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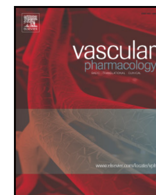

# Anti-thrombotic and pro-fibrinolytic effects of levosimendan in human endothelial cells in vitro

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A B S T R A C T

*Aims:* Levosimendan is an inodilator for the treatment of acute decompensated heart failure (HF). Data from clinical studies suggest that levosimendan is particularly effective in HF due to myocardial infarction. After acute revascularization, no reflow-phenomenon is a common complication that may lead to pump failure and cardiogenic shock. Our aim was to examine whether levosimendan interferes with the pro-thrombotic phenotype of activated endothelial cells in vitro.

*Methods:* Human heart microvascular endothelial cells (HHMEC) and human umbilical vein endothelial cells (HUVEC) were treated with interleukin-1β (IL-1β) (200 U/mL) or thrombin (5 U/mL) and co-treated with or without levosimendan (0.1–10 μM) for 2–24 h. In addition, flow experiments were performed. Effects on plasminogen activator inhibitor-1 (PAI-1) and tissue factor (TF) expression and activity were measured by rt-PCR, specific ELISA and flow cytometry.

*Results:* Treatment with IL-1β or thrombin significantly increased the expression of PAI-1 and TF in endothelial cells. Co-treatment with levosimendan strongly attenuated the effects of IL-1β and thrombin on PAI-1 and TF mRNA by up to 50% and 45%, in a dose- and time-dependent manner. Similar results were obtained under flow conditions. Furthermore, co-treatment with levosimendan dampened the antigen production of PAI-1 and the surface expression of TF by 35% and 45%, respectively. Additionally, levosimendan diminished both TF and PAI-1 activity.

*Conclusion:* Levosimendan down-regulates the expression of the pro-thrombotic and anti-fibrinolytic biomolecules TF and PAI-1 in activated human endothelial cells. Our findings may, at least in part, explain some of the beneficial effects of levosimendan after myocardial reperfusion.

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## 1. Introduction

In patients suffering from acute ST-elevating myocardial infarction (STEMI), timely reperfusion of the coronary vessel using either primary percutaneous coronary intervention (PPCI) or thrombolytic therapy represents the most effective therapeutic strategy in order to limit infarct size and prevent HF [1]. Still, morbidity and mortality after myocardial reperfusion remain high, as, paradoxically, the restoration of blood flow itself can induce myocardial tissue damage [2]. This so-called myocardial reperfusion injury causes different types of cardiac dysfunction, from myocardial stunning, reperfusion arrhythmias and lethal reperfusion injury to the no-reflow phenomenon, defined as the lack of myocardial perfusion despite angiographically patent epicardial coronary vessels [3].

Different mechanisms responsible for no-reflow have been proposed and further investigated, including microvascular obstruction due to endothelial damage, the production of reactive oxygen species (ROS), leukocyte accumulation within the vessel and the re-perfused tissue and activation of the coagulation cascade [3]. The production of ROS is triggered by sudden reperfusion of the previously hypoxic tissue, further inducing the production of inflammatory cytokines such as TNF-

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α and IL-1ß, causing an inflammatory activation of the vessel wall [4]. Additionally, pro-coagulatory mediators, such as thrombin and tissue factor (TF) are being induced in the reperfused heart and vessels. In animal models, TF was causally related to coronary no-reflow and inhibition of the TF-thrombin pathway limited infarct size in a myocardial ischemia-reperfusion model [5,6]. On the other hand the inflammatory mediators and thrombin itself act also indirectly pro-thrombotically and anti-fibrinolytically by upregulating the endothelial expression of TF and plasminogen activator inhibitor-1 (PAI-1), a serine protease inhibitor that constitutes the physiologic inhibitor of tissue-type plasminogen activator (tPA), an activator of fibrinolysis [7–9].

Levosimendan is an inodilator for the treatment of acute decompensated heart failure, combining positive inotropic effects via calcium sensitization through binding to troponin C and vasodilatory effects via opening ATP-sensitive $K^+$-channels in the vasculature [10]. Additionally, levosimendan exhibits cardioprotective effects by opening ATP-sensitive, mitochondrial $K^+$-channels (mito$K^+_{ATP}$-channels) [11]. Levosimendan was studied extensively in the setting of acute decompensated heart failure of variable etiology resulting in conflicting outcome results [12–14]. Interestingly, levosimendan showed the greatest mortality benefit in patients with heart failure due to acute ischemia [15,16]. Additional data obtained from animal models of MI and ischemia-reperfusion injury demonstrating a reduction of tissue injury in animals treated with levosimendan further supported the notion of levosimendan as a cardioprotective agent [17,18].

Whether levosimendan affects the generation of pro-coagulatory agents in activated endothelial cells is currently unknown. Therefore, the aim of our study was to examine whether levosimendan interferes with the pro-thrombotic phenotype of activated human heart microvascular cells (HHMEC) and human umbilical vein endothelial cells (HUVEC) in vitro under both static and flow conditions.

## 2. Methods

### 2.1. Cell culture

Human heart microvascular endothelial cells (HHMEC) were obtained from ventricular tissue from patients undergoing heart transplantation at the General Hospital of Vienna and were characterized and treated as described elsewhere [19]. Human umbilical vein endothelial cells (HUVEC) were freshly isolated from umbilical cords by mild collagenase treatment and characterized and treated as described [20]. Only cells between passages 2 and 5 were used in this experiment. All human material was obtained and processed according to the recommendations of the hospital's ethics committee and security board. Furthermore, the study was approved by the local ethical committee and complies with the Declaration of Helsinki.

### 2.2. Treatment of cells

Medium of confluent monolayers of endothelial cells was switched to M199 medium containing 1.25% FCS immediately prior to experiments. Unless otherwise stated, cells were incubated with or without levosimendan (Orion Pharmaceuticals, Espoo, Finland) for 30 min at the concentrations indicated, followed by addition of 200 U/mL recombinant human (rh)-IL-1ß (R&D systems, Minneapolis, MN, USA) or 5 U/mL bovine α-thrombin (Sigma) and further incubation for the indicated time periods within the same medium. After treatment, cell culture supernatants were collected and stored at −80 °C for later analysis. A lactic dehydrogenase (LDH) based toxicology assay kit (Sigma Aldrich, St Louis, MO, USA) was used to exclude any toxic effects. All experiments were performed in triplicate and were repeated three times with cells from three different donors.

### 2.3. Flow experiments

For the evaluation of the effects of levosimendan on IL-1ß-induced TF and PAI-1 expression under flow conditions, HUVEC were grown on "μ-slides I 0.4 Luer" (ibidi®, Martinsried, Germany). After reaching confluence (usually within 24 h), endothelial monolayers were incubated with fresh M199 medium containing 20% FCS with or without levosimendan (10 μM) for 30 min and afterwards stimulated with 200 U/mL IL-1ß and exposed to a laminar shear stress of 5 dyn/cm$^2$ for 4 h. After that, cells were lysed and respective mRNA was measured as described within the respective sections "mRNA purification and cDNA preparation" and "Real-time polymerase chain reaction" at the end of the section "Methods".

### 2.4. Quantification of PAI-1 antigen

PAI-1 antigen was measured in cell culture supernatants by a specific enzyme-linked immunosorbent assay (ELISA) using monoclonal antibodies (Technoclone, Vienna, Austria; limit of detection 0.5 ng/mL). For the measurement of active PAI-1 antigen, the TECHNOZYM® PAI-1 Actibind® ELISA Kit (Technoclone, Vienna, Austria) was used.

### 2.5. Flow cytometry

For the measurement of TF surface expression on endothelial cells, flow cytometry was used (FACS Canto II, Becton Dickinson, Franklin Lakes, NJ, USA). In brief, after incubating the cells as indicated, medium was removed and replaced with ice-cold PBS. Cells were gently detached by scratching and incubated at 4 °C in the dark for 30 min with a primary antibody against tissue factor (APC, clone HTF-a, mouse anti-human CD142; eBioscience, San Diego, CA, USA) that was diluted 1:20 in antibody diluents solution (DAKO North America Inc., Carpinteria, CA, USA) or with respective isotype-matched control antibody (Beckman Coulter). Afterwards, cells were washed once in 1× PBS, resuspended in a fixative solution (FACS Flow, reagent-grade water and BD Cellfix™) and mean fluorescent intensity (MFI) was analyzed by FACS Diva software.

### 2.6. Measurement of TF activity

For the determination of TF procoagulant activity, the Actichrome® TF chromogenic assay was used (Sekisui Diagnostics, Stanford, CT, USA, limit of detection 85 pg/mL).

### 2.7. mRNA purification and cDNA preparation

After incubation of cells as indicated, medium was removed, cells were incubated in lysing solution and mRNA was isolated using High Pure RNA Isolation Kit (Roche, Mannheim, Germany). For cDNA preparation, reverse transcription was performed using Transcriptor First Strand cDNA Synthesis Kit (Roche) as previously described [21].

### 2.8. Real-time polymerase chain reaction

Real time-PCR was performed using LightCyclerTaqMan Master (Roche) according to manufacturer's instructions. Primers were designed using the Roche Universal ProbeLibrary Assay Design Centre (http://www.universalprobelibrary.com): **GAPDH** (forward primer: 5′-agccacatcgctcagacac-3′, reverse primer: 5′-gcccaatacgaccaaatcc-3′, UPLprobe #60; Amplicon Size [bp] 66) – **TF** (forward primer: 5′-cagacagcccggtagagtgt-3′, reverse primer 5′-ccacagctccaatgatgtagaa-3′, UPLprobe #2; Amplicon Size [bp] 75) - **PAI-1** (forward primer: 5′-tccagcagctgaattcctg-3′, reverse primer 5′-gctggagacatctgcatcct-3′, UPLprobe #15; Amplicon Size [bp] 102) – The amplification conditions consist of an initial incubation at 95 °C for 10 min, followed by 45 cycles of 95 °C for 10 s, 63 °C for 20 s and 72 °C for

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