



Bivalirudin Inhibits Thrombin-Mediated Tissue Factor Expression in Human Endothelial Cells

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Highlights: Highlights. Thrombosis in Acute Coronary Syndromes (ACS) involves the activation of platelets and of Tissue Factor (TF) extrinsic coagulation pathway. Unperturbed endothelial cells, being in contact with circulating blood, do not express TF activity. However, several stimuli are able to induce TF in endothelial cells, including thrombin that in an acute "scenario", sustains a prothrombotic milieu. Bivalirudin (BIVA) is a direct thrombin inhibitor used in the setting of ACS and percutaneous coronary intervention to avoid acute thrombotic events. In the present study we demonstrate that thrombin induces TF-mRNA transcription as well TF expression/activity on endothelial cells shifting them to a procoagulant phenotype. BIVA, by inhibiting thrombin, was able to prevent these thrombin deleterious effects. Data of the present study, although in vitro, suggest that BIVA, in the context of ACS, might significantly reduce thrombogenicity not only by acting as direct thrombin inhibitor but through its effects on TF expression/activity too.

Keywords: Bivalirudin, Endothelial Cells, Thrombosis, Tissue Factor

Citation: Cirillo P, Pellegrino G, Conte S et al. Bivalirudin Inhibits Thrombin-Mediated Tissue Factor Expression in Human Endothelial Cells. *J.ATAMIS* 2017;2:24-26. <http://j-atamis.org/index.php/jatamis/article/view/363>

Thrombus formation is the key event in the pathophysiology of Acute Coronary Syndromes (ACS), in which the activation of platelets and of Tissue Factor (TF)-dependent extrinsic coagulation pathway play a pivotal role [1]. Tissue Factor (TF) is a 47-kD membrane-bound glycoprotein, constitutively expressed by cells that are not in contact with blood and it is widely represented in atherosclerotic plaques. After plaque rupture, TF exposed to coagulation factors contained in the blood stream, complexes with factors VII and VIIa, permitting enzymatic activation of factors X and IX, the substrates for factor VIIa, and ultimately leading to the generation of thrombin. Once generated, thrombin interacts with fibrinogen, forms fibrin, induces activation of factors V, VIII and XII, and finally amplifies the coagulative cascade. Moreover, thrombin formed during the initial phases of coagulative process contributes to enhance thrombosis by triggering platelet activation. TF-mRNA and antigen are detectable in the adventitia of normal vessels; little TF immunoreactivity is measurable in the smooth muscle cells and unperturbed endothelial cells (ECs), being in contact with circulating blood, usually do not express significant TF activity [2]. However, several stimuli, including thrombin, are able to induce

TF-mRNA and activity in ECs too [3]. Thus in an acute "scenario", thrombin might be responsible for creating a pro-thrombotic milieu in which activated ECs might be actively involved.

Bivalirudin is a synthetic engineered protein of 20-amino acids, bivalent analogue of hirudin able to specifically inhibit thrombin. Thus, this molecule is a valuable alternative to heparins in patients in whom antithrombotic therapy is mandatory such as in the setting of ACS specially when associated to percutaneous coronary intervention [4].

In the present study, we have investigated the hypothesis that bivalirudin might inhibit thrombosis not only by direct effects on thrombin, but also by reducing the thrombin-mediated expression of TF in human ECs.

TF-mRNA, TF expression and activity were evaluated as previously described [5]. Briefly, Human Umbilical Vein Endothelial Cells (HUVECs), once at confluence, were starved in serum-free medium. Twenty-four hours later, cells were incubated with Thrombin (1 U/ml) or with the coagulation factors FVIIa/Xa

(80/180 nM) for 60 minutes. After incubation, cells were washed and then, TF-mRNA levels were measured at 2 hrs by real-time PCR while TF expression was measured at 6 hrs in cell lysates by Western Blot and on cell surface by FACS analysis.

Finally, TF activity was evaluated by a pro-coagulant assay. In another set of experiments, HUVECs were pretreated with Bivalirudin (1.5 mg/ml) 60 minutes before being stimulated as above and finally processed to evaluate TF-mRNA, TF expression and activity. All reagents were analyzed founding endotoxin level to be <0.125 EU/mL (<12.5 pg/mL) by Limulus assay (Bio Whittaker, Walkersville, USA). TF-mRNA levels significantly increased in HUVECs stimulated with Thrombin (Figure 1, Panel A). Similarly, TF protein levels were almost undetectable in unstimulated HUVECs and Thrombin caused significant increase of TF protein levels (Figure 1, panel B). TF expression on cell surface evaluated by FACS analysis showed that TF antigen was almost undetectable on unstimulated HUVECs, at baseline and Thrombin caused significant increase of TF expression (Figure 2, panel A). Similarly, TF procoagulant activity, almost undetectable at baseline, significantly increased after stimulation with Thrombin, indicating that TF was functionally active (Figure 2, panel D). On the contrary, FVIIa/Xa did not cause any change in TF-mRNA levels as well as TF expression and activity. Interesting, when HUVECs were pre-incubated with Bivalirudin, thrombin did not exert any effect on TF expression and activity (Figure 1 and 2, Panels A and B).

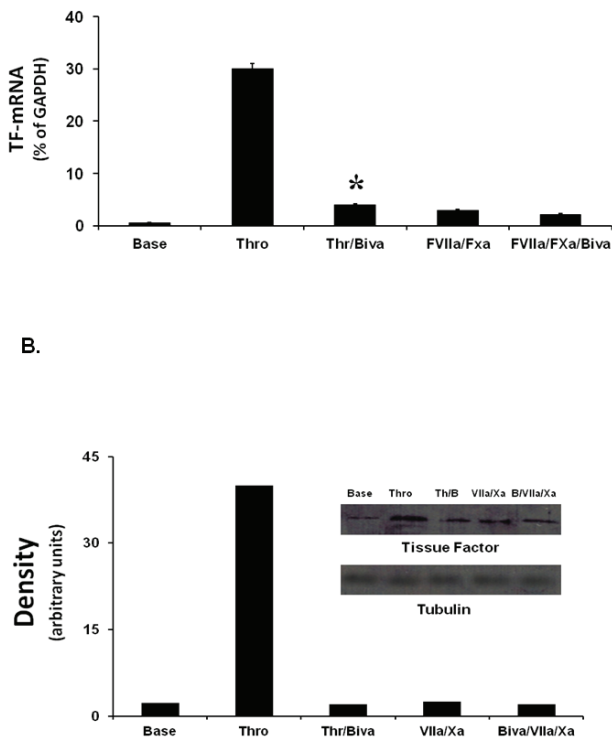
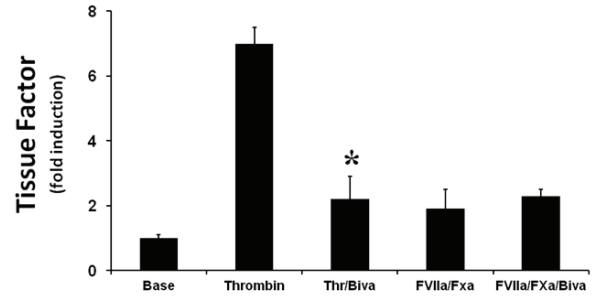


Figure 1. Panel A. Thrombin induced TF transcription in human endothelial cells assessed by Real Time quantitative PCR. Bivalirudin (BIVA) inhibited thrombin effects. Activated coagulation factors FVIIa and FXa did not have any effect on TF-mRNA transcription. Data are expressed as % change versus control gene represented by GAPDH. Each bar represents the mean \pm SD of 3 different experiments. Panel B. Effects of Bivalirudin on Thrombin-induced TF protein levels in HUVEC, evaluated by Western Blot analysis. Graphs are summary of data from 3 separate experiments. Insert shows result of a representative experiment. * = $p < 0.005$ vs thrombin stimulated cells.



D.

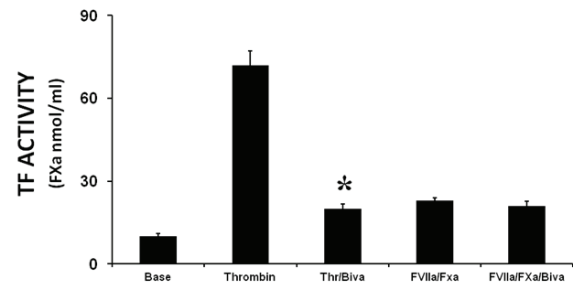


Figure 2. Panel A. Effects of Bivalirudin on Thrombin-induced TF expression on human endothelial cells, determined by FACS analysis. Panel B. Effects of Bivalirudin on Thrombin-induced TF activity determined by a two-step colorimetric assay. TF activity reflects results observed for TF expression, confirming that TF was functionally active. Each bar represents the mean \pm SD of 6 different experiments. * = $p < 0.005$ vs thrombin stimulated cells.

Thrombin has a fundamental role in thrombotic events occurring in ACS. Thus, a targeted antithrombotic therapy aimed to antagonize its effects has a strong rationale. Bivalirudin, as a direct thrombin inhibitor, significantly helps in this clinical setting and specifically when PCI is performed [4]. TF has a pivotal role in ACS pathophysiology and its expression is up-regulated by thrombin, suggesting that this protein might play a role in the perpetuation of the thrombogenic state of the vessel wall after an acute event [1, 2]. In the present report, we point out that thrombin, and not other activated coagulation factors, such as activated FVII and FX, is able to induce TF gene and protein expression. These results might be explained by considering that Thrombin-induced TF expression is regulated through the Protease Activated Receptor-1 (PAR-1) that is abundantly expressed by endothelial cells [6]. On the contrary, VIIa and Xa can induce TF by activating PAR-2 but only when they are already bound to TF [7].

On HUVEC these conditions are met only when TF expression has been previously up-regulated by exposing the cells to other stimuli such as thrombin [7]. Previous report has indicated that Bivalirudin inhibits TF expression in smooth muscle cells that constitutively express this glycoprotein [8]. In the present manuscript, we demonstrate that Bivalirudin is able to inhibit thrombin-induced TF expression in a cell population of the vessel wall never investigated before in this setting such as endothelial cells. These data, although in vitro, have important pathophysiological implications since these cells, being in



contact with blood stream, do not express TF constitutively, but thrombin appears able to shift them to a pro-coagulant phenotype. Moreover, these data, considering that TF belongs to the family of immediate-early genes and taken together with those indicating that TF expression is still persistent 1 month after angioplasty [9], permit to speculate that, in the ACS setting, thrombin might cause a long-lasting pro-thrombotic effect by involving endothelial cells too. In this hypothetical scenario, Bivalirudin plays a pivotal anti-thrombotic role by specific “direct” thrombin inhibition and by “indirect” action on TF expression and activity.

Acknowledgments

This study is dedicated to my daughter Camilla. Honey, “*you are the sunshine of my life*”.

The authors have abided by requirements for ethical publishing in biomedical journals in line with Shewan et al. [10].

Conflict of Interest.

The authors have no conflicts of interest to disclose.

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