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The adenylosuccinate synthetase-1 gene is activated in the hypertrophied heart

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Abstract

Adenylosuccinate synthetase 1 (ADSS1) functions as an important component in adenine nucleotide biosynthesis and is abundant in the heart. Here we report that the *Adss1* gene is up-regulated in two *in vivo* rodent models of surgically induced cardiac hypertrophy. In addition, we examined an *in vitro* hypertrophy system of rat neonatal cardiomyocytes treated with angiotensin II to study *Adss1* gene regulation. We show that this stimulus triggers a signaling cascade that results in the activation of the *Adss1* gene. The induction of *Adss1* gene expression was blocked by cyclosporin A *in vitro*, suggesting that calcineurin, a calmodulin activated phosphatase, is involved in this signaling pathway. Consistent with this view we provide evidence that the induction of *Adss1* by angiotension II requires the presence of an NFAT binding site located 556 base pairs upstream of the *Adss1* transcription start site. We propose that ADSS1 plays a role in the development of cardiac hypertrophy through its function in adenine nucleotide biosynthesis.

Keywords: cardiac hypertrophy - adenylosuccinate synthetase 1 - gene regulation - signaling pathway - calcineurin - nuclear factor of activated T cells - angiotensin II - rodent

Introduction

Cardiac hypertrophy is an adaptive response of the heart to stress, including hypertension, mechanical overload, myocardial infarction and genetic mutations in cardiac contractile protein genes [1,2]. In response to stress, intracellular Ca^{2+} concentrations increase [3-6], consistent with a role for Ca^{2+} in coordinating physiologic responses with enhanced cardiac output, both acutely and chronically. Thus Ca^{2+} provides an important signal for the development of cardiac hypertrophy. Although changes in the expression of contractile and structural genes are well known in cardiac hypertrophy, the genes encoding enzymes of cardiac metabolism and their signal pathways in the cardiac hypertrophy are less well studied. In the current study we were especially interested in changes in cardiomyocyte nucleotide metabolism in response to cardiac hypertrophy, and have focused on the muscle specific isoform of adenylosuccinate synthetase 1

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(*Adss1*), an enzyme of adenine nucleotide metabolism. *Adss1* functions at a critical branch point in purine nucleotide metabolism where it controls the synthesis of adenine nucleotides (AMP, ADP, and ATP) from the purine nucleotide intermediate IMP. There is increased demand for and biosynthesis of purine nucleotides in hypertrophied mice heart induced by pressure overload [7,8]. In view of the importance of *Adss1* in the synthesis of adenine nucleotides we examined whether *Adss1* gene expression is enhanced in cardiac hypertrophy, and if so, what is the intracellular signaling pathway regulating this change. We show that the *Adss1* gene is up-regulated in cardiac hypertrophy both *in vivo* (surgically induced cardiac hypertrophy in rats and mice) and *in vitro* (angiotension II induced hypertrophy in neonatal cardiomyocytes). The latter was utilized to more easily identify and characterize the signaling pathways controlling *Adss1* gene expression in cardiac hypertrophy. We also show that cyclosporin A or deletion of an NFAT binding site in the *Adss1* 5' flanking region both block Ang II induced *Adss1* induction in vitro. These findings indicate that the induction of *Adss1* is mediated by NFAT dephosphorylation as a consequence of calcineurin activation. We therefore propose that the *Adss1* gene may play an important role in the development of cardiac hypertrophy through its function as an important enzyme in adenine nucleotide synthesis.

Materials and methods

Surgically induced cardiac hypertrophy in rats and mice

The ascending aortas of rats and mice were constricted by 50% for 7 or 14 days, respectively, resulting in an increase in heart weight to body weight ratio of approximately 25%. Sham operated animals were used as controls. These are well-established procedures for inducing cardiac hypertrophy in both rat [9] and mouse [10].

Cardiomyocyte cell culture and angiotensin II stimulation

Cardiac myocytes were isolated from 2-day old rats and plated in 35 mm six well plates at a density of 5 X 105 cells per well [11]. Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Hyclone). Some cells were plated onto coverslips for fixation and morphological analysis. After 24 h, the 10% serum medium was removed, and the cells were washed and maintained in serum free DMEM containing 1% bovine serum albumin in the absence or presence of Ang II (10 nM). In other experiments cyclosporin A (CSA, 1 mM) was added to both control and Ang II treated cells at the initiation of Ang II stimulation.

RNA isolation and Northern blot analysis

RNA was isolated from cultured cardiomyocytes and from isolated heart tissue from aortic banded and sham operated rats using the Trizol isolation system according to the directions provided by the supplier (Gibco BRL). For Northern analysis, total RNA was fractionated by electrophoresis on 1% agarose gels containing 0.6% formaldehyde at 50 V for 3h. Fractionated RNA was transferred onto a Duralon-UV nylon membrane (Stratagene, La Jolla, CA) by capillary action overnight. After blotting, the RNA was cross-linked to the filter by UV irradiation. Following pre-hybridization (50% formamide, 25 mM potassium phosphate, 5×SSC, 5× Denhart's solution and 100 μg/ml salmon sperm DNA) overnight at 42 °C, the blots were hybridized in the prehybridization solution plus 1% dextran sulfate and the labeled DNA fragment. The StyI-BamH1 genomic fragment spanning exon 1 of the Adss1 gene [12] was used as probe and labeled using the random primer labeling kit from Promega. After washing, the blots were visualized by autoradiography.

Quantitative RT-PCR analysis

Specific quantitative assays were employed for rat α-MHC, and β-MHC as described previously [13]. In each case, 100ng of total RNA (prepared as described above) was reverse transcribed 30 min at 42°C, with 400 nM specific reverse primer, 2.5 mM $MgCl_2$ 50 mM KCl, 10 mM Tris buffer (pH8.3 at 20°C), 500 mM deoxynucleotides and 0.1 mU reverse transcriptase (superscript II, Life Technologies) in a total volume of 10 ml. The RT reaction was used for quantitative two-step PCR at 95°C for 1 min, followed by 40 cycles of 95°C 12 second, 60°C 1 min, in the presence of 400 nM specific forward and reverse primers, 100 nM specific fluorogenic probe, 2.5 mM $MgCl_2$ 50 mM KCl, 10 mM Tris buffer (pH 8.3 at 20 °C), 200 mM deoxynucleotides and 1.2 U Taq polymerase (Boehringer) in a final volume of 50 ml. The level of transcripts for constitutive housekeeping gene cyclophilin was quantitatively measured in each sample, to control for sample-to-sample differences in RNA concentration.

Preparation of plasmids and transient transfections

The reporter construct, 1.9 *Adss1*/CAT [12] has been described before. The putative NFAT binding site within 1.9 *Adss1*/CAT was disrupted using a PCR mutagenesis procedure [14]. The primers were designed to replace the NFAT consensus sequence (TGGAAAGT) in the *Adss1* promotor with a Kpn I cutting site as CCATGG. The primers that were used for PCR-based mutagenesis of the NFAT site are as follows (mutated nucleotides are capitalized): P1, 5'-CCATGGattgaatcctctgctcctgtgtcct-3'; P2, 5'-aagagacctcggggtcgatggtttct-3'. Rat cardiac myocytes were cultured as described above, transfected with plasmid vectors and assayed for CAT activity and β-galactosidase as described [11]. Cells in six-well dishes were transfected with a 1.9 *Adss1*/CAT reporter plasmid (2 mg) or a 1.9 *Adss*/CAT reporter with the NFAT site mutation (2 mg).

Fluorescent microscopic techniques

The morphological changes in cardiac myocyte size and sarcomere alignment were determined in both control and Ang II treated cells in the absence and presence of 1 mM of cyclosporin A for 24 h. Cells were stained for actin with BODIPY FL phallacidin (Molecular Probes, Inc., Eugene, OR). The fluorescent images of the myocytes on the coverslips were viewed and taken using an Olympus BX 60 fluorescent microscope equipped with dark field optics with a green filter and photographed using a SPOT digital camera (Diagnostics).

Statistical analysis

The significance of the experimental differences were determined using Student's t Test (two groups) or ANOVA (three groups or more) ($p<0.01$). Data are presented as the mean ± standard error (SE).

Results

The Adss1 gene is induced by pressure overload in aorta banded rats and mice.

To determine if *Adss1* gene expression is induced in response to pressure overload *in vivo*, the aortas of adult rats were constricted 50% for 7 days. As a result of this experimentally induced cardiac hypertrophy, at day 7, the ratio of heart weight to bodyweight of rats had reached its maximal increase of approximately 25%. Sham operated rats were used as controls. Total RNA was isolated from control and hypertrophied hearts and *Adss1* mRNA was detected by northern blot hybridization using an *Adss1* DNA probe. Fig. 1 shows a significant up-regulation of cardiac *Adss1* expression in the rat model of experimentally induced hypertrophy. To test the generality of *Adss1* gene activation in cardiac hypertrophy we also examined RNA from hypertrophied mouse heart. Hypertrophy was experimentally induced by aorta banding (50% constriction) for 14 days, resulting in an increased ratio of heart weight to body weight of approximately 25%. Heart from sham-operated mouse was used as control. The results (Fig. 1) show a significant increase in the abundance of *Adss1* RNA in the hypertrophied mouse hearts relative to sham-operated controls. Thus, *Adss1* gene expression is significantly up regulated in response to pressure overload in hypertrophied hearts of rats and mice.

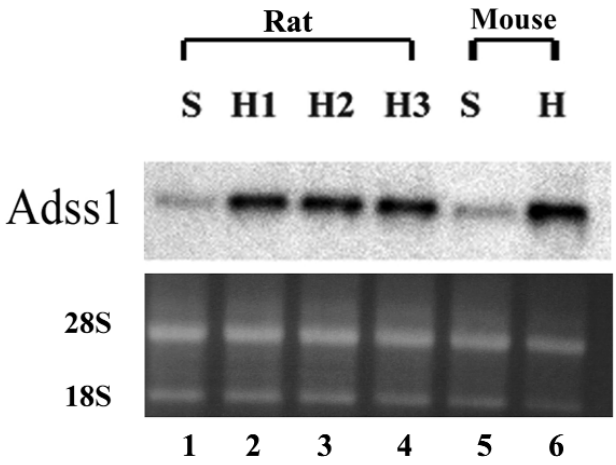

Fig. 1 ***Adss1*** **mRNA abundance in control and hypertrophied hearts**. The aortas of rats and mice were constricted 50% for 7 and 14 days, respectively. As a result of this experimental procedure the ratio of heart weight to body weight increased approximately 25%. Total RNA was isolated from the hearts of control (sham operated) and aorta banded animals and *Adss1* mRNA abundance determined by Northern blot analysis using an *Adss1* DNA probe. Ethidium bromide stained rRNA is shown as a loading control. S=sham operated hearts. H=hypertrophied hearts.

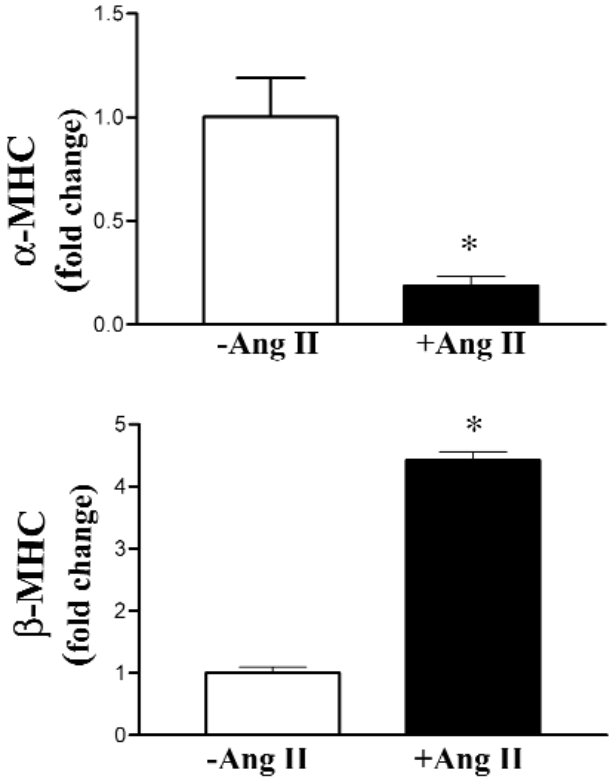

Fig. 2 The effect of Ang II stimulation on the relative abundance of a-MHC and b-MHC mRNA in rat neonatal cardiomyocytes. Total RNA was isolated from neonatal cardiomyocytes that were incubated in the presence or absence of Ang II for 24 hr. a-MHC and b-MHC mRNA abundance were determined by quantitative PCR and expressed as fold change relative to the abundance of that of control cardiomyocytes without Ang II treatment. The results are expressed as the mean ± S.E. of three independent determinations. * Indicates significant difference compare to that of control cardiomyocytes in the absence of Ang II ($p < 0.01$).

β-MHC mRNA was induced and α-MHC mRNA was repressed in Ang II treated cultured cardiac myocytes.

To facilitate the analysis of *Adss1* gene expression in hypertrophied myocardium we employed an *in vitro* model of cardiomyocyte hypertrophy. For this purpose we used Ang II to increase the force of contraction in cultured rat neonatal cardiac myocytes. In this assay neonatal rat cardiac myocytes were cultured for 24 h in the presence or absence of Ang II. The cardiac myocytes were collected and total RNA was extracted. We determined the levels of α-MHC and β-MHC mRNA using quantitative RT-PCR (QPCR). In Ang II treated cardiac myocytes, we observed a reciprocal change in the abundance of α-MHC and β-MHC mRNA (Fig. 2). β-MHC, the fetal isoform, increased significantly while α-MHC, the mature isoform, decreased significantly in response to Ang II. The results of quantitative RT-PCR confirmed MHC isoform switching in the Ang II treated cardiac myocytes. These data lend further support to the concept that Ang II treated cardiomyocytes are a useful model for cardiac hypertrophy.

The Adss1 gene is activated in Ang II treated rat cardiac myocyte cultures.

To detect *Adss1* gene expression in cardiac hypertrophy, rat cardiac myocytes were cultured for up to 24 h in the presence or absence of Ang II. The cardiac myocytes were collected at 12h and 24h and total RNA was extracted. Northern blot data (Fig. 3) showed that the *Adss1* mRNA was significantly more abundant by 24 h of Ang II stimulation. Thus, the *Adss1* gene is induced in this Ang II induced cardiac hypertrophy culture system.

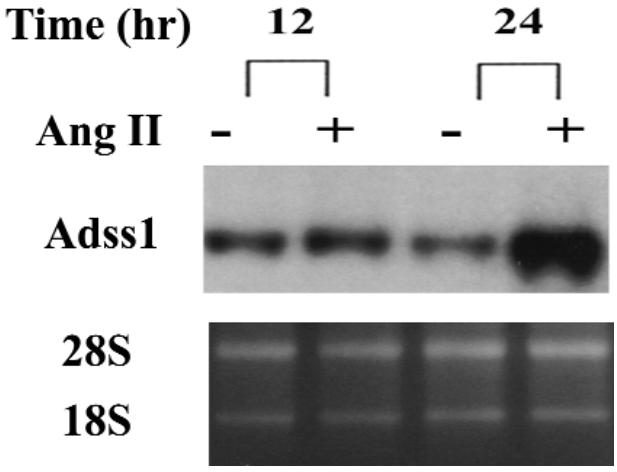

Fig. 3 The effect of Ang II stimulation on *Adss1* mRNA abundance in rat neonatal cardiomyocytes. Total RNA was isolated from neonatal cardiomyocytes that were incubated in the absence (-) or presence (+) of 10 nM Ang II for 12 or 24 hr. *Adss1* mRNA abundance was determined by northern blot analysis using an *Adss1* DNA probe. Ethidium bromide stained rRNA is shown as a loading control.

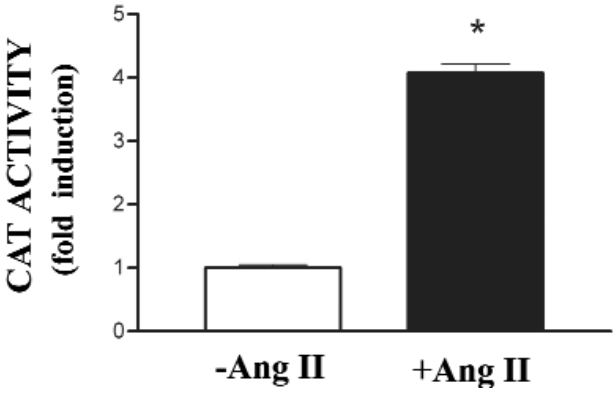

Fig. 4 The effect of Ang II stimulation on *Adss1* gene expression in cultured cardiomyocytes. The 1.9*Adss1*/CAT reporter construct was transfected into control and Ang II treated cardiomyocytes. After 24 hr cardiomyocytes were harvested, extracts prepared and CAT activity determined. CAT activity is expressed as a fold induction over the level of CAT activity in control cardiomyocytes without Ang II treatment. The results are expressed as the mean ±S.E. of three independent determinations. * Indicates significant difference compare to that of control cardiomyocytes without Ang II treatment ($p < 0.01$).

Expression of the 1.9 Adss1/CAT reporter construct was significantly activated in Ang II stimulated cardiac myocytes.

Previous studies have shown that the immediate 1.9 kb of the 5'-flanking sequence of the mouse *Adss1* gene contains all the cis-regulatory elements required to achieve proper activation and expression in the cardiac lineage [14]. To determine whether this regulatory region also contains genetic recognition signals that respond to Ang II induction, we measured the expression of 1.9 *Adss1*/CAT reporter construct following transfection in control and Ang II stimulated cardiac myocytes. We found that the expression of the 1.9 *Adss1*/CAT reporter construct was significantly increased in the Ang II stimulated cardiomyocytes (Fig. 4). These results indicate that the *Adss1* promoter and 1.9 kb of 5'-flanking sequence contain regulatory sequences that respond to Ang II induced changes in cardiac energy demand.

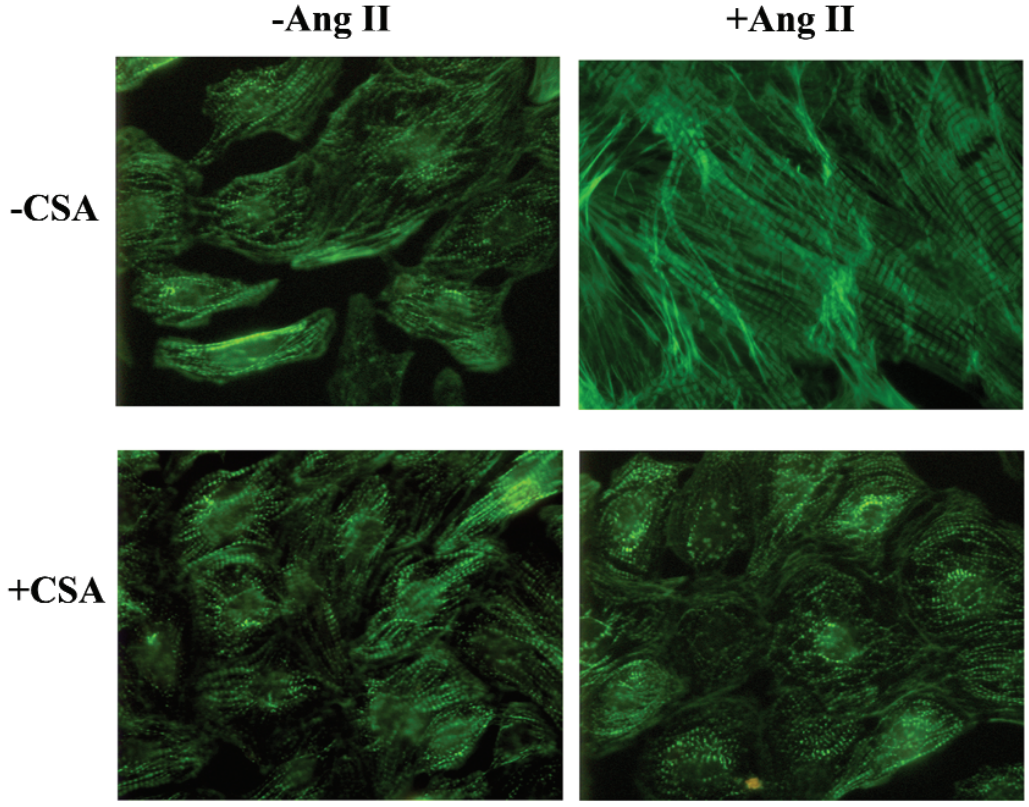

Fig. 5 The effect of cyclosporin A on Ang II induced changes in cardiomyocyte morphology. Control and Ang II treated cardiomyocytes were maintained in the presence or absence of cyclosporin A (CSA). After 48 hr cardiomyocytes were fixed and stained with BODIPY FL phallacidin to visualize actin. The results show that cyclosporin A blocks Ang II induced morphological changes in neonatal cardiomyocytes.

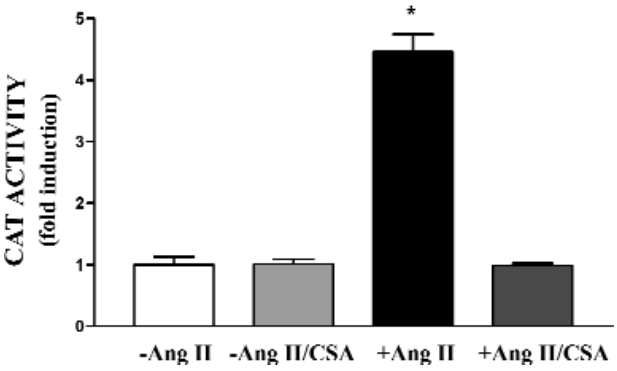

Fig. 6 The effect of cyclosporin A on Ang II induced activation of *Adss1* gene expression. The 1.9*Adss1*/CAT reporter construct was transfected into control and Ang II treated cardiomyocytes in the presence or absence of cyclosporin A (CSA). After 24 hr cardiomyocytes were harvested, extracts prepared and CAT activity determined. CAT activity is expressed as a fold induction over the level of CAT activity in control cardiomyocytes without Ang II and cyclosporin A (CSA) treatment. The results are expressed as the mean ±S.E. of three independent determinations. * Indicates significant difference compare to that of control cardiomyocytes without Ang II and CSA treatment ($p < 0.01$).

The increase in Adss1 gene expression following Ang II stimulation is blocked by Cyclosporin A.

Ang II stimulation of rat neonatal cardiac myocytes results in a significant increase in intracellular calcium [1]. Calcium can influence cardiac gene expression and the development of cardiac hypertrophy by a calcium/calmodulin-mediated activation of calcineurin, an intracellular phosphatase. Calcineurin activation and the cellular consequences of this activation, i.e. hypertrophy, are prevented by cyclosporin A. To determine whether Ang II stimulation regulates *Adss1* gene expression through a calcineurin-dependent pathway, we examined *Adss1* gene expression in control and Ang II stimulated cardiomyocytes in the presence and absence of cyclosporin A. Gene transfection experiments were conducted to determine whether cyclosporin A could block the induction of *Adss1* gene expression seen in the Ang II stimulated cardiomyocytes. For this purpose, the 1.9*Adss1*/CAT construct was introduced into control and Ang II stimulated cardiac myocytes in the presence or absence of cyclosporin A. The level of CAT activity in the cell extracts was determined 24h following transfection. As shown in Fig. 5, the cyclosporin A completely blocked the Ang II induced activation of the 1.9*Adss1*/CAT reporter construct in the Ang II stimulated cardiomyocytes. These results strongly suggest that Ang II induces *Adss1* gene expression through a calcineurin-dependent pathway.

Morphological changes induced by Ang II stimulation were blocked by Cyclosporin A.

We also examined the effect of cyclosporin A on the morpholocical changes induced by Ang II in the cardiac myocytes. After 24h, control and Ang II treated cardiomyocytes, without and with cyclosporin A, were fixed and examined following labeling with the actin stain, BODIPY FL phallacidin. Ang II treated myocytes in the absence of cyclosporin A fused and formed myotubes. They became much larger and more mature than control cells, and the actin fiber staining demonstrated a high degree of actin fiber organization (Fig. 6). However, these changes were blocked by the presence of cyclosporin A in the Ang II treated cultures (Fig. 6). Cyclosporin A had no effect on control cultures (Fig. 6). These data further support the view that the cellular response to Ang II is mediated through a calcineurin-dependent pathway.

The Ang II mediated induction of Adss1 gene expression requires the presence of an NFAT binding site in the 5'-flanking region of Adss1 gene.

It is well known that NFAT transcription factors translocate from the cytoplasm to the nucleus following dephosphorylation by calcineurin, a calcium/calmodulin activated phosphatase [1, 15-17]. Cyclosporin A is known to prevent the activation of calcineurin by binding to cyclophilins and competing for the calcium/calmodulin binging site [1,15]. Thus the fact that cyclosporin A blocks the induction of the *Adss1* gene following Ang II stimulation suggests that this induction may be mediated through the action of NFAT. An NFAT consensus sequence ((A/T)GGAAAN(A/T/C)) is located at 556 bp upstream of the *Adss1* transcription start site within 1.9 kb *Adss1* cardiac control region. To test the importance of the NFAT consensus sequence, we prepared a mutationally altered 1.9 *Adss1*/CAT reporter construct in which the NFAT

binding site was destroyed by site-directed mutagenesis. The control 1.9*Adss1*/CAT construct and a 1.9*Adss1* construct with the mutant NFAT binding site (MNFAT) were transfected into control and Ang II stimulated cardiomyocytes. After 24h, the transfected cells were harvested and extracts were tested for CAT activity. The results (Fig. 7) indicate that mutation of the NFAT consensus sequence prevented induction of the reporter gene in the Ang II stimulated cardiomyocytes. However, there was no difference in reporter gene activity between 1.9*Adss1*/CAT promoter construct and NFAT mutant construct in untreated cardiac myocytes (Fig. 7). Therefore, the NFAT consensus sequence in the *Adss1* 5'-flanking region is required for enhanced *Adss1* gene expression in the response to Ang II stimulation, but it is not required for basal expression.

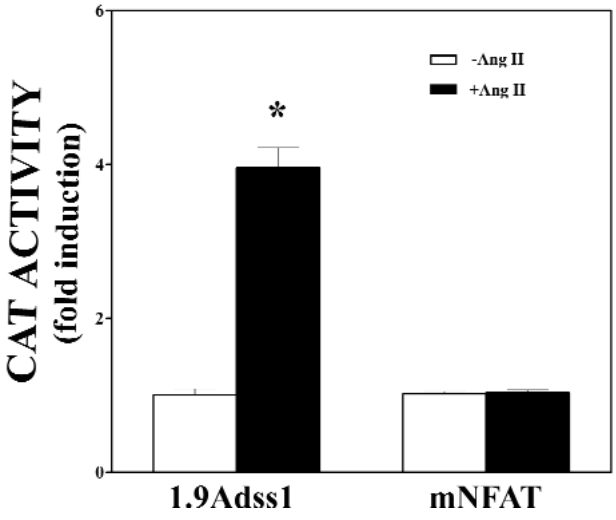

Fig. 7 The effect of an NFAT binding site mutation on Ang II induced activation of *Adss1* gene expression. The wild type (*Adss1*) and NFAT mutant (mNFAT) 1.9*Adss1*/CAT reporter constructs were transfected into control and Ang II treated cardiomyocytes. After 24 hr cardiomyocytes were harvested, extracts prepared and CAT activity determined. CAT activity is expressed as a fold induction over the level of CAT activity in control cardiomyocytes transfected with the wild type *Adss1*/CAT construct without Ang II treatment. The results are expressed as the mean ±S.E. of three independent determinations. * Indicates significant difference compare to that of control cardiomyocyte without Ang II treatment ($p < 0.01$).

Discussion

Restriction of aortic outflow in the hearts of mice and rats leads to cardiac hypertrophy with a substantial increase in the ratio of heart weight to body weight. We report here that each rodent model of cardiac hypertrophy is accompanied with a major increase in *Adss1* gene expression. To more easily manipulate, identify and characterize the signaling pathways controlling *Adss1* gene expression during cardiac hypertrophy we employed an *in vitro* model system that retains many of the important features seen in the surgically induced *in vivo* models. In cultured rat neonatal cardiac myocytes stimulated with Ang II, the β-MHC gene, which is the fetal isoform, is re-expressed and highly induced; α-MHC, the adult isoform, is repressed. This feature of myosin heavy chain isoform switching is commonly seen in cardiac hypertrophy [18,19]. Morphological analysis of Ang II treated cardiomyocytes revealed an increased size and actin filament organization, characteristic of mature cardiomyocytes. We show here that Ang II triggers a signaling cascade that results in the activation of the *Adss1* gene, thereby providing us a convenient opportunity to identify the signaling pathways linking *Adss1* gene expression with cardiac hypertrophy.

We have also shown that both the enlargement and differentiation of cardiomyocytes and the induction of the *Adss1* gene in response to Ang II treatment is blocked by cyclosporin A, indicating that the activation of calcineurin, a cellular phosphatase, is involved in the signaling cascade [1,15]. Activated calcineurin is known to catalyze the dephosphorylation of cytoplasmic NFAT, allowing the translocation of the latter into the nucleus, where it binds to its specific DNA sequences, resulting in synergistic activation of cardiac gene transcription. Transgenic mice that express activated forms of calcineurin or NFAT in the heart develop cardiac hypertrophy and heart failure that mimic important aspects of human heart disease. Cyclosporin A inhibition of calcineurin activity blocks hypertrophy both *in vivo* and *in vitro* [1]. We have shown here that an NFAT binding site located 556 bp upstream of the *Adss1* transcription start site is required for activation of the *Adss1* gene in response to Ang II stimulation of neonatal cardiomyocytes. Since *Adss1* gene expression is up-regulated in cardiac hypertrophy,

and both *Adss1* gene up-regulation and cardiac hypertrophy are inhibited by cyclosporin A, this strongly suggest that increased *Adss1* gene expression is closely associated with the development of cardiac hypertrophy. However, the absence of the NFAT binding site did not affect the basal level of *Adss1*/CAT reporter gene expression in untreated cardiomyocytes. These results indicate that the NFAT site is essential for the *Adss1* gene response to Ang II stimulation, but that it is not required for basal cardiac expression. The calcineurin-NFAT pathway has also been implicated in the activation of β-MHC, skeletal α-actin and b-type natriuretic peptide (BPN) during development of cardiac hypertrophy [1,20]. Although additional signaling pathways may be important, the evidence presented here indicates that calcium-mediated signaling pathways involved the activation of calcineurin play a very important role in the cardiac hypertrophy process through its activation of a specific set of genes, including β-MHC, skeletal α-actin, BPN and *Adss1*.

Why is *Adss1* gene expression enhanced in the development of cardiac hypertrophy? It is very likely related to its function in adenine nucleotide biosynthesis [21-24]. Adenine nucleotide biosynthesis increases several folds in the hypertrophied rodent heart and this enhancement of adenine nucleotide synthesis occurs very early and precedes the increase of protein synthesis [7,8,21-25]. It is known that the RNA content of cardiac hypertrophied rodent is increased [26]. In addition, a polyadenylate tail of about 250 adenylate residues is added to most mRNA precursors to increase RNA stability. For this reason, a greater dependance on ATP results, compared to other nucleotides in the synthesis and post transcriptional modification of mRNA. Consistant with the observations in the pressure overloaded expression of *Adss1* and other enzymes of adenine nucleotide synthesis are up-regulated in the diabetic heart [23]. These observations have lead us to hypothesize, that increased adenine nucleotide production meets the need for increased RNA synthesis in the hypertrophied heart, and that increased *Adss1* gene expression is involved in the development of cardiac hypertrophy through its function in the adenine nucleotide synthesis.

Our previous work has shown that the *Adss1* gene is activated at the beginning of cardiac lineage in the early embryo and is then massively up-regulated after birth and remains highly expressed in the adult heart [27]. We have shown here that the *Adss1* gene is further up-regulated in response to cardiac hypertrophy, both *in vivo* and *in vitro*. We have also shown that the *Adss1* gene is induced through the NFAT-calcineurin pathway in the development of cardiac hypertrophy. It is likely that NFAT may interact with or recruit other transcription factors to assist in the activation of the *Adss1* gene. In the hypertrophied heart, NFAT interacts with GATA4 to activate β-MHC and b-type natriuretic peptides (BPN) [1,20]. Calcineurin-dependent gene regulation in skeletal myocytes is mediated by both NFAT and MEF2C [28]. MEF2C, GATA4 and Nkx2.5 play important role in *Adss1* gene expression during cardiac development [27]. Furthermore, the β-MHC gene is also expressed as part of the cardiac myogenic program under the control of Nkx2.5, MEF-2C and GATA4 protein during embrygenesis [29,30]. These suggest that one or more of these transcription factors may play a role by interaction with NFAT to regulate the *Adss1* gene up-regulation in the development of cardiac hypertrophy.

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