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Inhibition of the Proliferation of Smooth Muscle Cells From Human Coronary Bypass Vessels by Vasonatrin Peptide

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Summary

Abnormal proliferation of vascular smooth muscle cells (VSMCs) is known to be a key event in the development of atherosclerosis and restenosis. The present study examined the effect of a novel synthetic natriuretic peptide, vasonatrin peptide (VNP), on norepinephrine (NE)-induced proliferation of VSMCs from coronary bypass vessels. Human VSMCs were isolated from an internal mammary artery (IMA) and saphenous vein (SV) by explant culture and stimulated with NE. MTT assay and [^{3}H] thymidine-incorporation were undertaken to analyze cell proliferation and radioimmunoassay was used to determine the level of intracellular cyclic 3',5'-guanosine monophosphate (cyclic GMP). NE (10^{-8} - 10^{-7} mol/l) had a mitogenic effect in human VSMCs from both SV and IMA. However, NE-stimulated proliferation of VSMCs from SV was greater than that from IMA. Furthermore, low concentration of NE (10^{-10} mol/l) promoted cell growth in SV-derived cells but not in IMA-derived cells. VNP (10^{-8} - 10^{-6} mol/l) reduced NE-induced cell proliferation and increased intracellular cyclic GMP, which were abrogated by HS-142-1. In addition, the growth inhibition of VNP was mimicked by 8-bromo-cGMP. These results indicate that VNP has a significant inhibitory effect on NE-stimulated proliferation of human VSMCs from both IMA and SV, which is mediated by guanylate cyclase-linked receptors by increasing cyclic GMP.

Key words

Vasonatrin peptide • Vascular smooth muscle cells • Proliferation

Introduction

Autogenous saphenous vein (SV) and internal mammary artery (IMA) are the most frequently used grafts in coronary artery bypass grafting (CABG). However, IMA has been shown to have a significantly superior long-term patency rate when compared with the SV graft. The differences have been attributed to a number of causes, many of which are associated with the intrinsic difference between veins and arteries including their growth capacity, structural features, mechanical and contractile properties (Cameron *et al.* 1996, Canham *et al.* 1997, Del Rizzo *et al.* 2002). Abnormal proliferation of vascular smooth muscle cells (VSMCs) plays an important role in intimal thickening in atherosclerosis and restenosis and is crucial for long-term patency of bypass

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grafts (Schwartz *et al.* 1995, Majack *et al.* 1996, Li *et al.* 1999). The growth of VSMCs is controlled by a balance of growth inhibitors and growth promoters, and in normal adult vessels this balance results in a very low rate of VSMCs growth. However, this balance is shifted when the proliferation of VMSCs occurs following vascular injury by either mechanical or chemical insults. Among various substances that are considered as possible VSMCs mitogens, the potent vasoconstrictor agent norepinephrine (NE) is recognized to contribute directly to normal vascular growth and deterioration of hypertrophy, atherosclerosis and restenosis (Mancia *et al.* 1997, Parenti *et al.* 2001). The identity of the factors that can effectively down-regulate the abnormal proliferation has been the subject of intense investigation.

The natriuretic peptides (NPs) are a family of structurally similar but genetically distinct peptides known to play important roles in the control of cardio-renal homeostasis, which includes atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP) and C-type natriuretic peptide (CNP). Recently, increasing attention has been paid to their inhibitory effects on cardiac hypertrophy and vascular remodeling in both basic research and clinical application (Silberbach *et al.* 2001). ANP is a 28-amino acid peptide, whereas BNP contains 32 amino acids and CNP contains 22. All of them have a 17-amino acid ring structure which has been identified as essential for their pharmacological activity. Unlike ANP and BNP, CNP lacks the COOH-terminal amino acid extension from the ring structure. ANP and BNP bind to the natriuretic peptide receptor (NPR)-A, and mediate natriuresis, vasodilation and renin inhibitory properties. CNP lacks natriuretic actions, but causes venodilation and exerts growth inhibiting action *via* NPR-B. Most functions of NPs appear to be mediated through the elevation of intracellular cyclic 3',5'-guanosine monophosphate (cyclic GMP) after their binding to NPR-A and NPR-B which are coupled to the particulate guanylyl cyclase. Vasonatrin peptide (VNP), the man-made novel member of the NP family (Wei *et al.* 1993), is a chimera of CNP and ANP. This synthetic peptide possesses the 22-amino acid ringed structure of CNP and the COOH terminus of ANP. It has been demonstrated both *in vitro* and *in vivo* that VNP have venodilating effects of CNP, the natriuretic action of ANP, and unique arterial vasodilating actions which are not associated with either ANP or CNP. Previous studies have shown that VNP exerts an important negative influence on the growth of cardiac myocytes and fibroblasts induced by hypoxia (Guo *et al.* 2001, Lu *et al.* 2002). However, little is known regarding the effect of this peptide on VSMCs growth. Since excessive proliferation of human VSMCs is a common phenomenon in many vascular diseases such as atherosclerosis and restenosis, the potential antimitogenic role of VNP on human VSMCs appears to be very intriguing. We have therefore investigated in the present study the influence of VNP on NE-stimulated growth of human VSMCs from both IMA and SV and the possible mechanisms involved.

Methods

Reagents

VNP was synthesized at the Shanghai Institute of Biochemistry. Norepinephrine HCl (NE), HS-142-1, 8-bromo-cGMP, 3-isobutyl-1-methylxanthine (IBMX), trypsin and mouse monoclonal antibody against human SMC α-actin were purchased from Sigma Chemical Co. (St. Louis, MO, USA). DMEM, FBS, antibiotics and all other cell culture reagents were obtained from GibcoBRL (Grand Island, NY, USA). [methyl 1-1',2',-^{3}H] thymidine was obtained from Shanghai Nuclear Technique Company of the Chinese Academy (Shanghai, China). [^{125}I]-cyclic GMP assay kit was from Shanghai Chinese Medical University (Shanghai, China). Stock solutions of drugs were prepared using sterile saline and diluted to working condition in DMEM.

Human VSMCs culture

VSMCs were cultivated from SVs and IMAs obtained from patients undergoing coronary artery bypass surgery by an explant technique (Campbell and Campbell 1993), with the approval of the local Ethical Committee. All comparisons of IMA- and SV-derived cells were made on paired explants from the same patients. The tissue was dissected free of fat and excess adventitial tissue and then opened along its longitudinal axis, and the endothelium was removed by scraping of the luminal surface. Small fragments (~ 1 mm^{3}) were transferred to a flask and cultured in DMEM containing 20 % FCS supplemented with L-glutamine 2 mmol/l, HEPES 15 mmol/l, benzylpenicillin 100 kU/l and streptomycin 100 g/l in a humidified atmosphere (95 % air/5 % CO_2) at 37 ^{o}C. After VSMCs outgrowth, a confluent monolayer was obtained and passaged. Cells were used between passages 2 and 6 after 24 h serum deprivation. VSMCs were characterized by their typical morphological pattern

(multilayer sheets, "hills and valleys") and by immunohistochemistry staining using specific mouse monoclonal antibody against human SMC α-actin.

Cell proliferation assay

Evaluation of DNA synthesis

DNA synthesis was determined by measuring the incorporation of [^{3}H]-thymidine into DNA of cells cultured in 96-well plates at the density of 1×10^3 cells/well. After the induction of quiescence, the cells were stimulated with NE in the presence or absence of experimental agents for 24 h and [methyl-^{3}H]-thymidine (18.5 KBq/ml) was added to the growth medium of each well for the last four hours. The radioactivity for each sample was counted by a liquid scintillation counter (LS-6500, Beckman, U.S.A).

Evaluation of cell number

The number of viable cells in each well was estimated by measurement of mitochondrial metabolism rate of tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT). MTT is selectively taken up and converted to a dark blue product by living but not by dead cells. Cells were used after the induction of quiescence in 96-well plastic culture plates at the density of 1×10^3 cells/well and stimulated with experimental agents for 24 h. Twenty microliters of MTT (2.5 g/l) per well was added to the culture medium subsequently, and incubation continued for additional 4 h at 37 ^{o}C. Thereafter, 150 μl DMSO were added to each well, and absorbance at 570 nm was read on a multiwell scanning spectrophotometer (Beckman, U.S.A).

Measurement of intracellular cyclic GMP

Cells were plated onto 90-mm culture dishes (1×10^6 cells/dish) for radioimmunoassay. After the induction of quiescence, the cells were incubated in a serum-free medium containing the phosphodiesterase-inhibitor 3-isobutyl-1-methyl-xanthine (IBMX 5×10^{-4} mol/l, to prevent degradation of cyclic GMP) and increasing concentration of VNP with or without HS-142-1 (10^{-5} mol/l), the antagonist of the particulate guanylyl cyclase-coupled natriuretic peptide receptors, for 15 min. After the incubation, the medium was rapidly removed, and 1 ml of ice-cold trichloroacetic acid (0.24 mmol/l) was added to the cells and then the cells were scraped from the plates. Cell samples were centrifuged to remove precipitated proteins, and the supernatant fractions were extracted with 1 ml water-saturated ether three times. The cyclic GMP level was determined by radioimmunoassay performed with a [^{125}I]-cyclic GMP assay kit. The sensitivities of the cyclic GMP radioimmunoassay were 50 fmol per assay tube.

Statistics

The results are expressed as means + S.D. and are representative of cells from four to five patients each assayed in triplicate. Statistical analyses were carried out with the Origin 5.0 statistical package. All values were analyzed by one-way analysis of variance (ANOVA) followed by Scheffé's test. The significance level was chosen for $p<0.05$.

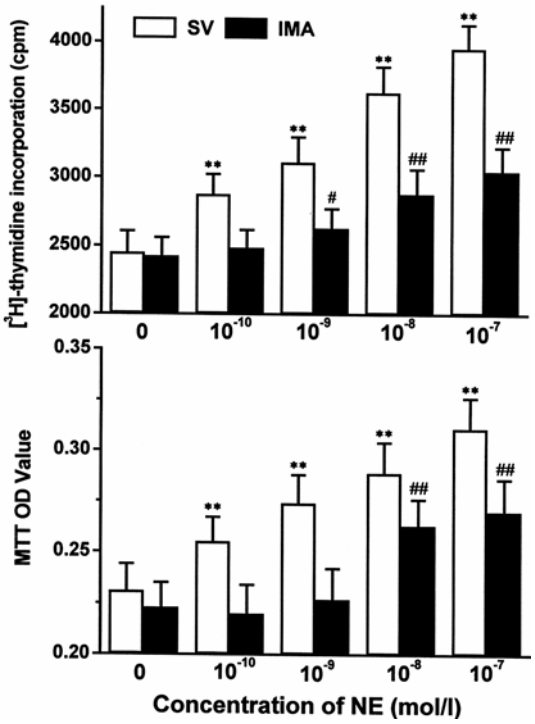

Fig. 1. Proliferative response of human VSMCs from IMA and SV to norepinephrine. Quiescent VSMCs were treated with indicated concentration of NE for 24 h. DNA synthesis and cell number were determined by [^{3}H]thymidine incorporation (top) and MTT OD value (bottom), respectively. Data represent means ± S.D. for cells from four patients each assayed in triplicate: ** $p<0.01$ vs. zero concentration of NE in SV-derived cells; $^{\#}$, $^{\#\#}$ $p<0.01$ vs. zero concentration of NE in IMA-derived cells.

Results

Inhibitory effects of VNP on NE-induced proliferation of human VSMCs

VSMC proliferation was quantified by the cell number and DNA synthesis. As shown in Figure 1, NE induced a significant increase in MTT OD value and

[^{3}H]-thymidine incorporation into serum-deprived, quiescent VSMCs from both IMA and SV at the concentration of 10^{-8} and 10^{-7} mol/l. These increases were greater for SV-derived cells than those for IMA-derived cells. Furthermore, at a lower concentration of NE (10^{-10} mol/l), both the MTT OD value and [^{3}H]thymidine incorporation were increased in SV- but not in IMA-derived cells.

Addition of VNP (10^{-8}–10^{-6} mol/l) to VSMCs from both SV and IMA reduced NE (10^{-8} mol/l)-stimulated [^{3}H] thymidine incorporation and MTT OD value in a concentration-dependent manner (Fig. 2).

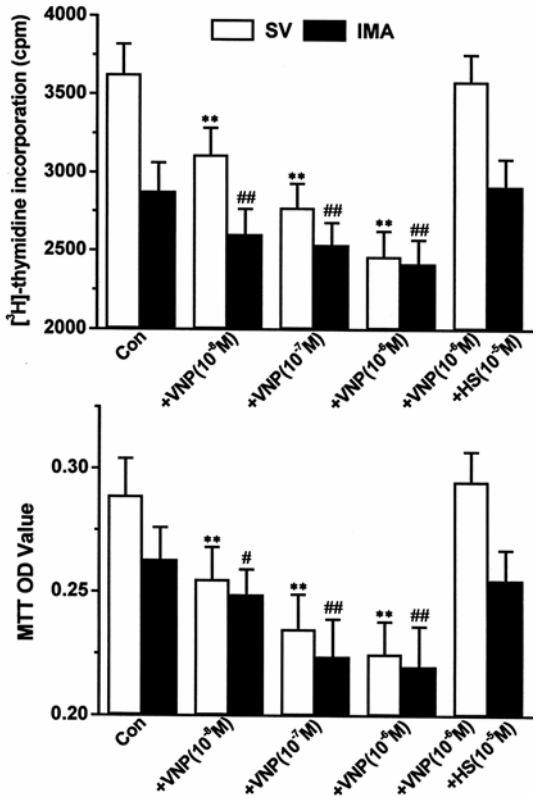

Fig. 2. Effects of VNP on NE-induced proliferation of human VSMCs from IMA and SV. VSMCs were incubated with 10^{-8} mol/l NE without (control group, Con) or with indicated concentration of VNP for 24 h. 10^{-5} mol/l HS-142-1 (HS) abolished the response of VSMCs to VNP (10^{-6} mol/l). Cell number and DNA synthesis was determined by [^{3}H] thymidine incorporation (top) and MTT OD value (bottom), respectively. Data represent means ± S.D. for cells from five patients each assayed in triplicate. ** p<0.01 vs. control group in SV-derived cells; $^{\#}$, $^{\#\#}$ p<0.05, p<0.01 vs. control group in IMA-derived cells.

Role of cyclic GMP in VNP mediated antimitogenic effects

The natural NPs are known to exert their biological action by enhancing particulate guanylate cyclase and by a subsequent elevation of intracellular cyclic GMP concentration. Due to the similar structure, we suggested that VNP might function through a similar pathway. In order to verify whether the intracellular cyclic GMP was involved in the signaling pathway of VNP, we studied the effect of VNP on cyclic GMP accumulation in VSMCs from coronary bypass vessels. There was no difference in the basal concentration of cyclic GMP between cells derived from SV and IMA (14.3±1.9 vs. 16.3±1.8 pmol/ml, respectively). Figure 3 shows the response of intracellular cyclic GMP to VNP in VSMCs. VNP significantly increased cyclic GMP formation at concentrations associated with a suppressed cell proliferation in VSMCs derived from both SV and IMA. The addition of HS-142-1 (10^{-5} mol/l), a well-known antagonist of a particulate guanylyl cyclase-coupled natriuretic peptide receptors (Morishita *et al.* 1991), completely abrogated the effects of VNP (10^{-6} mol/l) on both cyclic GMP formation (Fig. 3) and VSMCs proliferation (Fig. 2). In addition, the treatment of VSMCs with 8-bromo-cGMP (10^{-6} mol/l), a cell-permeable analog of cyclic GMP, resulted in significant inhibition of cell growth, which was similar to the action of VNP (Fig. 4).

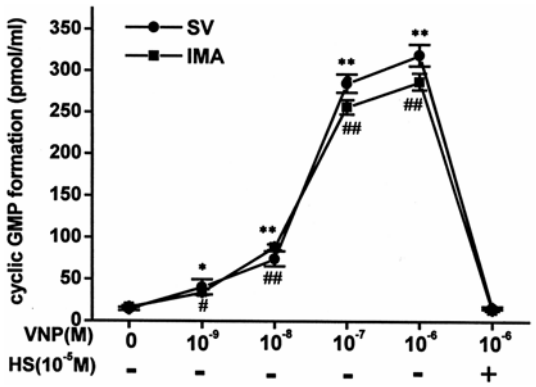

Fig. 3. Effects of VNP on intracellular cyclic GMP accumulation in human VSMCs from IMA and SV. Quiescent VSMCs were incubated with indicated concentration of VNP without (–) or with (+) 10^{-5} mol/l HS-142-1 (HS) for 15 min in serum-free medium supplemented with IBXM (5×10^{-4} mol/l). HS (10^{-5} mol/l) abolished the response of VSMCs to VNP (10^{-6} mol/l). Data represent means ± S.D. for cells from five patients each assayed in triplicate. *, ** p<0.05, p<0.01 vs. zero concentration of VNP in SV-derived cells; $^{\#}$, $^{\#\#}$ p<0.05, p<0.01 vs. zero concentration of VNP in IMA-derived cells.

Discussion

To find effective procedures or drugs controlling the proliferation of human VSMCs is of considerable importance due to the prevalence of vascular proliferative

diseases in which smooth muscle cells migrate and proliferate, with a subsequent occlusion of the lumen of blood vessels. Catecholamines have been shown to aggravate atherosclerosis in animals and humans, suggesting that they are likely to play a prominent role in the pathogenesis of atherosclerosis and restenosis. In the present study, NE was found to stimulate the proliferation of cultured human VSMCs from both IMA and SV, which is consistent with previous studies about the mitogenic effect of NE on rat VSMCs (Yu *et al.* 1996, Parenti *et al.* 2001). In addition, our data have shown that the VSMCs derived from human SV exhibit more pronounced proliferative activity in response to NE than that from IMA. This provides further evidence for explaining a higher patency of IMA grafts compared to SV grafts.

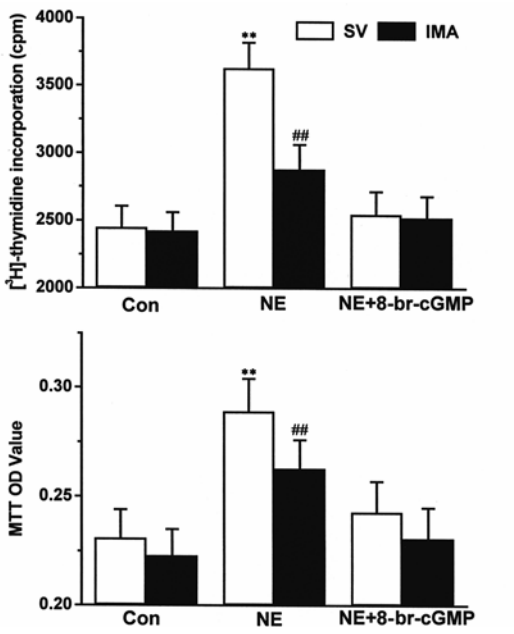

Fig. 4. Effects of 8-bromo-cGMP on NE-induced proliferation of human VSMCs from IMA and SV. Cells were incubated with vehicle (control group) or 10^{-8}mol/l NE in the absence or presence of 10^{-6} mol/l 8-bromo-cGMP for 24 h. Cell number and DNA synthesis was determined by [^{3}H]thymidine incorporation (top) and MTT OD value (bottom), respectively. Data represent means $\pm$ S.D. for cells from four patients each assayed in triplicate. ** $p<0.01$ vs. control group in SV-derived cells; ## $p<0.01$ vs. control group in IMA-derived cells.

The major finding of this study is that VNP, a new member of the NP family, has marked inhibitory effects on NE-induced proliferation of VSMCs from both IMA and SV. The NP family plays an important role in regulating mammalian blood volume and blood pressure. A potential action of natriuretic peptides in modulation of cardiac hypertrophy and cell proliferation is now beginning to be recognized. VNP, a unique peptide, is a chimera of CNP and ANP (Wei *et al.* 1993). This synthetic peptide possesses the 22-amino acid ringed structure of CNP and is structurally related to ANP because it also possesses the five amino-acid COOH terminus of ANP. In our previous study, we demonstrated that VNP prevented the development of cardiac hypertrophy through inhibition of the proliferation of cardiac fibroblasts and the protein synthesis in cardiac myocytes induced by chronic hypoxia (Yu *et al.* 1999, Guo *et al.* 2001, Lu *et al.* 2002). The current study extends these studies to demonstrate the possibility of regulating the proliferation of human VSMCs.

Natural NPs exert their biological effects through activation of two biologically active receptors: NPR-A, which shows high affinity to ANP and BNP, and NPR-B, which is specific for CNP (Suga *et al.* 1992). Various studies have demonstrated the existence of both NPR-A and NPR-B in human VSMCs. Nevertheless, ANP acts primarily on arteries and CNP predominately on veins due to the preferential expression of NPR-A in arteries and NPR-B in veins (Ikeda *et al.* 1996, Bonatti *et al.* 2000). The fact that VNP in this study had significant antiproliferative effects on VSMCs from both arterial and venous grafted vessels suggested that VNP might act through both NPR-A and NPR-B receptors in the vasculature. Therefore, VNP might have a special advantage over ANP or CNP in the prevention and treatment of restenosis after CABG since most patients undergoing CABG need both an arterial and venous conduit.

Due to the similar molecular structure and cardiovascular effects, VNP might act through the same signaling transduction pathway as ANP and CNP to enhance the intracellular cyclic GMP. Several lines of evidence from this study confirm that the growth inhibition of VSMCs by VNP is mediated by a cyclic GMP-dependent process. First, VNP significantly up-regulated the formation of intracellular cyclic GMP at doses associated with a suppression of cell proliferation in VSMCs derived from both SV and IMA. Second, the agent which increases intracellular cyclic GMP (8-bromo-cGMP) inhibited the growth of VSMCs, suggesting that up-regulation of cyclic GMP could inhibit cell growth. Finally, HS-142-1, the well-known antagonist of the particulate guanylyl cyclase-coupled natriuretic peptide receptors, blocked the antimitogenic effects of VNP and its promoting effects on cyclic GMP accumulation.

In conclusion, our present study shows that VNP, a synthetic member of the NP family, has a marked inhibitory effect on the proliferation of human VSMCs derived from both IMA and SV, which gives it a potential advantage in the therapy of restenosis after CABG. The antimitogenic effect appears to be mediated *via* the particulate guanylyl cyclase-coupled receptor by increasing the intracellular cyclic GMP.

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Reprint requests

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