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Cardiac ankyrin repeat protein, a negative regulator of cardiac gene expression, is augmented in human heart failure☆

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Abstract

The technique of representational difference analysis of cDNA has been applied to screen for differentially expressed genes in a canine model of pacing-induced heart failure. We identified the canine homolog of the cardiac ankyrin repeat protein (CARP) which has been shown to be involved in the regulation of the transcription of cardiac genes. To confirm the significance for human heart failure, cardiac tissue specimens obtained from non-failing donor hearts and from explanted hearts from patients with end-stage heart failure were investigated. CARP mRNA and protein levels were markedly increased in failing left ventricles. Interestingly, alterations in CARP expression were restricted to ventricular tissue and were not observed in atria. Fractionation experiments revealed that CARP was expressed predominantly in the nuclei consistent with the proposed function of CARP as a modulator of transcription. Together, these findings raise the possibility that augmented ventricular CARP expression may play a role in the pathogenesis of human heart failure.

Keywords: CARP; Representational difference analysis of cDNA; Gene expression; Cardiac muscle; Ischemic cardiomyopathy; Dilated cardiomyopathy; Heart failure

Congestive heart failure is a final common pathway of a variety of heart diseases. Numerous molecular responses have been described in association with heart failure, but our knowledge of the triggers, underlying mechanisms, and genes expressed is far from comprehensive. Differential screening has been used to identify more of the genes that are regulated during heart failure and therefore may serve as targets for drug development [1–5]. In the present study this approach has been applied to the canine model of pacing induced heart failure. Using representational difference analysis (RDA) of cDNA, a PCR-based subtractive hybridization technique, we identified the canine homolog of the cardiac ankyrin repeat protein (CARP) among genes that were upregulated in the failing canine myocardium. This result is of particular interest because CARP mRNA has been demonstrated to be upregulated in left ventricular myocardium in distinct animal models of cardiac hypertrophy like spontaneously hypertensive rats, Dahl salt-sensitive rats, the rat aortic constriction model, and MLP deficient mice with dilated cardiomyopathy [6,7]. Moreover, CARP has been proposed to act as a negative regulator of gene expression and thus could play a central role in the pathophysiology of heart failure. The present study recognized CARP upregulation in an experimental model of heart failure by means of differential gene expression analysis. The significance of this finding

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for human heart failure was unknown. Therefore, the expression pattern of CARP in failing left ventricular myocardium from patients with dilated and ischemic cardiomyopathy compared to non-failing control hearts was investigated.

Methods

Canine model of pacing induced heart failure. Rapid ventricular pacing was used to induce heart failure in dogs as previously described [8,9]. Briefly, mongrel dogs were instrumented with corkscrew electrode in the left ventricle (LV) attached to an external pacemaker. Hearts were paced at 250 bpm for 4 weeks ($n = 3$). Control dogs were instrumented but not paced ($n = 3$). The study conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Representational difference analysis. Total RNA was isolated from shock-frozen left ventricular tissue disrupted by the guanidinium thiocyanate method, followed by centrifugation in a cesium chloride gradient (Pharmacia, LKB, Freiburg, Germany). mRNA was isolated from total RNA with the Dynabeads mRNA purification kit (Dynal, Norway). Three failing hearts (testers) were compared to three healthy controls (drivers) as well as pools of those to overcome individual alterations in gene expression. After reverse transcription (cDNA Synthesis System, Gibco) double-stranded cDNAs were digested with *Dpn*II, ligated to R-Bam linkers, and amplified to generate representations according to the protocols of Hubank and Schatz [10,11] with minor modifications [12]. Three rounds of subtractive hybridization were performed with tester: driver ratios of 1:100, 1:800, and 1:400,000, respectively. The resulting difference products 2 and 3 were cloned into pBluescript II-SK+ in DH5alpha *Escherichia coli*. To analyze for redundancy, inserts of the clone libraries were amplified by PCR and analyzed by agarose gel electrophoresis. Inserts were sequenced by standard procedures on an ABI 377 sequencer. Sequence homologies were detected using BLAST routines at the NCBI. Representations and the difference products were analyzed by Southern blotting using a random primed canine CARP cDNA fragment, which was generated by RT-PCR. For RT-PCR, RNA isolated from canine hearts was reverse transcribed

Table 1
Patient details

Patient #	Age	Gender	Diagnosis[a]	NYHA class	LVEF[b] (%)	CI[c] (L/min/m^2)	Drugs[d]
1	52	M	NF (ICB)				nd
2	44	M	NF (SAB)				nd
3	50	F	NF (SAB)				nd
4	49	F	NF (CIC)				nd
5	31	M	NF (HT)				nd
6	35	M	NF (HT)				nd
7	36	M	NF (HT)				nd
8	45	M	NF (HT)				nd
9	52	M	NF (ICB)				nd
10	19	M	NF (HT)				nd
11	36	M	NF (ICB)				nd
12	50	M	DCM	III–IV		2.8	DGCNA
13	16	M	DCM	IV		2.2	DGNAO
14	65	M	DCM	IV			DNR
15	33	M	DCM	III	40	3.5	DGNA
16	35	F	DCM	IV	25	1.9	DGNARO
17	59	M	DCM	III		1.7	DGNA
18	62	M	DCM	IV	<20	2.2	DGAR
19	47	M	DCM	IV	25	2.2	DGNAR
20	43	M	DCM	III	26	2.7	GDCNA
21	48	M	DCM	III–IV		1.7	GDCNA
22	59	M	DCM	III	24	1.7	GDNA
23	61	F	ICM	III–IV	20		nd
24	64	M	ICM	III–IV	25		DNAO
25	46	M	ICM	III–IV			nd
26	54	M	ICM	III–IV			nd
27	57	M	ICM	III–IV			DGNR
28	66	M	ICM	III–IV	16	1.9	DCA
29	62	M	ICM	IV			DGNR
30	64	M	ICM	III–IV		1.8	DGNARO
31	50	M	ICM	IV	<20	1.2	DGNA
32	62	M	ICM	III–IV		3.1	DGNA
33	54	M	ICM	III	20	1.5	DGNAR

[a] ICB, intracerebral bleeding; SAB, subarachnoidal bleeding; CIC, cerebral ischemia; HT, head trauma; DCM, idiopathic dilated cardiomyopathy; ICM, ischemic cardiomyopathy; NF, non-failing donor.
[b] Left ventricular ejection fraction.
[c] Cardiac index.
[d] A, angiotensin converting enzyme inhibitors or angiotensin II receptor antagonists; C, calcium channel blockers; D: diuretics; G, cardiac glycosides; N, nitrates; R, antiarrhythmics (except β-AR blockers); O, dopamine/dobutamine; nd, unknown.

(cDNA synthesis system, Gibco) and a 255 bp CARP and a 348 bp cyclophilin A fragment were amplified using primers specific for CARP (5′-cgc gat atg ctt gaa tcc ac-3′ and 5′-ctt gta acg gtt cag cct cac t-3′) and cyclophilin A (5′-aga gaa ggg att cgg taa caa ag-3′ and 5′-atg gtg atc ttc ttg ctg gtc t-3′), respectively.

Human myocardial tissue. Failing hearts were obtained from patients undergoing orthotopic heart transplantation due to end-stage heart failure (NYHA functional class III–IV) resulting from idiopathic dilated cardiomyopathy (DCM) or with ischemic cardiomyopathy (by coronary angiography, ICM). All patients gave written informed consent before operation. Myocardial tissue from 11 non-failing donor hearts (NF) that could not be transplanted because of surgical reasons or blood group incompatibility was studied for comparison. Neither the donor patient histories nor 2-dimensional echocardiography had revealed signs of heart disease. Patient details are displayed in Table 1.

Northern and Western blot analyses. RNA was isolated from 100 mg cardiac tissue with 1 ml of Trizol (Gibco). Ten-microgram aliquots was electrophoresed, transferred onto Hybond N membranes, and UV-cross-linked. Northern blots were prehybridized for 2 h at 42 °C in a buffer containing 50% deionized formamide, 0.5% SDS, 6× SSC, 10 μg/ml denatured salmon sperm DNA (Sigma–Aldrich Chemicals), and 5× Denhardt's solution and were then hybridized for 15 h at 42 °C with a random-primed, [^{32}P]dCTP-labeled cDNA probes in the same buffer but without Denhardt's solution. A 608 bp fragment of human GAPDH cDNA and the human CARP cDNA, respectively, was used to generate specific probes. All Northern blots were subjected to stringent washing conditions (0.2 SSC, 0.1% SDS at 65 °C) prior to autoradiography with intensifying screen at −80 °C for 6–24 h. In addition, blots were scanned with a Molecular Imager System (BioRad) and Hybridization intensities were determined using Gel Doc software (BioRad).

Proteins from isolated rat cardiac myocytes and from human myocardium were extracted in SDS homogenization buffer (25 mmol/L Tris–HCl, pH 7.5, 250 mmol/L sucrose, 75 mmol/L urea, 1 mmol/L DTT). Fractionation of nuclear, cytosolic, and membrane proteins was performed as described [13]. The proteins were separated by PAGE on SDS-10% polyacrylamide gels and subsequently transferred to nitrocellulose (Schleicher & Schuell, Germany). The membrane was blocked with 5% non-fat dried milk in TBS-T (20 mmol/L Tris–HCl, pH 7.4, 150 mmol/L NaCl, 0.1% Tween 20) at 22 °C for 1 h prior to incubation with a polyclonal antibody against human CARP (raised against recombinant human CARP, 1:1000) at 4 °C overnight and horseradish peroxidase-conjugated anti-rabbit IgG for 1 h at room temperature. The CARP protein was visualized using the ECL chemoluminiscence detection system (Amersham). The blots were also probed with a polyclonal calsequestrin antibody (1:2500; Affinity Bioreagents) as a control for loading.

Statistical analysis. Data are presented as means ± SEM. Statistical analysis was performed using Student's t test to compare two groups and analysis of variance (ANOVA) with post hoc Newman–Keuls test for multiple comparison. A value of $P < 0.05$ was considered statistically significant.

Results

Characteristics of failing canine hearts

Tachycardia-pacing of adult mongrel dogs induced the hemodynamic changes characteristic for dilated cardiomyopathy with elevated left ventricular end diastolic pressure (LVEDP) and decreased dP/dt_{max} (Table 2). These hemodynamic changes resemble those observed previously in this model [8,9]. Tachypnea, ascites, pulmonary congestion, and pleural effusion were present in these animals.

Identification of CARP by RDA

Difference products 2 and 3 of four RDA experiments that were enriched for genes being upregulated in the failing canine myocardium were cloned into a bacterial vector. To determine the redundancy of the libraries, inserts were amplified and analyzed on a 1.5% agarose gel (Fig. 1A). Finally, 101 clones were sequenced. The most prominent and abundant sequence was a fragment homologous to the human gene for CARP (also known as cardiac adriamycin response protein). A Southern blot of tester- and driver-representations and difference products 1–3, hybridized with a CARP cDNA probe, demonstrated the enrichment-process for this fragment by RDA (Fig. 1B). CARP upregulation in the failing canine ventricles has been verified by RT-PCR (Fig. 1C) using constitutively expressed cyclophylin A as an endogenous control.

CARP expression is increased in human heart failure

The protein levels of CARP were analyzed in LV myocardium from donor hearts and in failing LV myocardium from patients with dilated or ischemic cardiomyopathy. Expression of CARP related to the internal standard protein calsequestrin is illustrated in Fig. 2. Human CARP was represented by a single band at 36 kDa and co-migrated with the recombinant human CARP loaded as a positive control. Cardiac failure due to dilated or ischemic cardiomyopathy resulted in a 1.8-

Table 2
Hemodynamics in dogs with congestive heart failure

	LVSP[a]	LVEDP[b]	dP/dt	MAP[c]	HR[d]
Prepace	129.8 ± 7.0	8.3 ± 1.1	3064.3 ± 394.3	108.4 ± 5.6	105.9 ± 7.1
Postpace	102.4 ± 5.3*	17.5 ± 3.8*	1513.2 ± 114.0**	89.2 ± 4.8*	126.7 ± 8.5

Values are means ± SEM; $n = 3$ congestive heart failure dogs.
[a] Left ventricular systolic pressure.
[b] Left ventricular end-diastolic pressure.
[c] Mean arterial pressure.
[d] Heart rate.
* $p < 0.05$.
** $p < 0.005$.

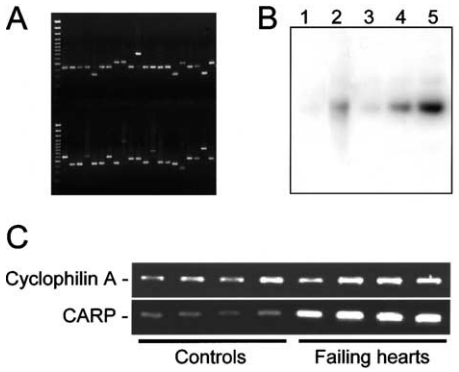

Fig. 1. Analysis of differentially expressed genes in canine heart failure by cDNA-RDA. (A) Cloned and amplifed RDA Difference Product III. Most fragments of the same size turned out to be a part of a homolog of the human CARP gene. (B) Enrichment of CARP in cDNA-RDA. A CARP cDNA fragment has been used as a probe for Southern hybridization. Tester representation (failing heart—lane 2) exhibits upregulation of CARP in comparison to the driver representation (healthy control—lane 1). The difference products generated within the course of cDNA-RDA demonstrate enrichment of the fragment (lanes 3–5 correspond to difference products 1–3). (C) RT-PCR to verify upregulation of CARP in failing canine myocardium from three individual animals compared to control animals. Cyclophilin A, which is constitutively expressed, serves for normalization.

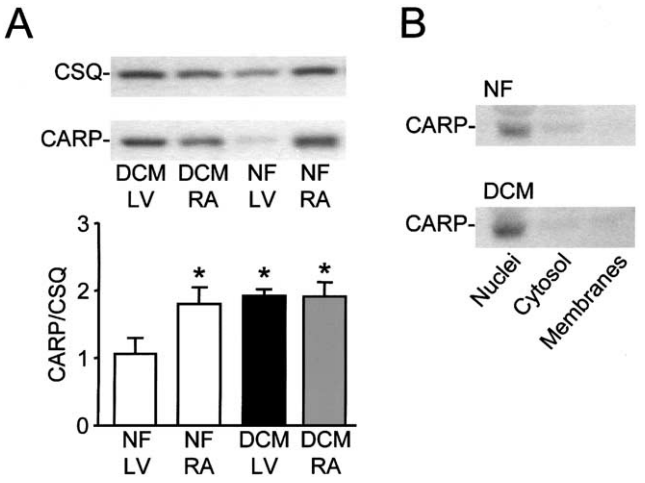

Fig. 3. (A) Western blot analysis comparing CARP expression levels in left ventricular and right atrial myocardium from failing hearts (DCM) and non-failing donor hearts (NF). In atria from donor hearts, CARP expression is markedly higher than in non-failing left ventricular myocardium. Atrial CARP levels do not differ between DCM and NF hearts. (B) Western blot analysis of subcellular CARP localization. CARP is extracted together with nuclear proteins. Small amounts are detectable in the cytosolic fraction and obviously no CARP protein is found in the membrane fraction.

fold increase in CARP expression in LV myocardium (NF: 1.0 ± 0.2, $n = 11$; DCM: 1.8 ± 0.1, $n = 11$, $p < 0.05$; ICM: 1.8 ± 0.2, $n = 8$). Notably, no difference in CARP protein concentrations was observed between atria from NF and DCM hearts. Like protein expression, gene expression was significantly increased about 1.7-fold in DCM and ICM compared to NF controls (NF: 0.68 ± 0.14, $n = 11$; DCM: 1.17 ± 0.10, $n = 11$; ICM: 1.13 ± 0.08, $n = 11$). Thus, CARP expression in left ventricular myocardium seems to be regulated at the transcriptional level. Fractionation experiments revealed that CARP was mainly localized in nuclei. Only weak expression was observed in the cytosolic fraction, and CARP was not detected in the membrane fraction prepared from human left ventricular myocardium (Fig. 3).

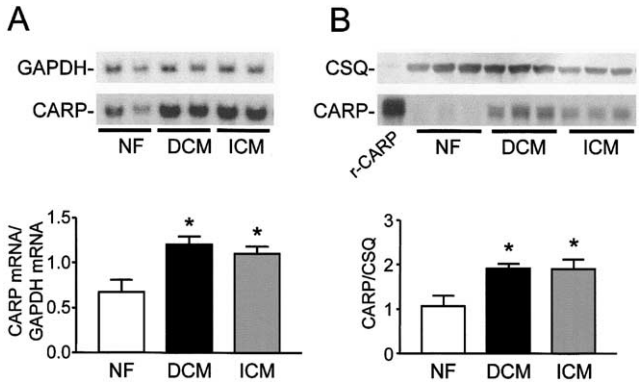

Fig. 2. CARP expression in left ventricles from patients with dilated cardiomyopathy (DCM) or ischemic cardiomyopathy (ICM) and from non-failing donor hearts (NF). (A) Northern blots were probed for CARP and for GAPDH as an internal control. CARP mRNA concentrations are significantly increased in failing hearts. (B) Western blot analysis with recombinant CARP (r-CARP) as a positive control. The internal standard calsequestrin (CSQ) was used to normalize for equal protein loading. CARP protein concentrations are significantly upregulated in failing hearts.

Discussion

RDA, like other subtractive hybridization technologies, was developed to identify differences between two cDNA representations from diseased, 'tester,' and healthy control populations, 'driver.' By this method, a wide range of expression differences, 2–80-fold, can be detected [11]. We successfully applied the cDNA-RDA to identify and isolate genes that are induced in failing left ventricular myocardium from dogs after cardiac overpacing. Ventricular pacing at 220–240 bpm resulted in profound low output, biventricular, edematous failure after four weeks. Positive features of this model, as characterized in numerous previous publications, include etiological relevance to patient tachycardia-induced heart failure and neurohumoral and functional profile similar to human heart failure [14]. cDNA-RDA led to the identification of CARP among genes previously known to be modulated and others that had not been previously associated with heart failure. Our finding was in particular interesting because previous studies demonstrated that gene expression of CARP, which was originally described as a cytokine-inducible

protein in human endothelial cells [15], was augmented in hypertrophic left ventricular myocardium from spontaneously hypertensive rats, Dahl salt-sensitive rats, rats with aortic coarctation, and muscle LIM protein deficient mice with dilated cardiomyopathy [6,7]. Moreover, treatment of cardiac myocytes with the antitumor agent doxorubicine lead to a downregulation of CARP transcript expression [16]. From the latter observation it has been proposed that decreased CARP may play a role in cardiac toxicity of doxorubicine. Overall, these findings suggest that CARP may be involved in cardiac pathophysiology.

Because of limitations of the canine heart failure model such as the rapid onset of heart failure and the absence of hypertrophy, the relevance of increased CARP expression for human heart failure was unclear. Therefore, CARP expression was directly investigated in failing and non-failing human myocardium. The major result was that CARP mRNA and protein levels were significantly increased in left ventricular myocardium from patients with end-stage heart failure. Augmented CARP expression in human heart failure was observed in both dilated and ischemic cardiomyopathy and therefore seems to be independent of etiology. Interestingly, CARP expression was constitutively higher in atria, but expression was induced selectively in ventricular myocardium during congestive heart failure. The expression pattern resembles that of ANF, which is restricted to the atrium in adult mammals and is induced in ventricular myocardium during various pathologic forms of hypertrophy and heart failure. CARP protein was shown to localize in the nucleus of rat neonatal cardiaomyocytes when cultured in serum-containing medium, but, interestingly the protein was distributed in the cytoplasm under serum-free conditions. This finding suggests that CARP translocation into the nucleus may be promoted by some components in the serum. The present study demonstrates that CARP protein is predominantly localized in the nuclear fraction in nonfailing as well as in failing hearts, suggesting that expression levels but not the subcellular distribution is changed in human heart failure.

CARP is a nuclear protein predominantly expressed in the heart although expression at much lower levels in other tissues, like vasculature or skeletal muscle, has been demonstrated [6,17]. Previous studies suggested that CARP plays a negative role in cardiac-specific gene expression. Jeyaseelan et al. [16] tested the effect of CARP overexpression on several cardiac-specific promoter–reporter constructs in primary rat neonatal cardiomyocytes, and demonstrated that the cardiac troponin-C and the atrial naturetic factor promoters were both significantly inhibited by CARP. Although promoter studies are suitable to investigate the mechanisms of CARP action, the physiological significance remains unclear. Several mechanisms by which CARP negatively regulates gene expression have been proposed. Co-transfection assays using a CARP expression vector and a luciferase reporter gene driven by an HF-element suggests that CARP may serve as a negative regulator of HF-1 dependent pathways [18]. The regulatory element HF-1 in turn is required to maintain ventricular chamber-specific expression of the myosin light-chain 2v gene [18]. In addition, it became obvious that CARP itself contains a transcriptional inhibitory domain [19]. Interestingly, adenoviral over-expression of CARP in C2/2 cells inhibits DNA synthesis probably through induction of $p21^{WAF/CIP1}$ [17].

In summary, we have demonstrated that CARP, initially identified by cDNA-RDA in a canine heart failure model, is augmented in failing human ventricular myocardium. This raises the possibility that increased CARP expression in human heart failure may have an impact on disease progression.

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