Bivalirudin decreases NO bioavailability by vascular immobilization of myeloperoxidase†

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Running title: Bivalirudin deteriorates endothelial function.

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Abbreviations: ACE, angiotensin converting enzyme; AT, angiotensin; BAEC, bovine aortic endothelial cells; BMI, body mass index; ESI-LC-MS, electrospray ionization liquid chromatography mass spectrometry; FMD, flow-mediated dilation; HBSS, Hanks' balanced salt solution; HDL, high density lipoprotein; IR, interquartile range; LDL, low density lipoprotein; MPO, myeloperoxidase; MS, mass spectrometry; MRM, multiple reaction monitoring, NO, nitric oxide, TMB, tetramethylbenzidine

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ABSTRACT

Bivalirudin - a direct thrombin inhibitor - has emerged as an important alternative to heparin in patients undergoing percutaneous coronary intervention. However, it remains elusive if potentially adverse extra-coagulant properties are responsible for the fact that its favorable effects in clinical studies are mainly driven by a reduction in bleeding events. The aim of the current study was to determine the effects and mechanisms of acute treatment with bivalirudin in comparison to heparin on NO bioavailability, an important factor for the pathogenesis of ischemic events. In particular, we studied the interaction between bivalirudin and myeloperoxidase (MPO) a leukocyte-derived enzyme which consumes endothelial derived nitric oxide (NO) and modifies a variety of biological targets and thus affects the integrity of the vessel wall. In patients undergoing elective percutaneous coronary intervention bivalirudin in contrast to heparin exhibited a significant decrease in plasma MPO levels (p=0.03) accompanied by a deterioration of flow-mediated dilation (p=0.02), a surrogate for endothelial NO bioavailability. In vitro experiments revealed avid binding of bivalirudin to both bovine aortic endothelial cells (BAEC) and MPO. Methylation of bivalirudin carboxyl groups at the carboxyterminal end revealed the specific binding site of bivalirudin to MPO. Bivalirudin-facilitated binding of MPO to BAEC resulted also in functional changes in terms of increased NO consumption as well as enhanced MPO-mediated redox modifications. These results illustrate dichotomous extra-coagulant properties of heparins and thrombin inhibitors and suggest that bivalirudin acutely impairs endothelial NO bioavailability thereby underscoring the potentially critical role of MPO as a mediator of vascular function.
Introduction

The direct thrombin inhibitor bivalirudin is thought to display various advantages in the treatment of acute coronary syndromes and during percutaneous coronary intervention as compared to unfractionated heparin. Thus, bivalirudin in contrast to heparin is able to inhibit fibrin-bound thrombin by displacing fibrin from its binding site (Bates and Weitz, 2000). Furthermore, bivalirudin is not inhibited by platelet factor 4 which is released from activated platelets and binds with high affinity to heparin thereby abrogating its activity (Weitz and Crowther, 2002). It has also been shown that bivalirudin in contrast to heparin does not lead to formation of platelet-monocyte (Harding, et al., 2006) and platelet-leukocyte aggregates (Keating, et al., 2006).

The results of the ACUITY trial, a large scale clinical trial including patients with moderate to high risk acute coronary syndromes suggest that treatment with bivalirudin may indeed be superior to a regimen of heparin plus glycoprotein IIb/IIIa inhibitor (Stone, et al., 2007). However, the advantages observed concerning the net clinical end point in this trial were not explained by a reduction in ischemic events but by reduced rates in major bleeding (Stone, et al., 2007). In the REPLACE-2 trial which demonstrated that bivalirudin is not inferior to heparin plus glycoprotein IIb/IIIa inhibitor in patients undergoing urgent or elective percutaneous coronary intervention bivalirudin treatment again was shown to be accompanied with a lower rate of major bleeding but a trend towards an even higher rate of peri-procedural non-Q-wave-infarctions (Lincoff, et al., 2004). A recently published study in high risk patients with ST-segment elevation myocardial infarction who were treated with heparin plus glycoprotein IIb/IIIa or bivalirudin alone for primary percutaneous coronary intervention revealed similar findings; the observed reduced net adverse clinical events were due to a lower rate of major bleeding but not to a reduction in ischemic event- in contrast an increased rate of stent thrombosis with bivalirudin was observed.
in this trial (Stone, et al., 2008). Thus, the theoretical pharmacokinetic and pharmacodynamic advantages of bivalirudin do not translate into a clinical reduction in ischemic events. This could easily be explained by the fact that bivalirudin was compared to the combination of heparin and glycoprotein IIb/IIIa inhibitor, instead of, for example, to heparin alone. However, it can also not be excluded from the results of these studies that extra-coagulant effects of bivalirudin as reported herein may contribute to this observation.

Reduced vascular nitric oxide bioavailability and concomitant endothelial dysfunction due to oxidative stress has often been linked to initiation and progression of acute and chronic coronary artery disease (Valgimigli, et al., 2003; Warkentin, 2004). The beneficial clinical effects of several cardiovascular drugs – for example 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors (John, et al., 1998; Laufs, et al., 1998), ACE inhibitors (Mancini, et al., 1996), clopidogrel (Heitzer, et al., 2006), and AT1 receptor blockers (Sudano, et al., 2006) - have been in part attributed to their pleiotropic positive effects on increasing vascular NO-bioavailability via different mechanisms. In addition, our group has recently shown that unfractionated heparin acutely improves systemic NO-bioavailability as assessed by flow mediated dilation (Baldus, et al., 2006). As a possible mechanism for this phenomenon we found that heparin electrostatically interacts with myeloperoxidase (MPO) - an enzyme which is abundantly expressed by polymorphonuclear neutrophils and has been shown to decrease endothelial NO-bioavailability (Eiserich, et al., 2002) - and thus mobilizes the enzyme from the vessel wall (Baldus, et al., 2006).

The objective of the current study was to determine whether bivalirudin has also an impact on endothelial function in patients undergoing elective percutaneous coronary intervention and whether this impact might also be explained by electrostatic
interactions between bivalirudin and MPO as could be expected by its chemical structure.
Methods

Patients. The study was conducted in accordance with the principles outlined in the declaration of Helsinki, was approved by the Ethics Committee of Hamburg, and all patients gave written informed consent prior to enrolment. Thirty consecutive patients, electively admitted to undergo elective percutaneous coronary intervention and diagnosed for lesions suitable for percutaneous coronary intervention were included into this double-blinded randomized trial using unfractionated heparin and bivalirudin, respectively. Patients with acute coronary syndromes within one month prior to study entry, congestive heart failure, impaired renal function (creatinine > 2.0 mg/dl) and intake of long-acting anti-anginal medication and oral anticoagulant drugs were excluded. After a 12 hour overnight fast, patients received either unfractionated heparin (70 U/kg) or bivalirudin (0.75 mg/kg) in a randomized fashion. Blood samples were taken from each subject before and 15 minutes after administration of the study medication and plasma was frozen at –80°C until further analysis. All patients underwent assessment of flow-mediated dilation of the brachial artery prior to and 15 minutes after administration of study medication. Thereafter patients were transferred into the catheterization laboratory. A research nurse not involved into the study unblinded the study arm and reassessed the activated clotting time. The percutaneous coronary intervention itself - executed by an independent operator - was performed by continuation of either heparin or bivalirudin, respectively according to current guidelines (Smith, Jr., et al., 2006).

Vascular function testing. Ultrasound determination of flow-mediated dilation (FMD) was performed as previously described (Rudolph, et al., 2007b) according to expert guidelines proposed by the International Brachial Artery Reactivity Task Force (Corretti, et al., 2002). Briefly, a Siemens Sonoline G50 ultrasound system with 12 MHz linear array transducer was used to record 2D cine-sequences of the brachial
artery over 4 seconds at baseline and 1 minute after induction of reactive hyperemia by 5-minute cuff occlusion of the forearm. Mean flow velocity was assessed by pulsed wave Doppler with correction of insonation angle at baseline and peak hyperemic flow to calculate the flow ratio. Subjects were instructed not to eat, drink or smoke within 12 hours prior to testing. 2D sequences were analyzed using an edge detection software (Brachial Analyzer, Medical Imaging Applications LLC). The operators were blinded to patients’ treatment.

**Assessment of plasma MPO levels.** Plasma myeloperoxidase levels were determined by ELISA according to the manufacturer’s recommendations (CardioMPO Assay, Prognostix, Cleveland, Ohio, USA).

**Effect of bivalirudin on MPO content in matrix proteins and BAEC.** To assess the effect of bivalirudin on MPO binding to extracellular matrices, fibronectin (13 µg/cm², Sigma-Aldrich, Inc, St Louis, Mo, USA) was exposed to MPO (5 nmol/l, Planta Natural Products, Vienna, Austria) for 2 hours at room temperature in phosphate-buffered saline and co-incubated for 20 minutes with bivalirudin (4 µg/ml, Nycomed, Zurich, Switzerland) or heparin (5 U/ml). Subsequently, supernatants were collected and analyzed for MPO activity by tetramethylbenzidine (TMB)-assay (Sigma, St. Louis, MO, USA) and fibronectin was washed to remove non-adherent enzyme. Thereafter, matrix-adherent MPO was detected by Western blotting with the use of a polyclonal anti-MPO antibody (1:10000, Calbiochem, EMD Biosciences, Inc, Merck KGaA, Darmstadt, Germany) and enhanced chemiluminescence used for detection.

Additionally, bovine aortic endothelial cells (BAEC, purchased from Clonetics, passage 4-8, grown to confluence on 6-well plates) were exposed to MPO (13 nmol/l, 2 hours at room temperature in Hanks’ balanced salt solution [HBSS]) and co-incubated with bivalirudin (0, 5 and 15 µg/ml). After 15 minutes supernatant was
collected and stored on ice, cells were washed twice with HBSS to remove non-adherent enzyme, scraped in 200 μl of lysis buffer (100 mmol/l NaH₂PO₄, 0.01% Triton X-100 containing 100 μl protease inhibitor [Protease Inhibitor Cocktail Set 1 Calbiochem EMD Biosciences, Inc, Merck KGaA, Darmstadt, Germany]), sonicated and stored on ice until analysis. Finally, MPO activity was assessed in supernatant and cells by TMB assay.

**Electrospray ionization liquid chromatography mass spectrometry analysis (ESI-LC-MS).** In order to demonstrate the capacity of bivalirudin to bind to endothelial cells an ESI-LC-MS method was developed for quantification of bivalirudin using a hybrid triple-quadrupole linear ion-trap mass spectrometer (Applied Biosystems/MDS Sciex, Thornhill, Canada). Two major species of bivalirudin having m/z of 1090.5 and 727.4 were detected by MS analysis, corresponding to the double (M+2H) and triple (M+3H) charged peptide, respectively. EPI analysis revealed the fragmentation pattern shown in Supplemental Figure 1A and 1B. The MRM transition 1090.5/356.3 was used for quantification because this transition provided the most consistent results in cell culture samples. The peptide eluted after 3.56 minutes (see Supplemental Fig. 1C). The standard curve used for quantification of results is shown in Supplemental Figure 2. The limit of detection, defined as a signal-to-noise-ratio of 1:10 was 1 ng/ml, the limit of quantification, defined as a signal-to-noise-ratio of 1:3, was 3.3 ng/ml. A more detailed description of the ESI-LC-MS-analysis of bivalirudin can be found in the supplemental data.

**Binding of bivalirudin to BAEC.** BAEC grown to confluence on 6-well plates were incubated with bivalirudin (0, 5, 10, 15 and 20 μg/ml) for 15 minutes at 4°C. Subsequently, supernatants were collected and stored on ice for ESI-LC-MS analysis. Cells were washed twice, scraped in 200 μl of lysis buffer. For separation of
bivalirudin from cell debris, 400 µl of acetone was added to cell lysate after sonication, samples were acidified to pH 3.0 using 0.1 mol/l HCl and centrifuged at 2000g. Supernatants were recovered and analyzed by ESI-LC-MS.

**Methylation of bivalirudin.** 1.5 ml of BF_3_-methanol (Pierce, Rockford, IL, USA) were added to bivalirudin (50 mg) and heated over a steam bath for 20 minutes in a capped vial. Solutions were concentrated in vacuo to a volume of 0.5 ml and extracts were loaded onto a PrepSep™ column (500 mg/3mL, Fisher Scientific, Pittsburgh, USA). The column was equilibrated with 0.1% formic acid. After washing, samples were eluted with 0.1% formic acid in acetonitrile-water (1:1). Methylation of bivalirudin was verified by ESI-LC-MS analysis using the same approach as described for non-modified bivalirudin.

**Binding of MPO to bivalirudin.** Bivalirudin and methylated bivalirudin, respectively were first coupled to agarose beads (HOOK™ amine reactive activated agarose, G-Biosciences, St. Louis, MO, USA) via formation of a stable amine linkage between the aldehyde group and the aminoterminal end of bivalirudin according to manufacturer’s instructions. Briefly, the peptide solution (1 mg/ml) was incubated with agarose and sodium cyanoborohydride (NaCNBH₃, Sigma, St. Louis, MO, USA) over night. On the next day the supernatant was collected to test coupling efficiency by comparing the concentration of the original peptide solution and the supernatant via absorbance measurement at 280 nm. Ethanolamine (12 mg/ml, Sigma, St. Louis, MO, USA) was added to quench the reactivity of the agarose. MPO (13 nmol/l) was added to each vial and allowed to incubate for 1 hour at 37°C. After incubation agarose was washed thoroughly and bivalirudin-bound MPO was measured by Western blotting using polyclonal anti-MPO-antibody (1:200 Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). To rule out non-specific binding of MPO to agarose, reactions were carried out in absence of peptide addition.
**NO-Consumption in vitro.** Solutions of nitric oxide (NO) were prepared by bubbling of NO gas (Matheson, Madison, WI) for 30 min into argon-saturated deionized water. BAEC were grown to confluence on 6-well plates and incubated with 50 nM MPO for 15 minutes at 37°C and co-incubated with either control, heparin (5 U/ml) or bivalirudin (15 μg/ml). Subsequently cells were washed, scraped in 1.2 ml of phosphate-buffered saline and transferred to the NO measuring chamber. 0.2 μl of NO solution was added to the cell lysate to a final concentration of 20 nM. H₂O₂ (50 μmol/l) was injected once the response had stabilized. NO consumption was measured continuously with a NO-specific electrode (Free Radical Analyzer, World Precision Instruments, Hertfordshire, United Kingdom). NO consumption per second was determined by using provided software (Apollo 4000).

**Immunofluorescence.** Confluent BAEC were incubated with MPO (13 nmol/l, 15 minutes at 37°C) and co-incubated with either control, heparin (5 U/ml) or bivalirudin (15 μg/ml). After washing, cells were incubated for 5 minutes with 100 μM nitrite and 50 μM H₂O₂ at 37°C. Subsequently cells were washed, fixed with 4% paraformaldehyde and blocked in 10 % goat serum. Nitrotyrosine and chlorotyrosine was visualized by immunofluorescence using a nitrotyrosine rabbit monoclonal antibody (Upstate, Temecula, CA, USA) and a chlorotyrosine rabbit polyclonal antibody (Hbt, Netherlands) followed by Alexa Fluor 488 (Invitrogen, Oregan, USA) and Alexa Fluor 546 (Molecular probes, Oregan, USA) conjugated anti-rabbit secondary antibody. Nuclei were stained with Hoechst (10 mg/ml, Sigma-Aldrich, Inc, St Louis, Mo, USA). Images were obtained using an Olympus fluorescence microscope.

**Statistical analysis.** Categorical data are presented as frequencies and percentages and were compared by χ² test and the Fisher’s exact test. Continuous variables were
tested for normal distribution using the Kolmogorov-Smirnov test. Data with normal
distribution are presented as mean±standard deviation; non-normally distributed data
are presented as median and interquartile range (IR). For normally distributed data
Student’s paired and unpaired $t$ test or one-way ANOVA followed by Bonferroni post
hoc test were used, as appropriate. Comparisons for non-normally distributed data
were performed by Mann-Whitney $U$ test and Wilcoxon’s signed rank sum test,
respectively. For assessment of the association between FMD and MPO,
Spearman’s rho was employed because of the non-normal distribution of MPO. A
value of $p < 0.05$ was considered statistically significant. All calculations were
performed with SPSS version 13.0.
Results

Patients’ characteristics. A total of 30 patients were included into the study. Patients were randomly assigned to either receive bivalirudin or unfractionated heparin, respectively. As shown in Table 1 there were no significant differences between patients receiving bivalirudin or heparin with respect to age, gender, medication, lipid profile and cardiovascular risk factors. Both drugs, given as a single bolus, were equipotent in affecting coagulation as assessed by the activated clotting time measured 15 minutes after drug administration (Table 1).

Plasma myeloperoxidase levels. Patients receiving heparin exhibited a significant increase in plasma MPO levels (2.63 [IR: 1.94-3.62] to 4.24 [IR: 2.44-5.34] ng/ml, p<0.01). In contrast, patients receiving bivalirudin displayed a significant decrease in plasma MPO levels (3.65 [IR: 2.36-5.03] to 3.22 [IR: 2.06-4.27] ng/ml, p=0.03; Fig. 1A).

Flow-mediated dilation (FMD). Whereas heparin led to a significant increase in FMD, FMD decreased significantly after administration of bivalirudin (Fig. 1B). These changes were also reflected by absolute changes in vessel diameter (heparin: 0.30±0.12 to 0.41±0.14mm, p<0.01; bivalirudin: 0.33±0.13 to 0.28±0.15mm, p=0.04). No significant difference was observed in baseline FMD (p=0.63), baseline diameter of the brachial artery (4.47±0.87 vs. 4.95±0.72; p=0.12), and flow ratios at baseline or after drug administration (baseline: 2.71±0.92 vs. 2.34±0.94; p=0.29; after drug administration: 2.50±0.90 vs. 2.32±1.03; p=0.63) between patients receiving heparin and bivalirudin. The change in FMD after administration of bivalirudin and heparin correlated with the differences between plasma MPO levels prior to and following administration of the study medication (Spearman’s rho: 0.40; p<0.01).

MPO content and activity in matrix proteins and BAEC. After co-incubation of fibronectin with MPO and 4 µg/ml bivalirudin, a 32% reduction of MPO activity was
observed in the supernatant (Fig. 2A), consistent with a 116% increase in fibronectin-adherent MPO, as detected by western blot analysis (Fig. 2B). In contrast, incubation with heparin significantly increased MPO activity in the supernatant and decreased fibronectin-adherent MPO. Similarly, co-incubation with MPO and bivalirudin on BAEC showed a decrease of MPO activity in the supernatant and an increase in cell-adherent MPO activity which was dependent on the dose of bivalirudin (Fig. 2C and 2D). Direct inhibition of MPO activity by bivalirudin was not observed (data not shown).

**Binding of bivalirudin to BAEC.** After incubation of BAEC with different concentrations of bivalirudin for 15 minutes recovery of bivalirudin in the cells and supernatant was dose dependent. Quantitative assessment of bivalirudin in cells and supernatant after 15 minutes of incubation is given in figures 3A and B. 15 minutes after incubation of BAEC with bivalirudin the recovery rate in the supernatant lay around 5 percent while an additional 0.1 percent were recovered from the cells. This reduced recovery can certainly be explained by proteolysis of bivalirudin by the endothelial cells. However, it appears also likely that bivalirudin was to a certain extent lost during precipitation of the cell lysate with acetone and that thus levels of cell-bound bivalirudin are even underestimated.

**Binding of MPO to bivalirudin.** To evaluate potential binding interactions between MPO and bivalirudin, MPO was incubated with bivalirudin coupled to agarose via its aminoterminal end. Western blot analysis showed that MPO specifically interacted with the carboxyterminal end of bivalirudin. To further characterize the binding site of bivalirudin for MPO, bivalirudin was methylated at the carboxylic acid residues of constituent glutamic acids hypothesized to interact with the cationic heparin-binding motifs of MPO. Methylation of the four glutamic acid residues and of the carboxyl
group the carboxyterminal end of the peptide was confirmed by ESI-LC-MS (Supplemental Fig. 1D-1F) and was shown to blunt binding of MPO (Fig. 4).

**NO consumption in vitro.** To evaluate whether bivalirudin-facilitated binding of MPO to BAEC translates into increased NO consumption in vitro, real-time continuous measurements of NO consumption were performed using a NO-specific electrode. Co-incubation of BAEC with bivalirudin and MPO resulted in a significantly increased NO consumption compared to control and heparin in combination with MPO (Figure 5A and 5B).

**Formation of chlorotyrosine and nitrotyrosine.** To identify MPO-derived oxidation of tyrosine residues BAEC, treated with MPO and control, heparin or bivalirudin, were stained for chlorotyrosine and nitrotyrosine. As shown in Fig. 6, treatment with bivalirudin led to a considerable increase in chlorotyrosine and nitrotyrosine formation in comparison to control. In contrast, heparin reduced formation of chlorotyrosine and nitrotyrosine.
Discussion

Herein we report that 1.) bivalirudin in opposition to heparin impairs flow-mediated dilation and decreases MPO levels in patients undergoing elective percutaneous coronary intervention 2.) bivalirudin avidly binds to endothelial cells and also binds to myeloperoxidase via its negatively charged carboxyterminal end 3.) bivalirudin reduces NO-bioavailability and increases formation of oxidized tyrosine residues by facilitation of MPO binding to endothelial cells.

Myeloperoxidase has traditionally been viewed as an enzyme of host defense, but has recently emerged as a potential participant in the promotion and propagation of acute and chronic cardiovascular disease (Baldus, et al., 2003; Brennan, et al., 2003; Rudolph, et al., 2007a; Eiserich, et al., 2002; Eiserich, et al., 1998). Thus, MPO oxidizes LDL cholesterol (Carr, et al., 2000; Heinecke, 1997), activates metalloproteinases (Fu, et al., 2001), promotes post-translational oxidative modification of various target proteins (Baldus, et al., 2001) and of importance, upon secretion from activated neutrophils, MPO binds to endothelial cells, undergoes transcytosis and accumulates in the sub-endothelial space, where it catalyzes the consumption of endothelial derived NO, thereby impairing vascular relaxation (Baldus, et al., 2001; Baldus, et al., 2002).

MPO is known to have several heparin binding sites consisting of positively charged Arginin-Lysin-residues which interact with the multiple anionic sulfate-residues of heparin and which physiologically bind to heparin/heparan-containing glycosaminoglycans on the surface of endothelial cells and thus enable its transcytosis to the sub-endothelial space (Baldus, et al., 2001). Heparin can compete with binding of MPO to vessel wall glycosaminoglycans by promoting electrostatic interactions between the strongly anionic polysaccharides of heparin and the multiple cationic residues of MPO (Daphna, et al., 1998). This concept has been supported by
the effect of various heparin derivatives on reversing the vascular deposition of MPO and on the associated increase in vascular NO-bioavailability (Baldus, et al., 2006; Baldus, et al., 2001).

The 20 amino acid polypeptide bivalirudin is a direct thrombin inhibitor which binds to thrombin in a bivalent fashion. Whereas its aminoterminal end interacts with the active site of thrombin its carboxyterminal end interacts with exosite 1, a positive domain of thrombin which physiologically binds to fibrin (Warkentin, 2004). This interaction is explained by the fact that the carboxyterminal end of bivalirudin consists of a dodecapeptide with predominantly negatively charged amino acids. These multiple anionic amino acid side chains of the carboxyterminal dodecapeptide-domain of bivalirudin also suggested interactions with cationic amino acid residues of MPO (Eikelboom, et al., 2003; Weitz and Buller, 2002).

In the current study bivalirudin is found to interact with MPO by the interaction of the heparin binding sites of MPO with the negatively charged carboxyterminal end of bivalirudin. However, in contrast to heparin bivalirudin does not mobilize MPO from the sub-endothelial space but instead appears to intensify sequestration of MPO in the vascular wall as demonstrated by the findings of our study showing an increase of fibronectin-adherent MPO and cell-adherent MPO activity along with a decrease in MPO activity in the supernatant. This observation is also supported by the mass spectrometric data, which revealed that bivalirudin avidly binds to endothelial cells. Of note, we also demonstrated that bivalirudin-facilitated binding of MPO to endothelial cells affected their humoral and structural integrity: Bivalirudin treatment not only increased MPO-dependent NO consumption in vitro but also reduced NO-bioavailability in patients undergoing elective percutaneous coronary intervention as suggested by the observed deterioration in flow mediated dilation. Moreover, circulating MPO plasma levels were reduced upon administration of bivalirudin; this
underscores increased vascular deposition of MPO which remains catalytically active; the inverse correlation between the changes in MPO plasma levels and endothelial function corroborates that the different degree of MPO deposition in the vessel following bivalirudin versus heparin administration drives the changes in vascular NO bioavailability.

In line with these observations on vascular NO bioavailability, the divergent formation of endothelial nitro- and chlorotyrosine upon co-incubation with bivalirudin as opposed to heparin further underscores the dichotomous effects of both anticoagulants with respect to the extent of endothelial sequestered MPO.

Obviously the current study has important limitations: the question, whether the oppositional acute effects of heparin and bivalirudin on endothelial function impact the long term clinical course of patients undergoing percutaneous coronary interventions, remains unanswered; in fact, the robust changes in vascular homeostasis due to percutaneous coronary interventions are much more profound than the subtle differences in NO bioavailability provoked by administration of heparin and bivalirudin, respectively: even in patients receiving heparins endothelial function deteriorates following percutaneous coronary intervention illustrating that a vigorous generation of reactive oxygen species overrides the MPO distracting effects of heparins (Rudolph, et al., 2007b). Furthermore, while the NO-oxidizing properties of vessel-wall immobilized MPO are well established (Eiserich, et al., 2002) and while our in vitro data clearly show that bivalirudin leads to an enhanced consumption of NO by increasing endothelial sequestration of MPO our in vivo data only provide indirect evidence of vascular MPO immobilization (i.e. decreased plasma MPO levels).

In conclusion, the present study revealed that bivalirudin impairs vascular NO bioavailability in vitro as well as in vivo. Our data suggest that a possible mechanism
is facilitation of vascular sequestration of MPO. These data importantly complement observations made for heparin and underscore the significance of extra-coagulant properties of these established anticoagulants. The fact that the modulation of MPO deposition in both cases is mediated by electrostatic interactions may point towards novel pharmacological interventions aiming to interfere with the vascular burden of MPO in both directions. The data presented here not only reinforce the notion that circulating MPO levels are a potentially poor read-out of the entire and in particular vascular immobilized MPO pool but also reveal that pharmacological interventions yielding a decline in MPO plasma levels may in fact reflect increased deposition of the enzyme in extra-luminal compartments. Additional studies are needed to further characterize the clinical significance of shifting the degree of vascular deposited myeloperoxidase in patients with vascular inflammatory disease.

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References


Footnotes

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Legends for Figures

**Fig. 1.** *In vivo* effect of heparin and bivalirudin on plasma MPO levels and FMD. A, In patients with stable coronary artery disease (n=30), bolus injection of heparin significantly increased plasma MPO levels, whereas injection of bivalirudin significantly decreased plasma MPO levels. Center line indicates median, box and whiskers interquartile range and 2.5 and 97.5 percentiles, respectively. Mann-Whitney *U* test assessed differences between groups (**p<0.01 vs. baseline; *p=0.03 vs. baseline). B, FMD significantly increased after heparin bolus injection (**p=<0.01 vs. baseline), whereas bivalirudin injection was associated with a decrease in FMD (*p=0.02). Values are presented as mean ± S.E.M. Student’s paired *t* test was used to assess differences between groups.

**Fig. 2.** Effect of heparin and bivalirudin on MPO binding to matrix proteins and endothelial cells. Heparin releases MPO from matrix proteins and BAEC, bivalirudin augments its binding. A, Fibronectin was pre-treated with MPO. Co-incubation with heparin increased MPO activity in the supernatant (**p<0.01 vs. control), whereas co-incubation with bivalirudin significantly decreased MPO activity (*p=0.04 vs. control) as assessed by TMB assay. B, Western blot analysis demonstrated reduced matrix-adherent MPO upon heparin treatment (*<0.05 vs. control), whereas binding of MPO to fibronectin was increased with bivalirudin (**p<0.01 vs. control). C, MPO activity was assessed by TMB assay. Addition of bivalirudin decreased MPO activity in the supernatant of cultured BAEC in a dose-dependent manner (*p<0.01 vs. control, #p<0.01 vs. 5 μg/ml bivalirudin). D, Conversely, there was a dose-dependent increase in cell-bound MPO-activity (**p<0.01 vs. control, #p<0.01 vs. 5 μg/ml bivalirudin). Data were obtained from three independent experiments (n=3-5 per
experiment). Data were evaluated using one-way ANOVA. Differences between groups of observations were assessed by Bonferroni post hoc test. Values are presented as mean ± S.D.

**Fig. 3.** Measurement of bivalirudin by mass spectrometry. BAEC were treated with 0, 5, 10 and 20 μg/ml of bivalirudin for 15 minutes. A, In a dose-dependent manner bivalirudin was detected in BAEC and in the supernatant B. Concentrations were calculated using a standard curve. Representative ion chromatograms (m/z 1090.5/356.3) are shown in the inserts.

**Fig. 4.** Binding of MPO to bivalirudin and methylated bivalirudin. Agarose beads were coupled with bivalirudin, methylated bivalirudin and vehicle. MPO (13 nM) was added for 1 hour, agarose was washed thoroughly and bound MPO was measured by western blot. The western blot showed a significant reduction of bound MPO when agarose beads were coupled with methylated bivalirudin or vehicle compared to bivalirudin (***p<0.001 vs. bivalirudin). Data were obtained from 3 independent experiments (n=3 per experiment). Data were evaluated using one-way ANOVA. Differences between groups of observations were assessed by Bonferroni post hoc test. Values are presented as mean ± S.D.

**Fig. 5.** Catalytic consumption of NO in vitro. BAEC were treated with MPO (50 μM) and co-incubated with either bivalirudin (15 μg/ml), heparin (5 U/ml) or control. A, Continuous measurement of NO with a NO-specific electrode revealed that adding NO to the cell lysate resulted in a fast peak. After stabilization of response injection of H₂O₂ (50μM) induced rapid loss of NO. Traces are representative for 3 separate
experiments. B, Bivalirudin significantly increased NO consumption compared to control or heparin (\(**<0.01\) vs. control and vs. heparin). Data were obtained from three independent experiments (\(n=6\) per experiment). Data were evaluated using one-way ANOVA. Differences between groups of observations were assessed by Bonferroni post hoc test. Values are presented as mean ± S.D.

**Fig. 6.** MPO-derived tyrosine modifications. BAEC were treated with MPO and co-incubated with either control, heparin or bivalirudin. BAEC were labelled for chlorotyrosine (red), nitrotyrosine (green), and nuclei (blue). Bivalirudin led to an increase in nitrotyrosine and chlorotyrosine labelled cells. x10 magnification.
Table 1: Patients’ characteristics.

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<th>Heparin (n = 15)</th>
<th>Bivalirudin (n = 15)</th>
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<td>Gender, male</td>
<td>9 (60.0%)</td>
<td>13 (86.7%)</td>
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</tr>
<tr>
<td></td>
<td>6 (40.0%)</td>
<td>2 (13.3%)</td>
<td></td>
</tr>
<tr>
<td>Age (y)</td>
<td>66.8 ± 9.6</td>
<td>66.1 ± 7.9</td>
<td>0.82</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>27.0 ± 4.9</td>
<td>28.1 ± 3.5</td>
<td>0.48</td>
</tr>
<tr>
<td>Hypertension</td>
<td>15 (100%)</td>
<td>15 (100%)</td>
<td>1.0</td>
</tr>
<tr>
<td>Hyperlipidemia</td>
<td>10 (73.3%)</td>
<td>11 (73.3%)</td>
<td>1.0</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>3 (20%)</td>
<td>7 (46.7%)</td>
<td>0.25</td>
</tr>
<tr>
<td>Current Smoker</td>
<td>3 (20%)</td>
<td>0 (0%)</td>
<td>0.22</td>
</tr>
<tr>
<td>Family history</td>
<td>1 (6.7%)</td>
<td>2 (13.3%)</td>
<td>1.0</td>
</tr>
<tr>
<td>LDL cholesterol (mg/dl)</td>
<td>91.5 ± 20.5</td>
<td>103.0 ± 38.9</td>
<td>0.32</td>
</tr>
<tr>
<td>HDL cholesterol (mg/dl)</td>
<td>48.1 ± 10.5</td>
<td>48.3 ± 8.5</td>
<td>0.97</td>
</tr>
<tr>
<td>Triglycerides (mg/dl)</td>
<td>103.7 ± 32.8</td>
<td>93.3 ± 30.5</td>
<td>0.38</td>
</tr>
<tr>
<td>Activated clotting time (sec)</td>
<td>312.2 ± 41.7</td>
<td>301.5 ± 39.4</td>
<td>0.56</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>131.3 ± 15.2</td>
<td>131.2 ± 15.6</td>
<td>0.98</td>
</tr>
<tr>
<td>Diastolic blood pressure (mmHg)</td>
<td>80.3 ± 6.7</td>
<td>78.3 ± 8.4</td>
<td>0.48</td>
</tr>
<tr>
<td>Heart rate (beats/min)</td>
<td>68.1 ± 8.0</td>
<td>65.2 ± 12.0</td>
<td>0.45</td>
</tr>
<tr>
<td>Acetylsalicylic acid</td>
<td>15 (100%)</td>
<td>15 (100%)</td>
<td>1.0</td>
</tr>
<tr>
<td>Beta-Blockers</td>
<td>14 (93.3%)</td>
<td>15 (100%)</td>
<td>1.0</td>
</tr>
<tr>
<td>Statins</td>
<td>12 (80%)</td>
<td>13 (86.7%)</td>
<td>1.0</td>
</tr>
<tr>
<td>ACE inhibitor</td>
<td>13 (86.7%)</td>
<td>12 (80%)</td>
<td>1.0</td>
</tr>
<tr>
<td>AT1 receptor blocker</td>
<td>2 (13.3%)</td>
<td>1 (6.7%)</td>
<td>1.0</td>
</tr>
</tbody>
</table>

Values are given as total numbers and percentages or mean ± SD.
Figure 1

A

Plasma MPO [ng/ml]

before  after  before  after

Heparin (70 IU/kg)  Bivalirudin (0.75 mg/kg)

B

Flow-mediated dilation [%]

before  after  before  after

Heparin (70 IU/kg)  Bivalirudin (0.75 mg/kg)
Figure 2

A

Supernatant MPO activity [% control]

Control | Bivalirudin (4 µg/ml) | Heparin (5 U/ml)
---|---|---

B

Fibronectin-bound MPO [% control]

Control | Bivalirudin (4 µg/ml) | Heparin (5 U/ml)
---|---|---

C

Supernatant MPO activity [% control]

Control | Bivalirudin (5 µg/ml) | Bivalirudin (15 µg/ml)
---|---|---

D

Cell MPO activity [% control]

Control | Bivalirudin (5 µg/ml) | Bivalirudin (15 µg/ml)
---|---|---

* | ** | *

** | # | **

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Figure 3

A

B

Bivalirudin (ng/ml)

Bivalirudin (μg/ml)

Bivalirudin (ng/ml)

Bivalirudin (μg/ml)
Figure 4

MPO binding (% control)

0 20 40 60 80 100 120

Bivalirudin  methylated bivalirudin  No bivalirudin

MPO

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Figure 5

A

Nitric oxide concentration

Time (sec)

H$_2$O$_2$

Heparin
Control
Bivalirudin

B

NO consumed [nmol/l/s]

Bivalirudin (15 μg/ml)
Control
Heparin (5 U/ml)

**