

# APPENDIX A

## **Effects of enoxaparin and its molecular fractions on endothelial Tissue factor Pathway Inhibitor: Role of 1, 6 anhydro**

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**Abstract:** Endotoxin (LPS)-induced inhibition of endothelial TFPI release is significantly ( $P < 0.01$ ) reversed by the various enoxaparin fractions to different degrees. The degree of reversal is shown to be affected by the chain length and % of 1, 6 anhydro group within the fractions. A greater reversal of endothelial TFPI release inhibition-induced by LPS is shown for heparanoid fractions with greater % of 1, 6 anhydro based on fractions 5-8 (% 1, 6 anhydro = 15-25%) versus 1-4 (% 1, 6 anhydro < 7%). Additionally, this was also shown with enoxaparin-like fraction with greater % of 1, 6 anhydro as compared to enoxaparin-like with lesser % of 1, 6 anhydro. These data indicated the key role of 1, 6 anhydro in the regulation of endothelial TFPI. Initial studies also showed that CRP impairs endothelial TFPI release, an effect that is effectively reversed by enoxaparin. Further studies are needed to define the role and mechanisms of 1, 6 anhydro in endothelial TFPI under various conditions.

**Introduction:** Tissue factor (TF), a membrane-bound glycoprotein that initiates blood coagulation by allosteric activation of factor (f) VII, is regulated predominantly by tissue factor pathway inhibitor (TFPI). TFPI is a potent inhibitor of the extrinsic coagulation system constitutively synthesized by endothelial cells. A major portion of intravascular TFPI is stored associated with endothelial cells and administration of heparin in vivo causes a prompt mobilization of TFPI into the circulation (1- 4). The pentasaccharide was shown to be ineffective in modulating endothelial cell TFPI release while LMWH demonstrated greater potency in releasing endothelial TFPI (3, 4). TFPI plays a key role as a vascular protective mediator in thrombosis, angiogenesis, and inflammation (4, 5).

### **Aim of the study:**

The present investigation determined the in vitro effects of enoxaparin and its molecular weight fractions with different % of 1, 6 anhydro on the release of TFPI from human endothelial cells.

## **Material and Methods:**

**Structure Information as provided by the manufacturer:** The characteristics of the products are the following: The compounds # 1,2,3,4 are respectively hexasaccharide, octasaccharide, decasaccharide and dodecasaccharides fraction of a LMWH (Enoxaparin like) bearing less than 7% of 1, 6 anhydro group. In contrast, compounds # 5,6,7,8 are respectively hexasaccharide, octasaccharide, decasaccharide and dodecasaccharides fraction of Enoxaparin (bearing between 15 and 25% of 1, 6 anhydro group). Compound # 9 is a batch of Enoxaparin. Compound # 10 is an "Enoxaparin like" compound bearing below 7% of 1, 6 anhydro groups and compound 11 is an "Enoxaparin like" compound bearing more than 40% of 1, 6 anhydro groups. Products 12, 13 and 14 are octasaccharides displaying different affinity to ATIII. Compound # 15 is the heptasaccharide fraction of enoxaparin.

**Endothelial Cells:** Confluent HUVECs ( $1 \times 10^6$ /ml) were re-suspended, and 200  $\mu$ L were plated in 48-well plates coated with fibronectin polymer. Cells were allowed to attach to fibronectin polymer for 3 hours (95% O<sub>2</sub>/5% CO<sub>2</sub>, at 37°C). After washing, fresh medium with and without LPS at 0.1  $\mu$ g was added to the endothelial cells and the volume in each well was adjusted to 1 mL final volume. At 4 hours post-incubation, TFPI released in the medium (50  $\mu$ L) was measured using a commercially available ELISA kit for total TFPI antigen (IMUBIND; American Diagnostica Inc., Greenwich, CT). In parallel assays, wells containing HUVECs and LPS were treated with enoxaparin or its fractions at 1.0  $\mu$ g/ml. The inter- and intra-sample variation ranged from 1% to 5% for this assay.

**Statistical Analysis:** Statistical analysis was performed by one-way analysis of variance (ANOVA) comparing treated with respective control groups and statistical significance was based on  $P < 0.05$ .

## **Results:**

### **1. Reversal of Endothelial TFPI release inhibition-induced by LPS with heparanoid fractions:**

LPS resulted in significant inhibition of endothelial TFPI release. This LPS-induced inhibition of TFPI release is shown to be reversed by the different fractions to different degrees. The degree of

reversal is shown to be affected by the chain length and % of 1, 6 anhydro group within the fractions. A greater reversal of endothelial TFPI release inhibition-induced by LPS is shown for heparanoid fractions with greater % of 1, 6 anhydro based on fractions 5-8 (% 1, 6 anhydro = 15-25%) versus 1-4 (% 1, 6 anhydro < 7%). Additionally, this was also shown with enoxaparin-like with greater % of 1, 6 anhydro as compared to enoxaparin-like with lesser % of 1, 6 anhydro (Table 1).

Table 1: Heparanoids and LPS-mediated impairment of Endothelial TFPI-release

Heparanoid (1.0 ug/ml)	LPS (0.1ug/ml) Mean TFPI (ng / 10 <sup>5</sup> cells) ± SD
Control	4.03 ± 0.4
LPS	0.54 ± 0.1
+ 1	1.22 ± 0.2
2	2.02 ± 0.3
3	2.33 ± 0.2
4	4.12 ± 0.4
5	3.26 ± 0.2
6	2.92 ± 0.3
7	3.26 ± 0.3
8	4.92 ± 0.3
9	7.51 ± 0.6
10	7.16 ± 0.7
11	8.99 ± 0.6
12	4.28 ± 0.4
13	3.94 ± 0.3
14	3.77 ± 0.4
15	6.49 ± 0.5
Enoxaparin	7.94 ± 0.6

*Data represent mean ± SD, n = 3. 0.5 x 10<sup>6</sup> cells (EGM media) were plated onto 0.2% gel coated 12 well plates. Final density of cells 1 x 10<sup>6</sup> / well/ml. Experimental media: MCDB-131+ 15mM HEPES buffer + 1% FBS + 2 mM Glutamine. Cells were treated with Heparanoids (1.0 ug) + LPS (0.1ug/ml). Cell supernatants were collected after 6 hr and stored at - 80 °C. TFPI was measured using IMUBIND total TFPI ELISA kit from American Diagnostica product.*

### **Conclusions:**

LPS resulted in significant inhibition of endothelial TFPI release. This is shown to be reversed by the different fractions to different degree. The degree of reversal is shown to be affected by the

chain length and % of 1, 6 anhydro group within the fractions. These data indicated the key role of 1, 6 anhydro in the up-regulation of endothelial TFPI and suggest the differential benefits of enoxaparin as compared to other LMWH preparations that do not contain 1, 6 anhydro.

**Inhibitory Effect of C - reactive protein (CRP) on the Release of Tissue Factor Pathway Inhibitor from Human Endothelial Cells: Reversal by Enoxaparin:**

**Preliminary Data:**

*Background:* The effects of C-reactive protein (CRP) and low molecular weight heparin (LMWH) on the release of tissue factor pathway inhibitor (TFPI) from human umbilical vein endothelial cells (HUVECs) were examined. *Methods:* Confluent HUVECs were resuspended and plated in 48-well plates coated with fibronectin polymer. Cells were allowed to attach for 3 hours; then washed, and fresh medium with or without CRP at different concentrations (0 to 20 ng/mL) was added. At 8 hours, TFPI released in the medium was measured using a commercial TFPI ELISA kit for total TFPI antigen. In parallel assays, wells containing HUVECs and CRP were treated with enoxaparin at 1.0 µg/mL. *Results:* Data showed that CRP significantly inhibited TFPI release from HUVECs in a concentration-dependent manner. Enoxaparin effectively reversed the inhibitory effects of CRP on TFPI release from HUVECs (Table 2). *Conclusions:* These findings support the hypothesis that CRP may play a direct role in promoting a hypercoagulable state by decreasing the release of the natural anticoagulant TFPI, which can be counteracted by LMWH. Additional studies are needed to define the role of 1, 6 anhydro on CRP-mediated suppression of endothelial TFPI.

TABLE 2: Inhibitory effect of CRP on endothelial TFPI release: Reversal by LMWH

Treatment group	Mean TFPI release (ng/10 <sup>5</sup> cells) ± SD
Control (PBS)	5 ± 1
CRP 1 ng/mL	2 ± 0.5*
CRP 10 ng/mL	0.15 ± 0.1**
CRP 20 ng/mL	0.01 ± 0.01**
PBS + Enoxaparin (1 µg/mL)	11 ± 2**
CRP (10 ng/mL) + enoxaparin (1 µg/mL)	9 ± 2**

Data represent mean ± SD, n = 3. \* P < 0.05, \*\* P < 0.01

CRP = C-reactive protein; HUVEC = human umbilical vein endothelial cell; LMWH = low molecular weight heparin; TFPI = tissue factor pathway inhibitor. LMWH and CRP were dissolved in phosphate buffered saline (PBS). Cells were incubated for 8 hours and media were removed for TFPI measurements.

### References:

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