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Dominant negative Ras attenuates pathological ventricular remodeling in pressure overload cardiac hypertrophy

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ABSTRACT

The importance of the oncogene Ras in cardiac hypertrophy is well appreciated. The hypertrophic effects of the constitutively active mutant Ras-Val12 are revealed by clinical syndromes due to the Ras mutations and experimental studies. We examined the possible anti-hypertrophic effect of Ras inhibition in vitro using rat neonatal cardiomyocytes (NRCM) and in vivo in the setting of pressure-overload left ventricular (LV) hypertrophy (POH) in rats. Ras functions were modulated via adenovirus directed gene transfer of active mutant Ras-Val12 or dominant negative mutant N17-DN-Ras (DN-Ras). Ras-Val12 expression in vitro activates NFAT resulting in pro-hypertrophic and cardio-toxic effects on NRCM beating and Z-line organization. In contrast, the DN-Ras was antihypertrophic on NRCM, inhibited NFAT and exerted cardio-protective effects attested by preserved NRCM beating and Z line structure. Additional experiments with silencing H-Ras gene strategy corroborated the antihypertrophic effects of siRNA-H-Ras on NRCM. In vivo, with the POH model, both Ras mutants were associated with similar hypertrophy two weeks after simultaneous induction of POH and Ras-mutant gene transfer. However, LV diameters were higher and LV fractional shortening lower in the Ras-Val12 group compared to control and DN-Ras. Moreover, DN-Ras reduced the cross-sectional area of cardiomyocytes in vivo, and decreased the expression of markers of pathologic cardiac hypertrophy. In isolated adult cardiomyocytes after 2 weeks of POH and Ras-mutant gene transfer, DN-Ras improved sarcomere shortening and calcium transients compared to Ras-Val12. Overall, DN-Ras promotes a more physiological form of hypertrophy, suggesting an interesting therapeutic target for pathological cardiac hypertrophy.

1. Introduction

Small GTP binding-proteins are a superfamily of proteins acting as molecular switches between extracellular signals and molecular pathways [1,2]. The Ras subfamily is the most important of the GTPases and includes three main homologous variants: H-Ras, K-Ras and N-Ras. The 3 genes are expressed ubiquitously with some variation in patterns of expression and function in developing and adult tissues [3,4].

H-Ras hyperactivity in the cardiomyocyte is associated with hypertrophy [2,5]: *i)* In Costello's syndrome, patients with activating mutations of Ras develop hypertrophic cardiomyopathy with atrial tachycardia [6]. *ii)* Transgenic mice expressing the constitutively active mutant Ras-Val12 demonstrate ventricular hypertrophy similar to human hypertrophic cardiomyopathy [7]. *iii)* Mouse models of inducible cardiac overexpression of Ras-Val-12 have shown pathological features of cardiac hypertrophy [8]. Hypertrophy, deranged myofibril structure and impaired calcium transients are demonstrated in NRCM treated with Ras-Val-12 [9].

Abbreviations: SR, sarcoplasmic reticulum; DAG, diacylglycerol; IP3, inositol 1,4,5,triphosphate; PLC, phospholipase C; PI3K, phosphatidyl,inositol 3,kinase; AKT, protein kinase B; GSK, Glycogen synthase kinase,3beta; CaN, calcineurine, protein phosphatase 2B; NFAT, nuclear factor of activated T cells; MEKK, mitogen, activated protein kinase; ERK, extracellular signal-regulated kinase

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On the other hand, Ras deficiency or Ras dominant negative mutants are associated with cardiac hypotrophy or anti-hypertrophic effects: *i)* K-Ras knock-out mice embryos die by day 15.5 of development, with very thin walls of cardiac ventricles as the likely cause of embryonic death [3]. *ii)* Thorburn et al. [10] showed that Ras-Ala15 (a negative interferent mutant of Ras) inhibits NRCM hypertrophy activated by phenylephrine (PE). Fuller et al. showed that N17-DNRas (DN-Ras) inhibits the hypertrophic effect of the active mutant of Src-Phe527 [11]. *iii)* Pracyk et al. [12] demonstrated the anti-hypertrophic effect of the DN-Ras in neonatal cardiomyocytes at baseline, but not under stimulation by endotheline 1. *iv)* Recently, Nagalingam et al. [13] demonstrated the in vitro anti-hypertrophic effect of miR-378, a MicroRNA that negatively regulates Ras signaling in cardiac hypertrophy.

However, the study of the effects of Ras on cardiac hypertrophy is made difficult by the complex and poorly understood regulatory balance between proteins of the Ras superfamily; moreover, there are several examples of paradoxical responses to Ras-dependent stimuli, such as Ras and Rap1 divergent actions [1,14].

Ras mutants exert different actions at baseline or under ligand stimulation [12]. Duquesnes et al. [15] have shown that the ERK signaling pathway was down-regulated by DN-Ras in basal conditions but not under mechanical stimulation and Harris IS et al. [16] demonstrated apparently paradoxical actions of the Raf1-MAPkinase cascade. In mice expressing Ras-Val12 in the ventricles, the phenotype was variable and heterogeneous [5,7,17].

In this study, we hypothesized that the DN-Ras mutant N17 exerted an anti-hypertrophic effect in vivo, but we found a mixed agonist–antagonist action on the Ras pathway leading to cardiomyocyte hypertrophy or anti-hypertrophic effects, and the balance between the agonist and antagonist effects of DN-Ras was stimulus-dependent. Furthermore, and beyond the hypertrophic response, we investigated the impact of DN-Ras and active Ras-Val12 mutants on the pathologic character of cardiomyocyte hypertrophy. To that end, studies were conducted in vitro with and without pro-hypertrophic ligands, and in vivo in the setting of pressure-overload left ventricular (LV) hypertrophy (POH). To corroborate the results obtained by overexpressing dominant negative and active Ras mutants, a set of experiments was performed using siRNA-H-Ras on NRCM.

2. Materials and methods

2.1. Recombinant adenovirus vectors

All recombinant adenoviruses were constructed using a cytomegalovirus (CMV) promoter. Cells were infected with adenovirus at 50 to 100 MOI (multiplicities of infection) per cell. We used recombinant adenoviral vectors encoding two variants of the H-Ras gene: the dominant negative mutant S17N (Ad-DNRas) and the oncogenic active mutant G12V (Ad-Val12). The DN-Ras mutant of Ras (substitution of asparagine for serine at the 17th amino-acid) is locked in its inactive form (Ras-GDP), due to a reduced affinity for GTP and preserved affinity for GDP [18]. The active mutant Ras Val12 (a substitution of glycine for valine, G12V, in the 12th amino-acid) was used as a positive control for DN-Ras [5]. Ad-βGal, encoding β-galactosidase under CMV promoter followed by target gene-IRES-reporter gene (a green fluorescent protein (GFP)) construct translated as two independent proteins [19] was used as an additional control; Ad.NFAT-Luc virus, carrying the luciferase reporter gene controlled by NFAT- responsive promoter [20] was used to measure NFAT activation. Ad-VIVIT, encoding NFAT competing peptide VIVIT and GFP under CMV promoter [21,22] was used as an additional control for NFAT-reporter experiments.

2.2. Isolation and culture of neonatal rat cardiomyocytes (NRCM)

Spontaneously beating NRCM were isolated from 1 to 2-day-old Sprague–Dawley rat pups using the Worthington Neonatal Cardiomyocyte Isolation System (Worthington Biochemical Corp.) as previously described [23]. Cardiomyocytes were plated in 12-well plates for 36 h in serum-free medium then transduced with the recombinant adenoviruses (Ad-DNRas, Ad-Val12, or Ad-βGal) or no virus as control group.

For experiments using siRNA-H-Ras, NRCM were plated in 6-well plates in triplicates and transfected with 50 nM Hras-siRNA (Santa Cruz Biotechnology) with RNAiMAX Transfection Reagent (Invitrogen) for 24 h. Control cells were treated with only RNAiMAX Transfection Reagent for 24 h. Scramble siRNA had no effect on NRCM compared to control in observations published elsewhere [24] where scramble siRNA does not affect calcium transient and contractility in NRCM compared to control.

Cells were then serum starved for 30 h before they were stimulated with phenylephrine (PE) (1 μM) for an additional 24 h. Cells were then harvested and homogenized for RNA and proteins preparation.

2.3. Protein synthesis assay

Protein synthesis rate was measured using the [^{3}H]-Leucine incorporation assay as previously described [23]. Briefly, NRCM were transduced with the relevant adenovirus for 48 h. Then, NRCM were stimulated for 7 h with PE (1 μM) or angiotensin-II (1 μM) in the presence of [^{3}H]-Leucine. Protein synthesis was quantified by [^{3}H]-Leucine incorporation measured by scintillation counting (MicroBeta Trilux, Perkin Elmer).

2.4. In vitro and in vivo cardiomyocyte size measurement

Cell surface areas were measured on micrographs using the ImageJ software (NIH). At least 50 individual cells were analyzed for each experimental condition. For in vivo experiments, the size of cardiomyocytes was evaluated on cross sections using Wheat germ agglutinin (WGA) Alexa Fluor® 488 conjugate (Invitrogen).

2.5. In vitro beating analysis

We designed a protocol to keep NRCM beating for prolonged periods of time, and analyzed the influence of experimental genes on beating. NRCM were maintained with 2% serum in the medium instead of serum-free (0.1% serum) medium. After 24 h with 2% serum, gene transfer was performed. Subsequent daily measurement of beating frequency was performed during 2 weeks. The beating frequency (beat/min) was counted in three different points of the well. The in vitro beating analysis was repeated three times, and with two wells per group each time.

2.6. Immunofluorescence

The sarcomeric structure of NRCM was analyzed by immunofluorescence with anti alpha-actinin (Sigma A2543), using standard protocol. Secondary antibody was a-rabbit IgG conjugated with Alexa 555 (Invitrogen). Slides were analyzed by confocal microscopy (Leica TCS-SP confocal microscopy).

2.7. Quantitative Real time PCR

Total RNA was isolated with TRIzol reagent (Invitrogen). Expression of H-Ras, beta-Myosin-heavy-Chain (β-MHC), atrial natriuretic factor (ANF) and B-type natriuretic peptide (BNP) mRNA in NRCM and LV tissue was quantified using real time RT-PCR (RT-PCR) analysis (7500 real-time PCR system, Applied Biosystems) performed according to standard procedures. Quantification of relative expression used the delta-delta-Ct approach. The following primers were used:

Rat-18S-F: 5′-GTTGGTTTTCGGAACTGAGGC-3′
Rat-18S-R: 5′-GTCGGCATCGTTTATGGTCG-3′