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Evaluation of Endothelium-Protective Effects of Drugs in Experimental Models of Endothelial Damage

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Summary

Endothelium-protective properties of pharmacological agents may be assessed by using different experimental models of endothelial dysfunction or injury. The model of endothelial dysfunction induced by vessel perfusion with polymorphonuclear leukocytes (PMN) was used for evaluation of pentoxifylline (PTX) effects on vasoconstrictor responses to noradrenaline (NA) in the rabbit renal artery. Addition of PMN into the perfusion solution significantly increased the responses to NA at all doses. PTX administration (10^{-5} mol.l^{1}) significantly diminished the constrictor responses to NA in vessels perfused with PMN+PTX when compared to the responses in PMN-perfused vessels (at dose 0.1 μg: 32.25 vs. 14.25, at dose 1 μg: 51 vs. 27.75 ($p<0.01$), at dose 10 μg 74.25 vs. 39.75 ($p<0.05$), all values expressed as median of perfusion pressure in mm Hg). The model of endothelial damage induced by repeated NA administration in 5 doses (10-50 μg of NA) was used for evaluation of the endothelium-protective effect of sulodexide (SLX). It was found that SLX (120 U/l) significantly decreased the number of desquamated endothelial cells (EC) compared to the control group (controls: 131.4±20.1 EC, +SLX: 83.3±13.8 EC, $p<0.01$). These results confirmed the favorable endothelium-protective effects of pentoxifylline and sulodexide in the two experimental models.

Key words

Models of endothelial dysfunction and damage • Endothelium-protective effects • Sulodexide • Pentoxifylline

Introduction

There is a growing body of evidence that endothelial dysfunction and/or injury are involved in numerous cardiovascular diseases such as hypertension, atherosclerosis and myocardial ischemia (Lüscher 1993, Angus 1996). Therefore, protection of the endothelium against injury and restoration of the integrity and function of endothelial cells is of particular interest for investigators and clinicians. Recent data from human and animal studies support the beneficial effect of some pharmacological agents on endothelial function, e.g. NO donors, ACE inhibitors, statins, estrogens and glycosaminoglycans (Guo *et al.* 1994, Gould *et al.* 1994, Drexler *et al.* 1995, Gaddi *et al.* 1996, Venkov *et al.* 1996, Kristová and Kriška 1998). A major effect of some pharmacological agents is exerted on the vascular endothelium resulting in improvement of endothelium-dependent vasodilatation, i.e. in reversal of the endothelial dysfunction. Such observations suggest that

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these drugs provide protection against endothelium-damaging stimuli and may act protectively.

It was recently found that endothelial dysfunction is mediated by cell adhesion molecules expressed on the surface of activated endothelial cells and leukocytes (Murohara *et al.* 1994). Drugs with the ability to inhibit the expression of adhesion molecules, e.g. monoclonal antibodies (Adams *et al.* 1997) and pentoxifylline (Ritter *et al.* 1996), might exert their beneficial effect on impaired function of the endothelium. Moreover, there is still a need of further studies clarifying these effects under experimental and clinical conditions.

To study the proposed endothelium-protective effects of drugs, we developed two original experimental models of endothelial dysfunction and injury. Selected drugs with different mechanisms of action were chosen for this assessment: pentoxifylline (PTX), a methylxantine with hemorrheologic properties, and sulodexide (SLX), a glycosaminoglycan composed of heparan and dermatan fractions. The aim of our study was to evaluate the effect of both pentoxifylline and sulodexide in prevention of endothelial dysfunction or damage using experimental *in vitro* models.

Method

Male and female rabbits (Cincilla, 2.5-3 kg) were sacrificed by cervical dislocation. In the experiments both renal and femoral arteries were employed. The arteries were excised and placed into Tyrode's solution (in mmol/l): NaCl 137, KCl 2.7, $MgCl_2$ 1.1, NaH_2PO_4 0.32, $CaCl_2$ 0.9, $NaHCO_3$ 11.9, glucose 5.5. After isolation and cannulation, vessel segments were cut to a constant length of 5 mm. Two paired vascular preparations from the same animal were transferred into a vessel chamber and perfused parallely at a constant flow rate with Tyrode's solution maintained at 37 °C and pH 7.25-7.35, saturated with 95 % O_2/5 % CO_2. The reactivity of vessels was expressed as pressure changes in the perfusion system and measured by a tensometric transducer with registration recorder. After an equilibration period of 30 min, the basal perfusion pressure was adjusted to 18-20 mm Hg with a flow rate of 22 ml/min in the renal artery and to 20 mm Hg with a flow rate of 25 ml/min in the femoral artery.

Model of endothelial dysfunction induced by adhesion of polymorphonuclear leukocytes (PMN) to endothelial cells

Rabbit renal arteries were perfused in parallel. Noradrenaline (NA) in standard doses of 0.1, 1.0 and 10 μg was administered directly into the cannula in a volume of 0.1 ml at 5 min intervals. Constrictor responses to NA were evaluated in the following experimental arrangement:

a) before (control) and after PMN application into the perfusate (concentration $2.75 \cdot 10^7$ cells/l)

b) before (control) and after application of PMN+PTX in a 10^{-5} mol/l concentration.

Perfusion with both PMN or PMN+PTX lasted 10 min. Activated PMN were obtained from the peritoneal cavity after repeated saline intraperitoneal administration (on day 1, 3 and 5) and diluted to a final concentration of $2.75 \cdot 10^7$ cells/l. At the end of the experiment, the vessel segments were fixed in 10 % formalin solution and stained by routine methods for histological evaluation.

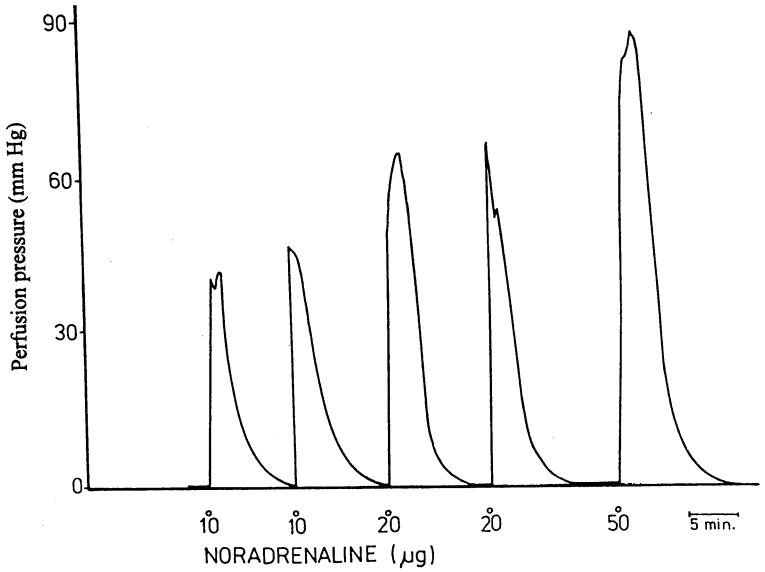

Fig. 1. *Constrictor responses of rabbit femoral artery to increasing noradrenaline doses (characteristic recording from the experiment).*

Model of endothelial injury induced by repeated noradrenaline administration

Two paired vessel segments of the rabbit femoral artery were perfused in parallel: the first served as a control, the second was continuously perfused with sulodexide (120 U/l). After equilibration (phase I) NA was administered in 5 doses of 10-50 μg (Fig. 1) at 5-min intervals (phase II). The perfusate from both phases was collected separately and filtered through Millipore filters with 5 μm pores. Endothelial cells (EC) caught on the filters were fixed in formalin, stained with hematoxylin and eosin and quantified by light microscopy (Babál *et al.* 1992).

Quantitative evaluation of endothelial loss

Endothelial cells were calculated in 5 standard microscopic fields along a spiral line from periphery to the center of the Millipore filter at 20 x magnification.

Drugs

Noradrenaline hydrogentartarate inj. (Léčiva, Czech Republic), pentoxifylline subst. (Slovakofarma, Slovak Republic), sulodexide inj. (Vessel Due F, Alfa Schiaparelli Wassermann SpA, Italy).

Statistics

Data from the renal arteries were evaluated using the Wilcoxon test for pairwise comparison. The Mann-Whitney U-test was used for the comparison of two independent samples. All values are expressed as median and confidence limits of the median in mm Hg. Endothelial cells from the femoral arteries are expressed as mean ± S.E.M. The paired Student's t-test was used for statistical analysis.

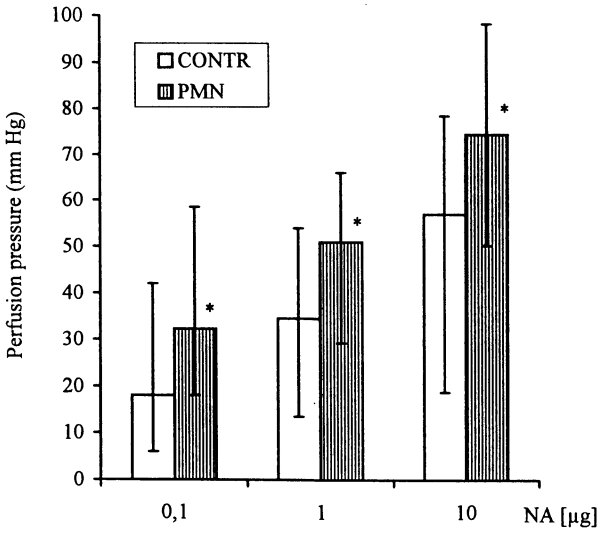

Fig. 2. *Constrictor responses of rabbit renal artery to single doses of noradrenaline (NA) before (CONTR) and after the application of polymorphonuclear leucocytes (PMN). All values are expressed as medians and their confidence limits.*

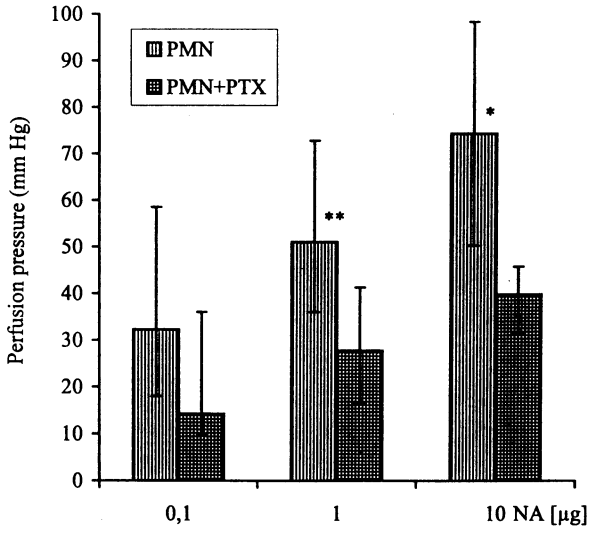

Fig. 3. *Comparison of constrictor responses of rabbit renal artery to noradrenaline (NA) affected by polymorphonuclear leucocytes (PMN) alone or in combination with pentoxifylline (PMN+PTX) applied into perfusion fluid. All values are expressed as medians and their confidence limits.*

Results

The constrictor responses to NA in rabbit renal artery perfused with PMN were significantly enhanced as compared to the controls (control vs. PMN): at dose 0.1 μg: 18 (6; 42) vs. 32.25 (18; 58.5), at dose 1 μg: 34.5 (13.5; 54) vs. 51 (36; 72.75) at dose 10 μg: 57 (18.79; 78.35) vs. 74.25 (50.25; 98.25), expressed as median (95 % CL) of perfusion pressure in mm Hg , n=6; $p<0.05$ (Fig. 2). In vessels perfused with PMN + PTX the vasoconstrictor responses to NA decreased when compared to PMN perfused preparations (PMN vs.

PMN+PTX): at dose 0.1 μg: 32.25 (18; 58.5) vs. 14.25 (9.75; 36) NS, at dose 1 μg: 51 (36; 72.75) vs. 27.75 (16.5; 41.25) $p<0.01$, at dose 10 μg: 74.25 (50.25; 98.25) vs. 39.75 (31.5; 45.75), n=6; $p<0.05$ (Fig. 3). Adhesion of PMN to endothelial cells in the renal artery is shown in Figure 4.

Noradrenaline administered in five increasing doses elicited dose-dependent constrictor responses in the rabbit femoral artery (Fig. 1). No significant differences in responses to NA between the control and SLX-perfused vessels were found. Endothelial cell loss significantly decreased in SLX-perfused vessels during phase II (Fig. 5) when compared to control vessels perfused without SLX (controls: 131.4±20.1 EC, +SLX: 83.3±13.8 EC, $p<0.01$, n=8). The differences in the number of desquamated endothelial cells during phase I were not significant.

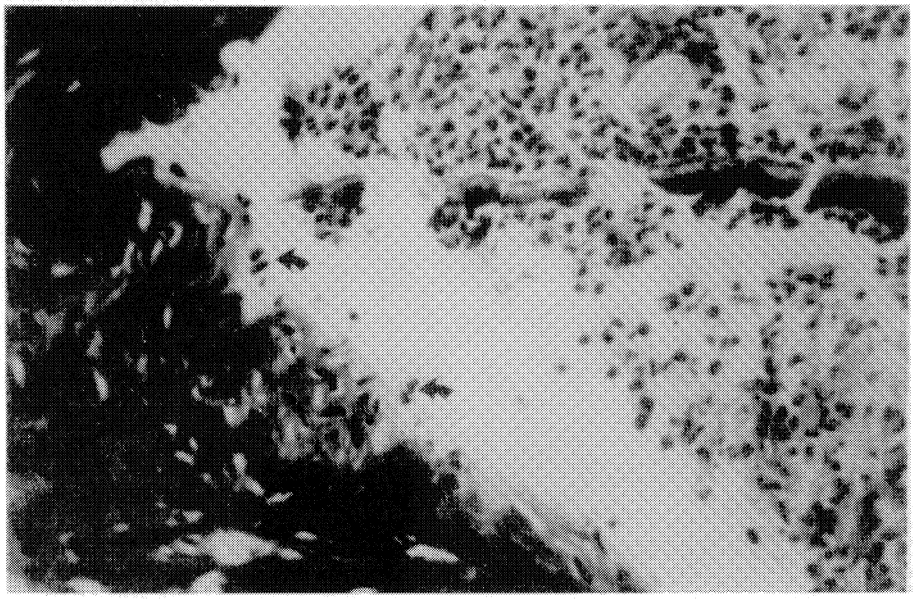

Fig. 4. *Polymorphonuclear leucocyte adhesion to the endothelium of rabbit renal artery. There is a suspension of PMN in the lumen. Stained by Giemsa. 200x.*

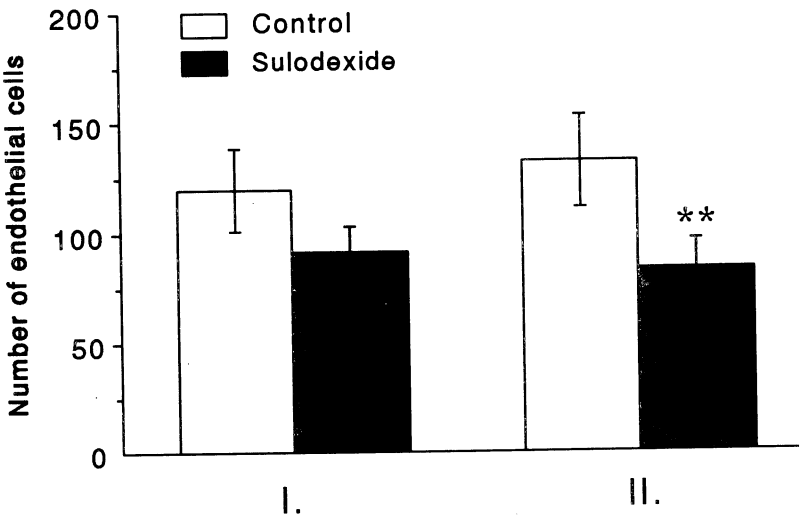

Fig. 5. *Endothelial loss in phase I and II of perfused rabbit femoral artery (n=8) before (Control) and after sulodexide administration ($p< 0.01$). The number of endothelial cells is expressed as mean ± SEM.*

Discussion

It has been shown that pentoxifylline diminished the constrictor responses to noradrenaline in the model of endothelial dysfunction and sulodexide decreased endothelial loss in the model of endothelial damage. These results suggest a favorable effect of the examined drugs on vascular endothelium. Although several experimental *in vivo* models have been used for evaluating the protective effects of drugs on the vascular

wall, simple and suitable models for *in vitro* evaluation are lacking.

Isolated mesenteric and coronary vascular beds (Lefer and Lefer 1996) as well as isolated coronary artery rings or endothelial cultures (Murohara *et al.* 1994) have been used as models of endothelial dysfunction induced by interaction with polymorphonuclear leukocytes. We have not found any data in the literature about a similar experimental approach used in vessels perfused *in vitro*. Under these conditions, we preferred the perfusion with activated PMN obtained from the peritoneal exudate because we expected increased adhesivity of PMN to endothelial cells. Histological examination supported this expectation, indicating that leukocyte-endothelial interaction may also occur using perfusion in the absence of plasmatic factors. Endothelial dysfunction induced by leukocytes is manifested by decreased production of vasodilator factors, thus enhancing the responses to vasoconstrictor agents and attenuating the relaxation responses (Murohara *et al.* 1994). The finding that constrictor responses to NA were potentiated after vessel perfusion with PMN confirmed the role of leukocytes in impaired endothelial function. Our results suggest that already subtle changes without serious morphological damage of endothelial cells may enhance the vascular responses.

The administration of pentoxifylline prevented the increased vasoconstriction induced by PMN which indirectly suggests its protective effect in the mechanism of leukocyte-induced endothelial dysfunction. Pentoxifylline is a hemorrheologic agent with a weak vasodilator effect. In additional experiments, we did not observe a decrease of vasoconstrictor responses to NA or vasodilation after pentoxifylline application. Recent data support novel properties of pentoxifylline as a leukocyte inhibitor in microcirculation (Ritter *et al.* 1996) suggesting further therapeutic implications of this drug (Švec and Kuželová 1997).

When comparing the above endothelial dysfunction with the morphological damage or removal of the endothelium, the latter represent more serious insults into vascular tone regulation. Besides the mechanical deendothelisation by scraping or air bubbles, catecholamines, especially noradrenaline, may damage the endothelial cells (Joris and Maino 1981). This is in agreement with our results that repeated administration of submaximal noradrenaline doses progressively damages the endothelium and increases vasoconstrictor responses (Kristová *et al.* 1993). Therefore, the *in vitro* model induced by repeated noradrenaline administration leading up to 50 % loss of total endothelium was found to be suitable for the assessment of endothelium-protective properties of sulodexide.

Sulodexide, composed of heparan (80 %) and dermatan fractions (20 %), is a glycosaminoglycan with a complex effect on the vascular wall (antithrombotic, fibrinolytic, antiatherogenic, antiproliferative properties). Glycosaminoglycans are produced in the functional endothelium and are capable of repairing or preventing the endothelial damage. For the complexity of its effects sulodexide is used in the treatment of some vascular diseases (Lunetta and Salanitri 1992, Gaddi *et al.* 1996). Although some clinical studies indicate its effectiveness in patients, only little is known about its protective effect on the endothelium *in vitro*. We found that sulodexide significantly lowered the number of endothelial cells during noradrenaline-induced contractions (phase II), suggesting endothelium-protective properties of sulodexide in this model. The finding that sulodexide did not change the magnitude of constrictor responses to noradrenaline was not surprising, since it was reported that it did not exhibit any vasodilator activity (Kristová *et al.* 1995).

The present results have shown that, although pentoxifylline and sulodexide have a different mechanisms of action, both drugs exert protective effects on the endothelium in experimental conditions. Our experimental *in vitro* models of endothelial dysfunction or damage can be used for evaluation of endothelium-protective effects of drugs.

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Reprint requests

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