Down-regulation of C-type natriuretic peptide receptor by vasonatrin peptide in cardiac myocytes and fibroblasts[1]

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KEY WORDS C-type natriuretic peptide; myocardium; fibroblasts; hypoxia

ABSTRACT

AIM: To investigate the regulatory effects of vasonatrin peptide (VNP) on the expression of C-type natriuretic peptide receptor (NPR-C) in cultured neonatal rat cardiac myocytes and fibroblasts. **METHODS:** Quantitative RT-PCR was undertaken to evaluate the levels of NPR-C mRNA and radioimmunoassay was used to determine the formation of intracellular cGMP. **RESULTS:** Twenty-four hours hypoxic exposure increased the level of NPR-C mRNA in cardiomyocytes, while did not alter the expression of NPR-C in cardiac fibroblasts. VNP (1×10^{-8}-1×10^{-6} mol/L) reduced the levels of NPR-C mRNA in cardiac myocytes induced by hypoxia in a concentration-dependent manner, and with high concentration (1×10^{-6} mol/L) also decreased the expression of NPR-C in cardiac fibroblasts and air-control cardiac myocytes. The inhibitory effects of VNP on the expression of NPR-C was mimicked by 8-bromo-cGMP 1×10^{-6} mol/L (a membrane permeable analog of cGMP). VNP (1×10^{-8}-1×10^{-6} mol/L) increased the formation of intracellular guanosine-3',5'-cyclic monophosphate (cGMP) in both cardiac myocytes and fibroblasts. HS-142-1, the particulate guanylyl cyclase-coupled receptor antagonist, partially abrogated the above effects of VNP. **CONCLUSION:** Hypoxic exposure for 24 h up-regulated the expression of NPR-C in cultured neonatal rat cardiac myocytes. VNP decreased the expression of NPR-C in cardiac myocytes and fibroblasts under both air-control and hypoxic condition, which was at least partially mediated by guanylate cyclase linked natriuretic peptide receptors through increasing the intracellular cGMP.

INTRODUCTION

The natriuretic peptides (NP) are a family of vasoactive substances that are produced in a number of organs, most notably in the heart. Three different natriuretic peptide receptors (NPR) have been characterized, NPR-A, NPR-B, and NPR-C[1-3]. NPR-A and NPR-B are membrane-bound guanylate cyclase coupled receptors, which could produce 3',5'-cyclic guanosine monophosphate (cGMP)[1]. These receptors mediate most of the biological actions of natriuretic peptides. The NPR-C is a membrane-bound receptor half size of NPR-A and NPR-B that lacks the intracellular guanylate cyclase domains of the biologically active receptors and is therefore uncoupled from cGMP[2]. NPR-C functions as a clearance receptor by binding and internalizing natriuretic peptides[2,4]. Recent experiments have suggested that the alteration of NPR-C gene expression modulates the development of hypoxic pulmonary hypertension and right ventricular hypertrophy[5-8]. Li[6] and Klinger[9] have previously demonstrated that both acute and chronic hypoxia cause significant down-regu-

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Received 2002-11-05 Accepted 2003-12-24

lation of NPR-C binding and gene expression without affecting NPR-A or NPR-B gene expression in rat lung[6,9]. Studies have revealed the existence of NPR-C in both cardiac myocytes and fibroblasts[10,11]. However, the pathophysiological role of NPR-C in heart failure has not been fully defined and there is still some controversy about the effects of hypoxia on the expression of NPR-C in heart[7,8,12,13].

Vasonatrin peptide (VNP) is a man-made new member of natriuretic peptide family[14]. Our previous studies have demonstrated that it could both *in vivo* and *in vitro* prevent the development of hypoxic pulmonary hypertension and right ventricular hypertrophy[15-17]. However, the underlying mechanisms of the effects of VNP on hypoxic cardiovascular diseases are not well understood. There is no research about its regulation on the expression of NPR.

Therefore, in the present study, we investigated the regulation of VNP on the expression of NPR-C in both cardiac myocytes and fibroblasts induced by hypoxia and try to elucidate the possible intracellular pathway involved in this process.

MATERIALS AND METHODS

Reagents VNP was synthesized by Shanghai Institute of Biochemistry. HS-142-1, trypsin, and collagenase (type I) were purchased from Sigma Chemical Co (St Louis, MO, USA). Total RNA extraction kit was purchased from Sino-American Biotech Co (Luoyang, Henan, China). AMV Reverse Transcriptase was obtained from Promega (Madison, WI, USA). Taqman quantitative PCR kit was from PE Co (Foster City, CA, USA). All cell culture reagents were purchased from GibcoBRL (Grand Island, NY, USA).

Cell culture Enriched cultures of cardiac myocytes and fibroblasts were obtained by stepwise dissociation as described previously[18]. Briefly, the hearts from 1- or 2-d-old SD rats (Grade II, from the Center of Laboratory Animal of the Fourth Military Medical University with certificate No C98008) were minced and dissociated with 0.125 % trypsin and 0.075 % collagenase type I. To purify the cardiac myocytes from non-myocytes, dissociated cells were preplated for 90 min, during which the non-myocytes attached readily to the bottom of the culture dish. Non-attached myocytes were collected and seeded into 90-mm culture dishes (1×10^7 cells/dish) for quantitative RT-PCR or 75-cm^2 flask (5×10^5 cells) for radioimmunoassay. The DNA synthesis inhibitor, bromodeoxyuridine (100 μmol/L) was added during the first 48 h to prevent the proliferation of non-myocytes. Cells were incubated in Dulbecco's modification of Eagle's Minimal Essential Medium (DMEM) supplemented with 10 % neonatal calf serum (NCF), benzylpenicillin 100 kU/L and streptomycin 100 g/L for 48 h and then replaced with serum-free DMEM with 0.5 % bovine serum albumin (BSA) 24 h before experiments. Using this method, we routinely obtained myocyte-rich culture with 90 %-95 % myocytes as assessed by microscopic observation of cell beating and by immuno-chemistry staining with a monoclonal antibody against sarcomeric myosin.

To obtain fibroblast-enriched fraction, the culture dishes containing attached non-muscle cells were plated into an incubator and passaged 48 h later with 0.25 % trypsin, as described elsewhere[19]. These cells were used at passage 3 to 5. For experiments, cardiac fibroblasts were plated onto 90-mm culture dishes (1×10^6 cells/dish) for quantitative RT-PCR or radio-immunoassay. Following overnight attachment, cardiac fibroblast cultures were placed in serum-free medium as above, and experiments were performed 24 h later. The purity of cardiac fibroblasts used in experiment was above 95 % identified by immunohistochemistry staining with anti-vementin.

Each dispersion procedure yielded an average of 6 dishes from 30 neonatal rat ventricles, which were divided for the experiments. Each culture experiment was repeated with 4 separate samples obtained from 4 different preparations.

Hypoxia exposure The hypoxic condition was produced as reported previously[20]. Briefly, cardiac myocytes were placed in an air-tight desiccator, where the air could be completely replaced by a gas mixture of 2 % O_2/93 % N_2/5 % CO_2 to produce hypoxic condition. To measure the oxygen content, the medium cultured with cells were collected by a syringe immediately after 30 min, 6 h, and 24 h hypoxia, and pO_2 was measured using a pH/Blood Gas Analyzer (AVL OMNI, Instru-Med, Inc. GA, USA). pO_2 of the medium reached 15-25 mmHg as early as 30 min, and could be maintained up to 24 h. The medium was replaced with fresh hypoxic per 12 h to prevent the buildup of waste metabolites and maintain the pH of the medium[21]. At the end of incubation period, cell viability was evaluated by Trypan blue exclusion and intracellular lactate dehydrogenase (LDH) leakage into culture medium[22].

RNA extraction After treatment with drugs for 24 h under air-control or hypoxic condition, cells were homogenized and lysed in 1 mL Biotragent (mixture of phenol and Guanidine Thiocyanate) and were processed according to the manufacturer's protocol to obtain total cellular RNA. Total RNA concentrations were quantitated by ultraviolet (UV) spectrophotometry (Beckman DU640, USA).

Analysis of NPR-C expression by RT-PCR One microgram of total RNA was suspended in 20 μL of buffer containing $MgCl_2$ 25 mmol/L, 10× Buffer, dNTP Mixture 10 mmol/L with recombinant Rnasin ribonuclease inhibitor 0.5 μL, Oligo $(dT)_{15}$ primer 0.5 μg, and AMV reverse transcriptase (Promega, USA) 15 U and reverse transcribed at 42 ºC for 15 min. The reaction was stopped by heat inactivation for 5 min at 99 ºC and then chilled on ice.

Complementary DNA products were amplified by PCR using the following primers: NPR-C forward, 5'-CCT CCT CCA CGT TCT GGC T-3'; and NPR-C reverse, 5'-GAT TTT CCC CCC ATC CTT CTT-3'. PCR buffer (50 μL) contained 20 % glycerol 16 μL, 10× Taqman Buffer A 5 μL, 25 mmol/L Mg^{2+} 10 μL, 10 mmol/L A 1 μL, 10 mmol/L C 1 μL, 10 mmol/L G 1 μL, 20 mmol/L U 1 μL, TaqGlod™ polymerase 0.5 μL, AmpErase UNG 0.5 μL, 0.5 μL each of forward and reverse primer (20 μmol/L). Taqman fluorescence probe (20 μmol/L) 0.25 μL, Template DNA 4 μL, and nuclease free water. Forty cycles were performed under the following conditions: 50 ºC 2 min, 95 ºC 10 min, 95 ºC 15 s, 60 ºC 1 min. The house-keeping gene of β-actin was co-amplified with NPR-C as intra-control. Negative control was obtained by replacing Template DNA by ddH_2O. For quantification, the fluorescence emitted during PCR was analyzed by the ABI Prism 7700 real-time sequence detection system (PE Applied Biosystems)[23].

Measurement of cGMP levels After a 24-h hypoxic exposure, cells were treated with various concentration of VNP and/or HS-142-1 for 15 min under air-control or hypoxic condition. The medium was then rapidly removed, and 1 mL ice-cold thrichloroacetic acid (0.24 mmol/L) was added. Cell samples were centrifuged to remove precipitated proteins, and the supernatant fractions were extracted with 1 mL of water-saturated ether three times. The cGMP level was determined by radioimmunoassay performing with a cGMP assay kit (Shanghai University of Traditional Chinese Medicine). The sensitivities of the cGMP RIA are 50 fmol per assay tube. Average results of determinations were expressed as picomoles of cGMP per milliliter of cells.

Statistical analysis Results were expressed as mean±SD. Statistical analyses were carried out with the Origin5.0 statistical package. All values were analyzed by one-way analysis of variance (ANONA). The significance level was chosen as $P<0.05$.

RESULTS

After an incubation for 24 h, the percentage of viable cells and the concentration of LDH in culture media had no significant difference among all groups, ie, the cells could tolerant the hypoxic environment well.

Down-regulation of the expression of NPR-C in cardiac cells by vasonatrin peptide As shown in Fig 1, hypoxic exposure for 24 h did not alter the level of NPR-C mRNA in cardiac fibroblasts, but significantly increased that of cardiac myocytes to 298 % of air-

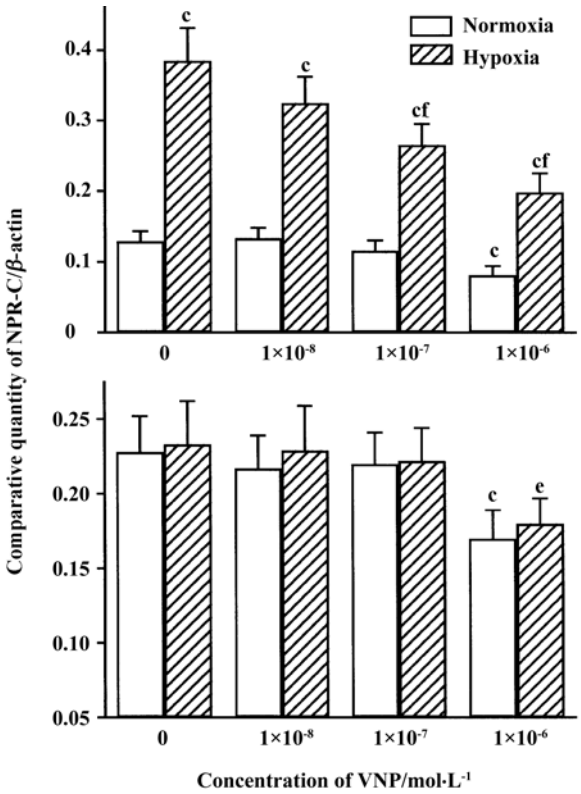

Fig 1. Effects of VNP on the mRNA levels of NPR-C induced by hypoxia for 24 h in cultured neonatal rat cardiac myocytes (top) and fibroblasts (bottom). *n*=4. Mean±SD. The data were presented as ratio of mRNA levels of NPR-C relative to β-actin mRNA. $^cP<0.01$ *vs* air-control group without VNP. $^eP<0.05$, $^fP<0.01$ *vs* hypoxic group without VNP by one-way ANOVA.

control level. VNP (1×10^{-8}-1×10^{-6} mol/L) concentration dependently decreased the expression of NPR-C induced by hypoxia in cardiac myocytes. What is more, high concentration of VNP (1×10^{-6} mol/L) reduced NPR-C in cardiac myocytes and fibroblasts under both hypoxic and air-control condition.

Effects of 8-bromo-cGMP on the expression of NPR-C in cardiac cells The natriuretic peptide family is known to exert its biological action through the activation of particulate guanylate cyclase and the subsequent elevation of intracellular cGMP concentration. Due to the similar structure, we postulated that VNP might function through similar pathway. The treatment of cardiac myocytes and fibroblasts with 8-bromo-cGMP 10^{-6} mol/L resulted in a significant decrease in the expression of NPR-C, which is similar to the action of VNP (Fig 2).

Effects of VNP on cGMP accumulation in cardiomyocytes and cardiac fibroblasts In order to further testify whether the intracellular cGMP was involved in the signaling pathway of VNP, we studied the effects of VNP on cGMP accumulation in cultured cardiac myocytes and fibroblasts. VNP 1×10^{-8}-1×10^{-6} mol/L markedly increased cGMP production in a concentration-dependent manner in both myocytes and fibroblasts. The addition of HS-142-1 (1×10^{-5} mol/L), the well-known antagonist of the particulate guanylyl cyclase-coupled natriuretic peptide receptors[24], completely abrogated the effect of VNP 1×10^{-6} mol/L on cGMP formation (Fig 3).

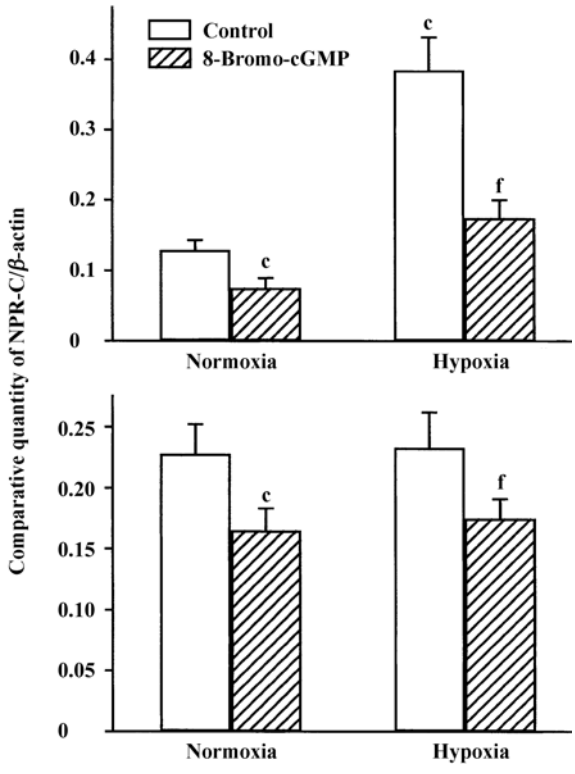

Fig 2. Effects of 8-bromo-cGMP on the mRNA levels of NPR-C in cultured neonatal rat cardiac myocytes (top) and fibroblasts (bottom). *n*=4. Mean±SD. The data were presented as ratio of mRNA levels of NPR-C relative to β-actin mRNA. Cells were incubated with 8-bromo-cGMP 1×10^{-6} mol/L for 24 h in 8-bromo-cGMP treated groups. $^{c}P<0.01$ *vs* air-control group (without 8-bromo-cGMP). $^{f}P<0.01$ *vs* hypoxic group (without 8-bromo-cGMP) by one-way ANOVA.

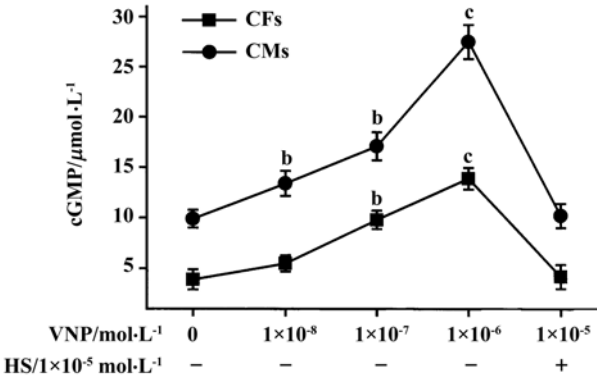

Fig 3. Effects of VNP on hypoxia-induced intracellular cGMP formation in cultured neonatal rat cardiac myocytes and fibroblasts. *n*=8. Mean±SD. Cells were incubated with indicated concentration of VNP in the presence (+) or absence (-) of HS-142-1 (HS) 1×10^{-5} mol/L for 15 min after an exposure to hypoxia for 24 h. $^{b}P<0.05$, $^{c}P<0.01$ *vs* 0 concentration of VNP.

Influences of HS-142-1 on the effects of VNP on the expression of NPR-C HS-142-1 is an inhibitor of the guanylyl cyclase-linked natriuretic peptide receptors, ie, NPR-A and NPR-B. VNP 1×10^{-6} mol/L significantly decreased the expression of NPR-C under both air-control and hypoxic condition. HS-142-1 1×10^{-5} -1×10^{-4} mol/L partly reversed the above effects of VNP. HS-142-1 alone did not produce detectable changes in the expression of NPR-C (Fig 4).

DISCUSSION

Increasing evidence suggest that the expression of NPR is altered to modulate hypoxic cardiopulmonary disease. However, the modulation of NPR-C, in relation to hypoxic heart disease, is not well defined. In the present study, we demonstrated that hypoxic exposure up-regulated the expression of NPR-C in cultured neonatal rat cardiac myocytes. VNP (a newly synthesized member of NPs) was shown to have negative regulation on the expression of NPR-C in cardiac

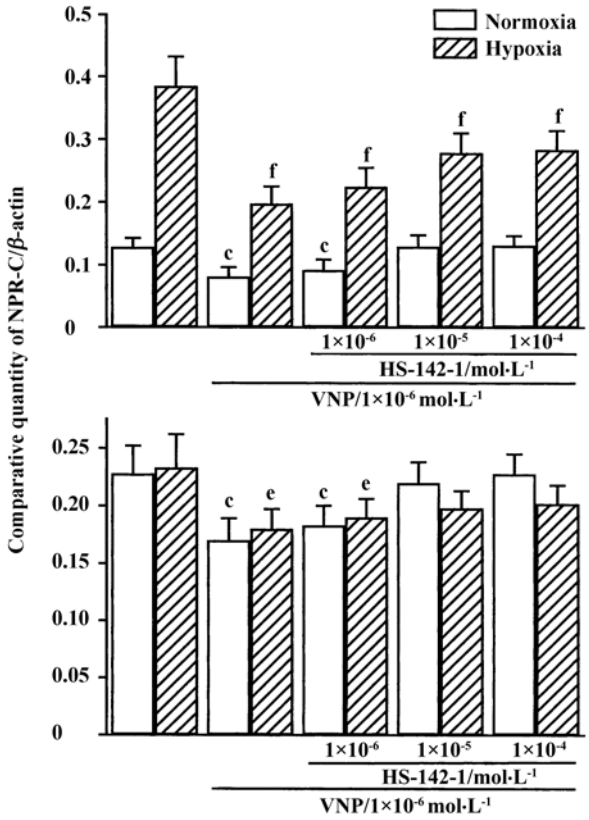

Fig 4. Effects of HS-142-1 on the levels of NPR-C mRNA induced by VNP under air-control and hypoxic condition in cardiac myocytes (top) and fibroblasts (bottom). *n*=4. Mean±SD. The data were presented as ratio of mRNA levels of NPR-C relative to β-actin mRNA. $^{c}P<0.01$ *vs* air-control group. $^{e}P<0.05$, $^{f}P<0.01$ *vs* hypoxic control group.

myocytes and fibroblasts under both hypoxic and air-control condition, which was partially mediated by guanylate cyclase coupled receptor through increasing the intracellular cGMP.

To assess the localization of NPR transcripts on a cellular level, we used quantitative RT-PCR, one of the most sensitive methods currently known for specific detection of mRNA in small tissue samples or isolated cells. Our results confirm the presence of NPR-C in both cultured rat cardiac myocytes and fibroblasts. As far as the regulation of hypoxia on cardiac natriuretic peptide receptors is concerned, to the best of our knowledge, this is the first study to show the direct effect of hypoxia, independent on the neural and humoral regulation, on the expression of NPR-C in cultured cardiac cells. The NPR-C contributes to the metabolic clearance of natriuretic peptides and is located in a number of different tissues and cells, including myocardial cells and smooth muscle cells, glomerular cells and pulmonary parenchyma[10,25,26]. Several studies have investigated the regulation of the NPR-C by stressful conditions, eg, pulmonary hypertension, myocardial infarction, and hypoxia[6,11]. A down-regulation of the NPR-C receptor has been suggested in these studies. In the present study, cardiac myocytes exposed to hypoxia for 24 h revealed up-regualtion of NPR-C mRNA expression, which is in contrast to the changes of NPR-C in other tissues such as lung, kidney, and brain. It is important to note that our data closely agree with the latest observation in heart after induction of myocardial infarction in rat[27]. The molecular mechanism underlying the different response of heart compared with other tissue is not known. It suggests that the regulation of NPR-C by hypoxia/ischemia has tissue specificity. The reduction of NPR-C in peripheral tissues such as kidneys and lungs, other than heart, makes contribution to the increased circulating plasma concentrations of BNP and ANP during hypoxia/ischemia. The up-regulation of "clearance" receptors in cardiac myocytes exposed to hypoxia may suggest a possible explanation for the resistance to biological effects of cardiac natriuretic hormones in chronic heart failure.

VNP, a unique synthetic peptide, is a chimera of CNP and ANP[14]. 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 possesses the venodilating actions of CNP, the natriuretic actions of ANP, and unique arterial vasodilating actions not associated with either ANP or CNP. 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[16,17,28]. In the present study, VNP was shown to reduce the expression of NPR-C in both cardiac myocytes and fibroblasts. Down-regulation of NPR-C is expected to raise both endogenous and exogenous NPs levels and make more NPs available to the guanylyl cyclase-linked receptors, thereby enhancing its local action in the heart.

Due to the similar molecular structure and cardiovascular effects, it is postulated that VNP might act through the same signaling transduction pathway as other members of natriuretic peptide to enhance intracellular cGMP. Our data showed that VNP (1×10^{-7}-1×10^{-5} mol/L) concentration dependently increased the production of cGMP in both cardiac myocytes and fibroblasts. What is more, the effects of VNP were

mimicked by 8-bromo-cGMP, a cell-permeable analog of cGMP. Therefore, the down-regulation of NPR-C by VNP obtained in the present study may be due to an increase in cGMP production. In order to confirm it, we observed the influences of HS-142-1, a competitive antagonist for the particulate guanylyl cyclase-coupled receptors[24], on the above effects of VNP. It turned out that HS-142-1 partly reversed the regulatory effects of VNP on the expression of NPR-C under both air-control and hypoxic condition. In addition, HS-142-1 completely abrogated the enhancement of cGMP induced by VNP. These results indicate that VNP reduces the expression of NPR-C partially by increasing the intracellular cGMP.

In summary, this study provided evidence that hypoxic exposure up-regulated NPR-C gene expression in cultured cardiac myocytes. VNP, a new member of natriuretic peptide family reduced NPR-C expression in cardiac myocytes and fibroblasts under both air control and hypoxic exposure, which was partially by increasing the intracellular cGMP. Down-regulation of NPR-C by VNP was likely to reduce the clearance of the natriuretic peptides, thus enhancing their biological effects, which might be one of the underlying mechanisms of its stronger cardiovascular effects compared to other NPs[15].

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