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Short communication

Treatment with platelet-derived growth factor (PDGF) and rock inhibitors is related to declined nerve growth factor (NGF) signaling in an experimental model of rat pulmonary hypertension

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ABSTRACT

Background: Recent studies reveal that nerve growth factor (NGF) plays a critical role in the pathobiology of pulmonary hypertension (PH). The aim of the present study is to clarify the relationship between NGF signaling and treatment with PDGF or ROCK inhibitors in an animal model of PH.
Methods: Lung tissues were obtained from animals with monocrotaline (MCT)-challenged PH which had been administered long term imatinib, fasudil or statin. Reversal of disease was indicated by decreases in right ventricle pressure (RVP) and hypertrophy. NGF expression was examined at the mRNA and protein levels using quantitative real-time PCR reaction and ELISA.
Results: MCT significantly increased NGF mRNA and protein content in lung tissue. ROCK inhibitor (fasudil) and PDGF inhibitor (imatinib) caused significant decreases in NGF mRNA and protein content when administered alone, with no further effects noted when used in combination.
Conclusion: The beneficial reversal of MCT-mediated effects in PH caused by PDGF or ROCK inhibition may be also partially mediated by decreased NGF signaling.

Introduction

Pulmonary hypertension (PH) is a rare, progressive cardiopulmonary disorder characterized by elevated pulmonary vascular resistance (PVR) and pulmonary artery pressure (PAP) leading to right-heart failure with right ventricle hypertrophy (RVH) and death [1]. The pathobiology of PH is very complex and multifactorial [2]. It is involved in the dysregulation of the vasoconstriction–vasodilatation balance, pulmonary vasoactivity, endothelial function, smooth muscle cell (SMC) proliferation, and vascular inflammation or thrombosis. Many mediators and signaling pathways with their downstream effectors are implicated in its pathobiology. Endothelial dysfunction leads to impaired production of vasodilator and anti-proliferative substances, i.e. nitric oxide (NO) and prostacyclin (PGI2), with overexpression of vasoconstrictor and proliferative agents such as thromboxane A2 (TXA2), endothelin-1 (ET-1). Excess growth factor and kinase pathway activation contribute to pulmonary vascular remodeling and proliferation, and hence growth factors such as platelet-derived growth factor (PDGF) [3] or fibroblast growth factor-2 (FGF2) [4] are considered to play a potential role in PH pathobiology. Very recent studies suggest that nerve growth factor (NGF) plays a critical role. It belongs to the neurotrophin family and plays an essential role in neuronal development, survival, and differentiation [5]. NGF and its receptors have also been demonstrated to stimulate proliferation and migration of human airway smooth muscle cells and lung fibroblasts. According to recent observations in animal and human PH, NGF can promote vascular cell proliferation and migration, as well as hyperreactivity or secretion of proinflammatory cytokines in pulmonary arteries [6]. Therefore, its blockade has been proposed as a novel method to treat pulmonary hypertension. It was suggested that NGF may contribute to increased activation of one of the receptor tyrosine kinases (RTKs) or RhoA/ROCK pathway. Hence, our study evaluates whether the beneficial role observed for inhibitors of platelet-derived growth factor (PDGF) and kinase RhoA/ROCK in PH can be linked to NGF signaling. NGF expression was assessed at the mRNA and protein level in lung tissue taken from animals with monocrotaline (MCT)-challenged PH; all samples demonstrated

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disease reversal by decrease in right ventricle pressure (RVP) and hypertrophy in response to chronic administration of imatinib, fasudil or statin.

Material and methods

Lung tissue isolation

The lung tissues were derived from young, 8–10-week-old, outbred Wistar male rats. The animals were treated as follows: (1) 1.0% methylcellulose (MC), 1 mL/kg bw (n = 5)–Sham; (2) 1.0% MC, 1 mL/kg bw after single, subcutaneous Monocrotaline – MCT (Fluorochem Ltd, UK) injection at 60 mg/kg bw – MCT + Vehicle (n = 6); (3) Fasudil (Fluorochem Ltd, UK) at 15 mg/kg bw (n = 6); (4) Imatinib (HBCChem Inc., USA) at 50 mg/kg bw (n = 6); (5) Imatinib at 50 mg/kg bw, i.g. plus fasudil at 15 mg/kg bw (n = 7); (6) Imatinib at 50 mg/kg bw, plus rosuvastatin at 10 mg/kg bw (n = 7). Drugs were administered intragastrically for 14 days, 14 days after MCT injection (groups 3–6). At the end of the study, the lung tissue was excised following euthanasia. The experimental procedures were carried out in accordance with the international guidelines for care and use of laboratory animals and were approved by the Ethics Committee.

RNA isolation and quality analysis

Total RNA from frozen rat lung tissues was extracted with TRIzol reagent (Ambion) [7]. The quality of isolated RNA was checked by separation in 1% agarose gel for the presence of intact 28S and 18S bands. The concentration and the purity of isolated total RNA was measured by a Picodrop spectrophotometer. High quality RNA was classified as a 260/280 ratio between 1.8 and 2.1.

Reverse transcription PCR

For each sample, 1 μg of total RNA was reversely transcribed using a High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific) based on the manufacturer's recommendations. The samples were incubated at 25 °C for 10 min and 37 °C for 120 min in a Biometra thermocycler. The reverse transcriptase was inactivated by heating at 85 °C for five minutes and the obtained cDNA was stored in temperature of −20 °C until use for real-time PCR.

Quantitative real-time PCR reaction

Expression levels of rat NGF and ACTB (endogenous reference gene, β-actin) were determined by using TaqMan Gene Expression Master Mix (Applied Biosystems) and Taqman Gene Expression Assays (Applied Biosystems; Rn01533872_m1 and Rn00667869_m1, respectively) by quantitative real-time PCR using an Agilent Technologies Stratagene Mx3000P System. The reactions were incubated at 95 °C for 10 min, followed by 45 cycles of 95 °C for 15 s and 60 °C for one minute. Each reaction was performed in triplicate, and the average of the three threshold cycles was used to calculate the amount of transcripts in the sample. The real-time PCR data were calculated using the $2^{-\mathrm{deltaCT}}$ method [8].

Enzyme-linked immunosorbent assay (ELISA)

Prior to the experiment, the lung tissues were homogenized, and PBS buffer (0.02 mol/L, pH 7.2) (Life Technologies) was added. Subsequently, the samples were centrifuged at 12 000*g* for 10 min at 4 °C, and supernatants were collected and stored at −80 °C until further use. Each sample was assayed in duplicate. NGF antigen levels were measured by commercial ELISA test kit (Life Science Inc.) according to the manufacturer's instructions. Absorbance was read using 450 nm as the primary wave length per micrograms of tissue protein. The amount of protein corresponding to albumin in the prepared homogenates was measured using the Lowry method [9]. The absorbance was read at 750 nm and unknown albumin concentrations were determined according to a prior standard curve (standard stock solution: 1 mg/ml of albumin bovine; concentration range: 0.15–0.35 mg/ml).

Statistical analysis

Statistical analysis was carried out using STATISTICA version 10.0. The statistical evaluation of differences between groups was performed using the one-way analysis of variance (ANOVA) and *post-hoc* comparisons were performed using the Student-Newman-Keuls test. The Shapiro-Wilk test was used to confirm whether a parameter had a normal distribution. The homogeneity of variance was tested by Brown-Forsythe test.

The validation of Lowry method was performed using the Measurement Methods Validation Module (Statsoft Poland, ver. 1.9.6). Regression analysis was performed when the normal distribution and homoscedasticity of residuals were confirmed. All parameters were considered statistically significantly different if $p < 0.05$.

Results

mRNA NGF level

Fig. 1 shows NGF expression at the level of mRNA as percentage of MCT-Vehicle. Monocrotaline caused a significant increase of NGF expression compared to Sham ($p < 0.0005$). NGF was fond to decrease in rats treated with fasudil and imatinib in monotherapy or in combination, compared to those treated with MCT ($p < 0.0001$). The simultaneous, chronic administration of fasudil and imatinib caused a significant decrease of NGF compared to MCT-treated rats ($p < 0.0001$). The mRNA level in lung tissue derived from rats receiving rosuvastatin and imatinib were significantly lower than those receiving MCT-Vehicle ($p < 0.0005$).

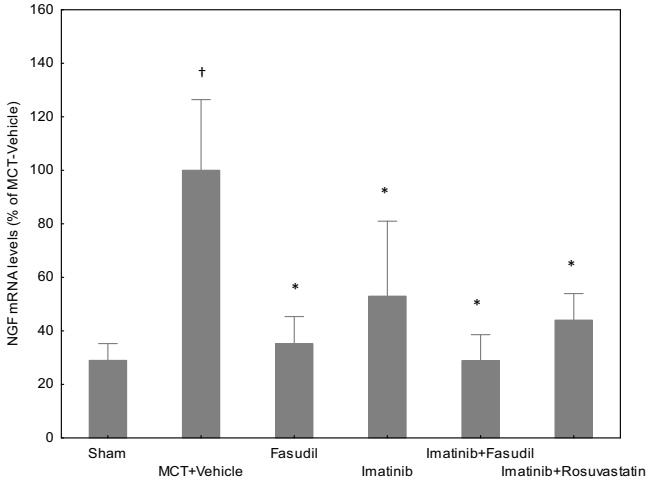

Fig. 1. NGF mRNA levels, measured by real-time PCR according to the delta CT method, in rat lung tissue expressed as percentage of MCT-Vehicle group: The histograms indicate: mean ± SD – † $p < 0.05$ *vs.* Sham; *$p < 0.05$ *vs.* MCT + Vehicle (Neuman-Keuls *post-hoc* test).