13 (87%)</td>
<td>15 (100%)</td>
<td>0.01</td>
</tr>
<tr>
<td>Hyperlipidemia†</td>
<td>16 (80%)</td>
<td>10 (67%)</td>
<td>12 (75%)</td>
<td>NS</td>
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<td>Smoker</td>
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<td>4 (27%)</td>
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<tr>
<td>Diabetes mellitus</td>
<td>7 (35%)</td>
<td>8 (53%)</td>
<td>5 (33%)</td>
<td>NS</td>
</tr>
<tr>
<td>Previous myocardial infarction</td>
<td>4 (20%)</td>
<td>4 (27%)</td>
<td>3 (20%)</td>
<td>NS</td>
</tr>
<tr>
<td>Previous coronary artery bypass graft</td>
<td>2 (10%)</td>
<td>3 (20%)</td>
<td>2 (13%)</td>
<td>NS</td>
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<tr>
<td>Previous PCI</td>
<td>7 (35%)</td>
<td>10 (67%)</td>
<td>5 (33%)</td>
<td>NS</td>
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<tr>
<td>Left anterior descending coronary artery</td>
<td>8 (40%)</td>
<td>8 (53%)</td>
<td>14 (93%)</td>
<td>0.01</td>
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<tr>
<td>Left circumflex coronary artery</td>
<td>8 (40%)</td>
<td>4 (27%)</td>
<td>3 (20%)</td>
<td>NS</td>
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<td>Right coronary artery</td>
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<td>NS</td>
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<td>Saphenous vein graft</td>
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<td>2 (13%)</td>
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<td>NS</td>
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<td>2 (13%)</td>
<td>0</td>
<td>NS</td>
</tr>
<tr>
<td>Intracoronary stent</td>
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<td>10 (67%)</td>
<td>4 (27%)</td>
<td>NS</td>
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<tr>
<td>Drug-eluting stent</td>
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<td>0</td>
<td>13 (87%)</td>
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<tr>
<td>Percutaneous transluminal coronary angioplasty</td>
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<td>8 (53%)</td>
<td>2 (13%)</td>
<td>NS</td>
</tr>
<tr>
<td>Percutaneous rotational coronary atherectomy</td>
<td>1 (5%)</td>
<td>0</td>
<td>2 (13%)</td>
<td>NS</td>
</tr>
<tr>
<td>Cutting balloon</td>
<td>2 (10%)</td>
<td>5 (33%)</td>
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<td>0.03</td>
</tr>
<tr>
<td>Intracoronary brachytherapy</td>
<td>0</td>
<td>2 (13%)</td>
<td>0</td>
<td>NS</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SD or number of patient (percentage).

* Blood pressure >140/90 mm Hg on ≥2 occasions or if patient was already receiving antihypertensive medication.

† Use of lipid-lowering drugs or history of total cholesterol >240 mg/dl.

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Bivalirudin mediates in vitro dose-dependent inhibition of platelet function: Bivalirudin demonstrated a significant dose-dependent decrease in platelet surface coverage (Figure 1) in healthy volunteers. Notably, 1,000 μg/ml (high concentration) decreased platelet surface coverage to 42 ± 8% (p = 0.02) and 12 μg/ml (steady-state concentration) decreased platelet surface coverage to 34 ± 3% (p = 0.05) compared with controls. In comparison, UFH demonstrated an increase in platelet function; 10 and 1 U/ml UFH led to 17 ± 5% (p = 0.05) and 32 ± 11% (p = 0.04) increases in platelet surface coverage, respectively, compared with controls (Figure 1).

**Bivalirudin inhibits platelet function in vivo:** In 20 PCI patients, treatment with bivalirudin resulted in 71 ± 5% platelet surface coverage compared with baseline (n = 464 images, p <0.001; Figures 2 and 3).

**UFH treatment leads to platelet aggregation and adhesion in vivo:** In 15 PCI patients, UFH demonstrated 129 ± 8% platelet surface coverage compared with baseline (n = 372 images, p = 0.004; Figures 2 and 3).

**Bivalirudin augments clopidogrel-dependent antiplatelet effects in vivo:** Mean temporal length of clopidogrel pretreatment was 4.7 ± 0.8 hours in this 15-patient PCI cohort. Clopidogrel significantly decreased platelet surface coverage to 77 ± 4% compared with baseline (p = 0.001; Figures 2 and 3). Bivalirudin administration demonstrated an additive effect in further decreasing platelet
surface coverage to 60 ± 5% compared with baseline (n = 597 images, p <0.0001; Figures 2 and 3); this was a 17 ± 4% platelet surface coverage decrease from the clopidogrel time point (p = 0.0008).

**Morphologic analysis of bivalirudin- or UFH-treated platelets by scanning electron microscopy:** Platelets incubated with bivalirudin did not demonstrate ultrastructural change consistent with platelet activation; platelets remained in a discoid state reflecting platelet inactivation (Figure 4). In contrast, UFH-treated platelets lost their normal discoid shape at rest and formed pseudopodia extending from the platelet central body, a morphology associated with platelet activation (Figure 4). UFH-treated platelets also demonstrated microparticle release in the local environment of the activated platelet (Figure 4).

**Bivalirudin decreases sCD40L in vivo:** Bivalirudin significantly decreased plasma levels of sCD40L from 509 ± 93 to 303 ± 42 pg/ml compared with baseline (p = 0.002) in those patients receiving only bivalirudin (Figure 5). UFH treatment decreased release of sCD40L from 270 ± 81 to 186 ± 24 pg/ml (p = NS; Figure 5). Periprocedural clopidogrel significantly decreased sCD40L from 405 ± 69 to 339 ± 85 pg/ml compared with baseline (p <0.001; Figure 5). Bivalirudin administration in patients pretreated with clopidogrel further decreased sCD40L to 245 ± 81 pg/ml compared with the clopidogrel time point (p <0.001; Figure 5); this was equal to a 96 ± 4 pg/ml decrease in sCD40L from the clopidogrel time point (p = 0.007).

**Discussion**

This prospective study analyzed the effects of bivalirudin on platelet function and sCD40L in the setting of PCI. Our findings suggest that bivalirudin may confer antiplatelet and anti-inflammatory effects during PCI for the
following reasons. First, under flow conditions, bivalirudin alone or coupled with periprocedural clopidogrel significantly decreased platelet surface coverage compared with UFH in patients undergoing PCI. Second, in platelet morphology studies, bivalirudin did not activate platelets, whereas UFH treatment resulted in marked activation (pseudopodia). Third, during PCI bivalirudin demonstrated an ability to significantly abrogate sCD40L levels and further decreased sCD40L levels after clopidogrel pretreatment.

Platelets play a paramount role in the development of acute thrombosis and its subsequent clinical sequelae. In contrast to previous reports using conventional agonist-mediated platelet aggregation to assess therapeutic antiplatelet effects, the CPA proffered an agonist-free, shear-induced flow environment, whereby the platelet surface coverage observed had physiologic relevance akin to that seen in a coronary artery. One mechanism for the decrease seen in platelet surface coverage by bivalirudin may be abrogation of thrombin-mediated endothelial-derived von Willebrand factor, thus decreasing circulating von Willebrand factor for soluble von Willebrand factor/GP Ib platelet adhesion. Another plausible reason is that bivalirudin, by inhibiting thrombin directly, may attenuate human protease-activated receptor-mediated activation of platelets and, hence, disable platelet transmembrane signaling by uncoupling the receptor from its intramolecular ligand. These postulates are congruent with studies demonstrating a decrease in platelet aggregation and platelet transmembrane calcium flux as a result of thrombin or protease-activated receptor inhibition.

In our study, UFH-treated patients exhibited significant augmentation in platelet surface coverage, and therapeutic-range UFH-treated platelets underwent a morphologic change consistent with platelet activation. These findings are in keeping with previous studies demonstrating platelet activation and aggregation after UFH treatment. One proposed mechanism for UFH-mediated platelet aggregation involves the notion of an integrin-dependent process, i.e., UFH binds the GP IIb/IIIa receptor and induces Rap2B translocation to the cytoskeleton. Further, this study showed enhanced platelet fibrinogen binding after UFH...
treatment, suggesting that UFH not only initiates signal transduction but also a functional conformational change consistent with GP IIb/IIIa activation. Our findings coupled with those of previous studies may necessitate further examination of the UFH’s molecular mechanisms with regard to platelet activation.

In our investigation, clopidogrel demonstrated an antiplatelet effect that was further enhanced by bivalirudin. Due to the necessity for adequate platelet inhibition and the ubiquitous practice of periprocedural clopidogrel loading during contemporary PCI, delineating the mechanism underlying this additive effect is important. The activity of the active metabolite of clopidogrel, which irreversibly antagonizes the platelet P2Y12 adenosine 5’-diphosphate receptor probably accounted for the antiplatelet effect under the shear stress observed and is confirmed by a previous report.19 Adding to the antiplatelet effect demonstrated by clopidogrel, bivalirudin significantly decreased platelet surface coverage. It is quite possible that direct thrombin inhibition in addition to P2Y12 receptor blockade suppresses platelet activation bifactorially, i.e., by blocking adenosine diphosphate-mediated stimulation initially and then by abrogating thrombin-mediated protease-activated receptor activation.

Intriguingly, bivalirudin alone or after periprocedural clopidogrel significantly decreased, whereas UFH numerically decreased plasma sCD40L. This finding is consistent with the notion that attenuating platelet activation by direct thrombin inhibition and, hence, platelet-associated CD40L expression results in lower levels of sCD40L. In addition, UFH has antithrombin activity that may have contributed to the observed decrease in sCD40L levels. Incongruent to our findings of significant early decreases in sCD40L, a recent study reported prothrombin fragment 1 + 2 and sCD40L trending lower and interleukin-1 receptor antagonist increasing after PCI.20 These differences may be explained by baseline blood-draw timing. Baseline blood was collected after coronary angiographic heparinization and cohort treatment initiation. It can be postulated that the delta change described in this study reflects a lower baseline value due to antithrombin and/or antiplatelet effects; therefore, although

Figure 4. Representative scanning electron photomicrographs of platelet morphologic change. Samples were derived from healthy volunteers. (A) A normal platelet at rest and (B) a platelet treated with 12 μg/ml bivalirudin. Note that there is no presence of pseudopodia extrusions. (C) Platelets treated with increasing concentrations of UFH. Platelet activation is demonstrated with UFH treatment by loss of the normal discoid shape and formation of distinct pseudopodia. (D) Release of platelet microparticles (arrows) distinct from the activated platelet body after UFH treatment. All platelet samples were treated with their respective antithrombin agent for 30 minutes at 37°C. Acquisition of platelet scanning electron photomicrographs was done by an investigator blinded to treatment group. All scanning electron photomicrographs were acquired at 4,000× magnification and are representative of 2 experiments performed.
a decrease/increase was noted, it was not significant due to a small delta change.

Of note, baseline values of sCD40L for the bivalirudin cohorts and the UFH cohort were markedly different. One plausible explanation for this may be platelet count differences (Table 1). Although the difference in platelet count across cohorts was statistically nonsignificant, the lower platelet count in the UFH cohort could have partly contributed to the relatively low baseline sCD40L reported. This notion is consistent with recent evidence that suggests platelet count is correlated with sCD40L.21

There are several limitations associated with this study. First, the cohort population was small and nonrandomized. Second, we were unable to formulate a soluble, therapeutic form of clopidogrel and did not perform scanning electron microscopic studies of clopidogrel pretreated plus bivalirudin platelets. Third, bivalirudin effects on inflammatory markers released upon endothelial CD40 engagement (i.e., soluble interleukin-1, etc.) were not investigated, which would have allowed determination of bivalirudin’s as an anti-inflammatory agent. Fourth, we did not assess quantitative or qualitative measurements of smoking, which would have allowed examination of the effect smoking has on platelets in relation to antithrombin and antiplatelet treatments.

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11. Goto S, Salomon DR, Ikeda Y, Ruggeri ZM. Characterization of the unique mechanism mediating the shear-dependent binding of soluble

Figure 5. In vivo quantification of plasma sCD40L in patients after PCI. Plasma sCD40L was (A) significantly decreased after bivalirudin administration (n = 20), (B) numerically decreased by UFH administration (n = 14), and (C) significantly decreased after clopidogrel pretreatment and bivalirudin administration (n = 14). Data are presented as mean ± SEM. *p <0.05 versus baseline; **p = 0.007 versus clopidogrel time point.


