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Sub-therapeutic doses of fluvastatin and valsartan are more effective than therapeutic doses in providing beneficial cardiovascular pleiotropic effects in rats: A proof of concept study

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ABSTRACT

Background: Statins and sartans can, in therapeutic doses, induce pleiotropic cardiovascular effects. Similar has recently been shown also for sub-therapeutic doses. We thus explored and compared the cardiovascular pleiotropic efficacy of sub-therapeutic vs. therapeutic doses.

Methods: Wistar rats were randomly divided into 7 groups receiving fluvastatin, valsartan and their combination in sub-therapeutic and therapeutic doses, or saline. After 6 weeks, the animals were euthanised, their hearts and thoracic aortas isolated, and blood samples taken. Endothelium-dependent relaxation of the thoracic aortae and ischaemic-reperfusion injury of the isolated hearts were assessed along with the related serum parameters and genes expression.

Results: Fluvastatin and valsartan alone or in combination were significantly more effective in sub-therapeutic than therapeutic doses. The sub-therapeutic combination greatly increased thoracic aorta endothelium-dependent relaxation and maximally protected the isolated hearts against ischaemia-reperfusion injury and was thus most effective. Beneficial effects were accompanied by increased levels of nitric oxide (NO) and decreased levels of asymmetric dimethylarginine (ADMA) in the serum (again prominently induced by the sub-therapeutic combination). Furthermore, nitric oxide synthase 3 (NOS3) and endothelin receptor type A (EDNRA) genes expression increased, but only in both combination groups and without significant differences between them. In the therapeutic dose groups, fluvastatin and valsartan decreased cholesterol values and systolic blood pressure.

Conclusion: Sub-therapeutic doses of fluvastatin and valsartan are more effective in expressing cardiovascular pleiotropic effects than therapeutic doses of fluvastatin and/or valsartan. These results could be of significant clinical relevance.

1. Introduction

Atherosclerosis-based cardiovascular diseases remain a major cause of morbidity and mortality. Therefore, new treatment strategies for their reduction are highly desired. Current treatment strategies are mainly focused on the control and/or treatment of traditional risk factors (arterial hypertension, hyperlipidaemia, obesity, smoking, etc.), but are obviously not sufficient. Consequently, a change in the prevention focus appears to be needed. Thus, it seems that optimal focus could be directed at the arterial wall per se, where the pathogenic process of atherosclerosis takes place. Functional and structural impairments of the arterial wall might therefore represent appropriate new targets. Of course, such treatment should be provided in parallel with current treatment strategies.

The pleiotropic effects of statins and/or sartans could be interesting in this regard. In therapeutic doses, statins, sartans and their combination have been shown to possess some beneficial pleiotropic cardiovascular effects [1–3], which could be attributed to both primary actions of those drugs (decrease of cholesterol levels and decrease of blood pressure) and independent of any intrinsic pleiotropic activity. On the other hand, the efficacy of statins and/or sartans in very low, and sub-therapeutic doses have also been studied lately. Thus,

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fluvastatin, valsartan and particularly their low, sub-therapeutic combination have been shown to effectively improve the functional and structural properties of the arterial wall [4–8] without affecting lipids or blood pressure. Importantly, these were pure pleiotropic effects, expressed beyond the primary action of these drugs. The pleiotropic capacity and efficacy of statins and/or sartans in sub-therapeutic vs. therapeutic doses have not yet been explored and compared.

The aim of the present study was to compare the pleiotropic effects of fluvastatin, valsartan and their combination in sub-therapeutic vs. therapeutic doses on the endothelium-dependent relaxation of the thoracic aortae, the ischaemia-reperfusion injury of the isolated hearts, the related plasma parameters and gene expression in rats. In this "proof of concept" study, we aimed to explore the feasibility of a new suggested concept: that sub-therapeutic doses of statin and/or sartan (fluvastatin and/or valsartan) are more effective than therapeutic doses.

2. Materials and methods

2.1. Animals and study design

Adult Wistar rats of both sexes (n = 35) weighing 235–280 g and 21–24 weeks of age were obtained from the Faculty of Medicine, Ljubljana, Slovenia. The animals were bred under constant housing conditions with a regular 12 h circadian cycle, at a controlled environmental temperature and fed with standard rat feed in the form of pellets (Altromin No. 1320, Lage, Germany). Five animals were kept in each cage.

The rats were randomly assigned to one of seven experimental groups as follows: 1) rats that received tap water (the control group); 2) rats that received sub-therapeutic doses of fluvastatin (4 mg/kg/day; *p.o.*); 3) rats that received sub-therapeutic doses of valsartan (6 mg/kg/day; *p.o.*); 4) rats that received therapeutic doses of fluvastatin (20 mg/kg/day; *p.o.*); 5) rats that received therapeutic doses of valsartan (30 mg/kg/day; *p.o.*); 6) rats that received a combination of fluvastatin and valsartan in sub-therapeutic doses (4 mg/kg/day *p.o.* and 6 mg/kg/day *p.o.*, respectively) and 7) rats that received a combination of fluvastatin and valsartan in therapeutic doses (20 mg/kg/day *p.o.* and 30 mg/kg/day *p.o.*, respectively). After 6 weeks of treatment, the animals were euthanised, blood samples were collected and their hearts and thoracic aortas were isolated. Each experimental group was composed of 3 male and 2 female rats.

Fluvastatin (fluvastatin sodium) was provided by Aurobindo Pharma (India). Valsartan was generously provided by Krka Pharmaceuticals (Krka, d. d., Novo mesto, Slovenia). The drugs were diluted in distilled water to prepare solutions that were applied daily, orally for 6 weeks, prior to the isolated organ experiments.

All experiments were conducted in accordance with the guidelines of the Veterinary Administration of the Republic Slovenia (permit No. 34401-23/2009/3), which conform to the Guide for the Care and Use of Laboratory Animals from the Institute for Laboratory Animal Research, National Research Council, Washington D.C. (National Academy Press, 1996).

2.2. Systolic blood pressure measurements

Systolic blood pressure measurements were performed at the beginning (0th day) and at the end (6th week) of the chronic treatment period. Conscious rats were placed in a chamber at 37 °C for 10 min, and then transferred to a restrainer and heating pad. The systolic blood pressure was measured by a non-invasive blood pressure module connected through a manometer and Powerlab module (both ADInstruments, Spechbach, Germany) to a computer. The final blood pressure value was calculated from five successive measurements. The same person performed all the measurements.

2.3. Isolated heart preparation and protocol

The rats were anaesthetised with an *i.p.* injection of urethane (130 mg urethane/100 mg body weight; Sigma-Aldrich, St. Louis, USA). Heparin (1000 I.U.; Krka, Novo mesto, Slovenia) was also injected *i.p.*. A thoracotomy was performed and a cannula filled with cold Krebs-Henseleit (K-H) solution with the addition of heparin (2500 I.U./100 mL K-H) was introduced into the aorta above the semilunar valve, followed by heart isolation. The hearts were mounted on a Langendorff apparatus and perfused with oxygenated K-H solution for isolated hearts (95% O_2 + 5% CO_2; pH 7.4 at 37.5 °C; composition in mM: 118.6 NaCl; 4.7 KCl; 11.1 glucose; 25 $NaHCO_3$; 1.66 $MgSO_4$; 1.2 NaH_2PO_4, and 2.52 $CaCl_2$; all Merck Darmstadt, Germany) under constant pressure, as previously described [9]. The hearts were protected with a glass cover and Parafilm to maintain constant temperature and humidity. The temperature of the experimental environment was kept at 23–25 °C.

The experiments lasted for 120 min. The hearts were perfused with oxygenated K-H solution during the first 30 min (perfusion period). After that, 40 min of global zero-flow ischaemia with complete flow cessation of K-H solution (ischaemia period) was performed. The hearts were then perfused with oxygenated K-H solution for 50 min (reperfusion period).

Several parameters were measured during the isolated heart experiments. In order to measure *coronary flow rate*, the coronary effluent was collected in a calibrated test tube at various time intervals during the experiments; coronary flow rate was expressed in mL/min. Effluents were further used for biochemical analysis of the *lactate dehydrogenase* (LDH) release rate. LDH release rate was determined by the modified Wroblewski-LaDue method [10] and expressed in $\mu kat\, g^{-1}\, min^{-1}$. *Heart rate* (HR) was obtained from oscillations detected in the electrocardiogram, aligned to ventricular pressure values and expressed in beats per min. *Left ventricular pressure* (LVP), expressed as the difference between systolic and diastolic pressures, was measured continuously with a Millar pressure catheter-transducer as previously described [9].

Data for all parameters measured in the isolated hearts were recorded and processed on a Dewetron acquisition system (Dewetron, Graz, Austria) after analogue-digital conversion (National Instruments, NI PCI-6013, Austin, USA) on a PC hard disk using Dewesoft 6.0 software (Dewetron, Trbovlje, Slovenia).

2.4. Thoracic aorta isolation and protocol

The thoracic aorta was rinsed of blood, dissected, cleansed of fat and connective tissue, and cut transversally into cylindrical rings (3–4 mm in length). The endothelium was preserved by cautiously dissecting the rings. On average, 4–6 arterial rings were prepared from the thoracic aorta of one animal. Aortic rings were immediately mounted in standard organ baths filled with K-H solution for arterial perfusion (composition in mM: NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $NaHCO_3$ 25, $MgSO_4$ 1.2, KH_2PO_4 1.2, and glucose 11; all Merck, Darmstadt, Germany), as described previously [11]. After mounting, the rings were allowed to equilibrate at 20 mN resting tension (found to be optimal in prior experiments) for 60 min. During this period, the tension was periodically adjusted to the desired level and the K-H solution was changed every 10 min. After the equilibration period, the rings were contracted three times with 60 mmol/L KCl to achieve stable contractions. Later, the rings were contracted with 60 mM KCl until reproducible contractile responses were obtained. After rinsing, we examined the relaxation of pre-contracted rings with 1 μmol/L phenylephrine by adding cumulative concentrations of the endothelium-dependent dilator acetylcholine (0.1 nmol/L–1 mmol/L). Phenylephrine and acetylcholine (both Sigma-Aldrich Chemie, Steinheim, Germany) were dissolved in distilled water prior to the experiments.

Vascular responses were processed and recorded on a Dewetron acquisition system (Dewetron, Graz, Austria) after analogue-digital