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Sulodexide modifies intravascular homeostasis what affects function of the endothelium

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ABSTRACT

Purpose: Sulodexide is a mixture of heparin and dermatan sulphate which has an antithrombotic action. It was shown that it has also direct effect on the endothelial cells. We tested the effect of sulodexide on the intravascular homeostasis in patients with peripheral vascular disease.

Methods: Sulodexide was infused iv. at a dose of 1200 Lipoprotein Lipase Releasing Units (LRU) in 10 patients with peripheral vascular disease. Blood samples were collected before the infusion and 1, 6 and 24 hours after the infusion. Inflammatory and fibrinolytic parameters were studied in the collected serum samples. Additionally, *ex-vivo* effect of the serum samples on *in vitro* function of the endothelial cells was studied.

Results: Infusion of sulodexide caused acute and transient peak of the Hepatocyte Growth Factor (HGF) concentration in blood and decrease of the Vascular Endothelial Growth Factor (VEGF) level, what, as we found in *in vitro* experiments, was due to adsorption of VEGF to endothelium. We found that HGF enhanced *in vitro* stimulating effect of VEGF on proliferation of the endothelial cells. Serum level of interleukin-6 was gradually decreased, whereas fibrinolytic activity of serum, reflected by t-PA/PAI-1 ratio, increased. Serum samples obtained from the studied patients suppressed oxidative stress and release of interleukin-6 in endothelial cells maintained in *in vitro* culture.

Conclusion: Sulodexide reduces intravascular inflammation and suppresses inflammatory reaction in the endothelial cells; both effects are desirable in patients with peripheral vascular disease.

Key words: Sulodexide, inflammation, endothelium, peripheral vascular disease

INTRODUCTION

Sulodexide is a mixture of natural glycosaminoglycans obtained from porcine digestive mucosa. It contains low molecular weight heparin sulphate catalyzing the antiprotease action of antithrombin III and dermatan sulphate with affinity for the heparin II co-factor, what explains strong antithrombotic effect of that drug [1]. In our previous studies, we found that sulodexide reduces inflammation in rats with peritonitis [2] and similar effect was observed *in vitro* in endothelial cells exposed to hyperglycemia or undergoing senescent changes [3,4]. Intravenous infusion of sulodexide in humans induces transient increase of HGF concentration in plasma [5] which is a potent anti-inflammatory cytokine [6]. Administering sulodexide continuously for 2 weeks suppresses in humans the baseline plasma level of transforming growth factor-beta1 (TGFbeta-1) [7]. Treatment with sulodexide attenuates myocardial ischemia and reperfusion injury and that effect is supposed to be due to inhibition of complement activation, possibly through inhibition of CRP [8]. One can

therefore speculate that intravenous infusion of sulodexide changes the properties of blood due to activation or inhibition of various metabolic pathways what may affect the function of the endothelial cells, independently of the direct effect of sulodexide on the endothelium.

In the present study, we show the changes in properties of serum, observed within 24 hours from the intravenous infusion of sulodexide in patients with peripheral vascular disease. Additionally, we tested how such modified serum affects function of the endothelial cells in *in vitro* culture.

MATERIAL AND METHODS

The study was performed in 10 patients treated in the Department of Vascular Surgery at the Poznan University of Medical Sciences (Poland). The patients were diagnosed with atherosclerotic changes in legs arteries. Study conforms to the ethical guidelines for human research and was approved by Bioethics Committee of our University. Mean age of the patients, all males, was 62.2 ± 2.1 years. Each patient was infused intravenously with Sulodexide (Vessel Due F, Alfa Wassermann SpA, Bologna, Italy) at a dose of 1200 Lipoprotein Lipase Releasing Units (LRU). Blood samples were obtained just before the infusion, as well as, 1 hour, 6 hours and 24 hours after the infusion of sulodexide. Collected blood samples were immediately span down and serum was stored at -86°C for further analysis.

Concentration of the following cytokines and proteins was measured in serum samples obtained at each time interval:

- Hepatocyte Growth Factor (HGF): ELISA assay (R&D Europe), sensitivity 20pg/ml;
- Interleukin 6 (IL-6): ELISA assay (R&D Europe), sensitivity 2 pg/ml;
- Monocyte Chemoattractant Protein-1 (MCP-1): ELISA assay, (R&D Europe), sensitivity 8 pg/ml;
- Tissue Plasminogen Activator (t-PA): ELISA assay, (Bender MedSystems GmbH Austria), sensitivity 6 pg/ml;
- Plasminogen Activator Inhibitor-1 (PAI-1): ELISA assay (R&D Europe), sensitivity 14 pg/ml.

In another part of the study, serum samples were tested on the human umbilical endothelial cells maintained in *in vitro* culture. Primary cell lines were purchased from Cascade Biologics (Paisley, UK). Cells were grown in medium 200PRF supplemented with fetal bovine serum 2%, Basic Fibroblast Growth Factor (1.5 μg/mL), human Epidermal Growth Factor (5μg/mL), and hydrocortisone (1μg/mL); all products purchased from Cascade Biologics (Paisley, UK). The cell culture was initially established in 75 cm^2 culture flasks and afterwards cells were reseeded into 24-well culture plates (Corning BV Life Sciences, Schiphol, Netherlands) and grown until reached confluence. Experiments in which proliferation of the endothelial cells was studied were performed on cells during their exponential growth.

In all experiments, cells were exposed to culture medium additionally supplemented with serum obtained from patients at each time interval; final patients' serum concentration in the sample was 25%. All experiments were repeated 4 times. We studied the effects of the serum samples on such functional properties of the endothelium in the *in vitro* culture, as proliferation, generation of free radicals and synthesis of interleukin 6.

Proliferation of the endothelial cells was measured on the pre-confluent cells during their exponential growth. Cells were exposed to culture medium supplemented with 25% patients serum, and ^{3}H-methyl-thymidine (Radioisotope Institute, Prague, Czech) which was added to medium to reach its final concentration of 1μCi/mL. Cells were cultured during 24 hours and afterwards, after removal of medium from the wells, were harvested with trypsin 0.05%–EDTA 0.02% solution and precipitated with 10% trichloroacetic acid (TCA). The precipitate was washed twice with TCA, and afterwards lysed with 1N NaOH. Radioactivity of the lysate was measured in a β- liquid scintillation counter (1209 Rackbeta LKB Wallac, Finland). Incorporation of the radioactive thymidine into the cellular DNA was used as an index of the cells growth.

Intracellular generation of reactive oxygen species (ROS) was measured in endothelial monolayers in 24-well culture clusters, with 2',7'-dichlorodihydrofluorescein diacetate (DCDHF) probe. After 48 hours, the culture of the endothelial cells in medium containing 25% of the patients serum, cells were exposed to the same medium for 60 minutes but supplemented with DCDHF. Afterwards, medium was removed from wells and the cells were lysed with lysis buffer (Promega, Madison Wis, USA). Fluorescence emitted by cell lysates was measured at a wavelength of 485 nm for excitation and 535 nm for emission in a Wallac Victor spectrofluorimeter (Perkin-Elmer, Turku, Finland). Protein concentration in the lysate was measured with Lowry method [9]. Generation of ROS was expressed per amount of cell protein.

Synthesis of IL-6 in the endothelial cells was studied in endothelial monolayers in 24-well clusters which were cultured during 48 hours in medium containing 25% of the patients serum. At the end of the incubation, medium samples were collected for measurement of IL-6 concentration (ELISA assay) and the cells were lysed with 0.1N NaOH for measurement of protein concentration with Lowry method [9]. Synthesis of IL-6 in the endothelial cells was expressed per amount of the cell protein.

We found, that iv. infusion of sulodexide, after 1 hour, caused increase of HGF concentration in plasma with simultaneous decrease of plasma VEGF. We supposed that HGF can enhance VEGF adsorption to the surface of the endothelium. Therefore we tested in *in vitro* experiments if

that is true. Endothelial monolayers were exposed during 1 hour to the following solutions:

- Medium supplemented with VEGF 1000 pg/ml
- Medium supplemented with VEGF 1000 pg/ml and HGF 5000pg/ml

After 1 hour incubation, medium from the wells was removed and the cells monolayers lysed with distilled water. Concentration of VEGF in the medium sample and in the cells lysate was measured and compared with amount of VEGF added to the well.

In the next series of experiments we studied if VEGF and HGF have synergistic effect of the endothelial cells growth. Endothelial cells during the exponential growth were exposed to the following solutions:

- Medium
- Medium supplemented with HGF 5000 pg/ml
- Medium supplemented with VEGF 1000 pg/ml
- Medium supplemented with HGF 5000 pg/ml and VEGF 1000 pg/ml

Additionally to all wells ^{3}H-methyl-thymidine (Radioisotope Institute, Prague, Czech Republic) was added to medium to reach its final concentration of 1μCi/mL. Proliferation of the cells was studied as described above.

Statistical analysis

Results are expressed as mean ± SD. Statistical analysis was performed with analysis of variance with *post hoc* Tukey test. The p value less than 0.05 was considered statistically significant.

RESULTS

Infusion of sulodexide caused several changes in the inflammatory and fibrinolytic blood parameters. Sulodexide induced transient rise in serum concentration of HGF concentration (*Fig. 1A*) and inverse change of the serum VEGF was observed at the same time (*Fig. 1B*). Serum concentration of IL-6 was gradually decreasing and at 6 hours was significantly lower, than before infusion (*Fig. 2A*). Similar trend was observed for blood MCP-1 level, but these changes did not reach statistical significance (*Fig. 2B*). After infusion of sulodexide, serum level of t-PA tended to increase, but these changes were not statistically significant (*Fig. 3A*). However, serum concentration of PAI-1 was significantly decreased (*Fig. 3B*) and therefore ratio of t-PA/PAI-1 increased (*Fig. 3C*).

In *in vitro* experiments, tested serum samples obtained after infusion of sulodexide did not change proliferation rate of the endothelial cells. Intracellular generation of free radicals in the endothelial cells was decreased when the cells were exposed to serum samples obtained 6 hours and 24 hours after infusion of sulodexide (*Fig. 4A*). All serum samples harvested after sulodexide injection inhibited *in*

Figure 1. **Plasma concentration of Hepatocyte Growth Factor (HGF) and Vascular Endothelial Growth Factor (VEGF) before iv. infusion of sulodexide (Start) and 1 hour, 6 hours and 24 hours after infusion of the drug.**

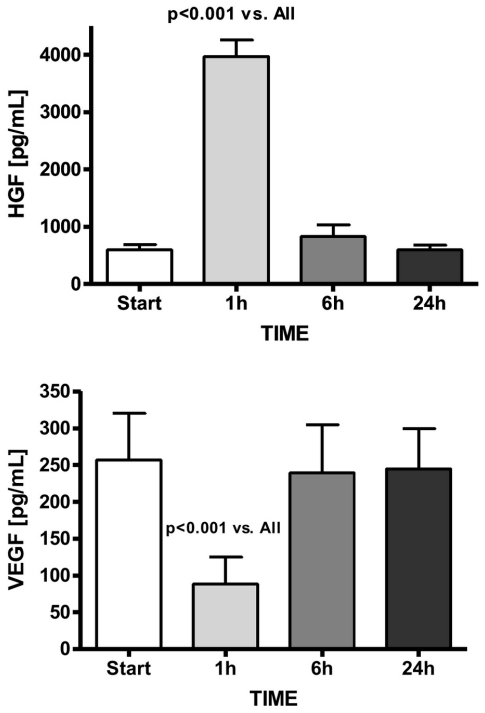


Figure 2. **Plasma concentration of Interleukin-6 [IL-6] and Monocyte Chemoattractant Protein-1 (MCP-1) before iv. infusion of sulodexide (Start) and 1 hour, 6 hours and 24 hours after infusion of the drug.**

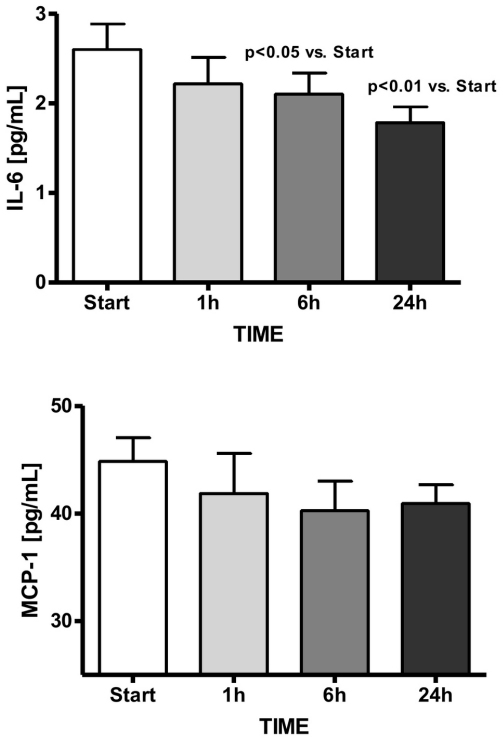
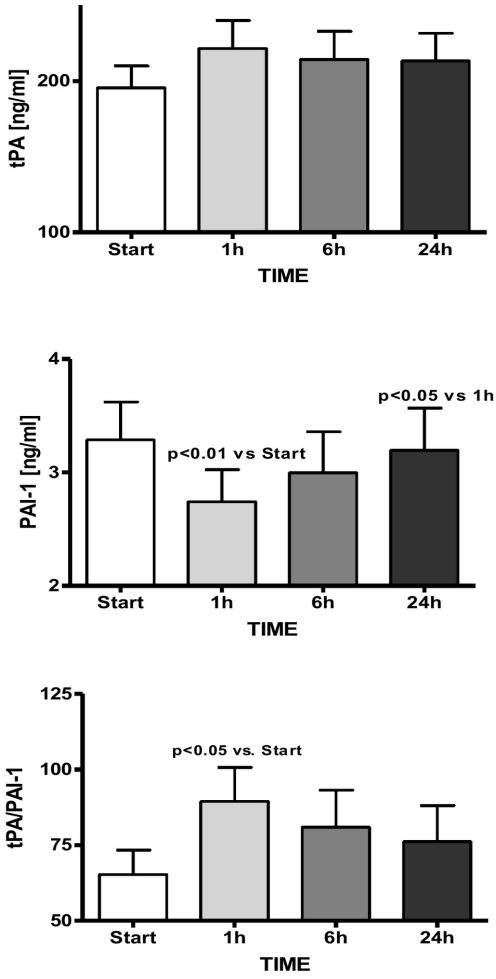


Figure 3. **Plasma concentration of Tissue Plasminogen Activator (t-PA), Plasminogen Activator Inhibitor-1 (PAI-1) and ratio t-PA/PAI-1 before iv. infusion of sulodexide (Start) and 1 hour, 6 hours and 24 hours after infusion of the drug.**



vitro IL-6 synthesis in the endothelial cells, but the effect was the strongest with the serum sample obtained 1 hour after injection of the drug (*Fig. 4B*).

Exposure of the endothelial cells to HGF enhanced *in vitro* adsorption of VEGF to the endothelium (*Fig. 5A*). VEGF dependent proliferation of the endothelial cells was stronger when the cells were simultaneously exposed to HGF (*Fig. 5B*).

DISCUSSION

We demonstrated, that intravenous infusion of sulodexide in humans changes the properties of blood. Inflammatory reaction is reduced as reflected by decreased concentration of IL-6 in serum (*Fig. 2A*) and a trend for decreased level of MCP-1 was observed (*Fig. 2B*). Anti-inflammatory action of sulodexide was previously described in conditions of

Figure 4. **Generation of free radicals [A] and synthesis of IL-6 [B] in the endothelial cells in vitro exposed during 48 hours to medium samples obtained before infusion (Start) and 1 hour, 6 hours and 24 hours after infusion of sulodexide.**

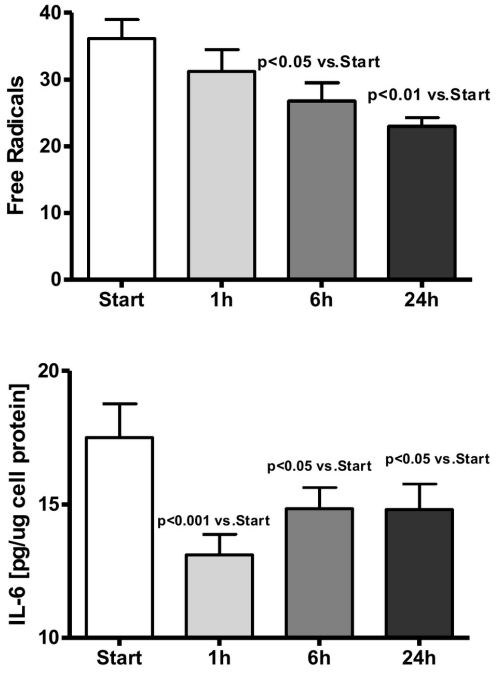
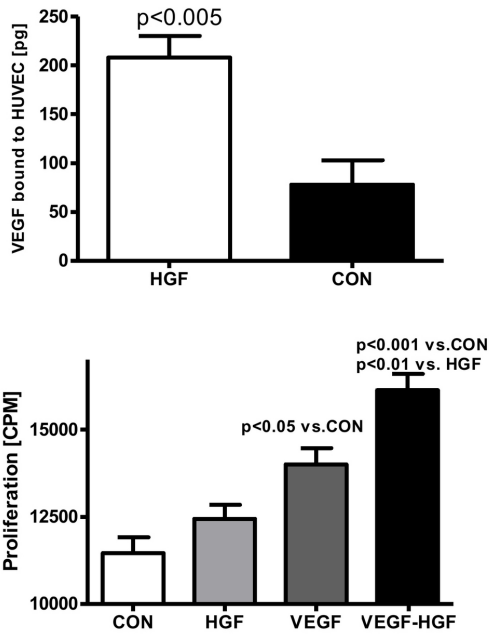


Figure 5. **Adhesion of VEGF to the endothelium in vitro during 1 hour incubation in control medium (CON) and in control medium supplemented with HGF 5000pg/ml and proliferation of the endothelial cells exposed in vitro to standard medium (CON) or to medium supplemented with HGF 5000 pg/ml (HGF) or with VEGF 1000 pg/ml (VEGF) or with VEGF 1000 pg/ml and HGF 5000 pg/ml (VEGF-HGF).**



peritoneal dialysis where chronic oral administration of that drug resulted in a significant decrease of the dialysate concentration of interleukin1-β, interleukin 6 and interleukin 8 [10]. We found, that sulodexide reduces intraperitoneal inflammation during acute peritonitis in rats [2]. An interesting observation in the present study was the prolonged effect of a single dose of sulodexide, which anti-inflammatory action was the strongest 24 hours after application of the drug (*Fig. 2*). However, changes of HGF in serum were similar in character, but weaker, as described previously by Borawski et al. [5]. That difference in the magnitude of the sulodexide effect could be due to the fact that our patients were older and with advanced atherosclerosis, contrary to Borawski et al. [5] who studied healthy volunteers. Concentration of HGF increased in serum 5-fold during the first hour after infusion of sulodexide, but at 6 and 24 hours was comparable to the preinfusion values (*Fig. 1A*). Such effect was already described for low molecular weight heparin which releases cytokines from the endothelial glycocalyx and the extracellular matrix to circulation [11,12]. Nevertheless, contrary to Salbach et al. [12], we found that parallel to HGF increase in serum, concentration of VEGF was decreased after 1 hour from 257 ± 201 pg/ml to 93 ± 113 pg/ml (*Fig. 1B*). Sulodexide-induced transient decrease of VEGF in blood may also prove the anti-inflammatory action of that drug. VEGF stimulates leukocytes rolling and adhesion to the endothelium [13] and progression of the atherosclerotic plaque [14].

We supposed, that rapid decrease of blood VEGF level, simultaneous with increased HGF concentration may be explained by HGF-induced enhanced binding of VEGF to the endothelium. We tested, that hypothesis in *in vitro* experiments which showed that indeed VEGF binding to the endothelial cells is significantly increased in presence of HGF (*Fig. 5A*). When we used in *in vitro* experiments HGF in concentration comparable to that observed in patients serum after sulodexide infusion no significant effect on proliferation of the endothelial cells was observed; only some visible trend for increase was noticed. (*Fig. 5B*). Heparin-induced angiogenesis is dependent on HGF [15] and HGF can induce angiogenesis independently of VEGF [16]. However, we found that serum samples obtained from patients infused with sulodexide had no effect on growth of the endothelial cells in *in vitro* culture. Discrepancy between our observations and the results from the other study may be due to a different type of the endothelial cells used during experiments, different type of the studied population and due to the specific properties of sulodexide which is a mixture of heparin and dermatan sulphate. However, we found that HGF enhanced VEGF dependent proliferation of the endothelial cells (*Fig. 5B*).

Another important observation in our study was increased fibrinolytic activity of serum after infusion of sulodexide (*Fig. 3*). However, we must stress that only t-PA and PAI-1 were measured in our study, which does not reflect all factors regulating the process of fibrinolysis. We measured both proteins in serum samples, what could affect their concentration, but because each patient served as its own control, we can neglect potential artifact due to applied methodology. In previous experimental and clinical studies enhanced fibrinolysis was observed after administration of sulodexide [17,18]. Our results suggest, that probable profibrinolytic action of sulodexide is due to decrease of blood PAI-1 level (*Fig. 3B*). Kim et al. [19] also found that sulodexide decreases D-dimers concentration in blood what may suggest its anticoagulant action.

We think that *ex-vivo* testing of serum from patients treated with sulodexide on endothelial cells in *in vitro* culture provides additional information about possible changes in the endothelial cells lining the blood vessels. Endothelial cells in *in vitro* culture offer a good experimental model, although conditions of such experiments do not completely reflect the *in vivo* environment. Growth factors or hydrocortisone in medium are necessary for adequate growth and viability of the cells, but may affect the results, especially those related to the inflammatory cellular response.

We confirmed the anti-inflammatory effect of sulodexide in our *in vitro* study in which endothelial cells were exposed for 48 hours to serum samples from patients treated with sulodexide. It is interesting that intracellular generation of free radicals was decreased in the presence of serum samples collected 6 hours and 24 hours after infusion of sulodexide (*Fig. 4A*). Therefore, the effect was probable not only dependent on the presence of sulodexide itself, but was related to sulodexide-induced changes in cytokines' composition in plasma. In our previous experiments on the *in vitro* cultured endothelial cells, we found, that sulodexide used as a supplement to medium reduced oxidative stress in senescent cells [4] or in cell exposed to hyperglycemia [3]. Antioxidative activity of sulodexide was observed also in clinical studies in patients with stable coronary artery disease [20] or diabetes mellitus [21]. Additionally. sulodexide upregulates the antioxidant mechanisms in the tissues [22].

Oxidative stress is one of the main factors responsible for stimulation of synthesis of the inflammatory cytokines [23,24]. We found, that endothelial cells exposed *in vitro* to serum samples harvested from patients infused with sulodexide showed reduced release of IL-6 (*Fig. 4B*). However, the effect on release of IL-6 was the strongest in the presence of samples collected 1 hour after infusion with sulodexide, which does not correlate with changes in the endothelial oxidative stress (*Fig. 4*). In our previous studies, we found, that sulodexide itself decreases synthesis of IL-6 [3,4]. Therefore, the observed changes in IL-6 synthesis in *in vitro* endothelial cells were probably mainly caused by sulodexide itself or could be also related to changes in the serum composition after infusion of that drug.

CONCLUSIONS

We conclude, that sulodexide infused intravenously changes the intravascular homeostasis. Inflammatory markers in the blood are reduced, whereas t-PA/PAI-1 ratio of serum increases. Both changes can contribute to better protection of viability of the endothelial cells lining the blood vessels what can result in delaying the progression of the atherosclerotic changes. Such effects might explain effectiveness of sulodexide treatment in patients with peripheral vascular disease [25]. One dose of sulodexide causes the effects which are stable at least during 24 hours. Results from experiments *in vitro* on the endothelial cells suggest that the beneficial, protective action of sulodexide may be cumulating after repeated doses of that drug. During the last decade, sulodexide, from an antithrombotic and profibrinolytic drug, has also become a strong anti-inflammatory drug, and due to a complex structure and properties of glycosaminoglycans, new therapeutic effects of sulodexide can be discovered in the near future.

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Conflict of interests

Sulodexide was provided by Alfa Wassermann Polska, Warsaw, Poland.

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