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Expression of C-type natriuretic peptide and its receptor NPR-B in cardiomyocytes

S. Del Ry[a,*], M. Cabiati[a], F. Vozzi[a], B. Battolla[b], C. Caselli[a], F. Forini[a], C. Segnani[b], T. Prescimone[c], D. Giannessi[a], L. Mattii[b]

[a] CNR Institute of Clinical Physiology Laboratory of Cardiovascular Biochemistry, Via Giuseppe Moruzzi 1, 56124 Pisa, Italy
[b] Department of Human Morphology and Applied Biology, Medical Histology and Embryology Section, University of Pisa, Pisa, Italy
[c] Department of Cellular Biology and Physiopathology, University of Siena, Italy

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ABSTRACT

C-type natriuretic peptide (CNP) was recently found in myocardium at the mRNA and protein levels, but it is not known whether cardiomyocytes are able to produce CNP. The aim of this study was to determine the expression of CNP and its specific receptor NPR-B in cardiac cells, both in vitro and ex vivo. CNP, brain natriuretic peptide (BNP) and natriuretic peptide receptor (NPR)-B mRNA expression were examined by RT-PCR in the H9c2 rat cardiac myoblast cell line, in neonatal rat primary cardiomyocytes and in human umbilical vein endothelial cells (HUVECs) as control. CNP protein expression was probed in cardiac tissue sections obtained from adult male minipigs by immunohistochemistry, and in H9c2 cells both by immunocytochemistry and by specific radioimmunoassay. The results showed that cardiac cells as well as endothelial cells were able to produce CNP. Unlike cardiomyocytes, as expected, in endothelial cells expression of BNP was not detected. NPR-B mRNA expression was found in both cell types. Production of CNP in the heart muscle cells at protein level was confirmed by radioimmunological determination (H9c2: CNP = 0.86 ± 0.083 pg/mg) and by immunocytochemistry studies. By immunostaining of tissue sections, CNP was detected in both endothelium and cardiomyocytes. Expression of CNP in cardiac cells at gene and protein levels suggests that the heart is actively involved in the production of CNP.
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1. Introduction

The natriuretic peptide system consists of three distinct endogenous peptides, atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP) and C-type natriuretic peptide (CNP), and their biological actions are mediated by three receptors: natriuretic peptide receptor (NPR)-A, NPR-B and NPR-C or clearance receptor [14,31]. This family of peptides plays several important roles in body fluid homeostasis and also has an important role in countering the influences of the renin–angiotensin system and in regulating vascular tone [14,31].

ANP and BNP are primarily cardiac hormones secreted mainly from the atria and ventricles, respectively. Although experimental evidence indicates that CNP may be an important new local autocrine and endocrine mediator in the heart [8,17], its production in cardiac cells and the local effect on the heart itself have not yet been fully elucidated.

CNP expression in endothelial cells was first shown by Suga et al. [32]. Since then, cell endothelial expression and release of CNP has been shown in a variety of studies on bovine endothelial cells [4,13,22]. CNP expression has also been detected in diverse cell types such as cardiac fibroblasts, chondrocytes, glial cells and macrophages in human and animal models [17,25,29,36,38] which implies that CNP is not limited to vascular roles. For example, CNP may be important in normal skeletal development [5] and in recent studies an increase in both myocardial production and plasma concentrations of CNP has been found in heart failure (HF) patients [6,7,20] suggesting the involvement of this peptide in pathophysiological cardiac remodeling, and supporting the hypothesis that CNP is produced directly within the myocardium in patients with HF. CNP appears to inhibit hypertrophy of cardiomyocytes and to have a hyperpolarizing action on different vascular beds. These physiological effects appear to involve the activation of the nitric oxide system via NPR-C [1,2]. Many recent studies have reported data on CNP and NPR-B expression in normal and failing heart myocardium of experimental animal models [9–12] indicating cardiac production of CNP and a possible autocrine and paracrine function.

On the other hand, the cellular heterogeneity of cardiac tissue, which is comprised of endothelial and smooth muscle cells in addition to cardiomyocytes, could suggest that CNP expression observed in myocardium reflects CNP expression in the endothelium of the vasculature and not in the cardiomyocyte.

* Corresponding author. Tel.: +39 050 3152793; fax: +39 050 3152166.
E-mail address: delry@ifc.cnr.it (S. Del Ry).

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Table 1
Sequence of RT-PCR primer pairs and optimal RT-PCR condition for glyceraldehyde 3-phosphate dehydrogenase (GAPDH), brain natriuretic peptide (BNP), C-type natriuretic peptide (CNP), natriuretic peptide receptor B (NPR-B). T_a: annealing temperature.

Gene	Primer sequence	GeneBank no accession	T_a (°C)	Cycles	[Primers] (mM)
GAPDH *Rattus norvegicus/Homo sapiens* (451 pb)	F: ACCACAGTCCATGCCATCAC R: TCCACCACCCTGTTGCTGTA	XM_576394	59	24	0.2
BNP *Rattus norvegicus* (202 pb)	F: ATCTGTCGCCGCTGGGAGGTR: TGGATCCGGAAGGCGCTGTC	NM_031545	58	30	0.2
BNP *Homo sapiens* (294 pb)	F: CTTGGAAACGTCCGGGTTA R: TAATGCCGCCTCAGCACTT	NM_002521	66	35	0.2
CNP *Rattus norvegicus*/*Homo sapiens* (172 pb)	F: GAAGCCAAGCCCGGGACACCR: TGTCCACACGCAGGTCCCGA	NM_053750	62	33	0.2
NPR-B *Rattus norvegicus* (127 pb)	F: ACGGGCTGGCTAGCTTCCGA R: GCCTTCTGCATGCCCGTGGT	NM_053838	66	34	0.2
NPR-B *Homo sapiens* (625 pb)	F: GTGGGGCTGCTGCTTTATCC R: CCTGGCTCTCGGGCATACAC	NG_009249	59	35	0.2

The aim of this study was to investigate whether cardiomyocytes are able to produce CNP, by evaluating CNP mRNA expression in a rat cardiac myoblast cell line (H9c2) and, as confirmation, in neonatal rat primary cardiomyocytes. NPR-B and BNP were also analyzed. Human umbilical vein endothelial cells (HUVECs) were used as control.

Immunocytochemistry and immunohistochemistry technique were also carried out, both in cells and in cardiac tissue fragments.

2. Methods

CNP, BNP and NPR-B mRNA expressions were examined by RT-PCR in HUVEC ($n = 5$), in H9c2 cells ($n = 7$), and for confirmation of CNP and NPR-B mRNA expression, in neonatal rat primary cardiomyocytes ($n = 2$). CNP protein was probed using immunostaining in cardiac tissue sections obtained from male adult minipigs, and in H9c2 cells. CNP protein levels were also determined in H9c2 cells by radioimmunoassay.

2.1. Cell cultures

2.1.1. HUVEC

Endothelial cells were obtained from human umbilical cord veins as previously described [19].

The umbilical vein was cannulated, washed with phosphate buffer solution (PBS) and filled with a solution of 0.1% collagenase type II (Sigma–Aldrich, St. Louis, MO, USA) in PBS. The cord was placed in an incubator at 37 °C for 15–20 min. After this step, the vein was washed with ECGM (Endothelial Cell Growth Medium, Promocell, Heidelberg, Germany) supplemented with Endothelial Supplement Mix (Promocell) to block the collagenase action. The mix of medium and cell was recovered and centrifuged at 900 rpm × 5 min; the supernatant was discarded, the pellet re-suspended in fresh media and cells were counted with a hemocytometer. The solution was then seeded in a 25 cm^2 flask pre-treated with 1% gelatin in PBS. Every 48 h the medium was replaced. Once confluence was reached, cells were trypsinized and seeded on a glass slide (20,000 cells/cm^2).

2.1.2. H9c2

The rat heart cell line H9c2 (ECACC, Salisbury, UK) was cultured in DMEM supplemented with 10% fetal bovine serum (FBS), 4 mM glutamine, 20 U/mL penicillin and 20 μg/mL streptomycin. Once at confluence, cells were detached with trypsin 0.25% in EDTA, counted with a hemocytometer (viability greater than 90%) and seeded on a glass slide (60,000 cells/cm^2).

2.1.3. Neonatal rat cardiomyocyte cell culture

Neonatal rat cardiomyocytes were isolated using a Worthington Neonatal Cardiomyocyte Isolation System according to the manufacturer's instructions, as previously described [24]. Briefly, rats were anesthetized and the beating heart was surgically removed. The heart was placed in ice, rinsed with 10 ml HBSS (Lonza Group Ltd., Switzerland) and transferred to the Petri dish where it was minced into 1-mm^3 pieces, keeping tissue in ice. After digestion with trypsin (50 μg/ml, Sigma) and collagenase IV (1 mg/ml), myocardial tissue fragments were dispersed into single-cell suspension and filtered through a Sigma Cell Strainer. Isolated cells were centrifuged at low speed (65 × g) to minimize non-cardiomyocyte contamination. The cell pellet was suspended in IMDM with 5% FBS and 1% antibiotics; after two 45′ preplating steps the supernatant, containing the cardiomyocyte-enriched fraction, was plated in cell culture medium at a density of 1.5×10^5 cells/ml. Cardiomyocytes were identified through immunofluorescent staining with an anti-alpha sarcomeric actin antibody (Sigma, A7811, clone EA-53) and they were used in the experiments when their yield reached 100%

2.2. Cardiac sample

Left ventricular (LV) tissue was collected from ($n = 3$) healthy male adult farm pigs (weight 35–40 kg). Left intercostal thoracotomy was performed under general anesthesia and sterile conditions. Tissue samples collected from LV of pigs were immediately placed in paraformaldehyde. These procedures conformed to the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Italian Ministry of Health.

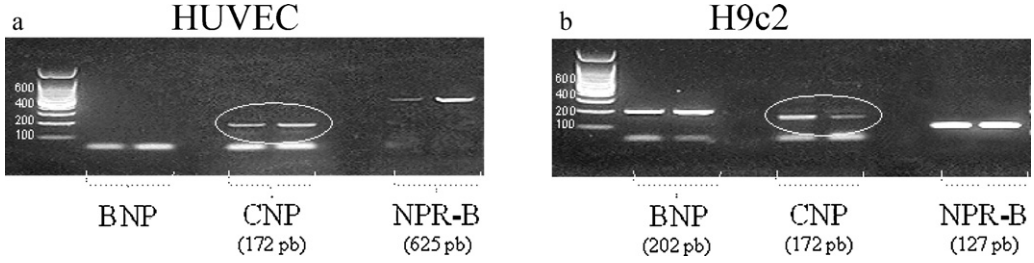

Fig. 1. Agarose gels representative of BNP, CNP and NPR-B mRNA expression in (a) HUVEC and (b) H9c2 cells.

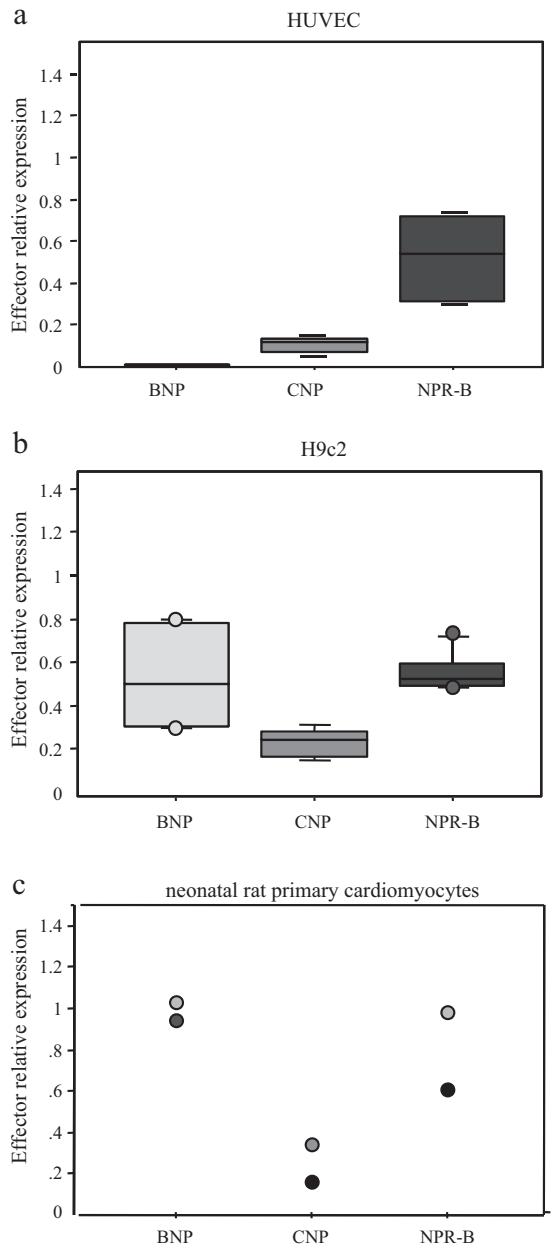

Fig. 2. BNP, CNP and NPR-B mRNA expression (mean value ± SEM) measured by RT-PCR in (a) HUVEC and (b) H9c2 cells. Each box consists of 5 horizontal lines displaying the 10th, 25th, 50th (median), 75th, and 90th percentiles of the variable. All values above the 90th percentile and below the 10th percentile are plotted separately. In (c) the single values obtained for BNP, CNP and NPR-B mRNA expression in neonatal rat primary cardiomyocytes were reported.

2.3. Molecular analysis

2.3.1. RNA extraction

Total RNA was extracted by acid guanidinium thiocyanate–phenol–chloroform (TRIREAGENT: Molecular Research Center, Cincinnati, OH, USA) method from HUVEC, H9c2, and from neonatal rat primary cardiomyocytes with Rneasy Mini kit (Qiagen S.p.A, Milano, Italy). Each sample was treated with DNase (Qiagen S.p.A, Milano, Italy) to remove the presence of further DNA contamination.

RNA concentration was determined spectrophotometrically (Biophotometer Eppendorf, Milan, Italy) at 260 nm. The ratio of readings at 260 nm and 280 nm (A_{260}/A_{280}) provided an estimate of the purity of RNA. The integrity and purity of total RNA was also detected by electrophoresis of samples on Gel Star stain agarose gels. Only samples that showed clear and distinct 28S and 18S ribosomal RNA bands and had spectrophotometric OD 260/280 ratios of 1.9–2.1 were used. A known amount of total RNA (Ambion) was used as marker. The RNA samples were stored at −80 °C for use in gene expression studies.

2.3.2. Protein extraction

Tri-reagent procedure (Molecular Research Center) allowed us to obtain RNA and proteins from a single sample by using a monophasic mixture of phenol and guanidine thiocyanate and isopropyl alcohol to precipitate RNA. Proteins were isolated by organic phase. The addition of ethanol and centrifugation allowed us to eliminate the lipid component. The subsequent addition of acetone and centrifugation (12,000 *g* × 5 min, 4 °C) created a protein pellet that was then washed and centrifuged three times with a wash buffer (guanidine, glycerol 25%, ethanol). After a final wash with a solution of glycerol 25% and ethanol, the pellet was resuspended with Tris (idrossi-methyl-aminomethane) HCl (4 mM) buffer (pH = 7.4) (NaCl-154 mM, phenyl methyl sulfonyl fluoride [PMSF] 0.1 mM, sodium dodecyl sulfate [SDS] 2%). The final protein preparations were frozen at −20 °C and the protein concentration was determined according to the method of Lowry using BSA as a standard [23].

Reverse transcriptase-polymerase chain reaction (RT-PCR): The reverse transcription of total RNA to cDNA was carried out using iSCRIPT cDNA Synthesis kit. Reverse transcriptase-polymerase chain reaction assay for CNP, BNP and NPR-B was performed in duplicate using the primers reported in Table 1.

The primer pair was designed to span one or more introns so that they could not give rise to genomic DNA template PCR products. They were obtained with the program Primer 3.

All sequences were analyzed using BLAST against GenBank database to determinate sequence identity.

GAPDH (Table 1) was used as housekeeping gene constitutively expressed to a constant amount in cells [39] and the data deriving from each sample were normalized with respect to the GAPDH expression. To check the sensitivity and linearity of the amplification, RT-PCR for each primer was performed in a range of different numbers of cycles, annealing temperatures and RNA concentrations.

The PCR conditions were optimized and the amplified products were electrophoresed on 1.5% (w/v) agarose gels, in parallel with a DNA Ladder 100 bp (Amersham Biosciences; Freiburg, Germany), stained with Gel Star Stain (Lonza Group) and visualized with an ultraviolet transilluminator at 260 nm.

2.3.3. CNP assay

CNP concentration was measured in H9c2 extracts by a commercial radioimmunoassay (C-type peptide-22 human, porcine, rat magnetic bead RIA kit, Phoenix Pharmaceuticals, Belmont, CA, USA). Each sample was assayed in duplicate and the experiment was carried out in an ice bath.

2.4. Immunocytochemistry and immunohistochemistry technique

To investigate CNP protein expression, HUVEC, H9c2 cells and myocardial tissue of normal pigs were subjected to immunocytochemical and immunohistochemical staining, respectively.

The cells grown to sub-confluence on slides were fixed in 1% formalin at 4 °C for 10 min. Left ventricle tissue samples were paraffin-embedded, cut into 5-μm-thick sections, and mounted serially on glass slides. Before use, slides were de-paraffinized, and rehydrated.

For antigen retrieval, samples were exposed to 0.2% Triton X-100 solution (Sigma) for 10 min (for cells) or to microwaves

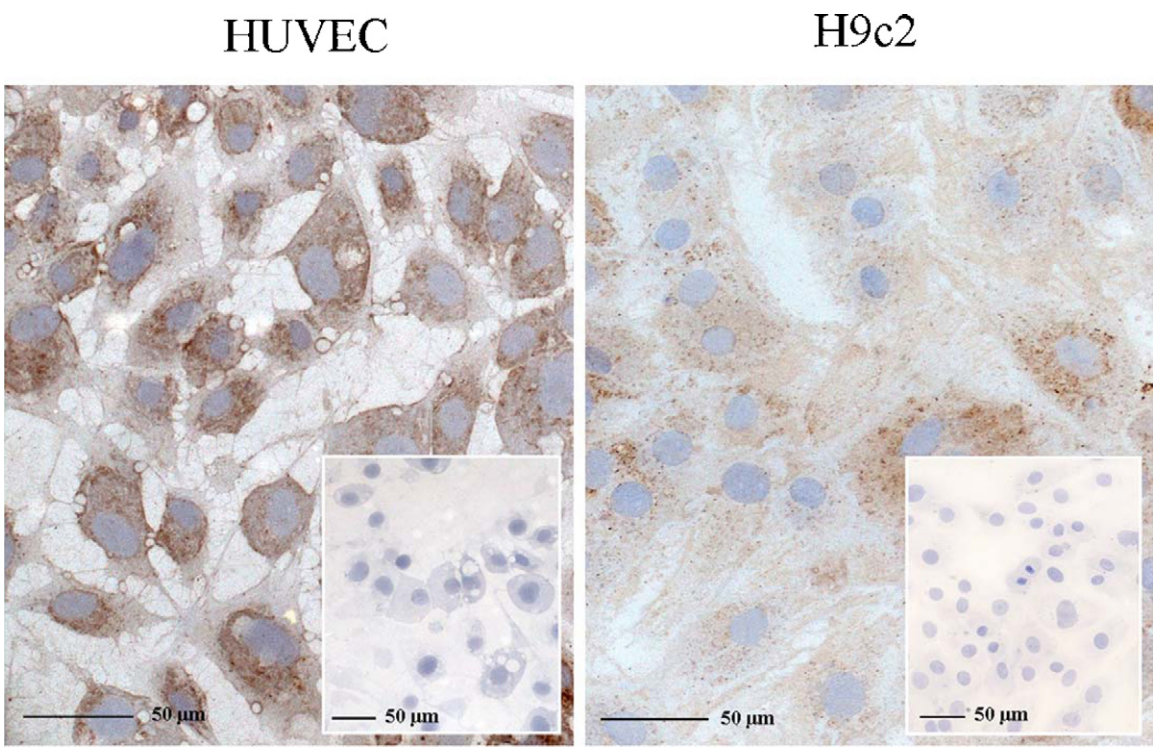

Fig. 3. Immunostaining of HUVECs and H9c2 cells positively stained for CNP. The negative controls are shown in the boxes.

for 3 × 5 min at 600 W in 10 mM sodium citrate (for sections), incubated with 3% H_2O_2 in distilled water for 20 min to block endogenous peroxidase, and finally with normal goat serum (1:20, Vector, Burlingame, CA, USA) to block non-specific binding. Samples were then incubated overnight at 4 °C with a rabbit anti-CNP antibody (CNP-53, Phoenix Pharmaceuticals) diluted 1:1000 (for sections) or 1:300 (for cells) in BSA–PBS, rinsed with phosphate-buffered saline, and sequentially treated with goat biotinylated anti-rabbit immunoglobulins (Vector), streptavidin–peroxidase complex (Vector), and 3,3′-diaminobenzidine tetrahydro-cloride (DAB, Dako Cytomation, Glostrup, Denmark). Specimens were counterstained with hematoxylin and examined using a Leica DMRB light microscope. Negative controls were obtained by incubating the specimens with BSA–PBS, omitting the primary antibody. Tissue sections of rat kidney were used as positive control [3]. Experiments were performed in triplicate. Representative photomicrographs were taken using a DFC480 digital camera (Leica Microsystem, Cambridge, UK).

3. Data analysis

The stained gels were photographed under ultraviolet illumination with a digital camera (Panasonic LC 40) and the optical density of the bands was analyzed by Quantity One Software (Bio-Rad Laboratories, CA, USA).

Differences between more than two independent groups were analyzed by Fisher's test after ANOVA. The results are expressed as mean ± SEM and *P*-value was considered significant when <0.05.

All sample values and other data for quality control of the RIA system were calculated by a previously described computer program [27]; the interpolation of the dose–response curves was computed using a four-parameter logistic function [27].

4. Results

4.1. mRNA expression of CNP, NPR-B and BNP by PCR

Using primer designed to amplify a 172-bp length of the gene sequence codifying for C-type natriuretic peptide in humans and rats, CNP was found to be expressed in HUVEC but also in H9c2 cells with a product size of between 150 and 200 bp, as compared against 1 kb DNA ladder (Fig. 1). Unlike cardiac cells, as expected, in endothelial cells expression of BNP was not detected while NPR-B mRNA expression was found in both cell types (Fig. 1). In Fig. 2a and b we report the mean value obtained for BNP, CNP and NPR-B with RT-PCR in HUVEC and in H9c2 cells.

To confirm these results, BNP, CNP and NPR-B mRNA expressions were also evaluated in samples of neonatal rat primary cardiomyocytes and values similar to those obtained with H9c2 were found (Fig. 2c).

4.2. Protein expression of CNP by immunostaining and immunometric tests

Assuming that brown staining indicated positive immunoreaction, HUVECs showed CNP immunoreaction, as expected (Fig. 3). Moreover, both H9c2 muscle cells (Fig. 3) and cardiomyocytes of pig hearts were CNP immunoreactive (Fig. 4A). In particular, staining intensity differed between cardiomyocytes, being more intense in some groups of cells than in others. It was noted that in tissue sections immunoreactivity of endothelial cells was also detectable (Fig. 4A). As evidence of the antibody specificity, rat renal tissue was used: CNP was localized in the renal tubules and in the visceral and parietal layer of glomeruli (Fig. 4B), as expected.

4.3. Immunometric determination

CNP concentration in H9c2 was measured by radioimmunologic assay and resulted 0.86 ± 0.083 pg/mg, confirming the production of CNP in the heart muscle cells.

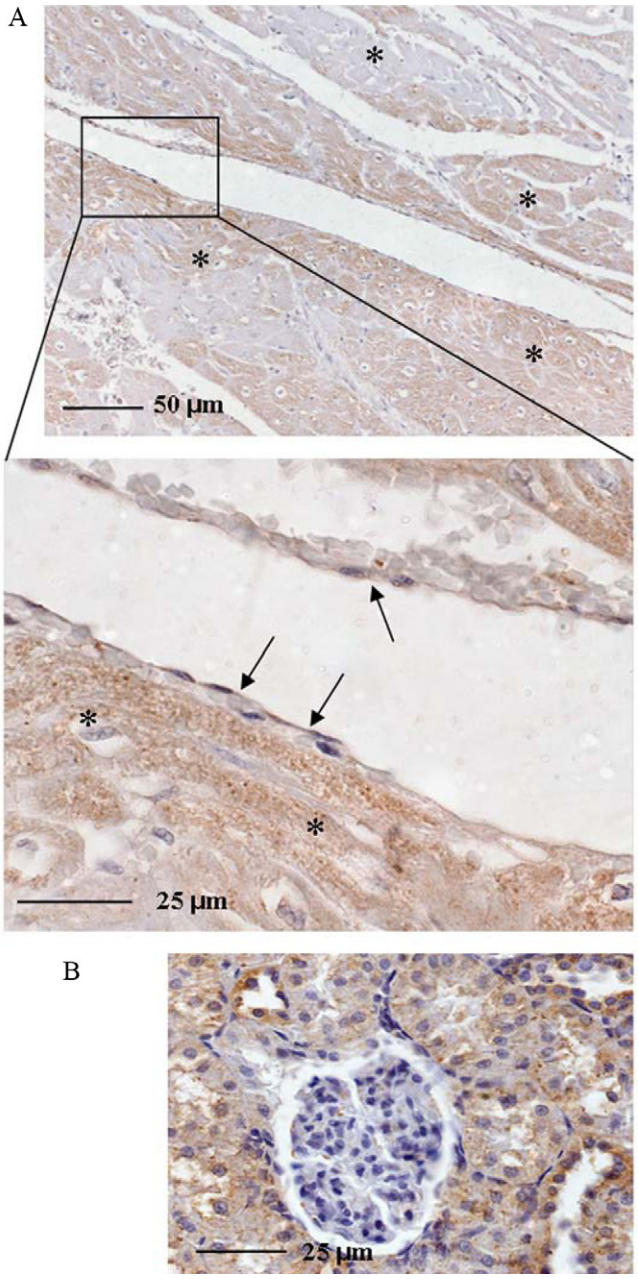

Fig. 4. A: Section of LV of normal pig stained for CNP. Some cardiomyocytes and endothelial cells are indicated by asterisks and arrows, respectively. B: Rat renal tissue stained for CNP used as positive control.

5. Discussion

This work is a novel investigation of CNP expression in cardiac cells. The major finding of this study is that the gene for CNP is present within cardiac cells where CNP protein is also constitutively expressed. The robustness of these findings is based on the use of three different approaches to determine the presence of CNP in myocytes. In fact, the determination of expression of CNP in cardiac cells, obtained by PCR, is validated by radioimmunometric determination and by immunocytochemistry studies. Moreover, by immunostaining tissue sections, CNP was detected in both endothelial cells and cardiomyocytes. Although CNP was initially thought to be distributed in the central nervous system and to act as a neuropeptide [21,30], CNP is localized or synthesized in many other sites such as the kidney [33], bone [15], vascular cells [32,37] and blood cells [18]. As for the heart, discrepant findings have been reported. Some studies have reported that ir-CNP or CNP mRNA was not detected in human or rat hearts [21,34]. On the contrary, CNP mRNA was also detected in rat and pig hearts using RT-PCR technique [10,11,26,35]. However, the latter studies had not clarified what type of cell synthesized CNP in the heart. The possibility that CNP gene expression was derived from cardiac endothelium could not be ruled out. Only later it was shown that CNP was synthesized and secreted from cultured adult cardiac fibroblasts [17], but no data are present in literature regarding CNP mRNA in cardiomyocytes. In our study, H9c2 cell line was used as a model of cardiomyocytes. It is well-known that H9c2 cells show morphological characteristics similar to those of immature embryonic cardiocytes, but have preserved several elements of the electrical and hormonal signal pathway found in adult cardiac cells, and this cell line may be useful as a model for cardiocytes [16,28]. However, to rule out any bias due to the use of H9c2 cells, neonatal rat primary cardiomyocytes were also analyzed and CNP mRNA expression was confirmed in these cells.

In conclusion, this study highlights the expression of CNP in cardiomyocytes at the gene and protein levels, and evaluates the co-localization of the peptide and its specific receptor NPR-B in these cells, suggesting that the heart is actively involved in the production of CNP, and reinforcing the involvement of CNP in cardiovascular physiology.

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