Receptor signaling and neutral endopeptidase are involved in the resistance of C-type natriuretic peptide to human mesangial proliferation and collagen-IV expression

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Accepted 24 December 2017
Published Online First 23 January 2018

ABSTRACT

C-type natriuretic peptide (CNP) is regarded as a local, paracrine hormone to regulate vascular tone and cell proliferation. Although several in vivo studies have documented that CNP exerts the inhibitory effects on mesangial cells (MCs) proliferation and collagen production, a limited number of studies exist about the resistance of CNP to MCs proliferation in vitro. Besides, whether its receptor signaling and neutral endopeptidase (NEP) are involved remains unclear. In the present study, human MCs were incubated in serum-containing medium in the absence or presence of CNP (0, 10 and 100 pM) for 24, 48 and 72 hours, respectively. CNP administration significantly suppresses MCs proliferation and collagen-IV (Col-IV) expression in a time-dependent and dose-dependent manner. As a down-stream signal molecule of CNP activation, the expressions of natriuretic peptide receptor (NPR)-B, cyclic guanosine monophosphate-dependent protein kinases II and NPR-C were obviously augmented, whereas NEP expression was significantly decreased after CNP treatment. In conclusion, receptor signaling and NEP are involved in the resistance of CNP to human mesangial proliferation and Col-IV expression.

Significance of this study

What is already known about this subject?

- C-type natriuretic peptide (CNP) is an endothelium-derived cytokine and exerts potent antiproliferative and antifibrotic properties in a paracrine manner.
- CNP and its receptors not only are expressed in the kidney, but possess several renoprotective properties.
- CNP has a marked inhibitory effect on mesangial proliferation and matrix generation; however, what on earth receptor signaling and neutral endopeptidase (NEP) are involved in the resistance of CNP to mesangial cells (MCs) proliferation and collagen-IV (Col-IV) expression remains unknown.

What are the new findings?

- CNP significantly suppressed MCs proliferation and Col-IV expression in a time-dependent and dose-dependent manner in vitro.
- CNP treatment was parallel with overexpressed CNP, whereas exogenous CNP did not disturb its endogenous expression in MCs.
- As a down-stream signaling of CNP activation, the expressions of natriuretic peptide receptor (NPR)-B, cyclic guanosine monophosphate-dependent protein kinases II and NPR-C were obviously augmented, whereas NEP expression was significantly decreased after CNP treatment.

INTRODUCTION

C-type natriuretic peptide (CNP), first isolated from the porcine in 1990, shares a similar molecular structure and physiological function with other members of natriuretic peptide family.[1] It comprises a ring structure of 17 amino acids linked by a cysteine disulfide bridge, which is essential for receptor binding and bioactivity. CNP selectively binds to the guanylyl cyclase-couple natriuretic peptide receptor (NPR)-B and subsequently leads to the activation of intracelluar cyclic guanosine monophosphate-dependent protein kinases (CGK) II. On the other side, CNP elimination occurs mainly by three different pathways, NPR-C, neutral endopeptidase (NEP) and urinary excretion.[2] CNP is an endothelium-derived cytokine and regulates body fluid homeostasis and vascular smooth muscle tone in a paracrine manner. More particularly, CNP exerts potent antiproliferative and antifibrotic properties.[3] In vitro, Furuyal *et al*[4] found that 5×10^{-7} M of CNP could inhibit serum-induced DNA synthesis of the rat vascular smooth muscle cells (VSMCs)

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To cite: Luo HH, Wu C, Hu P, *et al*. *J Investig Med* 2018;**66**:896–904.

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Significance of this study

How might these results change the focus of research or clinical practice?

- Exogenous CNP can ameliorate human mesangial proliferation and matrix excessive accumulation.
- Receptor signaling and NEP are involved in these renoprotective properties.
- Further studies on the antagonists of receptor signaling and NEP will clarify this issue more comprehensively in the near future.

in a dose-dependent manner and reduce the cell number by 2 per cent on 4 days post culture. Furthermore, another in vitro study conducted by Lu *et al*[5] also showed that CNP had inhibitory effects at the concentration from 10^{-7} to 10^{-5} M on cell proliferation and even collagen synthesis of VSMCs.

Currently, a growing body of evidence confirms that CNP and its receptors not only are expressed in the kidney, but possess several renoprotective properties including local vasorelaxation, acceleration of re-endothelialization, antagonism of the renin–angiotensin–aldosterone system, and even inhibition of renal remodeling.[6–8] Kimura *et al*[9] studied the therapeutic effects of CNP in a mouse model of cisplatin-induced nephrotoxicity and revealed that CNP infusion suppressed blood urea nitrogen and creatinine levels and ameliorated renal pathological damage. What's more, our recent study published in *Laboratory Investigation* demonstrated that CNP infusion significantly improved glomerular perfusion and relieved collagen-IV (Col-IV) accumulation in tubulointerstitium through downregulating tissue inhibitor of metalloproteinases in the unilateral ureteral obstruction (UUO) rats.[10]

Mesangial cells (MCs) occupy a central anatomical position in the glomerulus and possess smooth muscle-like characteristics. By regulating the metabolism of extracellular matrix (ECM), MCs play vital roles in glomerular structural stability. Besides, they are also capable of many other functions including inflammatory cytokine generation, immune complex clearance and close interaction with both endothelial cells and podocytes.[11 12] Since MCs are involved in a wide variety of glomerular diseases, modulation of MCs responses will be a promising therapeutic strategy.[13] In 1998, a pioneering study demonstrated that CNP had a marked inhibitory effect on mesangial matrix generation in vivo. Canaan-Kuhl *et al*[14] investigated the benefits of CNP infusion (1 µg/kg/min) on the rat model of anti-Thy 1.1 mesangioproliferative nephritis over 7 days and found that the glomerular expression of Col-IV was significantly decreased in the CNP-treated rats to 72 per cent of the buffer controls. Conformably, our latest study also showed that CNP could suppress MCs proliferation and Col-IV expression in vitro.[15] However, what on earth receptor signaling and NEP are involved in the resistance of CNP to MCs proliferation and Col-IV expression remains unknown.

MATERIALS AND METHODS

Cell culture

Renal tissue was obtained from the unaffected poles of kidneys surgically removed as part of treatment of renal carcinoma, and with the approval of the our institution's local medical ethics committee. Glomeruli were plated onto 6 cm dishes, dissociated with RPMI 1640 (Invitrogen, Carlsbad, California, USA) medium supplemented with 10 per cent fetal bovine serum, 50 U/mL penicillin, 50 U/mL streptomycin and 2 mM L-glutamine in a humidified atmosphere of 5 per cent CO_2 in air at 37°C. D-Valine was added to the cultures to inhibit the growth of fibroblasts. MCs appeared after 7–14 days in cultures and reached confluency by day 21. After reaching confluence, the cells were detached by treatment with 0.25 per cent trypsin and 1 mM EDTA in phosphate-buffered saline (PBS) acid; thereafter they were applied to subculture. Cells were used for the experiments between passages 4 and 6. Cultured cells did not show the morphological characteristics of macrophages and were stained positively with antifibronectin or anti-desmin antibody, but not stained with anti-cytokeratin or anti-IA antibody, indicating that these cells were identical to MCs.[16] Cells, grown in 96-well plates at the density of 1200 cells/well, were incubated in serum-containing medium in the absence or presence of CNP (0, 10 and 100 pM) for 24, 48 and 72 hours, respectively.

Cell proliferation assays

Total cell numbers were manually counted and dead cells were excluded with eosin red staining. The cells were suspended at a concentration of 2×10^6 cells/mL and incubated with 5 µM carboxyfluorescein diacetate succinimidyl ester for 5 min at 37°C and then subjected to extensive washing with PBS. Cell proliferation was evaluated with a proliferation assay kit (Invitrogen) based on the reaction of 3-(4,5-dimethylthiazol-2-yl)−5-(3-carboxymethoxyphenyl)−2-(4-sulfophenyl)−2H-tetrazolium (MTS) in metabolically active cells by flow cytometry.[17]

ELISA

The protein levels of CNP, NPR-B, CGK II, NPR-C, NEP and Col-IV in the culture supernatants were determined using commercially available ELISA kits (Invitrogen) according to the manufacturer's procedures.

Real-time (RT)-PCR

Total RNA was isolated from cultured MCs by Trizol reagent (Invitrogen). Ultraviolet spectrophotometer measuring absorbance and agarose gel electrophoresis confirmed that there was no degradation of RNA. Among this 4 µg of isolated RNA were reverse-transcribed into cDNA with an ExScript RT reagent kit (Takara Biotechnology, Dalian, PR China). RT-PCR was performed with the ABI 7900 sequence detection system (Applied Biosystems, Foster City, California, USA) using SYBR Green PCR Master Mix (Applied Biosystems) in accordance with the manufacturer's instructions. The primers of CNP, NPR-B, CGK II, NPR-C, NEP, Col-IV and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) are shown in table 1. Amplification conditions were as follows: pre-denaturing at 95°C for 2 min; denaturing at 94°C for 20 s, annealing at 65°C for 20 s,

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Table 1 List and sequence of primers

Variable	Sequence	Product length (bp)
C-type natriuretic peptide		
Sense	5′-AACAGAGGCTTCTCCCGAAAA-3′	149
Antisense	5′-GTCTTGCACTCTAGCAGCGT-3′	
Natriuretic peptide receptor-B		
Sense	5′-TGACCCCGACCTGCTGTTA-3′	150
Antisense	5′-CGAACCAGGGTACGATAATGG-3′	
Natriuretic peptide receptor-C		
Sense	5′-AGACTACGCCTTCTTCAACATTG-3′	90
Antisense	5′-GCTTCAAAGTCGTGTTTGTCTCC-3′	
Cyclic guanosine monophosphate-dependent protein kinase II		
Sense	5′-GAACCTCACCACTGATGCTCT-3′	104
Antisense	5′-GCTCCTTCAAATGGTACTCCCG-3′	
Neutral endopeptidase		
Sense	5′-AGAAGAAACAGCGATGGACTCC-3′	93
Antisense	5′-CATAGAGTGCGATCATTGTCACA-3′	
Collagen-IV		
Sense	5′-GGACTACCTGGAACAAAAGGG-3′	240
Antisense	5′-GCCAAGTATCTCACCTGGATCA-3′	
Glyceraldehyde-3-phosphate dehydrogenase		
Sense	5′-GGAGCGAGATCCCTCCAAAAT-3′	197
Antisense	5′-GGCTGTTGTCATACTTCTCATGG-3′	

extension at 72°C for 30 s; and finally one cycle of 5 min at 72°C. Gene expression was normalized using GAPDH as an endogenous control to correct for differences in the amount of total RNA originally added to each reaction. The average threshold cycle (Ct, the cycles of template amplification to the threshold) was worked out as the value of each sample. Relative quantitative $2^{-\Delta\Delta Ct}$ was used to compare the mRNA expression.[18]

Western blot analysis

Harvested MCs were homogenized in sodium dodecyl sulfate (SDS) sample buffer (2 per cent SDS, 10 mM Tris-HCl, pH 6.8, 10 per cent (vol/vol) glycerol). Cell homogenates (30 μg of protein per lane) were treated with Laemmli sample buffer, heated at 100°C for 5 min and electrophoresed in a 10 per cent acrylamide denaturing SDS-polyacrylamide gel. The protein bands were electrotransferred onto polyvinylidene difluoride membranes (Bio-Rad, Beijing, PR China), blocked with 5 per cent non-fat milk in Tris-buffered saline with 0.05 per cent Tween 20 buffer (TBST) overnight at 4°C. The membranes were then incubated with anti-CNP, NPR-B, CGK II, NPR-C, NEP, Col-IV and GAPDH primary antibodies (Medical Discovery Leader, Beijing, PR China) at 4°C overnight. The membranes were washed three times (10 min each) with TBST, followed by incubation with horseradish peroxidase-conjugated anti-rabbit IgG antibodies (1:3000 dilution; Medical Discovery Leader) at room temperature for 60 min. The reaction products were detected using the enhanced chemiluminescence detection system and exposed on radiographic films for variable time points.

Statistical analyses

All values are expressed as mean±SEM Comparison of mean values between groups was made using one-way analysis of variance, and post hoc analysis was calculated using the Student-Newman-Keuls test. A value of $P<0.05$ was considered significant. Statistical analysis was performed using the Statistical Package for Social Studies V. 17.0.

RESULTS

CNP expression in the culture supernatant and MCs

Figure 1 depicts the supernatant and MCs CNP levels. By ELISA, the baseline levels of supernatant CNP in the control group were 23.00±5.73, 29.75±4.21 and 37.35±6.94 pg/mL at 24, 48 and 72 hours after culture, respectively. CNP treatment was parallel with a significant increase of supernatant CNP in a time-dependent and dose-dependent manner. Supernatant CNP was the highest throughout the high-dose group compared with the corresponding low-dose group and the control group at each time point (figure 1A; $P<0.05$). By RT-PCR and western blot analysis, CNP expression was markedly elevated in MCs at each time point (figure 1B,C; $P<0.05$). Compared with the corresponding control group, CNP expression presented a 1.0-fold, 7.5-fold and 13.4-fold increase in the low-dose group at 24, 48 and 72 hours post treatment, respectively ($P<0.05$); and a 3.0-fold, 20.7-fold and 32.0-fold increase in the high-dose group at 24, 48 and 72 hours post treatment, respectively ($P<0.05$). Moreover, there was a significant elevation in CNP expression in the high-dose group at each time point compared with the corresponding low-dose group ($P<0.05$). According to the above findings, exogenous CNP did not disturb its endogenous expression in MCs.

MCs proliferation and Col-IV expression

MCs proliferation and Col-IV expression are shown in figure 2. Based on the result of MTS assays, CNP significantly inhibited the growth of MCs at both low and high doses ($P<0.05$). More specifically, compared with the corresponding control group, MTS activity experienced a 24 and 34 per cent decrease in the low-dose group at 48 and 72 hours post treatment, respectively ($P<0.05$); and a 32 and 57 per cent decrease at 48 and 72 hours post treatment in the high-dose group, respectively ($P<0.05$). Furthermore, the high-dose group showed an 11 per cent decrease and a 35 per cent decrease compared with the corresponding low-dose group at 48 and 72 hours after culture, respectively (figure 2A; $P<0.05$).

By ELISA, CNP treatment progressively decreased the supernatant level of Col-IV throughout the observational period (figure 2B; $P<0.05$). Compared with the corresponding controls, it was notable that supernatant Col-IV showed a 15, 43 and 70 per cent decrease in the low-dose group at 24, 48 and 72 hours post treatment, respectively; and a 34, 71 and 92 per cent decrease in the high-dose group at 24, 48 and 72 hours post treatment, respectively

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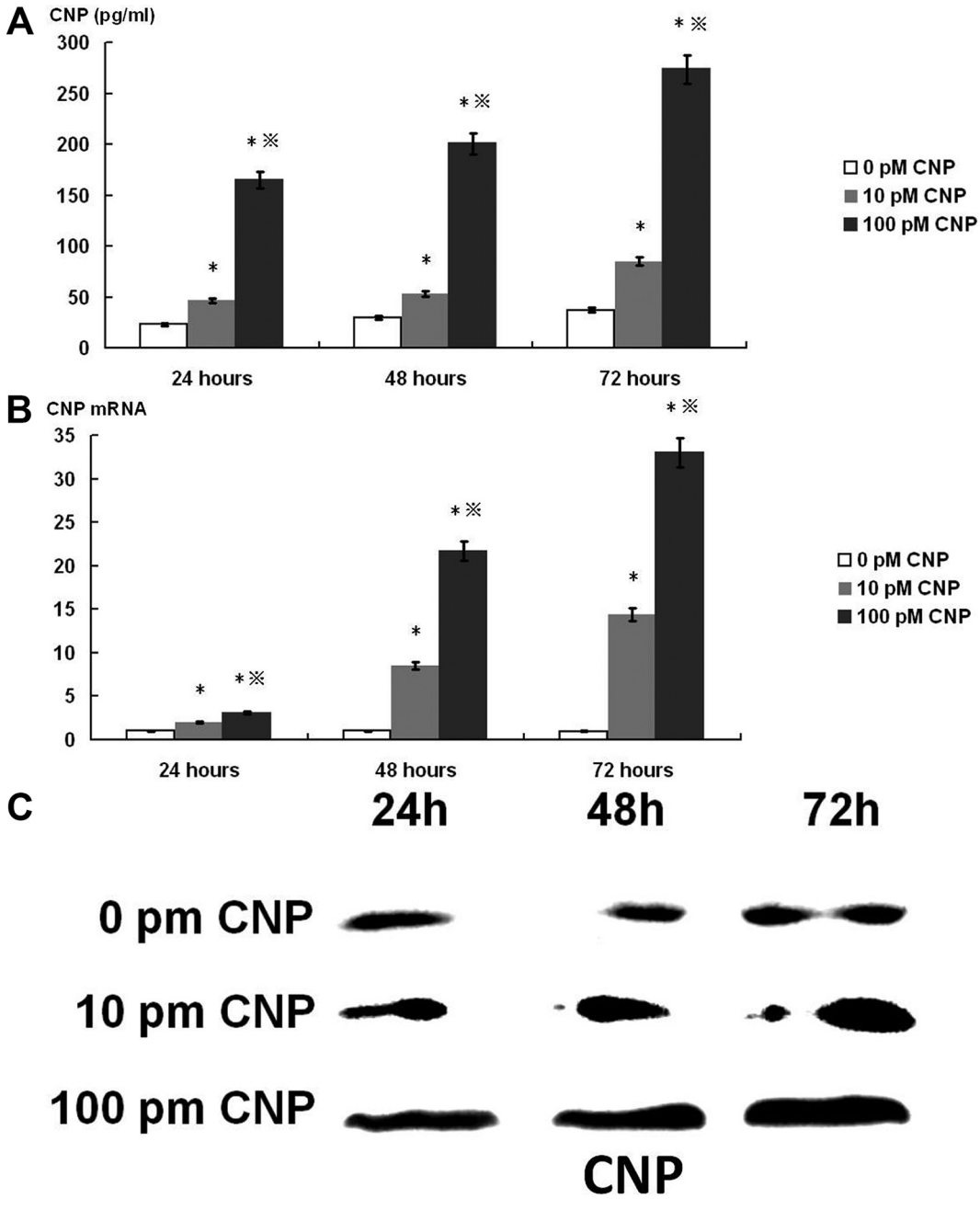

Figure 1 Exogenous did not disturb its endogenous expression after C-type natriuretic peptide (CNP) treatment in mesangial cells. (A) ELISA was used to detect the supernatant level of CNP; (B) real-time PCR was used to detect the mRNA level of CNP; (C) the protein expression of CNP was measured by western blot. *P<0.05, significantly different from the corresponding control group (0 pM CNP). ※ P<0.05, significantly different from the corresponding low-dose group (10 pM CNP).

(P<0.05). Besides, the high-dose group exhibited larger drops in supernatant Col-IV than that in the corresponding low-dose group at 24, 48 and 72 hours post treatment (a 23, 49 and 72 per cent decrease, respectively, figure 2B; P<0.05). Consistent with the supernatant changes, RT-PCR and western blot analysis of Col-IV expression were significantly suppressed by CNP in a time-dependent and dose-dependent manner (figure 2C,D; P<0.05). In more detail, Col-IV mRNA expression underwent a 52, 87 and 96 per cent decrease in the low-dose group at 24, 48 and 72 hours post treatment, respectively (P<0.05); and a 86, 92 and 97 per cent decrease in the high-dose group at 24, 48 and 72 hours post treatment, respectively (P<0.05). The high-dose group showed a 72, 39 and 31 per cent decease at 24, 48 and 72 hours post treatment compared with their low-dose counterparts, respectively (figure 2C; P<0.05).

Receptor signaling

The expression of CNP receptor signaling (NPR-B, CGK II and NPR-C) is presented in figure 3. By ELISA, supernatant NPR-B was significantly elevated after CNP treatment in

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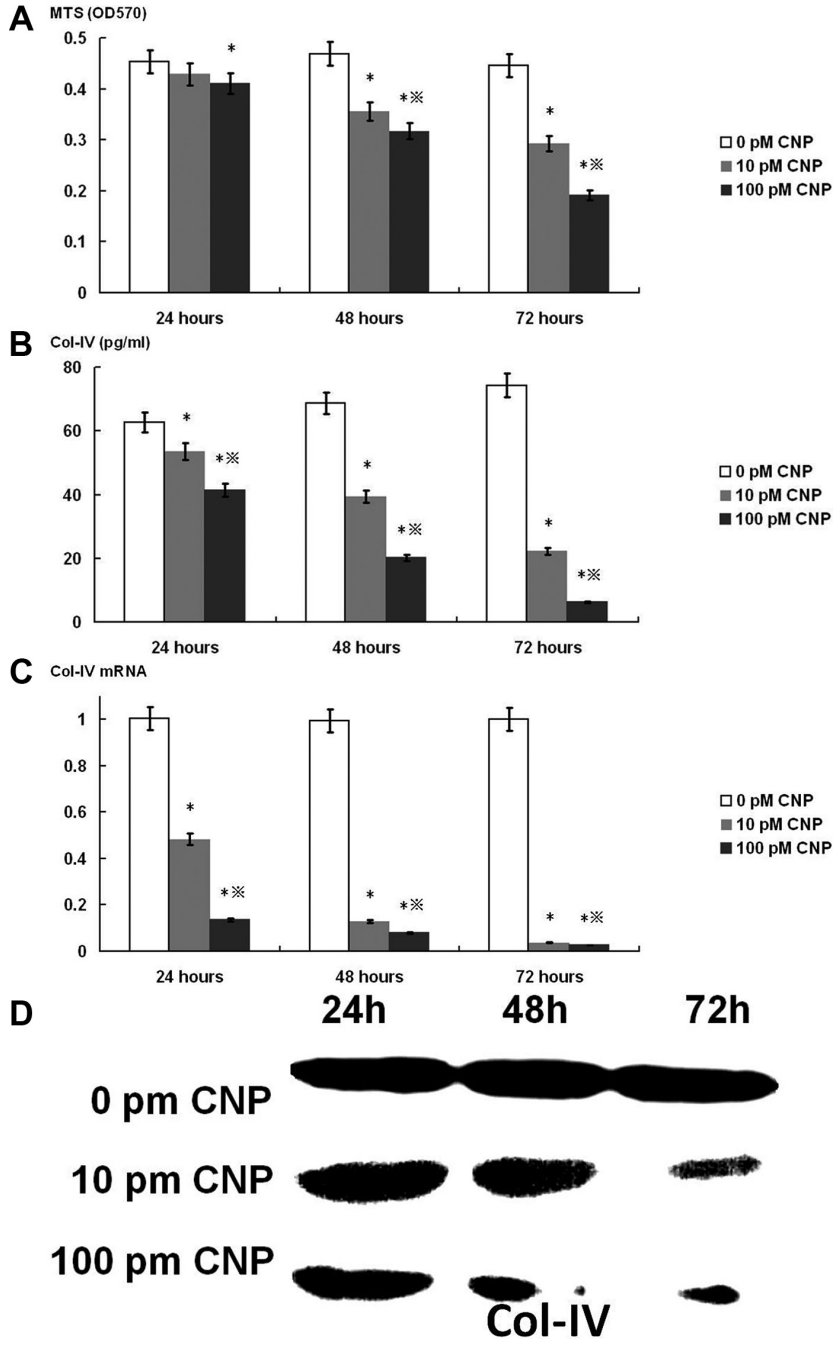

Figure 2 C-type natriuretic peptide (CNP) inhibited mesangial cells (MCs) proliferation and collagen-IV (Col-IV) expression. (A) 3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assays were used to measure the growth of MCs; (B) ELISA was used to detect the supernatant level of Col-IV; (C) Real-time PCR was used to detect the mRNA level of CNP; (D) the protein expression of CNP was measured by western blot. *P<0.05, significantly different from the corresponding control group (0 pM CNP). ※ P<0.05, significantly different from the corresponding low-dose group (10 pM CNP).

a dose-dependent manner (figure 3A; P<0.05). Compared with the corresponding controls, supernatant NPR-B showed a 2.0-fold, 3.8-fold and 5.5-fold increase in the low-dose group at 24, 48 and 72 hours post treatment, respectively (P<0.05); and a 3.6-fold, 5.9-fold and 9.4-fold increase in the high-dose group at 24, 48 and 72 hours post treatment, respectively (P<0.05). Moreover, the high-dose group showed a 54, 45 and 60 per cent increase at 24, 48 and 72 hours post treatment compared with their low-dose counterparts, respectively (figure 3A; P<0.05). Similarly, NPR-B expressions at mRNA and protein levels were markedly increased at each time point (figure 3B,C; P<0.05). Compared with the corresponding control group, NPR-B transcript exhibited a 0.7-fold, 15.9-fold and 24.5-fold increase in the low-dose group at 24, 48 and 72 hours post treatment, respectively (P<0.05); and a 6.7-fold, 24.4-fold and 32.1-fold increase in the high-dose group at 24, 48 and 72 hours post treatment, respectively (P<0.05). In

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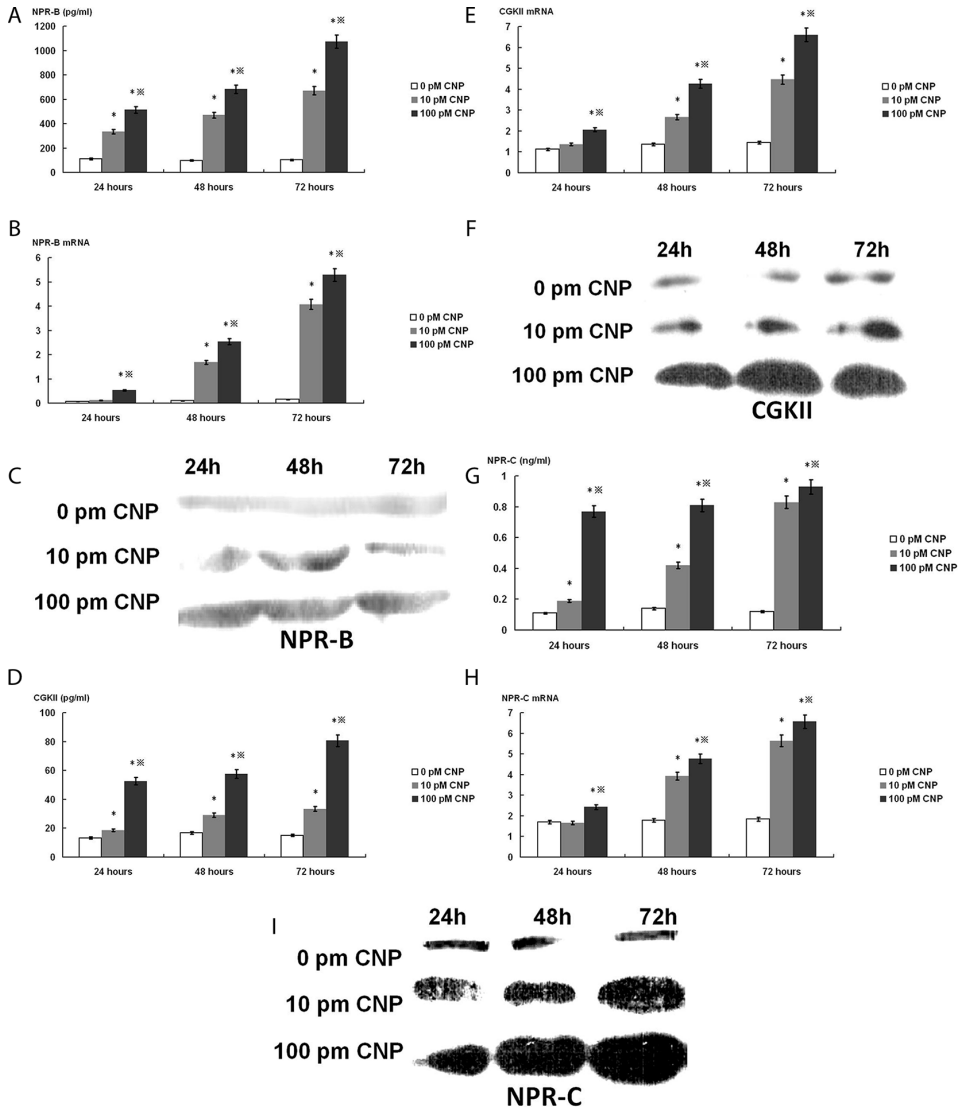

Figure 3 C-type natriuretic peptide (CNP) induced the elevation of the expressions of CNP receptor signaling (natriuretic peptide receptor (NPR)-B, cyclic guanosine monophosphate-dependent protein kinase (CGK) II and NPR-C) in mesangial cells (MCs). (A, D, G) ELISA was used to detect the supernatant levels of NPR-B, CGK II and NPR-C; (B, E, H) Real-time PCR was used to detect the mRNA levels of NPR-B, CGK II and NPR-C; (C, F, I) the protein expressions of NPR-B, CGK II and NPR-C were measured by western blot. *P<0.05, significantly different from the corresponding control group (0 pM CNP). ※ P<0.05, significantly different from the corresponding low-dose group (10 pM CNP).

addition, the high-dose group experienced a higher expression of NPR-B than that in the low-dose group at 24, 48 and 72 hours post treatment (a 3.5-fold, 0.50-fold and 0.30-fold increase, respectively, figure 3B; P<0.05).

As a down-stream signal molecule of CNP activation, supernatant CGK II was augmented after CNP treatment in a time-dependent and dose-dependent manner (figure 3D; P<0.05). Relative to the corresponding controls, it presented a 0.4-fold, 0.7-fold and 1.2-fold increase in the low-dose group at 24, 48 and 72 hours post treatment, respectively (P<0.05); and a 3.0-fold, 2.4-fold and 4.4-fold increase in the high-dose group at 24, 48and 72 hours post treatment, respectively (P<0.05). Furthermore, overexpressed supernatant CGK II was noted in the high-dose group at 24, 48 and 72 hours post treatment compared with their low-dose counterparts (a 1.8-fold, 1.0-fold and 1.4-fold increase, respectively, figure 3D; P<0.05). By RT-PCR and western blot analysis, CGK II expression was obviously upregulated throughout the whole observational period (figure 3E,F; P<0.05). Compared with the corresponding control group, CGK II expression exhibited a 0.2-fold, 1.0-fold and 2.1-fold increase in the low-dose group at 24, 48 and 72 hours post treatment, respectively (P<0.05); and a 0.8-fold, 2.1-fold and 3.6-fold increase in the high-dose group at 24, 48 and 72 hours post treatment, respectively (P<0.05). Besides, the high-dose group presented a 51, 60 and 48 per cent increase compared with the corresponding low-dose group at 24, 48 and 72 hours post treatment, respectively (figure 3E; P<0.05).

Figure 3G illustrates NPR-C levels in the culture supernatant. There was progressive elevation in supernatant NPR-C after CNP treatment at 24, 48 and 72 hours. Compared with

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the corresponding controls, supernatant NPR-C presented a 0.7-fold, 2.0-fold and 5.9-fold in the low-dose group at 24, 48 and 72 hours post treatment, respectively (P<0.05); and a 6.0-fold, 4.8-fold and 6.8-fold in the high-dose group at 24, 48 and 72 hours post treatment, respectively (P<0.05). In addition, the high-dose group exhibited a marked increment in supernatant NPR-C than that in the corresponding low-dose group at 24, 48 and 72 hours post treatment (a 3.0-fold, 0.9-fold and 0.1-fold, respectively; P<0.05). By RT-PCR and western blot analysis, NPR-C expression was obviously raised after CNP treatment at 48 and 72 hours (figure 3H,I); P<0.05). Relative to the corresponding controls, NPR-C mRNA experienced a 1.2-fold and 2.1-fold increase in the low-dose group at 48 and 72 hours post treatment, respectively (P<0.05); and a 0.4-fold, 1.7-fold and 2.6-fold increase in the high-dose group at 24, 48 and 72 hours post treatment, respectively (P<0.05). The high-dose group showed a 46, 21 and 16 per cent increase at 24, 48 and 72 hours post treatment compared with their low-dose counterparts, respectively (figure 3H; P<0.05).

Neutral endopeptidase

The expression of NEP is presented in figure 4. Supernatant NEP was significantly declined after CNP treatment (figure 4A; P<0.05). Compared with the corresponding controls, supernatant NEP experienced a 50 and 87 per cent decrease in the low-dose group at 48 and 72 hours

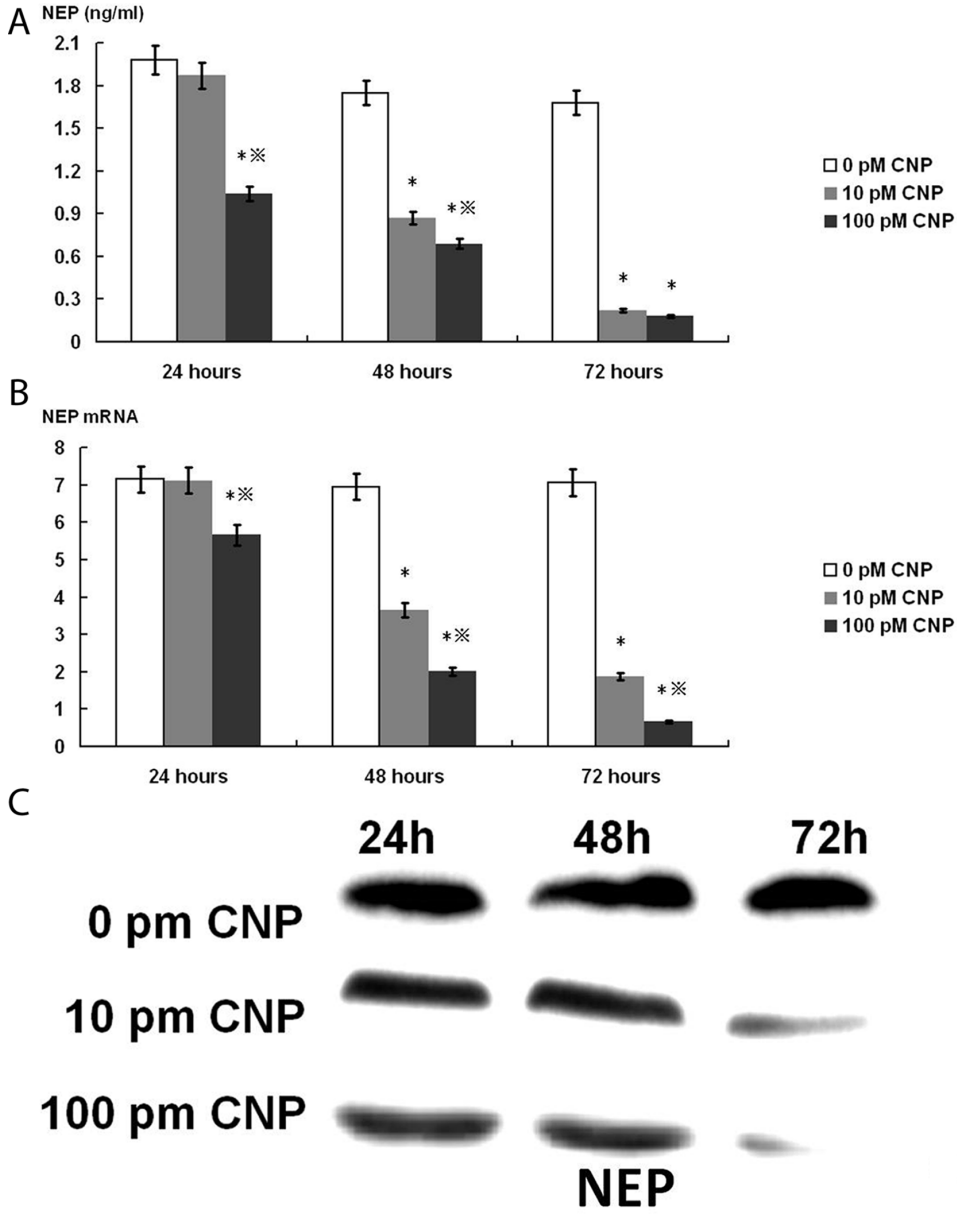

Figure 4 C-type natriuretic peptide (CNP) downregulated the neutral endopeptidase (NEP) expression in mesangial cells. (A) ELISA was used to detect the supernatant level of NEP; (B) real-time PCR was used to detect the NEP mRNA; (C) the protein expression of NEP was measured by western blot. *P<0.05, significantly different from the corresponding control group (0 pM CNP). ※ P<0.05, significantly different from the corresponding low-dose group (10 pM CNP).

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post treatment, respectively (P<0.05); and a 47, 61 and 89 per cent decrease in the high-dose group at 24, 48 and 72 hours post treatment, respectively (P<0.05). Moreover, the high-dose group showed a 44 and 21 per cent decrease at 24 and 48 hours post treatment compared with their low-dose counterparts, respectively (figure 4A; P<0.05). NEP expressions at mRNA and protein levels were also markedly decreased (figure 4B,C; P<0.05). Compared with the corresponding control group, NEP transcript exhibited a 47 and 74 per cent decrease in the low-dose group at 48 and 72 hours post treatment, respectively (P<0.05); and a 21, 71 and 91 per cent decrease in the high-dose group at 24, 48 and 72 hours post treatment, respectively (P<0.05). Furthermore, the high-dose group displayed a lower expression of NEP than that in the low-dose group at 24, 48 and 72 hours post treatment (a 21, 45 and 64 per cent -decrease respectively, figure 4B; P<0.05).

DISCUSSION

In the present study, our findings showed that CNP treatment was parallel with overexpressed CNP, whereas exogenous CNP did not disturb its endogenous expression in MCs. In 1995, Brandt *et al*[19] administrated CNP (10 ng/kg/min) to 14 dogs and observed a negative arteriovenous concentration gradient across the lung, suggesting that exogenous CNP did not suppress its endogenous synthesis in pulmonary tissue. Subsequently, Soeki *et al*[20] established a coronary ligation-induced myocardial infarction rat model and noted that CNP mRNA expression was increased either in the infarcted (4.0-fold increase) or normal (2.0-fold increase) left ventricular on day 14 after CNP treatment (0.1 μg/kg/min). More persuasively, Chen *et al*[21] studied the effects of CNP on calcified aortas in vivo and VSMCs in vitro simultaneously and revealed that a significant elevation of CNP expression occurred in both calcified aortas and VSMCs (73 and 23 per cent, respectively) after CNP treatment (500 ng/kg/hour).

During the past two decades, the inhibitory effects of CNP on proliferation and ECM accumulation have been documented in generous studies undertaken by our laboratory and the others. Our recent study showed that exogenous CNP significantly ameliorated the pathological changes of tubulointerstitial fibrosis and caused a 22 and 27 per cent decrease of Col-IV expression in the obstructed kidneys at 3 weeks and 1 month after CNP infusion, respectively.[10] Segawa *et al*[22] found that CNP exposure could dose-dependently inhibit [^{3}H]-thymidine incorporation into MCs via protein phosphorylation pathways. Osawa *et al*[23] discovered that 10 μM CNP elicited the maximal inhibitions on MCs proliferation as well as Col-IV secretion stimulated by serum in vitro. Herein, our study also indicated that CNP downregulated MCs proliferation and Col-IV expression in a time-dependent and dose-dependent manner.

NPR-B possesses a particulate guanylyl cyclase domain and is considered to specifically bind to CNP. Subsequently, the CNP/NPR-B complex results in CGK II activation, which is located predominantly in intestine, brain, kidney and bone.[24] In the present study, we showed for the first time that NPR-B and CGK II were significantly provoked by CNP exposure in MCs, which was identical to some previous studies on the other cells and tissues. Obata *et al*[25] observed the therapeutic potential of CNP for myosin-induced acute myocarditis and found that CNP infusion (0.05 μg/kg/min) induced the overexpression of NPR-B mRNA in myocarditic heart. Agoston *et al*[26] investigated the molecular mechanisms on endochondral bone growth by 1 μM CNP in vitro and revealed that CGK II expression is 4.4-fold and 2.5-fold higher in the hypertrophic zone versus the resting/proliferative and mineralized zones, respectively.

NPR-C is evident in the adrenal, brain, heart, kidney, mesentery and vascular smooth muscle tissue. It binds CNP with high affinity and clears CNP from the intracellular environment via a receptor-mediated internalization and degradation process.[27 28] Chen *et al*[21] infused continuous CNP (500 ng/kg/hour for 4 weeks) to a rat vascular calcification model and discovered that the upregulative NPR-C stimulated CNP clearance and inhibited CNP entering the circulation in a negative feedback manner. More directly, Kake *et al*[29] found that CNP transgenosis could trigger a 53 per cent increase of NPR-C mRNA expression in mouse heart in comparison with wild-type littermates. Therefore, the upregulative NPR-C secondary to CNP exposure mainly avoids the overgeneralization of CNP effects in MCs. Besides acting as a clearance receptor of CNP, NPR-C is also involved in antiproliferation. Hashim *et al*[30] cultured VSMCs with different concentrations of NPR-C ($10^{-9}-10^{-6}$ μM) for 24 hours and found that NPR-C inhibited DNA synthesis stimulated by angiotensin II or arginine vasopressin in a concentration-dependent manner, conformable to the antiproliferative action of CNP (10^{-7} M) in vitro. Another in vitro study from a British laboratory demonstrated that NPR-C antagonist M372049 (10 μM) significantly attenuated the inhibition of VSMCs proliferation by CNP (1 μM); whereas in VSMCs from NPR-C$^{-/-}$ mice the inhibitory effect of CNP was absent and the basal growth was significantly increased compared with wide-type mouse VSMCs.[31] In consistent with NPR-C, cGMP also exerts a potent inhibitory effect on cell proliferation. In rat MCs, 8-bromo-cGMP (10^{-1}– 10^{-3} μM) suppressed the [^{3}H]-thymidine incorporation and the numbers of cells in S and G2/M phases dose-dependently.[32] Currently, the further studies on NPR-C antagonist and cGMP analogue have been conducted in our laboratory to identify whether the antiproliferation of CNP is mediated by NPR-C or cGMP, and the preliminary data are being arranged.

NEP, a membrane-bound metallopeptidase, is widely expressed in the brain, kidney, liver, breast, lung and so on, thus distributed ubiquitously in the circulation. It efficiently cleaves CNP at multiple sites (Cys6-Phe7, Gly8-Leu9, Lys10-Leu11, Arg13-Ile14, Ser16-Met17 and Gly19-Leu20), and the ring structure of CNP is essential for hydrolysis, because reduction and alkylation of the peptide greatly influenced degradation.[2] Kim *et al*[33] observed the renal expression of CNP in a cisplatin-induced nephropathy rat model and found that the upregulative CNP was accompanied by the obvious decreased NEP expression. Our recent in vivo study demonstrated that renal CNP expression tended to be higher within 24 hours after UUO and declined thereafter.[6] The elevated expression of NPR-B may contribute to the overexpressed CNP in the early phase, whereas the sustained expression of NEP helps to, at

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least partly, account for the subsequent decline of CNP.[34 35] However, the present study showed that NEP expression was significantly decreased after CNP treatment in MCs in vitro. Therefore, NEP attenuation is mainly attributed to the missing origin from the circulation and may not participate in the intracellular clearance of CNP in MCs.

CONCLUSIONS

Taken together, this in vitro study demonstrated that CNP could suppress human MCs proliferation and Col-IV expression in a time-dependent and dose-dependent manner. As a down-stream signal molecule of CNP activation, the expressions of NPR-B, CGK II and NPR-C were obviously augmented, whereas NEP expression was significantly decreased after CNP treatment in MCs in vitro. Therefore, receptor signaling and NEP are involved in the resistance of CNP to human mesangial proliferation and Col-IV expression.

Contributors YFW, DDZ and SYL carried out ELISA. GMJ, HHL and YX carried out RT-PCR. YW, JJW and FFL carried out western blot analysis. PH conceived and designed the experiments. CW, BH and WW interpreted the experiments. HHL and PH wrote the manuscript. All authors approved the final version of the manuscript.

Funding This study was supported by the National Natural Science Foundation of China (nos. 81570637 and 81000306).

Competing interests None declared.

Provenance and peer review Not commissioned; externally peer reviewed.

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