
RESEARCH PAPER

IL-6/STAT3 signaling in mice with dysfunctional type-2 ryanodine receptor

Tai-Qin Huang,^{1,†} Monte S Willis,² and Gerhard Meissner^{1,*}

¹Department of Biochemistry & Biophysics; University of North Carolina; Chapel Hill, NC USA

²Department of Pathology and Laboratory Medicine; University of North Carolina; Chapel Hill, NC USA

ABSTRACT. Mice with genetically modified cardiac ryanodine receptor ($Ryr2^{ADA/ADA}$ mice) are impaired in regulation by calmodulin, develop severe cardiac hypertrophy and die about 2 weeks after birth. We hypothesized that the interleukin 6 (IL-6)/signal transducer and activator of transcription-3 (STAT3) signaling pathway has a role in the development of the $Ryr2^{ADA/ADA}$ cardiac hypertrophy phenotype, and determined cardiac function and protein levels of IL-6, phosphorylation levels of STAT3, and downstream targets c-Fos and c-Myc in wild-type and $Ryr2^{ADA/ADA}$ mice, mice with a disrupted IL-6 gene, and mice treated with STAT3 inhibitor NSC74859. IL-6 protein levels were increased at postnatal day 1 but not day 10, whereas pSTAT3-Tyr705/STAT3 ratio and c-Fos and c-Myc protein levels increased in hearts of 10-day but not 1-day old $Ryr2^{ADA/ADA}$ mice compared with wild type. Both STAT3 and pSTAT3-Tyr705 accumulated in the nuclear fraction of 10-day old $Ryr2^{ADA/ADA}$ mice compared with wild type. $Ryr2^{ADA/ADA}/IL-6^{-/-}$ mice lived 1.5 times longer, had decreased heart to body weight ratio, and reduced c-Fos and c-Myc protein levels. The STAT3 inhibitor NSC74859 prolonged life span by 1.3-fold, decreased heart to body weight ratio, increased cardiac performance, and decreased pSTAT-Tyr705/STAT3 ratio and IL-6, c-Fos and c-Myc protein levels of $Ryr2^{ADA/ADA}$ mice. The results suggest that upregulation of IL-6 and STAT3 signaling contributes to cardiac hypertrophy and early death of mice with a dysfunctional ryanodine receptor. They further suggest that STAT3 inhibitors may be clinically useful agents in patients with altered Ca^{2+} handling in the heart.

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*Correspondence to: Gerhard Meissner; Email: meissner@med.unc.edu

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[†]Present affiliation: Department of Medicine and Ion Channel Research Unit; Duke University Medical Center; Durham, NC USA

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KEYWORDS. cardiac ryanodine receptor, calmodulin, cardiac hypertrophy, IL-6, NSC74859, S3I-201, STAT3

ABBREVIATIONS. CaM, calmodulin; IL-6, interleukin 6; mTOR, inhibitor of mammalian target of rapamycin; RyR2, cardiac ryanodine receptor; SR, sarcoplasmic reticulum; STAT3, signal transducer and activator of transcription-3

INTRODUCTION

Type-2 ryanodine receptors (RyR2s) release Ca^{2+} from the sarcoplasmic reticulum (SR) to cause cardiac muscle contraction. The RyR2s are composed of 4 560-kDa RyR2 and 4 12.6-kDa FK506 binding protein subunits, and regulated by Ca^{2+} and associated proteins such as calmodulin (CaM).¹⁻⁴ CaM is a 16.7-kDa Ca^{2+} binding protein that modulates proteins through CaM-dependent protein kinases and phosphatases or through direct binding.⁵ In mouse cardiomyocytes, the majority of CaM is bound to RyR2⁶ which reduces the release of Ca^{2+} from SR by inhibiting RyR2.⁷⁻⁹

To determine the role of CaM in regulating RyR2 *in vivo*, a mouse model with 3 amino acid replacements in the CaM binding domain of RyR2 (RyR2-W3587A/L3591D/F3603A, RyR2^{ADA}) was generated.¹⁰ The triple mutation impaired inhibition of RyR2 by CaM at diastolic and systolic Ca^{2+} , and resulted in severe cardiac hypertrophy and death of mice within about 2 weeks after birth. The mutations did not appear to introduce other major structural and functional changes in RyR2. The mutant mice had reduced RyR2 content and SERCA2a activity, lower $\text{Ca}_v1.2$ current density and showed irregularities in local and global Ca^{2+} transients.¹⁰⁻¹¹ Ultrastructural analysis indicated only minor structural changes in myofibrillar organization, however, a reduction in SR/T-tubule junctions and RyR2 content were observed in mutant hearts which had a 2–3-fold increase in size compared to wild type.¹² Additionally, several signaling molecules were implicated in cardiac hypertrophy of RyR2^{ADA/ADA} mice. Phosphorylation levels of extracellular signal-regulated kinases 1 and 2, p90 ribosomal S6 kinase and glycogen synthase kinases 3 α and 3 β increased in

hearts of embryonic day 16.5 RyR2^{ADA/ADA} mice.¹³ Class II histone deacetylase/myocyte enhancer factor-2 signaling and nuclear factor of activated T cell transcriptional activity were up-regulated in the hearts from 1-day old RyR2^{ADA/ADA} mice but not hearts from E16.5 mice.^{10,13} Calcineurin A- β ablation decreased heart weight and improved cardiac contractility and extended the lifespan of RyR2^{ADA/ADA} mice by 2-fold, without suppressing the expression of genes associated with cardiac hypertrophy.¹³ Treatment with rapamycin, an inhibitor of mammalian target of rapamycin (mTOR), inhibited phosphorylation of mTOR and downstream targets ribosomal S6 kinase and ribosomal protein S6, decreased heart size, and improved cardiac function but did not extend the lifespan of RyR2^{ADA/ADA} mice.¹⁴ The results suggest that multiple signaling pathways are involved in the development of cardiac hypertrophy and heart failure in RyR2^{ADA/ADA} mice expressing cardiac RyR2 impaired in regulation by CaM.

The present study explores the role of interleukin-6 (IL-6) and signal transducer and activator of transcription 3 (STAT3) signaling in cardiac hypertrophy and heart failure in RyR2^{ADA/ADA} mice. In addition to its well-known role in inflammatory and immunological processes, increased circulating levels of IL-6 and related cytokines have been linked to heart failure in patients and animal models.¹⁵ Binding of IL-6 to membrane-bound and soluble receptors results in a complex with glycoprotein (gp) 130.¹⁵ In turn, formation of the IL-6/IL-6R/gp130 complex triggers 3 major downstream signaling pathways, janus kinase (JAK)/(STAT), mitogen activated protein kinase and phosphatidylinositol 3-kinase (PI3K)-AKT dependent pathways.^{15,16} Seven structurally related but functionally distinct isoforms of STAT¹⁷ are

expressed in cardiomyocytes.¹⁸ Among these, STAT3 has been implicated in the development of cardiac hypertrophy.^{15,19-24} Binding of IL-6 to its receptor and complex formation with gp130 causes phosphorylation of associated JAKs and recruitment and phosphorylation of STAT3. Following its phosphorylation, STAT3 translocates to the nucleus where it binds to response elements of c-Fos and c-Myc target genes.²⁵⁻²⁷ Absence of a hypertrophic response in the absence of soluble IL-6 receptor²⁸ and dependence of STAT3 phosphorylation on the soluble receptor²⁹ suggested that IL-6 mediates its effect on STAT3 through the soluble receptor in cardiomyocytes.

Here we show that IL-6 protein levels, STAT3 phosphorylation, and c-Fos and c-Myc protein levels are upregulated in Ryr2^{ADA/ADA} mice. Crossing Ryr2^{ADA/ADA} mice with *Il-6*^{-/-} mice or treatment of Ryr2^{ADA/ADA} mice with STAT3 inhibitor NSC74859 decreased pSTAT-Tyr705 phosphorylation and c-Fos and c-Myc protein levels, reduced heart size, prolonged life span, and improved cardiac performance of Ryr2^{ADA/ADA} mice. Our findings suggest that IL-6/STAT3 signaling contributes to the development of cardiac hypertrophy in Ryr2^{ADA/ADA} mice. A preliminary report of this work has been presented in abstract form.³⁰

MATERIALS AND METHODS

Materials

STAT3 mouse monoclonal antibody (catalog No. 9139), pSTAT3-Tyr705 rabbit monoclonal antibody (catalog No. 9145), pSTAT3-Ser-727 rabbit polyclonal antibody (catalog No. 9134), c-Fos rabbit monoclonal antibody (catalog No. 2250), and GAPDH rabbit monoclonal antibody (catalog No. 2118) were from Cell Signaling. c-Myc mouse monoclonal antibody (catalog No. sc-40) was from Santa Cruz, and IL-6 mouse monoclonal antibody (A3218) was from eBioscience. STAT3 inhibitor NSC74859 (also known as S3I-201) (catalog No. S1155) was from Selleck Chemicals. Primary antibodies were used at 1:1000 dilution, except

GAPDH (1:5000). For the secondary antibodies, the dilution was 1:10,000. Protease (catalog No. 11697498001) and phosphatase (catalog No. P5726) inhibitors were from Sigma-Aldrich. Other chemical reagents were from Sigma-Aldrich.

Animals

Ryr2^{+/+} and Ryr2^{ADA/ADA} mice were obtained by backcrossing Ryr2^{+/ADA} mice at least 10 times to 129/Svev background.¹⁰ To generate Ryr2^{+/+} and Ryr2^{ADA/ADA} mice with disrupted IL-6 gene, 129/Svev Ryr2^{+/ADA} mice were mated with B6.129S2-*Il-6*^{tm1Kopf}/J mice (Jackson Laboratory). The subsequent litters were backcrossed 3–4 times with 129/Svev Ryr2^{+/ADA} mice. Animal experiments were approved by the Institutional Animal Care and Use Committee of the University of North Carolina at Chapel Hill.

Primers for genotyping of mice were: Ryr2, sense (s) 5'-CAAGGCTTAGGA GCTACGAG-3' and antisense (a) 5'-TTCACGTTGCCCC TCGTTAC-3'; IL-6, (common) 5'-TTCCA TCCAGTTG CCTTCTTGG-3', (wild type reverse) 5'-TTCTCATTTC ACGATTTC CAG-3', (mutant reverse) 5'-CCGGAGAA CCTGCGTG CAATCC-3'.

STAT3 inhibitor treatment

STAT3 inhibitor NSC74859 was dissolved (1.5 mg/ml) in 0.15 M NaCl and 3% DMSO.³¹ One day old mice were separated randomly into 4 groups. NSC74859 (20 µg/g body weight) was injected intraperitoneally once every day. Vehicle injection served as the negative control. For mRNA analysis, animals were sacrificed and hearts collected 1 h after the last injection. For protein analysis, hearts were collected 4 h after the final injection.

2.3. Echocardiography

To determine left ventricular cardiac function in mice, transthoracic M-mode echocardiography was performed on restrained

unanesthetized 10-day old mice, using a 770 high resolution imaging system (VisualSonics) with a 40-MHz probe. Mice were restrained by taping down gently to on a warmed mouse board (Indus Industries for VisualSonics).

Quantitative RT-PCR

Total RNA was extracted from left ventricles of 10-day old mice using Trizol (Invitrogen) and reverse transcribed into cDNA using SuperScript First-Strand Synthesis System for RT-PCR Kit (Invitrogen). Real-Time PCR was performed using the following primers: IL-6, (s) 5'-CCGGAGAGGAGACTTC ACAG-3' and (a) 5'-TCCACGATTTCCAGGA GAAC-3'; STAT3, (s) 5'-GGAGGAG GCATTTG GAA AGT-3' and (a) 5'-GGCAGGTCA-ATGGTATTGCT-3'; c-Fos, (s) 5'-GTCC GGTTCCTTCTA TGCAG -3' and (a) 5'-TAAGTAGTG CAGCCCGGAGT -3'; c-Myc, (s) 5'-GCTGGAGAT GATGACCGAGT-3' and (a) 5'-AACCGCTCCACATACAGTCC-3'; ANP, (s) 5' ATCTGCCCTCTTGAA AAGCA-3' and (a) 5'- AAGCTGTTGCAGCC TAGTCC-3'; BNP, (s) 5'- CAGCTCTTGAAG GACCAAGG-3' and (a) 5'- CCGATCC GGTCTATCTTGTG-3'; 18S RNA, (s) 5'-CGTCTGCCCTAT CAAC TTTCG-3' and (a) 5'- CCTTGGATGTGGTAGC CGTT-3'. Real-time PCR was performed using Maxima[®] SYBR Green /Rox qPCR master mix (Thermo Scientific) and 7900 HT Fast Real Time PCR machine (Applied Biosystems). Cycling conditions were 95°C for 10 min, and 40 cycles of 95°C for 15 sec and 60°C for 30 sec. Melting curve analysis of products was performed following each amplification to verify the specificity of the PCR products. 18S RNA was used as an internal control for normalization.

Preparations of heart fractions

Whole heart homogenates were prepared in 20 mM imidazole, pH 7.0, containing 0.3 M sucrose, 0.15 M NaCl, protease and phosphatase inhibitor cocktails, 25 mM β -glycerophosphate, 5 mM NaF, and 2.5 mM NaVO₄ using

Tekmar Tissumizer for 3 × 7 s at a setting of 13,500 rpm. Cytosolic and nuclear fractions were prepared using Pierce Biotechnology NePer Nuclear and Cytoplasmic Extraction Reagents kit. Cellular fractions were stored in small aliquots at -80°C. Protein concentrations were determined using the bicinchoninic acid assay.

Immunoblot analysis

Homogenates (20 μ g protein/lane) were separated by 10% SDS/PAGE, transferred to nitrocellulose membranes, and probed with monoclonal or polyclonal antibodies. Western blots were developed using enhanced chemiluminescence and quantified using ImageQuantTL Analysis Software. GAPDH was the loading control.

Statistical analysis

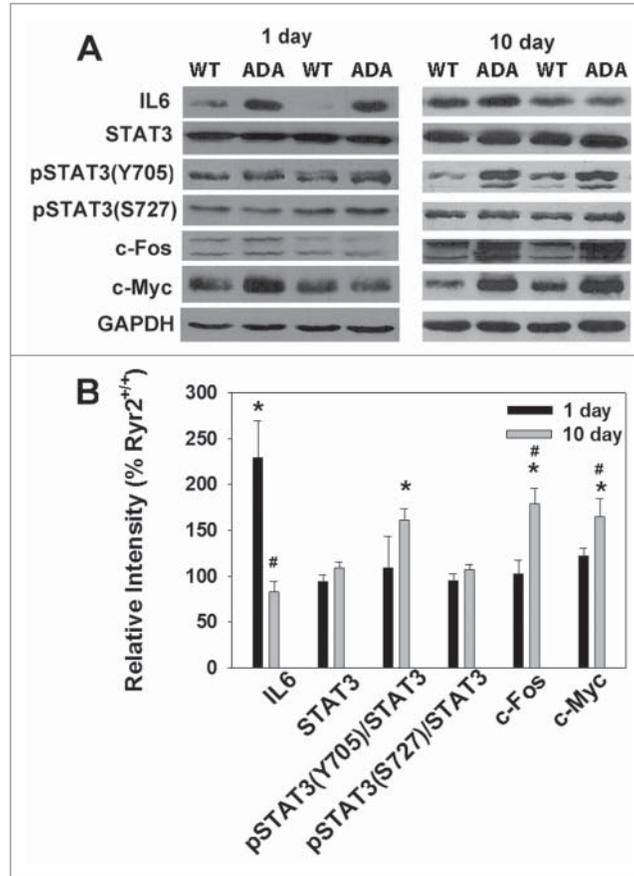
Statistical analysis was performed using Sigma-plot 11. T-test was used to compare 2 groups and ANOVA followed by Tukey's test to analyze the significance among multi groups. Statistical significance was taken at the $p < 0.05$ level.

RESULTS

Upregulation of IL-6 and pSTAT3-Tyr-705 in Ryr2^{ADA/ADA} mouse hearts

We reported previously that mice with impaired CaM inhibition of the cardiac ryanodine receptor (*Ryr2*^{ADA/ADA} mice) show signs of cardiac hypertrophy at postnatal day 1 and die within about 2 weeks after birth.¹⁰ To determine the mechanism of cardiac hypertrophy induced by the RyR2 mutation, protein levels of IL-6 and STAT3, phosphorylation levels of pSTAT3-Tyr705 and pSTAT3-Tyr727, and protein levels of c-Fos and c-Myc downstream transcription factors were determined. An increase in IL-6 protein was observed in 1-day old but not 10-day old *Ryr2*^{ADA/ADA} hearts, whereas STAT3 protein levels were similar in

FIGURE 1. IL-6 and downstream signaling molecule expression in hearts of *Ryr2*^{+/+} and *Ryr2*^{ADA/ADA} mice. (A) Immunoblots of heart homogenates from 1-day old and 10-day old *Ryr2*^{+/+} (WT) and *Ryr2*^{ADA/ADA} (ADA) mice. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was the loading control. (B) Relative protein and phosphorylation levels in 1-day old and 10-day old *Ryr2*^{ADA/ADA} compared to *Ryr2*^{+/+} mice. Data are the mean \pm SEM of 5–19 samples. **p* < 0.05 compared to *Ryr2*^{+/+}, #*p* < 0.05 compared to corresponding 1 day sample using one way ANOVA.

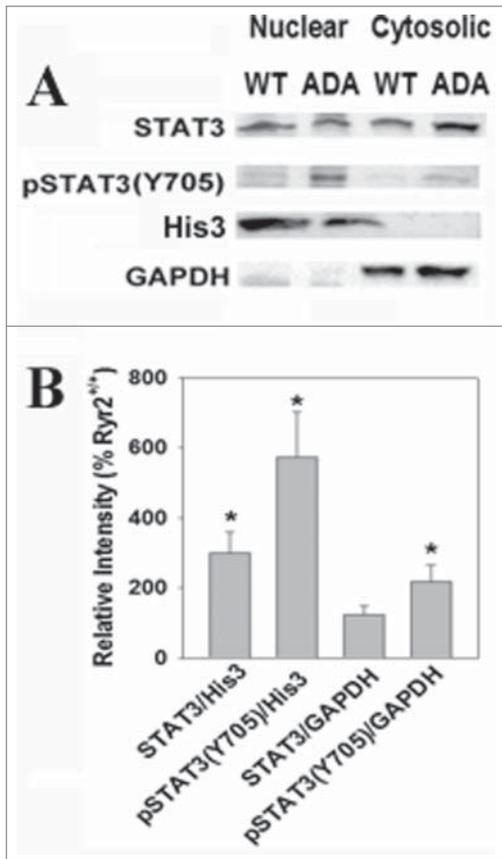


wild-type *Ryr2*^{+/+} hearts (Fig. 1). STAT3 is activated by phosphorylation at Tyr-705 following phosphorylation of janus kinase (JAK) in IL-6/IL-6R/p130-JAK complex, whereas phosphorylation at Ser-727 is mediated through the mitogen activated protein kinase and mTOR pathways.¹⁵ The pSTAT3-Tyr705/STAT3 ratio increased at postnatal day 10, but not at postnatal day 1 (Fig. 1) or days 3–4 (not shown). No change in the pSTAT3-Ser727/STAT3 ratio was observed at postnatal days 1 and 10. Consistent with the absence of an elevated pSTAT3-Tyr705/STAT3 ratio at postnatal days 1 and 3–4, c-Fos and c-Myc increased only during the later stages of cardiac

hypertrophy compared to wild-type hearts (Fig. 1). The data suggested the IL-6/STAT3 signaling pathway may have a role in development of the *Ryr2*^{ADA/ADA} cardiac hypertrophy phenotype.

To determine whether STAT3 and phosphorylated pSTAT3-Tyr705 translocated to the nucleus in hearts of *Ryr2*^{ADA/ADA} mice, cytosolic and nuclear fractions were prepared. GAPDH and histone 3 served as cytosolic and nuclear markers, respectively (Fig. 2). In agreement with the nuclear accumulation of unphosphorylated STAT3 in response to IL-6,³² STAT3 was enriched in the nuclear fraction of 10-day old *Ryr2*^{ADA/ADA} hearts.

FIGURE 2. Nuclear and cytosolic location of STAT3 and pSTAT3-Tyr705. (A) Immunoblots of nuclear (N) and cytosolic (C) fractions from hearts of 10-day old $Ryr2^{+/+}$ (WT) and $Ryr2^{ADA/ADA}$ (ADA) mice. GAPDH and histone 3 (His3) were markers for cytosolic and nuclear fractions, respectively. (B) Protein and phosphorylation levels of $Ryr2^{ADA/ADA}$ mice were normalized to $Ryr2^{+/+}$. Data are the mean \pm SEM of 5–6 samples. * $p < 0.05$ compared to $Ryr2^{+/+}$ using t-test.



pSTAT-Tyr705 was predominantly localized in the nuclear fraction of $Ryr2^{ADA/ADA}$ mouse hearts compared to wild-type hearts.

$Ryr2^{ADA/ADA}/IL-6^{-/-}$ mice. In addition to IL-6, other members of the cytokine family might have contributed to the $Ryr2^{ADA/ADA}$ cardiac hypertrophy phenotype.¹⁵ To obtain evidence that IL-6 signaling was associated with cardiac hypertrophy, we generated $Ryr2^{+/+}$ and $Ryr2^{ADA/ADA}$ mice carrying a disrupted IL-6 gene. 129/Svev $Ryr2^{+/+ADA}$

mice were mated with B6.129S2- $Il-6^{tm1Kopf/J}$ mice. The subsequent litters were backcrossed 3–4 times with 129/Svev $Ryr2^{+/+ADA}$ mice. Accordingly, littermates with a predominantly 129/Svev genetic background were obtained, out of which the 4 genotypes investigated were $Ryr2^{+/+}$, $Ryr2^{ADA/ADA}$, $Ryr2^{+/+}/IL-6^{-/-}$ and $Ryr2^{ADA/ADA}/IL-6^{-/-}$. $Ryr2^{+/+}/IL-6^{-/-}$ mice carrying an IL-6 gene disrupted in the first coding exon by insertion of *neo* cassette³³ were viable without change in body to heart weight ratio compared with $Ryr2^{+/+}$ mice (Table 1). However, loss of IL-6 significantly decreased heart weight to body weight ratio in $Ryr2^{ADA/ADA}/IL-6^{-/-}$ mice compared to $Ryr2^{ADA/ADA}$ mice (Table 1). $Ryr2^{ADA/ADA}/IL-6^{-/-}$ mice also lived longer compared to $Ryr2^{ADA/ADA}$ mice, with mean lifetimes of 25.1 ± 3.5 d and 16.9 ± 1.3 d ($p < 0.05$), respectively (Fig. 3). Echocardiography indicated that the IL-6 mutation did not significantly alter cardiac performance in $Ryr2^{+/+}$ and $Ryr2^{ADA/ADA}$ mice (Table 1).

Co-expression of $Ryr2^{ADA/ADA}$ and IL-6 mutants did not alter STAT3 protein levels and pSTAT-Ser-727/STAT3 ratios among the 4 genotypes (Fig. 4). The elevated pSTAT3-Tyr705/STAT3 ratio in the hearts of $Ryr2^{ADA/ADA}$ mice was modestly (not significant) decreased in the mutant mice carrying the IL-6 mutation, whereas the IL-6 mutant significantly decreased c-Fos and c-Myc protein levels in $Ryr2^{ADA/ADA}$ mice. No significant change was observed in $Ryr2^{+/+}/IL-6^{-/-}$ mice compared with $Ryr2^{+/+}$ mice (Fig. 4). Taken together, the results of Figure 4 and Table 1 suggest that IL-6 had a role in decreasing lifespan, increasing heart size and upregulating c-Fos and c-Myc protein levels in $Ryr2^{ADA/ADA}$ mice. However, the data also suggest that other mechanisms exist that contributed to the $Ryr2^{ADA/ADA}$ phenotype.

Treatment with STAT3 inhibitor NSC74859

To obtain more direct evidence for STAT3 regulation in cardiac hypertrophy of $Ryr2^{ADA/ADA}$ mice, newborn mice were daily

Table 1. Body and heart weights and echocardiography of 10-day old *Ryr2*^{+/+} and *Ryr2*^{ADA/ADA} mice.

	<i>Ryr2</i> ^{+/+} n = 8	<i>Ryr2</i> ^{ADA/ADA} n = 7	<i>Ryr2</i> ^{+/+} / <i>Il6</i> ^{-/-} n = 7	<i>Ryr2</i> ^{ADA/ADA} / <i>Il6</i> ^{-/-} n = 7
HW/BW ratio	0.58 ± 0.08	1.13 ± 0.04*	0.56 ± 0.01	0.89 ± 0.02*%#
IVSd	0.68 ± 0.06	0.63 ± 0.03	0.80 ± 0.08	0.70 ± 0.06
IVSs	1.23 ± 0.06	0.80 ± 0.04*	1.24 ± 0.07	0.92 ± 0.06*%
LVIDd	1.69 ± 0.06	2.96 ± 0.10*	1.37 ± 0.13	2.75 ± 0.14*%
LVIDs	0.54 ± 0.07	2.55 ± 0.09*	0.39 ± 0.09	2.24 ± 0.16*%
LVPWd	0.67 ± 0.04	0.57 ± 0.08	0.77 ± 0.06	0.68 ± 0.07
LVPWs	1.05 ± 0.06	0.59 ± 0.07	1.29 ± 0.10	0.71 ± 0.09*%
FS (%)	68.4 ± 3.45	13.85 ± 0.88*	73.0 ± 3.9	18.9 ± 2.5*%
EF (%)	94.7 ± 1.6	30.7 ± 1.8*	96.3 ± 1.6	40.1 ± 4.6*%
HR (bpm)	580 ± 21	327 ± 30*	527 ± 24	340 ± 36*%

BW and HW, body and heart weight, respectively; IVSd, interventricular septum diastolic thickness; IVSs, interventricular septum systolic thickness; LVIDd and LVIDs, left ventricular dimensions at end diastole and systole, respectively; LVPWd and LVPWs, left ventricular posterior wall at end diastole and systole, respectively; FS, fractional shortening; EF, ejection fraction; HR, heart rate; bpm, beats/min. Data are the mean ± SEM of indicated number of mice shown in the Table.

*p < 0.05 compared to *Ryr2*^{+/+} mice #p < 0.05 compared to *Ryr2*^{ADA/ADA} mice

%p < 0.05 compared to *Ryr2*^{+/+}/*IL-6*^{-/-} mice, using 2 way ANOVA.

injected with specific STAT3 inhibitor NSC74859.³⁴ Treatment with NSC74859 significantly decreased the heart to body weight ratio in *Ryr2*^{ADA/ADA} mice compared to no change in *Ryr2*^{+/+} mice. The lifespan of *Ryr2*^{ADA/ADA} mice increased, with mean lifetimes of 18.4 ± 1.8 d and 13.9 ± 0.5 d with or without NSC74859 (p < 0.05), respectively (Fig. 5). Echocardiography showed that NSC74859 significantly improved left

ventricular dimension at end systole (2.58 vs 3.16 without NSC), fractional shortening (17.2% vs 10.2% without NSC74859, as determined by t-test) and ejection fraction (36.7% vs 22.6% without NSC) in 10-day old *Ryr2*^{ADA/ADA} mice (Table 2). Values obtained for wild type remained similar with and without NSC74859 treatment (0.42 vs 0.50, 73.0% vs 68.9%, and 96.4 % vs 95.4%, respectively).

FIGURE 3. *Ryr2*^{ADA/ADA} and *Ryr2*^{ADA/ADA}/*IL-6*^{-/-} mice survival. Mean lifetimes ± SEM of *Ryr2*^{ADA/ADA} and *Ryr2*^{ADA/ADA}/*IL-6*^{-/-} mice of 16.9 ± 1.3 (n = 9) and 25.1 ± 3.5 (n = 7), respectively, were significantly different (p < 0.05).

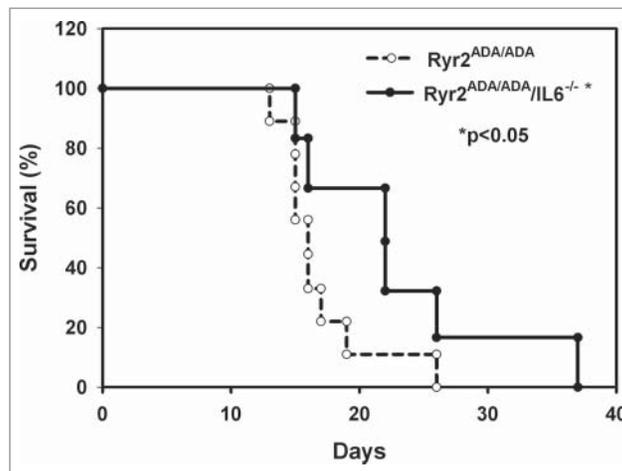
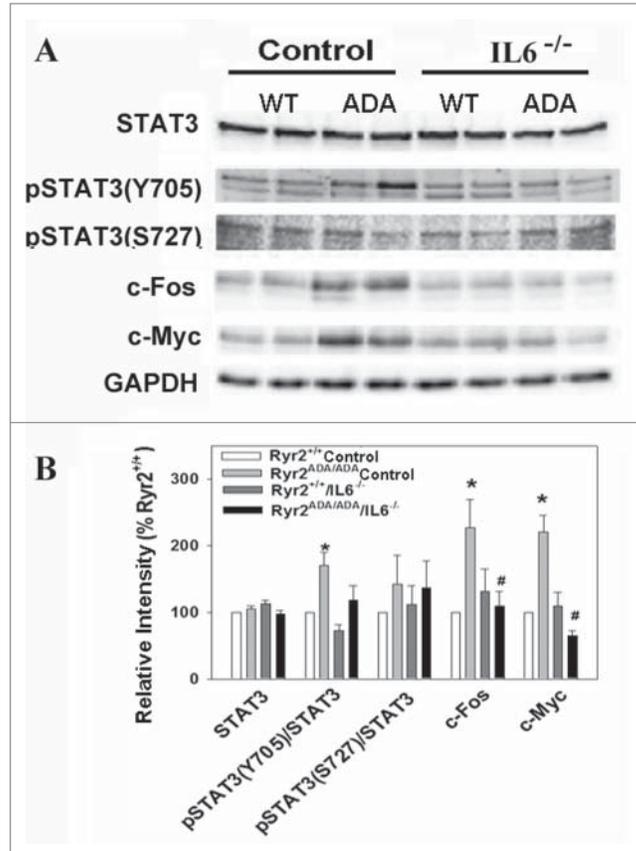


FIGURE 4. Expression of IL-6 downstream signaling molecules in mice targeted for $Ryr2^{ADA}$ and $IL-6^{-/-}$. (A) Immunoblots of heart homogenates from 10-day old $Ryr2^{+/+}$ (WT) and $Ryr2^{ADA/ADA}$ (ADA) mice with and without IL-6. GAPDH was the loading control. (B) Protein levels and phosphorylation ratios of $Ryr2^{ADA/ADA}$ mice were normalized to $Ryr2^{+/+}$. Data are the mean \pm SEM of 6–14 determinations. $p < 0.05$ compared with $Ryr2^{+/+}$, # $p < 0.05$ compared with $Ryr2^{ADA/ADA}$, using one way ANOVA.

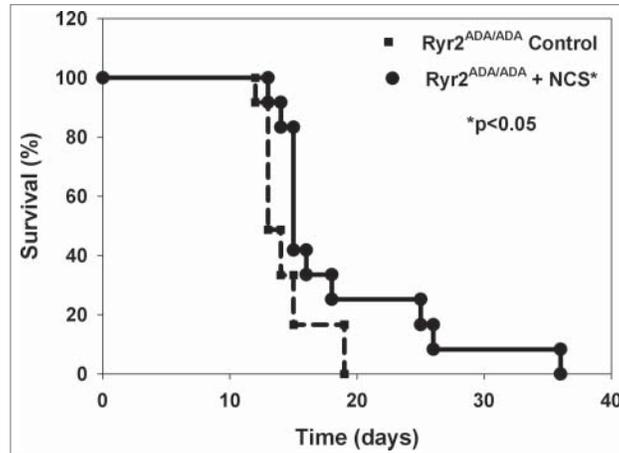


Treatment with NSC74859 significantly lowered IL-6 protein levels in both $Ryr2^{+/+}$ and $Ryr2^{ADA/ADA}$ hearts (Fig. 6). The pSTAT3-Tyr705/STAT3 ratio and the protein levels of c-Fos and c-Myc in $Ryr2^{ADA/ADA}$ mice were significantly reduced by NSC74859 without a change in $Ryr2^{+/+}$ mice. The results show that the $Ryr2^{ADA/ADA}$ phenotype is linked to elevated phosphorylation of pSTAT3 at Tyr-705 and protein levels of c-Myc and c-Fos.

Next we asked whether NSC74859 altered the mRNA levels of other cardiac hypertrophy associated genes. Quantitative RT-PCR showed that c-Fos and c-Myc mRNA levels increased in hearts from 10-day old $Ryr2^{ADA/ADA}$ mice compared with $Ryr2^{+/+}$ mice.

NSC74859 decreased 5-fold c-Fos mRNA levels in $Ryr2^{ADA/ADA}$ hearts without changing the levels in $Ryr2^{+/+}$ mice (Fig. 7). c-Myc mRNA levels were not significantly reduced in the mutant mice. In agreement with our previous report,¹⁰ brain (BNP) and atrial (ANP) natriuretic peptide mRNA levels were significantly increased in hearts from $Ryr2^{ADA/ADA}$ mice compared to $Ryr2^{+/+}$ mice. Interestingly, mRNA levels of cardiac hypertrophy associated genes BNP and ANP were not reduced by NSC74859 in hearts from $Ryr2^{ADA/ADA}$ mice. Indeed, in the case of ANP, NSC74859 increased rather than decreased the mRNA level in $Ryr2^{ADA/ADA}$ mice. The results suggest that, in addition to

FIGURE 5. Survival data from *Ryr2*^{ADA/ADA} mice treated with STAT3 inhibitor NSC74859. Mean lifetimes ± SEM of *Ryr2*^{ADA/ADA} mice treated without (Control) and with NSC74859 (NSC) of 13.9 ± 0.5 (n = 13) and 18.4 ± 1.8 (n = 13), respectively, were significantly different (p < 0.05).



IL-6/STAT3 signaling, other mechanisms are associated with the *Ryr2*^{ADA/ADA} phenotype.

various tissues including heart and is synthesized in fibroblasts and cardiomyocytes.³⁵ Elevation of IL-6 by chronic infusion was associated with left ventricular hypertrophy, fibrosis, and dysfunction,³⁶ overexpression of both IL-6 and IL-6 receptor developed myocardial hypertrophy,²⁸ and cardiac hypertrophy induced by angiotensin II infusion was attenuated by deletion of IL-6.³⁷ On the other hand, deletion of IL-6 did not attenuate left

DISCUSSION

Our findings suggest that aberrant Ca²⁺ handling in *Ryr2*^{ADA/ADA} mice increases IL-6 expression and activation of STAT3 through Tyr-705 phosphorylation. IL-6 is released by

Table 2. Body and heart weights and echocardiography of 10-day old *Ryr2*^{+/+} and *Ryr2*^{ADA/ADA} mice with and without NSC74859.

	Control		NSC74859	
	<i>Ryr2</i> ^{+/+} n = 7	<i>Ryr2</i> ^{ADA/ADA} n = 6	<i>Ryr2</i> ^{+/+} n = 7	<i>Ryr2</i> ^{ADA/ADA} n = 8
HW/BW ratio	0.58 ± 0.01	1.03 ± 0.03*	0.57 ± 0.01	0.94 ± 0.02*%#
IVSd	1.01 ± 0.06	0.75 ± 0.08	0.89 ± 0.7	0.57 ± 0.06*%#
IVSs	1.50 ± 0.05	0.88 ± 0.09*	1.37 ± 0.06	0.78 ± 0.07*%#
LVIDd	1.55 ± 0.14	3.52 ± 0.20*	1.52 ± 0.10	3.14 ± 0.13*%#
LVIDs	0.50 ± 0.11	3.16 ± 0.20*	0.42 ± 0.07	2.58 ± 0.13*%#
LVPWd	0.96 ± 0.15	0.68 ± 0.06	0.86 ± 0.08	0.74 ± 0.06
LVPWs	1.28 ± 0.09	0.79 ± 0.07*	1.24 ± 0.07	0.85 ± 0.09*%#
FS (%)	68.9 ± 3.6	10.2 ± 2.4*	73.0 ± 3.7	17.2 ± 1.7*%and
EF (%)	95.4 ± 1.7	22.6 ± 12.6*	96.4 ± 1.2	36.7 ± 3.4*%#
HR (bpm)	514 ± 35	340 ± 31*	598 ± 24	329 ± 32*%#

Abbreviations are as in Table 1. Data are the mean ± SEM of indicated number of mice.

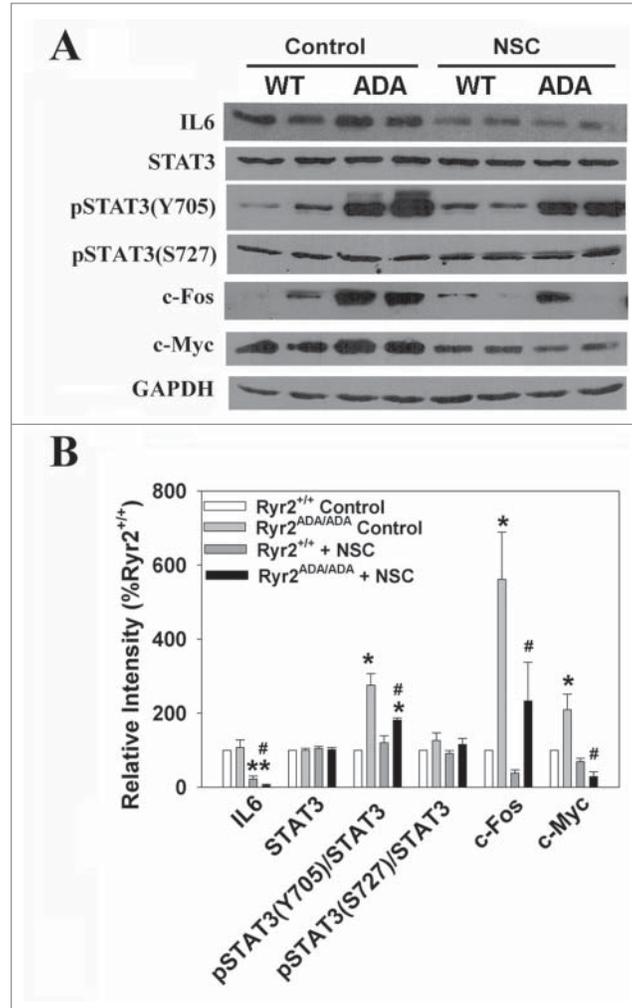
*p < 0.05 compared to wild-type mice without NSC74859

#p < 0.05 compared to *Ryr2*^{ADA/ADA} mice without NSC74859

%p < 0.05 compared to wild-type with NSC74859, using 2 way ANOVA

and#p < 0.05 compared to *Ryr2*^{ADA/ADA} mice without NSC74859, as determined by t-test.

FIGURE 6. IL-6 and downstream signaling molecule levels in 10-day old mice treated with or without STAT3 inhibitor NSC74859. **(A)** Immunoblots of heart homogenates from 10-day old *Ryr2*^{+/+} (WT) and *Ryr2*^{ADA/ADA} (ADA) mice treated with or without NSC74859 (NSC). GAPDH was the loading control. **(B)** Protein levels and phosphorylation ratios of *Ryr2*^{ADA/ADA} mice were normalized to *Ryr2*^{+/+} Control. Data are the mean \pm SEM of 4–10 samples. * $p < 0.05$ compared to *Ryr2*^{+/+} mice without NSC74859, # $p < 0.05$ compared to *Ryr2*^{ADA/ADA} mice without NSC74859, using one way ANOVA.

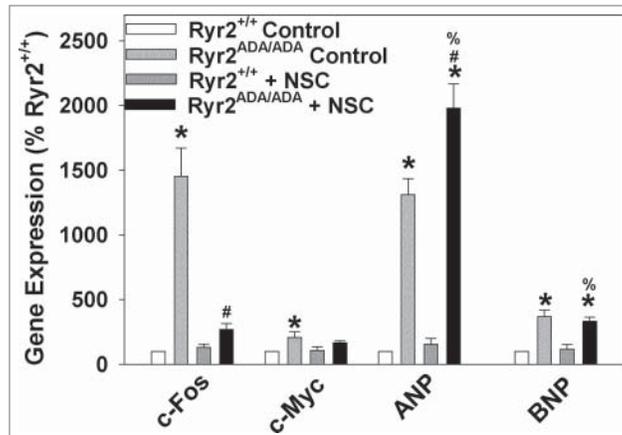


ventricular remodeling or dysfunction in pressure-overloaded hearts.³⁸ We find that IL-6 protein levels increased in 1-day old but not in 10-day old *Ryr2*^{ADA/ADA} mice. *Ryr2*^{+/+}/*IL-6*^{-/-} mice carrying a disrupted IL-6 gene lived longer and had a decreased heart size without significantly altered cardiac performance. In addition, loss of IL-6 decreased protein levels of c-Fos and c-Myc, 2 STAT3-responsive transcription factors^{25,27} that are rapidly induced in

the ventricular myocardium by pressure overload.³⁹

To obtain evidence that IL-6 mediated its effects through phosphorylation of STAT3 at Tyr-705, *Ryr2*^{ADA/ADA} mice were treated with the STAT3 inhibitor NSC74859 (S3I-201). NSC74859 interacted with the STAT3 SH2 domain and preferentially inhibited growth in cells that contained STAT3 activated by phosphorylation at Tyr-705 by inhibiting STAT3

FIGURE 7. c-Fos, c-Myc, ANP and BNP mRNA levels in hearts of *Ryr2*^{+/+} and *Ryr2*^{ADA/ADA} mice treated with or without STAT3 inhibitor NSC74859. mRNA levels were measured by quantitative RT-PCR and normalized to levels in hearts of *Ryr2*^{+/+} mice not treated with the inhibitor (*Ryr2*^{+/+} Control). Data are the mean \pm SEM of 5–6 samples. **p* < 0.05 compared to *Ryr2*^{+/+} mice without NSC74859, #*p* < 0.05 compared with *Ryr2*^{ADA/ADA} mice without NSC74859, %*p* < 0.05 compared with *Ryr2*^{+/+} mice with NSC74859, using one way ANOVA.



dimer formation and STAT3 DNA-binding and transcriptional activities.³⁴ NSC74859 was effective in inhibiting angiotensin-II induced oxidative stress, endothelial dysfunction and hypertension in 4–5 month old mice⁴⁰ and in decreasing heart size, improving cardiac function and inhibiting cardiac hypertrophy markers such as ANP, c-Fos and c-Myc in a renal artery ligated rat model.²⁶ In the present study, in agreement with the results from the rat model,²⁶ daily injection of NSC74859 significantly increased lifespan and decreased c-Fos and c-Myc protein levels in *Ryr2*^{ADA/ADA}/*Il-6*^{-/-} mice. However, at variance with ANP expression at the protein level in the renal artery ligated rat model,²⁶ ANP mRNA expression was increased in *Ryr2*^{ADA/ADA} by NSC74859 without a change in *Ryr2*^{+/+} mice. Thus the pathological effects of the *RyR2*^{ADA} mutation appear to be mediated in part by additional mechanisms that may include an upregulation of calcineurin A- β ¹³ and mTOR¹⁴ signaling. Another possibility is that nuclear accumulation of unphosphorylated STAT3 contributed to the *Ryr2*^{ADA/ADA} phenotype. NSC74859 was less effective in reducing growth and viability in normal fibroblast NIH3T3 cells than transformed NIH3T3 cells

that harbored activated pSTAT3-Tyr705,³⁴ which suggests that nuclear STAT3 may promote the activation of genes that do not respond directly to phosphorylated STAT3.⁴¹

An intriguing result from our studies was evidence that STAT3 inhibition by NSC74859 reduced the IL-6 protein level in hearts of *Ryr2*^{+/+} and *Ryr2*^{ADA/ADA} mice. This could suggest at first sight that the effects of NSC74859 are due to its direct effect on IL-6 expression. However, this appears unlikely, as there is no evidence of a direct interaction with IL-6. More likely, NSC74859 inhibited IL-6 expression by inhibiting STAT3 transcriptional activity.³⁴ Myocardial IL-6 expression is regulated by multiple factors. Hypoxia-induced activation of IL-6 was mediated by transcriptional factors NF-IL-6 and NF- κ B in primary cultures of myocytes prepared from 1-day old rat hearts.⁴² In skeletal muscle, activator protein-1, NF- κ B and inositol trisphosphate release of Ca²⁺ were involved in upregulation of IL-6 transcription.⁴³ In 8-week old mouse hearts, STAT1 promoted the expression of IL-6 and countered the suppressive role of p53.⁴⁴ In adult rat ventricular myocytes, IL-1 and β -adrenergic receptor synergistically increased IL-6 expression and secretion.²⁹ Further,

coordinate regulation of STAT3 and microRNAs suggests that IL-6 expression was regulated by microRNAs.⁴⁵ Future studies will be required to clarify the relationship between the effects of NSC74859 on STAT3 signaling and suppression of IL-6 expression in the hearts of young *Ryr2^{ADA/ADA}* mice.

In summary, our results indicate that impaired inhibition of RyR2 by CaM resulted in increased IL-6 expression, phosphorylation of STAT3 at Tyr-705, and nuclear accumulation of both phosphorylated and unphosphorylated STAT3. Generation of double targeted *Ryr2^{ADA/ADA}/IL-6^{-/-}* mice and treatment of *Ryr2^{ADA/ADA}* mice with STAT3 inhibitor NSC74859 prolonged life span, decreased heart size, and decreased the expression levels of proteins associated with IL-6/STAT3 signaling. The results suggest that upregulation of IL-6 and STAT3 signaling contribute to the rapid development of cardiac hypertrophy and early death of *Ryr2^{ADA/ADA}* mice.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

No potential conflicts of interest were disclosed.

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