Levosimendan exerts anti-inflammatory effects on cardiac myocytes and endothelial cells in vitro

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Summary

Levosimendan is a positive inotropic drug for the treatment of acute decompensated heart failure (HF). Clinical trials showed that levosimendan was particularly effective in HF due to myocardial infarction. Myocardial necrosis induces a strong inflammatory response, involving chemotactants guiding polymorphonuclear neutrophils (PMN) into the infarcted myocardial tissue. Our aim was to examine whether levosimendan exhibits anti-inflammatory effects on human adult cardiac myocytes (HACM) and human heart microvascular endothelial cells (HHMEC). Cardiac myocytes and endothelial cells were stimulated with interleukin-1β (IL-1β) (200 U/ml) and treated with levosimendan (0.1–10 µM) for 2–48 hours. IL-1β strongly induced expression of IL-6 and IL-8 in HACM and E-selectin and intercellular adhesion molecule-1 (ICAM-1) in HHMEC and human umbilical vein endothelial cells (HUVEC). Treatment with levosimendan strongly attenuated IL-1β-induced expression of IL-6 and IL-8 in HACM as well as E-selectin and ICAM-1 in ECs. Levosimendan treatment further reduced adhesion of PMN to activated endothelial cells under both static and flow conditions by approximately 50%. Incubation with 5-hydroxydecanoic acid, a selective blocker of mitochondrial ATP-dependent potassium channels, partly abolished the above seen anti-inflammatory effects. Additionally, levosimendan strongly diminished IL-1β-induced reactive oxygen species and nuclear factor-κB (NF-κB) activity through inhibition of SS36 phosphorylation. In conclusion, levosimendan exhibits anti-inflammatory effects on cardiac myocytes and endothelial cells in vitro. These findings could explain, at least in part, the beneficial effects of levosimendan after myocardial infarction.

Keywords

Levosimendan, endothelial cells, cardiac myocytes, granulocytes, myocardial infarction

Introduction

Levosimendan is a cardiovascular drug for the treatment of acute decompensated heart failure (HF). As an inodilator, it exhibits both positive inotropic effects by calcium sensitisation and vasodilatory effects by opening ATP-sensitive potassium channels in vascular smooth muscle cells (VSMCs) (1).

Several big clinical trials investigating levosimendan in patients suffering from acute decompensated heart failure (ADHF) of variable etiology demonstrated conflicting results in terms of mortality benefit (2–4). However, in the RUSSLAN trial, that recruited only patients suffering from acute HF due to myocardial infarction (MI), levosimendan treatment was associated with a reduction in short- and long-term mortality (5). Further smaller clinical trials confirmed the beneficial effects of levosimendan in patients with acute HF due to MI undergoing reperfusion therapy (6, 7). In animal models of MI and ischaemia-reperfusion injury, levosimendan reduced the size of injured tissue, thus supporting the notion that levosimendan exhibits additional cardioprotective effects (8–11). These cardioprotective effects of levosimendan might be explained in part by the fact that levosimendan opens mitochondrial ATP-sensitive K+-channels (mitoKATP-channels) (12, 13). In cell culture experiments, levosimendan protected rat myocytes against apoptotic cell death (14), induced nitric oxide (NO) production in endothelial cells (15) and inhibited the release of reactive oxygen species (ROS) in polymorphonuclear leukocytes in vitro (16).

Molecular and cellular events during myocardial ischaemia are complex. The sudden obstruction of a coronary vessel leads to tissue hypoxia and cell necrosis, triggering ROS generation. A cytokine cascade is initiated by tumour necrosis factor-α (TNF-α) and interleukin-1β (IL-1β), causing activation of the microvascular endothelium and leukocyte recruitment. Chemotactic cytokines such as IL-8, so-called chemokines, play a crucial role in attracting leu-
kocytes to the vessel wall. Once activated, endothelial cells express selectins, like E-selectin that results in loose attachment and rolling of leukocytes, while the expression of adhesion molecules like intercellular adhesion molecule-1 (ICAM-1) causes firm adhesion and subsequent migration of polymorphonuclear neutrophils (PMN) into the infarcted cardiac tissue leading to further tissue damage (17). Reperfusion of ischaemic cardiac tissue induces production of IL-6, a potent pro-inflammatory cytokine (18).

Several studies have shown that levsimendan may exhibit anti-inflammatory effects in vivo, as evidenced by reduced plasma levels of circulating IL-6 and TNF-α (19). In vitro treatment with levsimendan resulted in inhibition of lipopolysaccharide (LPS)-induced nuclear factor (NF)-κB activation in monocytes and TNF-α induced NF-κB activation in human umbilical vein endothelial cells (HUVEC) (20, 21). However, effects of levsimendan on IL-1β induced activation of cardiac myocytes and cardiac microvascular endothelial cells are unknown.

Therefore, the aim of our study was to examine whether levsimendan exhibits anti-inflammatory effects on human adult cardiac myocytes (HACM) and human heart microvascular endothelial cells (HHMEC) in vitro.

Methods

Cell culture

HHMEC were isolated from ventricular tissue obtained from patients undergoing heart transplantation in the General Hospital of Vienna and were characterised and cultivated as shown previously (22). Human aortic endothelial cells (HAEC) were isolated from pieces of the aortic outflow tract from hearts of patients undergoing heart transplantation by mild collagenase treatment as described elsewhere (22). HUVEC were isolated from fresh umbilical cords and characterised and cultured as previously described (23). Briefly, endothelial cells were cultivated in cell culture flasks coated with 1% gelatine (Sigma, St. Louis, MO, USA) in minimum essential medium (M199, Sigma) containing 20% fetal calf serum (FCS; HyClone, Logan, UT, USA), 100 U/ml penicillin, 100 µg/ml streptomycin, 0.25 µg/ml fungizone, and 1 mM L-glutamine (all Cambrex, East Rutherford, NJ, USA), 2 IU/ml heparin and 50 µg/ml endothelial cell growth supplement (ECGS; Technoclone, Austria) at 37°C in a humidified atmosphere of 5% CO₂:95% air. HACM were isolated from hearts of patients undergoing cardiac transplantation as described previously, resulting in a cell population consisting of >95% rod-shaped cells with a viability of >90% (24). HACM were then cultured as described previously (25, 26). In brief, cells were cultivated in cell culture flasks coated with 15% gelatine (Sigma) in minimum essential medium (M199, Sigma) containing 20% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin, 0.25 µg/ml fungizone, and 2 mM L-glutamine (all Cambrex) at 37°C in a humidified atmosphere of 5% CO₂:95% air. All cells used in this experiment were between passages 2 and 5. All human material was obtained and processed according to the recommendations of the hospital’s ethics committee and security board. The study was approved by the local ethical committee and complies with the Declaration of Helsinki.

Treatment of cells

Confluent HACM were starved in M199 medium containing 0.1% bovine serum albumin (BSA; Sigma) 24 hours (h) prior to experiments. Thereafter, fresh medium containing 0.1% BSA was added. Immediately prior to treatment of HUVEC and HHMEC, medium was switched to M199 medium containing 1.25% FCS. Cells were pretreated with or without levsimendan (Orion Pharmaceuticals, Espoo, Finland) for 30 minutes (min) (unless otherwise stated) at the concentrations indicated and recombinant human (rh)-IL-1β (R&D systems, Minneapolis, MN, USA) or rh TNF-α (R&D systems) was added. For blocking experiments, 5-Hydroxy-decanoic acid (5-HD; Sigma) a selective blocker of mitoK-ATP channels at the concentration of 10 µM was added 15 min prior to levsimendan treatment. At the end of the incubation period, conditioned media from these cultures were collected and stored at −80°C. To exclude any toxic effects of levsimendan, a lactate dehydrogenase (LDH) based in vitro toxicity assay kit (Sigma) was used according to manufacturers’ instructions. Key experiments were conducted in HHMEC. As results with HUVEC were similar and due to limited supply of myocardial tissue for isolation of HHMEC, HUVEC were used to perform further mechanistic studies. All experiments were performed in triplicate and were repeated three times with cells from three different donors.

Quantification of IL-6 and IL-8 protein

IL-6 and IL-8 antigen was measured in cell culture supernatants by specific enzyme-linked immunosorbent assays (ELISA) using monoclonal antibodies (R&D systems for IL-6, and eBioscience, San Diego, CA, USA for IL-8).

Flow cytometry

For the measurement of ICAM-1, VCAM-1 and E-selectin surface expression on HHMECs and HUVEC, flow cytometry was used (FACS Canto II, Becton Dickinson, Franklin Lakes, NJ, USA) as previously described (23). In brief, medium was removed and adherent cells were incubated with a detachment buffer (Dulbecco’s phosphate-buffered saline (PBS) without calcium and magnesium (PAA, Pasching, Austria) containing 25 mM HEPES (Boehringer Mannheim GmbH, Mannheim, Germany) and 10 mM EDTA (Pierce, Rockford, IL, USA) for 10 min. Afterwards, cells were gently removed from the wells and incubated at 4°C at dark with primary antibodies against ICAM-1 (FITC, mouse anti-human CD54, Beckman Coulter, Brea, CA, USA), VCAM-1 (PE-Cy5™ mouse anti-human CD106, BD Pharmingen, San Jose, CA, USA) and E-selectin (PE mouse anti-human CD62E; BD Pharmingen) that were diluted 1:40 in antibody diluents solution (DAKO North America Inc., Carpinteria, CA, USA) prior to use, or with respective isotype-matched control antibodies (PE-Cy5™ IgG1 BD; FITC and PE IgG1 Beckman Coulter, see Suppl. Figure 1, available on-
Afterwards, cells were washed with 1× PBS, resuspended in fixative solution (FACS Flow, aqua destillata and BD CellfixTM) and mean fluorescence intensity (MFI) was analysed by FACS Diva software (BD).

mRNA purification and cDNA preparation

After treatment of cells, culture supernatants were removed, cells were lysed and mRNA was isolated using High Pure RNA Isolation Kit (Roche, Mannheim, Germany) according to manufacturer's instructions. For obtaining cDNA, reverse transcription was performed using Transcriptor First Strand cDNA Synthesis Kit (Roche) as previously described (27).

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’-gcgccaat-agcacaaccac-3’, UPLprobe #60; Amplicon Size [bp] 66) – VCAM-1 (forward primer: 5’-gtatgcgatggcaatggag-3’, reverse primer: 5’-tgtatcgagacgaccaaac-3’, UPLprobe #39; Amplicon Size [bp] 69) – ICAM-1 (forward primer: 5’-ccctctctctctctctctc-3’, reverse primer: 5’-acccacacacacacacacacacacacac-3’, UPLprobe #71; Amplicon Size [bp] 90) – E-selectin (forward primer: 5’-accacacagggcagttc-3’, reverse primer: 5’-gcgccaat-agcacaaccac-3’, UPLprobe #86; Amplicon Size [bp] 89) – IL-6 (forward primer: 5’-gatgtagataaaagtctgtgacca-3’, reverse primer: 5’-ctgacgactcagtcgtgctc-3’, UPLprobe #40; Amplicon Size [bp] 130) – IL-8 (forward primer: 5’-agacagacag- cacacaacg-3’, reverse primer: 5’-catggtcttccggtcctg-3’, UPLprobe #72; Amplicon Size [bp] 62). The amplification conditions consist of an initial incubation at 95 °C for 10 min, followed by 45 cycles of 95 °C for 10 sec, 63 °C for 20 sec and 72 °C for 6 sec and a final cooling to 40 °C. LightCycler Software Version 3.5. (Roche) was used to perform data analysis.

Isolation of human polymorphonuclear neutrophils

For the isolation of human PMN heparinised (100 U/ml) peripheral venous blood was taken from healthy donors using thevacuette blood collection set (21G needle; Greiner bio-one, Kremsmünster, Austria) without tourniquet. For the positive isolation of PMN from whole blood, the Easy-Sep® human whole blood CD66b positive selection kit (Stemcell technologies, Grenoble, France) was used according to manufacturers’ instructions. Briefly, after lysis of erythrocytes, positive isolation was performed by adding CD66b antibodies conjugated to magnetic beads. The vial containing the cells with conjugated magnetic beads was placed into a magnetic field, the fluidic content was discarded and the remaining cells were resuspended with fresh M199 medium.

Figure 1: Time- and dose-dependent effects of levosimendan on IL-6 and IL-8 mRNA and antigen production. A-D) Human adult cardiac myocytes (HACM) were treated with media alone (full triangles), 10 µM levosimendan (open triangles), 200 U/ml IL-1 β alone (full circles) or 200 U/ml IL-1 β and 10 µM levosimendan (open circles). mRNA was analysed for IL-6 (A) and IL-8 (B), values represent mean ± SD x-fold control. IL-6 (C) and IL-8 (D) antigen was determined. Values represent mean ± SD. E-F) HACM were treated in the absence or presence of 200 U/ml IL-1 β and levosimendan for 4 h at the concentrations indicated. mRNA was analysed for IL-6 (E), IL-8 (F), values represent mean ± SD x-fold control. All experiments were performed three times with cells obtained from three different donors with similar results. Representative images are shown. * p<0.05 for IL-1 β vs IL-1 β + levosimendan.
Adhesion assay under static conditions

PMN adhesion to HUVEC under static conditions was determined as described previously (23). Briefly, medium was removed and the confluent HUVEC monolayer was incubated with 1 ml well medium alone or medium containing levosimendan (10 µM) for 30 min and subsequently with IL-1β (200 U/ml) with or without different concentrations of levosimendan (0.1–10 µM) as indicated. After 4 h of incubation, cells were washed three times with medium and subsequently, 1 ml medium containing 1×10⁶ PMN was added to the wells and incubated at 37°C in a 5% CO₂ atmosphere for the time periods indicated. Afterwards, cells were washed with PBS and cell adhesion was examined under a Zeiss Axiovert 40 CFL light microscope with 10× lens. A Zeiss Axiocam ICC3 camera was used to acquire images. The area covered by granulocytes was measured using the Image-J software (National Institute of Health, Bethesda, MD, USA).

Adhesion assay under flow conditions

For flow adhesion experiments, 48-well plates of the Bioflux200™ system (FluxionBio, San Francisco, CA, USA) were used. In brief, channels were coated with fibronectin (100 µg/ml; Sigma) and incubated at 37°C for 1 h. Afterwards, channels were washed with medium at 5 dyn/cm² for 10 min and again at 2 dyn/cm² for 15 min. HUVEC were mobilised by trypsinisation, washed twice with medium, resuspended in 20% FCS medium at a concentration of 3×10⁶/ml and seeded into the channels at 2 dyn/cm² for 5 seconds and afterwards at gravity flow (approx. 1 dyn/cm²) overnight to grow to confluence. The next day, cells were incubated with 10 µM levosimendan for 30 min and afterwards with IL-1β (200 U/ml) with or without levosimendan (10 µM) for 4 h, as indicated. After washing the channels, freshly isolated PMN at a concentration of 3×10⁶/ml dissolved in medium were added and a shear stress of 1 dyn/cm² was applied for 5 min. After perfusion, channels were washed with medium for 10 min, pictures were taken using a Zeiss Axiocam ICC3 and the amount of granulocytes adherent per viewing window was counted using the 10× lens.

Nuclear extraction and analysis of NF-κB DNA binding

HUVEC were incubated in M199 containing 1.25% FCS and HACM in M199 medium containing 0.1% BSA with or without 200 U/ml IL-1β and 10 µM levosimendan for 30 min. Nuclear extract preparation was performed using a Nuclear Extract kit (ActiveMotif, Rixensart, Belgium) according to manufacturer’s instructions. For the quantification of p50 and p65 NF-κB subunits we used an ELISA based TransAM™ NF-κB Kit (Active Motif, Rixensart, Belgium). In brief, the active NF-κB contained in nuclear extracts specifically bound to an oligonucleotide immobilised to a 96-well plate containing a NF-κB binding site. By using specific antibodies, the NF-κB subunits bound to the oligonucleotide were detected. The amount of bound subunits was quantified by spectrophotometry using a secondary antibody conjugated to horseradish peroxidase.

SDS-PAGE and Western blot analysis

Cell pellets were lysed in Laemmli buffer and subjected to three cycles of freeze/thaw. Equal amounts of protein extracts were separated under reducing conditions on 10% SDS-PAGE and transferred onto a nitrocellulose membrane (Millipore, Billerica, MA, USA) by semidy blotting. Membranes were blocked for 1 h in 5% skimmed milk powder (Sigma) in PBS containing 0.1% Tween-20 (Bio-Rad, Hercules, CA, USA). For protein detection membranes were incubated in PBS/0.1% Tween-20/5% milk powder containing a 1:1,000 diluted rabbit phospho-IκBα (Cell Signalling, Danvers, MA, USA) at 4°C overnight. Bound antibodies were detected by applying an anti-rabbit horseradish peroxidase-conjugated secondary antibody (GE Healthcare; diluted 1:5,000) for 1 h at room temperature followed by enhanced chemiluminescence (SuperSignal™ West Pico Substrate, Pierce, Rockford, IL, USA). As an internal control to evaluate loaded protein amounts, GAPDH was detected using a monoclonal mouse anti-GAPDH antibody at a dilution 1:5,000 (Millipore). As secondary antibody an anti-mouse horseradish peroxidase-conjugated antibody at a dilution 1:5,000 (GE-Healthcare, Cleveland, OH, USA) was applied.

Phospho p65

 Nuclear extracts were obtained as described in the methods section above. For the detection of p65 phosphorylation the following antibodies were used: rabbit anti-p-NF-κB p65 (Ser 356), rabbit anti-p-NF-κB p65 (Ser 276), rabbit anti-p-NF-κB p65 (Ser 529) and rabbit anti-p-NF-κB p65 (Ser 276) (all Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA) at a dilution of 1:500 in 5% skimmed milk powder in tris-buffered saline (TBS) containing 0.1% Tween-20. Incubation was performed at 4°C overnight. As secondary antibody an anti-rabbit horseradish peroxidase-conjugated antibody (GE Healthcare; diluted 1:5,000) was used for 1 h at room temperature. Blots were developed by chemiluminescence (SuperSignal™ West Femto, Pierce). As a loading control, we performed Ponceau S staining which showed equal loading in all lanes (data not shown).

Reporter gene assays

HUVEC and HACM confluent 70–80% were harvested and resuspended in RPMI 1640 medium containing 10% FCS at a concentration of 5×10⁶ per ml. A total of 20 µg plasmid DNA, 19 µg NF-κB reporter plasmid (constructed as previously reported [28], a kind gift of Prof. Brostjan, Department of Surgery, Medical University of Vienna) and 1 µg pCMV-lacZ (Clontech; Mountain View, CA, USA) as control plasmid was added to 400 µl of cell suspension. Thereafter, cells were electroporated in a 4 mm cuvette using a Gene PulserXcell system (Bio-Rad) at 200 V and 1200 µF and subsequently transferred into six-well plates (Cellstar® Greiner bio-one). After 24 h, cells were treated with IL-1β and levosimendan as indicated. Cell extracts were prepared after 4 h of stimulation and firefly luciferase as well as beta-galactosidase activity were determined by Dual Light Chemiluminescent Reporter Gene
Assay (Applied Biosystems, Foster City, CA, USA) in duplicate measurements with a Varioskan Flash Multimode Reader (Thermo Fisher Scientific Inc., Waltham, MA, USA). Relative light units of luciferase activity were normalized to beta-galactosidase values and were expressed in relation to untreated control samples set to 100%.

**Determination of reactive oxygen generation**

To determine effects of levosimendan on ROS-generation in endothelial cells, HUVEC were incubated in M199 medium containing 1.25% FCS, antibiotics and 20 µM 2',7' -dichlorofluoresceindiacetate (H<sub>2</sub>DCFDA; abcamMitoSciences®, Cambridge, MA, USA). DCFDA is a fluorogenic dye, that is being deacetylated after diffusion into the cell into a non-fluorescent compound, can later be oxidised by ROS into 2',7'-dichlorofluorescein (DCF), a highly fluorescent compound that can be detected by fluorescence spectroscopy. After staining with DCFDA, cells were treated with levosimendan with or without IL-1β for 4 h, as indicated. Fluorescent activity was detected and measured using the Synergy™ H1 Microplate Reader (Biotek, Winooski, VT, USA) set at 485 nm excitation and 535 nm emission wavelength.

**Immunoflourescence microscopy**

HHMEC were grown in eight-well µ-slides (ibidi GmbH, Martinsried, Germany) and pre-treated without or with levosimendan at a concentration of 10 µM for 30 min. Afterwards, rh-IL-1β (200 U/ml) without or with levosimendan (10 µM) was added for 4 h. Cells were washed with PBS twice and afterwards fixed in 4% paraformaldehyde (PFA) for 15 min at room temperature and then neutralised with 50 mM ammonium chloride for 10 min. Cells were incubated with indicated first step antibodies, diluted in PBS containing 0.1% TritonX-100 (Bio-Rad) for permeabilisation:

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**Figure 2: Effects of levosimendan on ICAM-1, E-selectin and VCAM-1 expression and mRNA in human heart microvascular endothelial cells.**

A-C) Human heart microvascular endothelial cells (HHMEC) were treated as indicated. Cells were stained with the respective fluorescence-labelled monoclonal antibodies and analysed with flow cytometry. Given are histograms of mean fluorescence intensity (MFI); histograms filled in grey represent cells treated with levosimendan and IL-1β as opposed to clear histograms that represent cells treated with IL-1β alone. D-F) Cells were treated for 8 h, as indicated. Cells were gently detached, stained with the respective fluorescence-labelled monoclonal antibodies and analysed by flow cytometry. Values are given in mean MFI and represent mean values ± SD. G-I) Cells were treated as indicated for 4 h, mRNA was prepared and analysed for ICAM-1 (G), E-selectin (H) and VCAM-1 (I); mRNA levels were normalised according to the respective GAPDH mRNA levels. Values represent mean ± SD x-fold control. All experiments were performed three times with cells obtained from three different donors with similar results. Representative images are shown. * p<0.05 for IL-1β vs IL-1β + levosimendan.

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Figure 3: Effects of levosimendan on expression of ICAM-1, E-selectin and VCAM-1 by human heart microvascular endothelial cells. Staining for ICAM-1 (A-D), E-selectin (E-H) and VCAM-1 (I-L) was performed as described in Methods. Cells were treated with media alone (A, E, I), 10 µM levosimendan (B, F, J), 200 U/ml IL-1β alone (C, G, K) or 200 U/ml IL-1β and 10 µM levosimendan (D, H, L) for the time periods indicated. Original magnification X63.

E-selectin (R&D Systems), ICAM-1 (R&D Systems), VCAM-1 (BD Pharmingen) or respective isotype control (1 µg/ml mouse IgG1; Cedarlane, Burlington, ON, Canada). Antibodies were incubated for 1 h at room temperature before cells were washed twice with PBS containing 0.05% Tween 20 (Bio-Rad). Second step antibody was Alexa Fluor® 488-labelled goat anti-mouse IgG (Invitrogen, Paisley, UK), diluted in PBS. Fluorescence-labelled antibodies were incubated for 1 h at room temperature followed by two washing steps with PBST. Nuclei were visualised using DAPI (Sigma) in a concentration of 1 µg/ml. After two washing steps, the eight-well chamber was filled up with 200 µl PBS, so fixed cells cannot dry out. Samples were analysed with the confocal laser scanning microscope LSM780 (Carl Zeiss, Jena, Germany) using the 20× lens (numeric aperture 0.8) or the 63× lens with oil (numeric aperture 1.4).

Statistical analysis
All data are presented as mean ± SD. Data were compared by ANOVA followed by Bonferroni correction. Values of p<0.05 (two-tailed) were considered as statistically significant. For all statistical analysis, PASW Statistics 18 (SPSS Inc., Chicago, IL, USA) was used.

Results
Levosimendan down regulates IL-1β-induced IL-6 and IL-8 production in human cardiac myocytes
As can be seen in Figure 1, only little basal expression of IL-6 and IL-8 was detected in untreated HACM and treatment with levosimendan alone (10 µM) did not alter the expression pattern of these cytokines. Treatment with IL-1β (200 U/ml) significantly increased mRNA levels of IL-6 and IL-8 after 2 h, 4 h and 8 h, reaching a peak at 4 h and returning nearly to basal levels after 24 h of treatment. Incubation with levosimendan significantly diminished up-regulation of IL-6 and IL-8 mRNA after incubation with IL-1β by up to 75% (n=12; p<0.01) and 65% (n=12; p<0.01), respectively (Figure 1A and B). Similar results were observed when the cells were treated with TNF-α at a concentration of 2,000 U/ml before incubation with levosimendan. Levosimendan reduced TNF-α-induced expression of IL-6 and IL-8 in these cells by 33% (n=6; p<0.05) and 42% (n=6; p<0.05), respectively. IL-1β increased IL-6 and IL-8 antigen production time-dependently as measured by specific ELISA. Comparable to data obtained from rt-PCR, treatment with levosimendan at a concentration of 10 µM diminished antigen production of IL-6 and IL-8 at all time-points measured by up to 60% (n=6; p<0.001) and 90% (n=6; p<0.001), respectively (Figure 1C and D). When HACM were co-treated with IL-1β and different concentrations of levosimendan ranging from 0.1 up to 10 µM for 4 h, a significant reduction in mRNA levels was ob-
served for 10, 5, 1 and 0.5 µM, but not for 0.1 µM (Figure 1E and F). An LDH-based toxicity assay showed no differences between treated and untreated samples, thus excluding any potential toxic effects of levosimendan (data not shown).

**Effects of levosimendan on IL-1β-induced ICAM-1, E-selectin and VCAM-1 expression in human heart microvascular endothelial cells**

Basal expression of ICAM-1, E-selectin and VCAM-1 in HHMEC was low, as determined by flow cytometry and real-time PCR (Figure 2). Addition of levosimendan alone (10 µM) had no effect on basal expression of the beforehand mentioned adhesion molecules. Treatment of cells with IL-1β (200 U/ml) for 8 h significantly increased cell surface expression of ICAM-1 seven-fold, E-selectin 20-fold and VCAM-1 four-fold, as measured by flow cytometry. When cells were treated with levosimendan (10 µM), IL-1β-induced expression of ICAM-1 was reduced by 40% (n=12; p<0.001), E-selectin by 70% (n=12; p<0.001), while expression of VCAM-1 (n=12; p=NS) remained unchanged (Figure 2A-F). These results were confirmed by rt-PCR in a similar experiment; cells were treated as described above for 4 h and ICAM-1, E-selectin and VCAM-1 mRNA was analysed by real-time PCR. Co-treatment with levosimendan for 4 h significantly decreased ICAM-1 mRNA by 32% (n=9; p<0.05) and E-selectin mRNA by 64% (n=9; p<0.05). In contrast, downregulation of VCAM-1 mRNA by levosimendan was only minimal which did not translate to a reduced cell surface expression of the protein (n=9; p=NS; Figure 2G-I). Additionally, when HHMEC were stained with fluorescence-labelled antibodies against ICAM-1 and E-selectin and analysed with immunofluorescence microscopy a markedly decreased expression of adhesion molecules was observed (Figure 3). As current evidence points to a heterogeneity of endothelial cells from different vascular beds we further tested the effects on levosimendan on HAEC. Treatment with IL-1β for 4 h strongly increased surface expression of ICAM-1, E-Selectin and VCAM-1 15-fold, 60-fold and 20-fold, respectively. Co-incubation with levosimendan (10 µM) strongly attenuated this effect by 50%, 78% and 30%, respectively (p<0.0001 for ICAM-1 and E-selectin and p<0.001 for VCAM-1; n=3), suggesting a slightly heterogenic reaction of ECs obtained from different vascular beds. Comparable results were found by rt-PCR (Suppl. Figure 2, available online at www.thrombosis-online.com). Similar effects were observed in HUVEC where levosimendan (10 µM) time-dependently inhibited the effects of IL-1β (200 U/ml) on antigen and mRNA expression of ICAM-1 and E-selectin, respectively (Figure 4A-D; n=6) while VCAM-1 expression remained unchanged (data not shown). Co-incubation of HUVEC with IL-1β and increasing concentrations of levosimendan led to a significant reduction in surface expression of ICAM-1 and E-selectin with 10, 5 and 1 µM, but not with 0.5 and 0.1 µM (Figure 4E, F). Similar results were observed when the stimulus for cells was treatment with TNF-α at a concentration of 2,000 U/ml. Levosimendan reduced the TNF-α-induced expression of ICAM-1 and E-selectin in these cells by 33% and 55%, respectively (p<0.05; n=3). To test whether levosimendan also exhibits beneficial effects when given after IL-1β, mimicking a possible real-life situation of a patient receiving medical treatment after MI, levosimendan was given at the same time as IL-1β or 30, 60 and 120 min after cytokine treatment for a total incubation time of 4 h and expression of adhesion molecules was measured with flow cytometry. As can be seen in Figure 4, the effects of levosimendan on ICAM-1 and E-selectin surface expression varied according to the time of administration, with the strongest effects seen when given together with IL-1β (Figure 4G and H). Notably, even when given up to 120 min after IL-1β, levosimendan exhibited attenuating effects on the expression of E-selectin.

**Effects of levosimendan on IL-1β-mediated adhesion of polymorphonuclear neutrophils to endothelial cells**

When HUVEC were pre-treated with IL-1β (200 U/ml) for 4 h and subsequently incubated with 1 ml of 1×10⁶ PMN for 5 min, a 17-fold increase in the area covered with PMN adherent to the endothelium was observed, when compared to untreated HUVEC as control (p<0.001). This IL-1β-induced granulocyte adhesion was significantly attenuated when cells were pre-incubated with levosimendan at concentrations of 10 µM and 1 µM by 90% and 60%, respectively (p<0.01 for both, Figure 5A-B). Incubation for 30 min resulted in similar effects, a 75% and 55% reduction of area covered was seen with 10 µM and 1 µM (p<0.001 for both, Figure 5C-D). Concentrations of 0.1 µM had no effect on granulocyte adhesion.

Under flow conditions (1 dyn/cm² for 5 min), pretreatment with IL-1β (200 U/ml) for 4 h induced a 10-fold increase in PMN adhesion to HUVEC monolayer, when compared to unstimulated cells (p<0.001). Co-treatment with levosimendan (10 µM) reduced the number of adherent granulocytes by 40% (p<0.001; Figure 5E and F).

**Figure 4: Effects of levosimendan on ICAM-1 and E-selectin surface and mRNA expression in HUVEC. A-D) HUVEC were treated with media alone (full triangles), 10 µM levosimendan (open triangles), 200 U/ml IL-1β alone (full circles) or 200 U/ml IL-1β and 10 µM levosimendan (open circles) for the time periods indicated. Cells were gently detached, stained with the respective fluorescence-labelled monoclonal antibodies and analysed by flow cytometry. Values are given in MFI and represent mean values ± SD. E-H) Treatment after MI, levosimendan was given at the same time as IL-1β, mimicking a possible real-life situation of a patient receiving medical treatment after MI, levosimendan was given at the same time as IL-1β or 30, 60 and 120 min after cytokine treatment for a total incubation time of 4 h and expression of adhesion molecules was measured with flow cytometry. As can be seen in Figure 4, the effects of levosimendan on ICAM-1 and E-selectin surface expression varied according to the time of administration, with the strongest effects seen when given together with IL-1β (Figure 4G and H). Notably, even when given up to 120 min after IL-1β, levosimendan exhibited attenuating effects on the expression of E-selectin.**

**Figure 5: The effects of levosimendan on ICAM-1 and E-selectin surface expression and mRNA expression in HUVEC. A-B) HUVEC were treated with media alone (full circles), 10 µM levosimendan (open circles), 200 U/ml IL-1β alone (full circles) or 200 U/ml IL-1β and 10 µM levosimendan (open circles) for the time periods indicated. Cells were gently detached, stained with the respective fluorescence-labelled monoclonal antibodies and analysed by flow cytometry. Values are given in MFI and represent mean values ± SD. C-D) Concentrations of 0.1 µM had no effect on granulocyte adhesion.**
Anti-inflammatory effects of levosimendan are partly dependent on mitoK$_{ATP}$-channels

To examine whether the above described anti-inflammatory effects rely on opening mitochondrial K$_{ATP}$-channels, HUVEC and HACM were incubated with 5-HD, a selective blocker of mitoK$_{ATP}$-channels, 15 min prior to levosimendan treatment. Pretreatment with 5HD partly abolished the anti-inflammatory effects of levosimendan on ICAM-1 and E-selectin expression by HUVEC (p< 0.05 and p< 0.01, respectively; ▶ Figure 6 A and B). However, effects on IL-6 and IL-8 mRNA by HACM remained unchanged (▶ Figure 6 C and D).

Levosimendan attenuates reactive oxygen production

Treatment with IL-1$\beta$ significantly induced ROS production (p<0.05) in HUVEC. This effect was completely abolished by treatment with levosimendan (p<0.01; ▶ Figure 6E).

Influence of levosimendan on the NF-$\kappa$B pathway

Treatment of HUVEC and HACM with IL-1$\beta$ strongly increased the levels of NF-$\kappa$B subunits p50 and p65 within nuclear extracts. Interestingly, pretreatment with levosimendan did not alter nuclear translocation of these two subunits (▶ Figure 7A-D). Furthermore, neither levosimendan nor 5-HD had any influence on IL-1$\beta$ induced phospho-I$k$B$\alpha$ protein levels as measured by Western blotting (▶ Figure 7E-F). On the contrary, levosimendan treatment attenuated NF-$\kappa$B reporter gene-activity in HUVEC and HACM transfected with luciferase reporter NF-$\kappa$B promoters (p<0.001 and p<0.05 for HUVEC and HACM, respectively; ▶ Figure 7G-H). Since phosphorylation at different sites of p65 have been found to influence its activity, we assayed some of them and found that levosimendan diminished the IL-1$\beta$-induced phosphorylation of S536, but not of S276 or S529. These findings suggest a possible involvement of specific phosphorylation sites in the transactivation domain of the p65 subunit of NF-$\kappa$B (▶ Figure 7I) in the effects of levosimendan described above.
Discussion

Myocardial reperfusion by primary PCI or thrombolysis is the most effective therapy for patients presenting with MI to limit infarct size and prevent HF. However, morbidity and mortality after myocardial reperfusion remain high, a fact that can be explained by a phenomenon termed “reperfusion injury”, a paradoxical myocardial tissue damage caused by blood flow restoration (17). Reperfusion of the infarcted myocardium induces a strong inflammatory response, involving increased expression of chemoattractants guiding neutrophils into the infarcted myocardial tissue (29). Levosimendan, a calcium sensitizer used for the treatment of acute decompensated HF, was shown to be particularly effective in patients suffering from acute MI (5, 7). These cardioprotective effects might be explained by the drug’s ability to open mitochondrial ATP-sensitive potassium channels in cardiomyocytes (1, 11).

Here we present evidence for the first time that levosimendan exhibits anti-inflammatory effects on human adult cardiac myocytes in vitro, by dampening the IL-1β-induced production of IL-6 and IL-8. Furthermore, we show that levosimendan significantly

Figure 6: Effects of levosimendan are partly dependent on opening mitoK_{ATP}-channels and influencing reactive oxygen production. A-B) Effects of levosimendan on HUVEC are partly dependent on opening mitoK_{ATP}-channels. HUVEC were treated for 4 h as indicated. mRNA for ICAM-1 (A) and E-selectin (B) was normalised according to the respective GAPDH mRNA levels. Values represent mean ± SD x-fold control. C-D) Effects of levosimendan on HACM are partly dependent on opening mitoK_{ATP}-channels. HACM were treated for 4 h as indicated. Cells were lysed and mRNA was analysed for IL-6 (C), IL-8 (D); mRNA levels were normalised according to the respective GAPDH mRNA levels. Values represent mean ± SD x-fold control. E) Effects of levosimendan on reactive oxygen production. HUVEC were incubated with DCFDA for 30 min and subsequently treated with levosimendan, IL-1β or IL-1β + levosimendan for 4 h, as indicated. Fluorescence intensity was measured. Values are given in relative fluorescence units and represent mean ± SD. * p<0.05 IL-1β vs IL-1β + levosimendan; § p<0.05 IL-1β + levosimendan vs IL-1β + levosimendan + 5HD.
attenuates the expression of ICAM-1 and E-selectin in activated human heart microvascular endothelial cells. Additionally, levosimendan treatment reduced granulocyte adhesion to endothelial cells under static and flow conditions.

IL-6, a potent pro-inflammatory cytokine, is rapidly expressed in the ischaemic myocardium, promoting ICAM-1 upregulation and is implicated in reperfusion injury of the myocardium (18, 30). The chemokine IL-8 is induced within 1 h after reperfusion of a myocardial infarct and represents a potent chemoattractant for neutrophils (31). We found that in cardiac myocytes, levosimendan attenuates IL-1β-induced expression of IL-6 and IL-8 in a time- and dose-dependent manner, with significant effects of levosimendan seen at a minimum dosage of 0.5 µM. This dose corresponds to plasma concentrations found in patients treated with

![Figure 7: Effects of levosimendan on the NF-κB-pathway after inflammatory activation. A-B) HUVEC (A) and HACM (B) were pre-treated with levosimendan and/or 5-HD for 30 min, followed by IL-1β for 5 min. Cells were lysed and western blot analysis was performed for the detection of GAPDH and phosphor-IκBα as described in Methods. Experiments were performed two times, representative images are shown. C-F) HUVEC (C-D) and HACM (E-F) were treated with IL-1β and levosimendan for 30 min as indicated and NF-κB subunits p50 (C, E) and p65 (D, F) within nuclear extracts were quantified using an ELISA based TransAmTM NF-κB Kit as described in Methods. Values represent OD measured at 492 nm ± SD. G-H) HUVEC and HACM were transfected with NF-κB reporter plasmid by electroporation, as described in Methods and subsequently treated with media alone, IL-1β or IL-1β + levosimendan for 4 h, as indicated. Values are given in relative light units of luciferase activity normalised to β-galactosidase activity and represent mean values ± SD from two experiments. I) HUVEC were treated as described for 30 min, nuclear extracts were obtained and analysed for phosphorylation of different p65 sites. * p<0.05 IL-1β vs IL-1β + levosimendan; § p=non-significant Control vs IL-1β + levosimendan.

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Levosimendan (32). As much of the reperfusion injury takes place in the microvasculature and is dependent on activation of the endothelial monolayer, we further tested possible anti-inflammatory effects of levosimendan on human microvascular endothelial cells. Activation of endothelial cells induces surface expressions of selectins, such as E-selectin, promoting initial tethering and rolling on the vessel wall, a prerequisite for later firm binding and transmigration. When microvascular endothelial cells were pretreated with levosimendan, the IL-1β induced expression of the adhesion molecules ICAM-1 and E-selectin, two important players in granulocyte-induced myocardial damage, was strongly attenuated on both mRNA and protein levels. It has to be addressed, that VCAM-1 expression that is involved in monocyte but not in PMN migration, with the exception of endothelial cells harvested from human aorta was barely changed. We could further demonstrate a dose- and time-dependent down-regulation of ICAM-1 and E-selectin in human endothelial cells pretreated with levosimendan with significant effects seen at a minimum dosage of 1 µM. However, in an every day real-life situation, a patient would receive medical treatment after a coronary thrombotic occlusion with subsequent inflammatory activation in the myocardium had occurred. Therefore, we tested the dampening effects of levosimendan when given after IL-1β and could demonstrate that the beneficial effects of levosimendan decreased when given at later time points. Still, even when given up to 1 or 2 h after IL-1β, levosimendan attenuated the expression of E-selectin and ICAM-1. However, when given 2 h after IL-1β, only the effects on E-selectin remained statistically significant.

Granulocyte adhesion to the vessel wall and subsequent extravasation into myocardial tissue represents a major detrimental step in reperfusion injury. In animal models, blocking the neutrophil-endothelium interaction by administering blocking antibodies directed against ICAM-1 on endothelial cells or against CD18, the correspondent receptor on granulocytes, resulted in a significant reduction in infarct size (33, 34). Here, we demonstrate for the first time that levosimendan treatment significantly attenuates granulocyte adhesion to activated endothelial cells by approximately 50%, both under static and flow conditions.

Pretreatment with 5-HD, a selective blocker of mitoK+ATP+ channels, partly abolished the above-mentioned anti-inflammatory effects, thus suggesting a participation of the cardioprotective potassium channels in the anti-inflammatory effects seen in our experiments.

The finding that levosimendan inhibits the production of ROS provided a first insight into its mode of action, since ROS have been demonstrated to lead to activation of NF-κB, a main regulator of ICAM-1 and other pro-inflammatory genes in endothelial cells (35). We have therefore investigated on which level levosimendan interferes with NF-κB signalling. Levosimendan did not prevent IkBα phosphorylation, nuclear translocation, or binding of p65 and p50 subunits of NF-κB to their target site. In contrast, NF-κB activity, as measured by reporter gene analysis, was inhibited, in line with previously published work demonstrating inhibition of NF-κB-activation in monocytes and HUVEC (20, 21). This suggests that a post-translational modification is affected, and since phosphorylations at several sites on p65 have been reported to regulate its activity we tested some of them for responsiveness to levosimendan. Indeed, levosimendan prevented the IL-1- and β2-induced phosphorylation of S536, which is located in the transactivation domain 1, but not of S276 or S529. It is interesting to note that S526 has been implicated in gene-specific regulation of NF-κB targets in macrophages (35), supporting our finding of differential inhibition of only certain cell adhesion molecules and interleukins.

After infusion of levosimendan, active metabolites are circulating for 7–10 days (36). We were not able to study the anti-inflammatory activity of these metabolites. However, mainly the short term effects of levosimendan treatment may be of importance to avoid reperfusion injury as it occurs within the first hours after acute MI. In addition, effective concentrations in our study are relatively high. However, we were able to demonstrate a dose-dependent effect on PMN adhesion as main functional readout. In addition, at least to our knowledge, tissue concentrations of levosimendan in comparison to plasma concentrations are not known.

In conclusion, we provide evidence for the first time that levosimendan dampens the expression of IL-6 and IL-8 in activated human cardiac myocytes. Furthermore, we illustrate that levosimendan attenuates granulocyte adhesion to endothelial cells by down-regulating the expression of ICAM-1 and E-selectin. These effects of levosimendan are partly dependent on mitoK+ATP+/channels and mediated by inhibition NF-κB activity and ROS production. If these anti-inflammatory actions are also effective in vivo, administration of levosimendan after acute MI could have beneficial effects due to a blunted reperfusion injury.
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Conflicts of interest
Walter S. Speidl received an unrestricted research grant from Orion Pharma. All the other authors declare no conflict of interest regarding this manuscript.

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