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Sequencing and cardiac expression of natriuretic peptide receptor 2 (NPR-B) in Sus Scrofa

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ABSTRACT

The cardiovascular actions of the C-type natriuretic peptide (CNP) are mainly mediated by the interaction with natriuretic peptide receptor-B (NPR-B). The aim of this study was to identify the sequence of NPR-B in Sus Scrofa, which is not present in GenBank, to verify the expression of NPR-B in the different cardiac chambers of normal pigs and evaluate its homology with murine and human species. Using the guanidinium thyocyanate-phenol-chloroform method, we extracted total RNA from samples obtained from heart of mouse and from the atrium, ventricle, and septum of normal pigs. Pig NPR-B mRNA was sequenced using polymerase chain reaction primers designed from mouse consensus sequences. Sus Scrofa natriuretic peptide receptor 2 mRNA, 1–396 bp, was submitted to GenBank (accession number DQ487044). The presence of NPR-B at mRNA level was detected in all the cardiac chambers; moreover, the bands obtained from pig cardiac tissue shared a 93% sequence homology with a region of the mouse NPR-B and a 95% sequence homology with *Homo sapiens*. Therefore, NPR-B sequencing provides a new tool to investigate the role of CNP under physiological and pathological conditions in the experimental and clinical setting.

1. Introduction

C-type natriuretic peptide (CNP) is the third member of the natriuretic peptide family and has considerable sequence homologies with atrial and brain natriuretic peptide (ANP and BNP), particularly within a 17-membered disulfide ring structure that is shared by all of the natriuretic peptides and which is essential for biological activity [21,11]. First isolated from porcine brain in 1990 [24], CNP is widely expressed throughout the vasculature and is highly concentrated in the endothelium, where it takes part in the regulation of vascular tone by exerting a vasodilator effect [4,1,22]. Recent studies have demonstrated that CNP is also produced in the myocardium of patients with chronic heart failure (HF) [8,14], and that its plasma level actually increases during HF [19,7,27], suggesting an important compensatory effect on cardiovascular remodeling.

The cardiovascular actions of this natriuretic peptide are mainly mediated by the natriuretic peptide receptor-B (NPR-B) [6], whose pathophysiological role on cardiovascular function remains unclear.

So far, at least three different subtypes of natriuretic peptide receptors (NPRs) have been identified: NPR-A, NPR-B and NPR-C. These are also known as GC-A, GC-B, and the clearance receptor, respectively, or as NPR-1, NPR-2 and NPR-3.

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Subtype A (NPR-A) preferentially binds ANP and BNP, whereas subtype B selectively binds to the peptide CNP. Lastly, the third subtype receptor, NPR-C, which has a high binding affinity for all natriuretic peptides, is thought to act as a clearance receptor. Its primary role is considered to be the regulation of plasma levels of natriuretic peptides.

Among the natriuretic peptides, CNP binds NPR-B with an affinity three fold that of either ANP or BNP [15,25,18].

NPR-A and NPR-B are transmembrane proteins containing an extracellular binding domain and an intracellular particulate guanylate cyclase domain. An important region of the natriuretic peptide receptor, called the kinase homology domain, normally inhibits guanylate cyclase. The binding of the natriuretic peptide to the receptor results in the release of inhibition of particulate guanylate cyclase by the kinase homology domain, enabling the formation of cyclic guanosine monophosphate (cGMP) from guanosine triphosphate, which acts as the second messenger, mediating the biological effects of natriuretic peptides [15,25].

The human NPR-B gene spans about 16.5 kb, contains 22 exons and is located on chromosome 9p21-12. The mouse gene NPR-2 is found on chromosome 4 and presents a high homology among the species [18]. No information is reported on pig NPR-B gene. Because the pig is a widely used animal experimental model [12,20,3,10], the knowledge of its NPR-B sequence will help understand how this receptor's expression is altered in different diseases and lead to the development potential ways for therapeutic intervention.

This study was carried out in the context of a wide study devoted to assessing the possible role and function of CNP and its main receptor NPR-B in a pig experimental model of pacing induced heart failure (HF). Pacing-induced heart failure presents many features common to clinical dilated cardiomyopathy, while ventricular pacing induces differences in regional left ventricular contraction similar to regional functional abnormalities seen in clinical ischemic heart disease [12]. Several studies [16,23,12] examined myocardial blood flow (MBF) in pacing-induced HF; it has been found that regional variations in MBF, an immediate consequence of rapid ventricular pacing, may play a role in the pathogenesis of regional and global dysfunction in pacing-induced HF. We believe that the mechanic dyssynchrony of the left ventricle wall can contribute to progression of dilated cardiomyopathy versus HF, and that the contractile dysfunction caused by a severe mechanic dyssynchrony by left ventricle pacing could be associated with regional differences of perfusion and metabolism. Indeed, a recent study of ours [9] found a correlation between coronary microvascular impairment and CNP, which supports the link between endothelial damage and impairment of myocardial perfusion and function. All together, these data seem to suggest that CNP, along with other natriuretic peptides, can be activated early in the presence of myocardial damage. In order to evaluate the variations and the possible differences of regional expression of CNP and of its main receptor NPR-B in a pig experimental model of pacing induced HF, it is first necessary to acknowledge the characterization of CNP and of NPR-B receptors in the different cardiac chambers of normal pigs.

Therefore, the main aim of this study was to sequence NPR-B in Sus Scrofa, whose sequence is not present in GenBank, and to evaluate its homology with murine and human species. Subsequently, the study assessed the NPR-B mRNA expression in the different cardiac chambers (right and left atrium, ventricle and septum) and in particular in the different regions of the left ventricle (lateral, anterior and intermedie wall) of normal pig. This evaluation represents a useful starting point for future studies devoted to characterize the expression of this receptor in the different cardiac regions during HF.

2. Methods

2.1. Tissue collection

Cardiac tissue was collected from male BALB/C mice of 6 weeks of age (mean body weight 30 g) and from adult male pigs.

Mice were sacrificed under anesthesia (0.1 ml 2.5% Avertin/10 g bw, intraperitoneally) and the heart was immediately placed in ice-cold RNA*later* (Qiagen S.p.A., Milano, Italy), an RNA preservation solution, and stored at −80 °C.

Pigs were first tranquillized with an intramuscular injection of 40 mg/kg of midazolam and tiletamine hypochloride (Zoletil®, Virbac laboratories BP 27-06511 Carros, France), induced and maintained in a surgical plane of inhalatory anesthesia with a mixture of isofluorane and oxygen. The animals were sacrificed with an intravenous injection of 10 ml of KCl and then the heart was removed from the mediastinal space. Right and left atrium, right ventricle, left ventricle (lateral, anterior and intermedie wall) and septum were immediately placed in ice-cold RNA*later* and stored at −80 °C.

These procedures were conform to the NIH Guide for the Care and Use of Laboratory Animals and approved by the Italian Ministry of Health.

2.2. Experimental protocol

Total RNA was extracted by acid guanidinium thiocyanate-phenol-chloroform method from tissue samples obtained from mouse and pig hearts with Rneasy Midi kit (Qiagen S.p.A.). The tissue was homogenized with a Mixer Mill MM300 (Qiagen S.p.A.). In order to remove contractile proteins, connective tissue and collagen which can interfere with the procedure, we modified the protocol to include proteinase K digestion. Briefly, samples were lysed in a guanidine-isothiocynate-containing lysis buffer. After dilution of the lysate, the samples were treated with proteinase K. Debris was pelleted by centrifugation. Ethanol was then added to the cleared lysate and RNA was bound to the Rneasy membrane. Contaminants were washed away, and total RNA was eluted in Rnase-free water. The presence of proteinase K and the tissue homogenization by Mixer Mill MM300 allowed us a high yield in RNA extraction.

RNA concentration was determined spectrophotometrically (Beckman DU 640) at 260 nm. The ratio of readings at 260 and 280 nm (A_{260}/A_{280}) provided an estimate of the purity of RNA with respect to contaminants that absorb in the UV spectrum, such as proteins and DNA. The integrity and purity of total RNA was also detected by electrophoresis of samples on ethidium bromide agarose gels. Only samples that showed clear and distinct 28S and 18S ribosomal RNA bands and had