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Short Communication

NATRIURETIC PEPTIDES REDUCE PLASMINOGEN ACTIVATOR INHIBITOR-1 EXPRESSION IN HUMAN ENDOTHELIAL CELLS

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Abstract: Plasma concentrations of natriuretic peptides increase in some pathological conditions, but very little is known about the effect of these vasodilator peptides on the regulation of the blood coagulation system. The fundamental role in the regulation of fibrinolysis is played by plasminogen activator inhibitor type 1 (PAI-1). Recent studies demonstrate that natriuretic peptides can modulate PAI-1 expression in bovine aortic smooth muscle cells and rat aortic endothelial cells. In this report, we tested the effect of natriuretic peptides on PAI-1 expression in the human endothelial cell line (EA.hy 926). For this purpose, we treated the cell cultures with ANP, BNP and CNP, and modulation of PAI-1 synthesis was evaluated. We compared the effect of natriuretic peptides on synthesis and release of PAI-1 in unstimulated cells, and after activation with tumour necrosis factor α (TNFα). Natriuretic peptides abolished TNFα- induced upregulation of PAI-1 expression at both the PAI-1 mRNA and the antigen levels. The inhibitory efficiency was higher in the case of CNP when compared to that produced by ANP and BNP, particularly when TNFα-stimulated cells were used. We observed an inhibition of stimulatory effect of TNFα on PAI-1 expression also at the level of the PAI-1 promoter in cells transfected with a PAI-1 promoter fragment (+71 to –800) [1]. The PAI-1 promoter activity was markedly inhibited by C-type natriuretic peptide, already at a very low (0.001 μM) concentration of the peptide.

Key Words: Plasminogen Activator Inhibitor, Natriuretic Peptide, Endothelial Cells

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INTRODUCTION

The natriuretic peptide family consists of atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), and C-type natriuretic peptide (CNP). ANP and BNP, mainly released from cardiac atria and ventricles, respectively, play an important role in the regulation of blood pressure and body fluids. CNP was identified as a natriuretic peptide of endothelium origin [2]. PAI-1 plays a fundamental role in the regulation of fibrinolysis, acting as a main inhibitor of t-PA and u-PA. Elevated PAI-1 concentration has an evidenced role in thrombotic diseases [3,4]. Several factors have been reported to increase PAI-1 expression, but there are few agents known with anti-PAI-1 inhibitory activity. It has been reported that natriuretic peptides were able to suppress angiotensin II - induced PAI-1 expression in rat aortic endothelial cells [5,6] and vascular smooth muscle cells [7]. This study is focused on the regulation of PAI-1 expression by ANP, BNP, and CNP in the human endothelial cell line (EA.hy 926). To examine the effect of natriuretic peptides, endothelial cells were exposed to 0.001 – 1 μM of the compounds, and then incubated in the presence or absence of 50 ng/ml TNFα. This cytokine plays an important role in the regulation of PAI-1 synthesis in endothelial cells [8]. Upregulation of PAI-1 expression by the activation of the cells with TNFα observed by measurement of the antigen and the mRNA levels, and PAI-1 promoter activity, was diminished in the presence of natriuretic peptides. Modulation of PAI-1 expression can play an important role in clinical control of the concentration of endogenous plasminogen activators in the circulation.

MATERIALS AND METHODS

Cell culture

The human endothelial cell line, EA.hy 926 cell line, was used and the cells were cultured in DMEM medium as described before [8]. Prior to the treatment with peptides, the cell medium was changed to DMEM supplemented with 0.1% FCS.

PAI-1 mRNA and antigen analysis

Total cellular RNAs were extracted from human endothelial cells using the Trizol reagent method, a single-step purification protocol [9,10]. The quality of the isolated total RNA was checked by 1% agarose- 2M formaldehyde gel electrophoresis. 1μg of total RNA was then used for cDNA synthesis by the SuperScript II RNase H Reverse Transcriptase System, using oligo (dT) 12-18 primers. cDNA was amplified with forward and reverse primers specific for PAI-1 or β-actin cDNA. The amplification products were resolved on 6% polyacrylamide gel. The PAI-1 antigen was determined by ELISA in experimental medium collected from the cell culture.

Transient transfection of EA.hy 926 cells

Semiconfluent cell cultures in 6-well tissue culture plates were transfected with DNA constructs, plasmid p800LUC containing a PAI-1 promoter fragment (+71 to -800), using the lipofectAMINE method [10]. For peptide studies, CNP was added to the wells, cells were incubated for 24 hours, washed, and harvested in lysis buffer. A luciferase assay kit (GIBCO) was used according to the manufacturer's instructions, using a VICTOR 1420 (EGG WALLAC) microplate reader. β-galactosidase activity from a constitutively expressed internal control was assayed with the β-galactosidase Enzyme Assay System (GIBCO) according to the manufacturer's instructions. In parallel experiments, cells were transfected with the luciferase reporter vector pGL3 (Promega) and used as control cells to test specificity of the effect of inhibitors for the PAI-1 promoter.

RESULTS AND DISCUSSION

Effect of natriuretic peptides on PAI-1 release from endothelial cells

EA.hy 926 cells were incubated in the presence of increasing concentrations of ANP, BNP or CNP for 15 minutes, and modulation of PAI-1 synthesis was evaluated by ELISA based on amounts of the PAI-1 antigen released into the medium. Treatment of the cells with peptides resulted in a decreased release of PAI-1 to the medium in a concentration-dependent manner. Treatment of the cells with TNFα (50ng/ml) resulted in an about two-fold increase in PAI-1 expression measured by antigen levels after 24 hours. Incubation with all the tested peptides inhibited a release of PAI-1 from endothelial cells with the highest effect of CNP (Fig. 1). The inhibitory efficiency was markedly higher in the case of TNFα-stimulated cells, compared to the basal level.

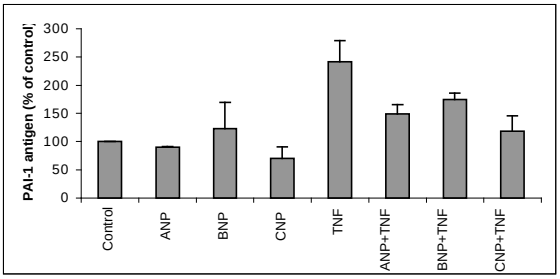

Fig. 1. Effect of natriuretic peptides on PAI-1 antigen release from endothelial cells. Data show the level of the PAI-1 antigen after preincubation of the cells with 1 μM of each peptide. Untreated cells were used as a control. Data are expressed as means ± SEM.

Effect of natriuretic peptides on PAI-1 mRNA

Inhibitory effect of natriuretic peptides on TNFα-induced expression of PAI-1 was also well documented by an RT PCR analysis of PAI-1 mRNA. The cells

were preincubated for 15 minutes with different concentrations of natriuretic peptides and treated for 4 hours with or without TNFα. Changes in PAI-1 mRNA were estimated and compared to the actin mRNA levels. Treatment of the cells with TNFα significantly increased expression of PAI-1 mRNA, but did not affect the actin mRNA level. Natriuretic peptides reduced the PAI-1 mRNA in a dose-dependent manner (not shown). CNP reveals the strongest inhibitory activity on TNFα-stimulatory effect on the PAI-1 mRNA level. (Fig. 2).

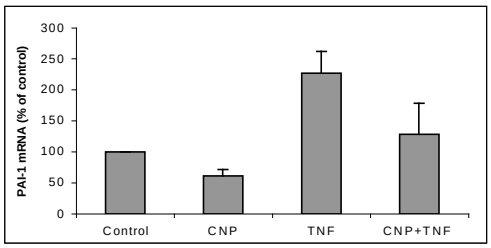

Fig. 2. Effect of CNP (0.01 μM) on PAI-1 mRNA in endothelial cells. Untreated cells were used as a control. Data are expressed as means ± SEM.

Effect of CNP on PAI-1 gene promoter activity

To study the responsiveness of the PAI-1 promoter to CNP, the cells were transfected with plasmid p800LUC containing a PAI-1 promoter fragment. The transfected cells were preincubated for 15 minutes in the presence of CNP, and then treated with or without TNFα. The PAI-1 promoter activity was suppressed by up to 80% by CNP (0.01μM) in TNFα-stimulated cells, without significant effect on its basic level (Fig.3). Thus, the inhibitory effect of CNP could be

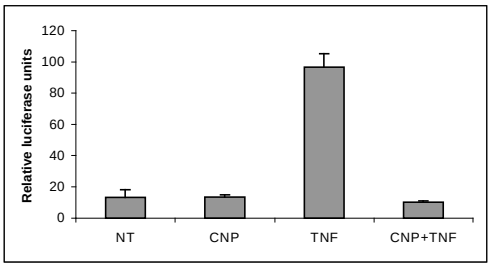

Fig. 3. Effect of CNP (0.01 μM) on PAI-1 gene promoter activity. NT – non-transfected cells. Data are expressed as means ± SEM.

detected at the level of the PAI-1 promoter in EA.hy 926 cells. These results suggest that natriuretic peptides are involved in a pathway leading to downregulation of the TNFα-stimulated level of PAI-1 expression in endothelial cells. In this study we provide an evidence that natriuretic peptides are effective modulators of PAI-1 synthesis and release from endothelial cells with stronger inhibitory activity for TNFα-stimulated cells.

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