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C-type natriuretic peptide transcriptomic profiling increases in human leukocytes of patients with chronic heart failure as a function of clinical severity

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ABSTRACT

The aim of this study was to evaluate the transcriptomic profiling of C-type natriuretic peptide (CNP) and of its specific receptor, NPR-B in human leukocytes of heart failure (HF) patients as a function of clinical severity, assessing the possible changes with respect to healthy subjects (C). mRNA expression was evaluated by Real-Time PCR and total RNA was extracted from leukocytes of C ($n = 8$) and of HF patients (NYHA I–II, $n = 7$; NYHA III–IV, $n = 13$) with PAXgene Blood RNA Kit. Significantly higher levels of CNP mRNA expression were found in HF patients as a function of clinical severity (C = 0.23 ± 0.058, NYHA I–II = 0.47 ± 0.18, NYHA III–IV = 2.58 ± 0.71, $p = 0.005$ C vs NYHA III–IV, $p = 0.017$ NYHA I–II vs NYHA III–IV) and NPR-B transcript levels resulted down-regulated in HF patients with higher NYHA class (C = 2.2 ± 0.61, NYHA I–II = 2.76 ± 0.46, NYHA III–IV = 0.29 ± 0.13, $p = 0.001$ C vs NYHA III–IV, $p < 0.0001$ NYHA I–II vs NYHA III–IV). A significant negative correlation between CNP and NPR-B mRNA expression ($r = 0.5$, $p = 0.03$) was also observed. These results suggest a co-regulation of NPR-B and CNP expression supporting the relevance of this receptor in human disease characterized by a marked inflammatory/immune component and suggesting the possibility of manipulating inflammation via pharmacological agents selective for this receptor.

1. Introduction

C-type natriuretic peptide (CNP), a vasoactive and anti proliferative peptide, shares sequence homology and biological actions with the endocrine cardiac peptides, atrial natriuretic peptide (ANP) and brain natriuretic peptides (BNP) [4,17,22]. After the isolation of CNP from porcine brain tissue [28], both CNP and CNP mRNA have been identified in vascular endothelium [27,29] and in cardiac [5–13,18,32], renal [19], skeletal [16], and reproductive tissues [20,31].

However CNP expression is not only confined to those tissues and organs but was also found in organs of the immune system (thymus, spleen, and lymph nodes) as well as in a variety of blood cells, including lymphocytes and monocytes [30]. Recently, mRNA expression of CNP was evaluated in human whole blood samples by Real-Time PCR study, and significantly higher levels of both CNP and NPR-B mRNA expression were found in heart failure (HF) patients (irrespective of severity) with respect to control subjects [2]. These findings are in agreement with those of several studies carried out in humans reporting increased NTpro-CNP [12,21,23] and CNP plasma levels in chronic HF patients compared to healthy subjects [5,6,8,9,12,32]. The progressive rise in CNP and NTpro-CNP plasma values with worsening of symptoms [6,12,32], and by the negative relation between plasma CNP values and left ventricular ejection fraction [6] suggest an increased secretion related to the severity of the disease.

Thus, while a increase in CNP plasma concentrations as a function of clinical severity is now well-documented, the behavior of CNP mRNA expression in the different NYHA classes is not known.

In order to evaluate the presence of increased CNP transcriptomic profiling in leukocytes of HF patients according to clinical severity, we measured mRNA levels of CNP in a population of HF patients at different stages of the disease and in a group of age-matched healthy subjects. Moreover, as CNP physiological actions are mainly attributable to activation of its specific receptor NPR-B, the study will be completed by the transcriptomic profile of this specific receptor.

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Table 1
Clinical and biochemical characteristics of heart failure patients.

	Patients NYHA I–II ($n=7$)	Patients NYHA III–IV ($n=13$)	p
Age, yrs	54 ± 5	53 ± 2	0.843
Male, *n* (%)	7 (100)	10 (77)	0.188
Diabetes, *n* (%)	1 (14)	3 (23)	1.000
Etiology, *n* (%)			0.249
IDC	7 (100)	9 (69)	
IHD	–	4 (31)	
LVEDV, ml	191 ± 43	262 ± 31	0.122
LVESV, ml	138 ± 36	197 ± 26	0.219
LVEF, %	32 ± 5	26 ± 2	0.449
MID, *n* (%)			0.224
0	1 (14)	–	
1+	3 (43)	2 (15)	
2+	2 (29)	7 (54)	
3+	1 (14)	4 (31)	
4+	–	–	
LA area, cm^2	21 ± 3	40 ± 8	0.039
Therapy, *n* (%)			
ACEi	5 (71)	8 (62)	1.000
β-Blocker	7 (100)	13 (100)	1.000
Statin	2 (29)	6 (46)	0.642
Antiplatelets	4 (57)	7 (54)	1.000
Diuretic	6 (86)	13 (100)	0.350
C-reactive protein, mg/dl	0.39 ± 0.11	0.82 ± 0.31	0.639

Data are expressed as mean and SE or frequency (percentage).
Abbreviations: IDC, idiopathic dilated cardiomyopathy; IHD, ischemic heart disease; NYHA, New York Heart Classification; LVEDV, left ventricular end-diastolic volume; LVEF, left ventricular ejection fraction; LVESV, left ventricular end-systolic volume; MID, mitral insufficiency grade; LA, left atrium; ACEi, angiotensin converting enzyme inhibitor.

2. Materials and methods

2.1. Blood collection and RNA extraction

The investigation conforms to the principles outlined in the Declaration of Helsinki (Br Med J 1964; ii:177). The study was approved by the local Ethics Committee and all patients provided signed informed consent.

Eight healthy adults (age, mean ± SEM 42 ± 3, 5 males, 3 females) with no history of cardiovascular disease, normal regional and global left ventricular function assessed by two dimensional echocardiography and under no medical treatment were studied as control group.

Human whole blood samples (2.5 ml) from healthy adults ($n=8$) and from 20 HF patients (New York Heart Classification – patients NYHA I–II, $n=7$ and NYHA III–IV, $n=13$) were collected into PAXgene blood RNA system (DIALAB ITALIA Srl) tubes.

Inclusion criteria consisted of a significantly depressed (<45%) left ventricular ejection fraction (EF), measured by echocardiography [25]. Exclusion criteria were acute myocardial infarction or unstable angina within 6 months before the examination, significant primitive pulmonary disease and renal failure (defined as a serum creatinine value above 2.0 mg/dl). Patients were treated with restriction of water–sodium intake (using a personalized, well-controlled diet with a sodium intake of 100–140 mmol/day) and were receiving homogeneous optimal medical treatment (furosemide, ACE-inhibitors, carvedilol, spironolactone – for patients in class NYHA ≥ III), not discontinued at the time of the study, for ethical reasons.

The PAXgene blood RNA system is a new, innovative methodology for the collection, storage and transport of blood, which efficiently stabilizes intracellular RNA and allows preservation of the samples at −20 to −70 °C, maintaining the same degree of purity and stability of the fresh blood.

Blood samples were processed with PAXgene blood RNA kit (Qiagen, Milan, Italy) to obtain total RNA as previously described [2].

In all cases, the integrity of total RNA was detected by electrophoresis on Gel-Star (Lonza, Germany) stained 1.5% Agarose (Bio-Rad, Milan, Italy) gel; total RNA purity and concentration were evaluated spectrophotometrically (NanoDrop, Celbio, Milan, Italy). The RNA samples were stored at −80 °C.

Moreover blood samples were collected in all the subjects by venipuncture for NT-proBNP determination. In order to minimize degradation, blood samples were collected in polypropylene tubes containing K_3EDTA, rapidly separated by centrifugation at 1500 × *g* for 15 min at 4 °C, and stored at −20 °C in 1-ml aliquots.

Table 1 reports the clinical and biochemical characteristics of HF patients.

2.2. NT-proBNP assay

NT-proBNP was measured by a fully automated electrochemiluminescence immunoassay on Roche/Hitachi 917 analyzer (Roche Diagnostics GmbH, Mannheim).

2.3. Reverse transcription and Real-Time PCR

A quantity of 0.5 μg of total RNA obtained from each sample was reverse transcribed with iScript cDNA Synthesis Kit (Bio-Rad) according to the manufacturer's instructions.

CNP and NPR-B primer pairs were designed with Primer Express Version 2.0 (Applied Biosystems) (Table 2). Specificity of each primer pair, i.e., absence of artifacts, multiple PCR products or primers–dimers, and PCR yield were checked by melting analysis.

RT-PCR reactions were performed in a 96-well CFX96 RT-PCR System (Bio-Rad). The reaction was carried out in a total volume of 25 μl per reaction. Reaction mixture included 2 μl of template cDNA, 1 μM of each primer, 2× QuantiFast SYBR Green SuperMix (Qiagen) and sterile water. Amplification protocol started with 95 °C for 3 min followed by 39 cycles at 95 °C for 10 s and 60 °C for 30 s. To assess product specificity, amplicons were checked by melting curve analysis. Melting curves were generated from 65 °C to 95 °C with increments of 0.3 °C/cycle.