Enhanced expression of DYRK1A in cardiomyocytes inhibits acute NFAT activation but does not prevent hypertrophy in vivo

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Aims

The calcineurin and nuclear factor of activated T cells (NFAT) pathway can mediate pro-hypertrophic signalling in the heart. Recently, it has been shown that dual-specificity tyrosine phosphorylation-regulated kinase 1A (DYRK1A) phosphorylates NFAT, which limits calcineurin/NFAT signal transduction in T cells and hypertrophy in cultured cardiomyocytes. The hypothesis tested in this study was that DYRK1A prevents calcineurin/NFAT-mediated cardiac hypertrophy in vivo.

Methods and results

In cultured rat cardiomyocytes, adenovirus-mediated overexpression of DYRK1A antagonized calcineurin-mediated nuclear NFAT translocation and the phenylephrine-induced hypertrophic growth response. To test the ability of DYRK1A to reduce hypertrophic cardiac growth in vivo, we created tetracycline-repressible Dyrk1a transgenic mice to avoid the cardiac developmental defects associated with embryonic DYRK1A expression. However, in the mouse model, histological determination of myocyte diameter, heart weight/body weight ratio, and echocardiographic measurements revealed that myocardial expression of DYRK1A failed to reduce hypertrophy induced via aortic banding or co-expression of calcineurin. This discrepancy is explained, at least in part, by insufficient long-term inhibition of NFAT and the activation of DYRK1A-resistant maladaptive genes in vivo.

Conclusion

Isolated augmentation of DYRK1A can be compensated for in vivo, and this may significantly limit anti-hypertrophic interventions aimed at enhancing DYRK1A activity.

Keywords

Hypertrophy • DYRK1A • Calcineurin • NFAT • Transgenic mice

1. Introduction

Although myocardial hypertrophy is thought to be initially compensatory to normalize ventricular wall stress, sustained cardiac hypertrophy correlates with the development of heart failure and sudden cardiac death.¹,² Such maladaptive hypertrophy is associated with pathological cardiac demand and unfavourable clinical outcome; therefore, there has been a search for molecular signals that drive maladaptive cardiomyocyte growth. Among several candidates,³,⁴ one broadly examined signal transduction pathway in cardiac hypertrophy involves calcineurin/calmodulin-activated phosphatase calcineurin and its most studied substrates, the transcription factors nuclear factor of activated T cells (NFAT). Activated calcineurin dephosphorylates NFAT, which mediates NFAT translocation into the nucleus. In cardiomyocytes, increased nuclear NFAT has been shown to initiate pro-hypertrophic and maladaptive gene expression.⁵ Genetic inhibition of calcineurin results in reduced cardiac hypertrophy in response to a wide variety of hypertrophic stimuli.⁶,⁷ Moreover, mice overexpressing a constitutively active NFATc4 mutant in the heart develop cardiac hypertrophy,⁵ and Nfatc2 knockout mice have significant attenuation of calcineurin-induced cardiac growth.⁸ These studies suggest that NFAT
is required and is sufficient to transmit signals inducing maladaptive hypertrophy in the heart. Consequently, NFAT protein kinases, which counter NFAT dephosphorylation by calcineurin, might protect against maladaptive hypertrophy.9 Currently, pharmacological inhibition of calcineurin in the heart is only accomplished at doses that cause deleterious systemic side effects.10 Thus, inhibition of NFAT may offer an alternative approach. Recently, two independent groups have identified dual-specificity tyrosine phosphorylation-regulated kinase 1A (DYRK1A) as an NFAT kinase.11,12 DYRK1A phosphorylates the conserved serine-proline repeat 3 motif of NFAT, which primes NFAT for subsequent phosphorylation by glycogen synthase kinase 3 beta and casein kinase 1. DYRK1A antagonizes calcineurin-mediated dephosphorylation of NFAT. DYRK1A, which is mainly located in the nucleus, has been demonstrated to drive nuclear export of NFAT.11 The Dyik1a gene lies within the Down syndrome critical region, and three different mouse models overexpressing DYRK1A have exhibited neurodevelopmental delay, motor abnormalities, and learning defects.13–15 Importantly, mice overexpressing DYRK1A also have displayed embryonic structural heart defects and heart failure, suggesting that DYRK1A plays a role in embryonic heart development.12 As these studies examined the effect of DYRK on NFAT in non-cardiomyocytes, it is tempting to examine whether DYRK1A may also counter cardiac NFAT activity and hypertrophy. Recently, a study in isolated cardiomyocytes showed that DYRK1A attenuates phenylephrine (PE)-induced cardiomyocyte growth.16 However, to model more physiological pro-hypertrophic stimuli, time course, and systemic compensatory effects, the effects of DYRK1A need to be investigated in a living organism. This may be of particular importance in the case of DYRK1A because it has been demonstrated that DYRK1A has varying control over NFAT activity depending on the expression level, tissue, and developmental stage.17 Hence, this study tested the hypothesis that DYRK1A prevents cardiac hypertrophy by inhibiting myocardial NFAT signalling in cultured cardiomyocytes and in Dyik1a transgenic mice.

2. Methods

2.1 Real-time reverse transcriptase–polymerase chain reaction

Total RNA was isolated from left ventricular tissue and isolated cardiomyocytes using the RNeasy Fibrous Tissue Mini Kit (Qiagen). Complementary DNA (cDNA) was synthesized from 200 ng of total RNA using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories), and real-time reverse transcriptase–polymerase chain reaction (RT–PCR) was performed using IQ SYBR Green Supermix and the iCycler iQ Detection System (Bio-Rad). Starting quantities were extrapolated from standard curves for each primer set and normalized to 18S or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) RNA levels.

2.2 Western blotting

The primary antibodies used were anti-GAPDH (BioTrend), anti-DYRK1A (Santa Cruz), anti-phospho-mitogen-activated protein kinase 1/2 (MEK1/2; Cell Signaling), and anti-MEK1/2 (Cell Signaling). Then the blots were incubated with a horseradish peroxidase-coupled secondary antibody (GE Healthcare). Visualization was achieved using a chemiluminescence kit (Thermo Scientific), and Alphaview software (Alpha Innotech) was used for image acquisition and densitometry.

2.3 Isolation and culture of neonatal cardiomyocytes and adenoviral gene transfer

An adenovirus expressing NFATc3 fused with green fluorescent protein (GFP) (NFATc3-GFP) was kindly provided by R. Marchase. Small interfering RNA (siRNA) sequences for knock-down of DYRK1A were used as described,16 and constructs were generated using the BLOCKit RNAi expression vector kit (Invitrogen). We generated adenoviruses by ligation of the cDNA-coding sequences of Dyik1a, Dyrk1aK188R (kindly provided by W. Becker), constitutively active calcineurin A (CnA), Dyrk1a-siRNA, control-siRNA or beta-galactosidase (LacZ) into the vector pDC515 and by Flippase-mediated recombination with pBHGfrE13FLP (Admax, Microbix) in HEK293 cells. The adenoviruses were then purified by a cesium chloride gradient. Neonatal ventricular cardiomyocytes were isolated from Wistar rats (1–2 days old). Hearts were minced in phosphate-buffered saline (PBS) containing 0.2% (w/v) trypsin (Biochrom AG) and 0.1% (w/v) collagenase type II (Worthington). Four digestion cycles were carried out to dissociate the cells. The cells were centrifuged and resuspended in Dulbecco’s modified Eagle’s medium:F12 medium with 10% (v/v) foetal calf serum and preplated onto tissue culture dishes. Finally, myocytes were plated on gelatine-coated tissue culture dishes, switched to serum-free media 16 h after plating, and transfected with adenovirus. For certain experiments, harmine (Roht, Germany) was added at a final concentration of 1 μM, and PE (Sigma) was added at a final concentration of 5 μM.

2.4 Planimetric measurements and α-actinin staining

To examine hypertrophy, adenoviral gene transfer was performed in neonatal ventricular rat cardiomyocytes. The cells were incubated with adenovirus at the indicated multiplicity of infection and treated with 5 μM PE (Sigma) or vehicle as a control. After 48 h, the cardiomyocytes were fixed, permeabilized with 0.1% (v/v) Triton X-100 in PBS, and blocked with 4% bovine serum albumin (w/v) in PBS. Staining was conducted with a monclonal mouse anti-alpha-actinin antibody (Sigma) followed by a Cy3-coupled anti-mouse antibody (Dianova) and 4′,6-diamidino-2-phenylindole (DAPI) staining for nuclei visualization. Cell surface areas were determined using Axio Vision and ImageJ software (Zeiss). The analysis was performed with the examiner blinded towards the group assignment.

2.5 Mice

The generation of Dyik1a transgenic mice was performed by ligating rat Dyik1a cDNA (kindly provided by W. Becker) with an amino-terminal FLAG-tag sequence into the tet-sensitive α-myosin heavy chain promoter construct (kindly provided by J. Robbins).17 The construct was micro-injected into the pronucleus of FVB/N mouse oocytes and implanted into pseudopregnant females according to standard techniques. Dyik1a transgenic mice were cross-bred with a trans-activator (tTA) mouse line (kindly provided by J. Robbins) to obtain doxycycline-repressed expression of DYRK1A in Dyik1a/tTA double transgenic animals. Suppression of DYRK1A overexpression during embryonic development was achieved by adding 0.2 g/L doxycycline to the drinking water of breeding pairs. Doxycycline administration was terminated at weaning to induce transgene overexpression. After the initial characterization of the four lines, one line, which exhibited no overexpression of DYRK1A during doxycycline treatment and five-fold overexpression following withdrawal of doxycycline, was selected. Transverse aortic constriction (TAC) was carried out in 8-week-old female mice as described.18 For all other in vivo experiments, male and female animals were used and matched for gender. In separate experiments, Dyik1a/tTA transgenic mice were cross-bred with CnA transgenic mice (kindly provided by J. Molkenst). Littermates were used as controls throughout the study. The investigation conforms with the Guide for the Care and Use of
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Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996) and was approved by the Animal Ethics Committee of the University of Göttingen and the German federal state government.

2.6 Echocardiography

The mice were anesthetized using 1.5% isoflurane, and echocardiography was performed using the Vevo 660 system (Visualsonics). M-mode images were recorded in the long-axis view at the left mid-ventricular level. The examiner was blinded towards group assignment.

2.7 Histological analysis

Following deep anaesthesia with isoflurane, the mice were sacrificed by cervical dislocation. The hearts were quickly excised and fixed with 4% (w/v) paraformaldehyde in PBS. After dehydration, 6 μm paraffin-embedded sections were stained with wheat germ agglutinin (WGA) tetramethyl rhodamine isothiocyanate-coupled lectin (Sigma). To measure myocardite diameter, Axio Vision and ImageJ software (Zeiss) were used with the examiner blinded towards group assignment. Identical areas of the left mid-ventricular walls were examined. Immunohistochemistry staining of the myocardial sections was performed using an antibody directed against DYRK1A (Santa Cruz) and SuperPicture HRP Conjugate (Zymed Laboratories).

2.8 In vivo imaging of PE-stimulated NFAT activity

To monitor NFAT-dependent transcriptional activity in vivo, transgenic mice expressing luciferase NFAT dependently (kindly provided by J. Molkentin) were used. Micro-osmotic pumps (Alzet, model 1002) were implanted for chronic infusion of PE (0.1 mg/kg/d). Before implantation, the pumps were primed in 0.9% sterile saline overnight at 37°C to ensure steady delivery at implantation. The luciferase signal was measured before implantation, 12 h after, and 7 days after implantation using the imaging system IVIS Lumina II (Caliper Life Sciences). In brief, the mice were anaesthetized using isoflurane; d-luciferin (150 mg/kg) was injected intraperitoneally and after 5 min d-luciferin bioluminescence was measured for 30 min. For signal quantification, we used Living Image R 3.1 Software (Caliper Life Sciences) and defined a region of interest (ROI) with a threshold of 10% and background subtraction. The intensity was recorded as the maximum amount of photons (s^-1·cm^-2·sr^-1) within an ROI.

2.9 Statistical testing

Statistical analysis was carried out using Prism software version 5.01 (Graphpad) with two-tailed unpaired Student’s t-test or one-way ANOVA with Bonferroni post-test correction where appropriate. Graphs represent the mean ± standard error. A P-value of <0.05 was considered statistically significant.

3. Results

3.1 DYRK1A reduced calcineurin-mediated NFAT nuclear localization and PE-stimulated hypertrophy in neonatal cardiomyocytes

We examined the influence of DYRK1A on intracellular localization of NFAT in isolated neonatal cardiomyocytes transduced with an adenovirus encoding an NFATc3-GFP fusion protein (Ad-NFATc3-GFP). NFATc3-GFP-expressing cardiomyocytes displayed predominantly cytoplasmic NFAT localization (Figure 1A). Additional expression of CnA promoted the translocation of NFATc3-GFP into the nucleus in 75.9 ± 6.2% of cells. Transduction with Ad-DYRK1A completely inhibited CnA-mediated nuclear localization of NFATc3-GFP in cardiomyocytes, whereas a catalytically inactive mutant of DYRK1A (Ad-DYRK1AⒸ188R) did not (CnA + DYRK1A: 2.4 ± 1.6% nuclear NFAT; CnA + DYRK1AⒸ188R): 66.6 ± 5.9% nuclear NFAT; P < 0.001, 200 cells per group, n = 3). In addition, NFATc3-GFP-overexpressing cardiomyocytes were treated for 48 h with 1 μM harmine, which is a specific inhibitor of DYRK1A.20 As expected, pharmacologic inhibition of DYRK1A resulted in increased nuclear translocation of NFATc3-GFP after CnA overexpression compared with the vehicle control. Moreover, down-regulation of DYRK1A by specific siRNA knock-down promoted NFATc3-GFP nuclear translocation when CnA was co-expressed (Figure 1B).

3.2 DYRK1A overexpression did not reduce aortic banding-induced hypertrophy

To examine whether enhanced expression of DYRK1A might also blunt hypertrophic growth in left ventricular cardiomyocytes in the intact organism, DYRK1A-overexpressing transgenic mice were generated and subjected to TAC. To circumvent the known embryonic developmental defects in DYRK1A-overexpressing mice, we generated a tetracycline-dependent cardiomyocyte-specific Dyrk1a transgenic mouse and cross-bred it with the cardiomyocyte-specific tetracycline-controlled tTA mouse line. Removing doxycycline after weaning induced cardiomyocyte-specific DYRK1A overexpression in Dyrk1a/tTA double transgenic mice. We generated several different mouse lines and tested these for their ability to suppress DYRK1A overexpression during doxycycline treatment and to induce transgene overexpression after doxycycline withdrawal. Two mouse lines fulfilled the criteria of sufficient suppression in the presence of doxycycline and activation after withdrawal. The line with lower DYRK1A overexpression (5.1-fold) was used in this study. As demonstrated, overexpression of DYRK1A began at 4 weeks of age after doxycycline withdrawal. After 8 weeks, a constant overexpression level was observed (Figure 2A). Overexpression of DYRK1A was maintained during the development of hypertrophy (Supplementary material online, Figure S2C and D). Cardiac-specific overexpression of DYRK1A in Dyrk1a/tTA double transgenic mice was also demonstrated using quantitative real-time RT-PCR. Immunohistochemistry staining with anti-DYRK1A antibody revealed enhanced expression of DYRK1A in the nuclei of cardiomyocytes but not in the nuclei of...
non-cardiomyocytic cells. This pattern confirmed the expected location within the cardiomyocyte nucleus (Supplementary material online, Figure S2A and B). Aortic banding for 4 weeks induced robust hypertrophy with reduced fractional shortening (FS) but without dilation as determined by echocardiography (Figure 2B). Notably, the response towards TAC was similar in DYRK1A-expressing Dyrk1a/tTA double transgenic mice compared with control mice expressing only tTA; therefore, enhanced DYRK1A expression does not prevent TAC-induced hypertrophic growth and functional decline in the animal model. Furthermore, both groups had similar myocyte diameters measured by WGA staining and similar heart weight/body weight ratios (Figure 2C and D). Moreover, expression of the hypertrophy marker B-type natriuretic peptide (Bnp) was significantly enhanced in TAC vs. sham surgery animals in both genotypes and was significantly higher in Dyrk1a/tTA vs. tTA mice. Thus, a functional benefit from enhanced DYRK1A expression in adult mice is unlikely. Finally, an isoform shift from alpha- to beta-myosin heavy chain after TAC was induced to a similar extent in DYRK1A-expressing vs. control mice (Figure 2E).

### 3.3 Lack of a substantial anti-hypertrophic effect of DYRK1A overexpression in calcineurin transgenic mice

As multiple signal transduction pathways in addition to calcineurin/NFAT will be activated by aortic ligation, Dyrk1a/tTA mice were cross-bred with CnA transgenic mice to enhance calcineurin-induced hypertrophy. CnA transgenic mice developed severe cardiac hypertrophy at 2 months of age, and dilated cardiomyopathy occurred by 3 months of age. CnA/tTA double transgenic mice were compared with Dyrk1a/CnA/tTA triple transgenic mice at 8 and 12 weeks of age. At 8 weeks, mice displayed significant septal hypertrophy while posterior wall (PW) thickness as well as end-systolic and end-diastolic ventricular diameters did not change significantly. Notably, there was no reduction in septal hypertrophy upon enhanced DYRK1A expression (CnA/tTA: 1.42 ± 0.08 mm, n = 12; Dyrk1a/CnA/tTA: 1.53 ± 0.11 mm, n = 10; P = 0.4; Figure 3A). At 12 weeks, CnA-expressing mice exhibited a marked cardiac enlargement and reduced FS, but these parameters were comparable with the triple

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**Figure 1** DYRK1A inhibits CnA-mediated NFATc3-GFP nuclear localization. (A) NFATc3-GFP expressing neonatal rat cardiomyocytes 48 h after transfection with Ad-LacZ control virus, Ad-CnA, Ad-DYRK1A, and the catalytically inactive mutant Ad-DYRK1A<sup>K188R</sup>. Expression of CnA resulted in nuclear NFATc3-GFP localization, whereas DYRK1A overexpression significantly inhibited CnA-mediated nuclear translocation. *P < 0.001, scale bar: 10 μm. (B) Nuclear translocation of NFAT can be augmented by the DYRK1A inhibitor harmine or by adenosinergic transduction with siRNA directed against DYRK1A. (C) DYRK1A attenuated PE-stimulated hypertrophic growth in cardiomyocytes. Neonatal rat cardiomyocytes were transfected with Ad-LacZ or Ad-DYRK1A and were examined in the presence or absence of 5 μM PE stimulation. There was a small pro-hypertrophic effect of DYRK1A expression. DYRK1A overexpression significantly reduced PE-induced hypertrophic growth. *P < 0.001, Actinin/DAPI stain, Scale bar: 5 μm.
transgenic (i.e. DYRK1A-expressing) mice. The WGA-stained histological sections from 12-week-old animals revealed a slight but significant enlargement of cardiomyocyte diameter in the Dyrk1a/tTA mice compared with the tTA transgenic mice. Of note, there was also a slight reduction in myocyte diameter in the triple transgenic mice compared with the CnA/tTA mice. Therefore, DYRK1A may indeed reduce hypertrophic growth in this model, albeit to a minor extent (Figure 3B). In particular, given the artificial induction of hypertrophy via selective expression of constitutively active calcineurin and that the heart weight/body weight ratio (Figure 3C), myosin heavy chain gene expression shift and Bnp expression did not improve (Figure 3D), the biological significance of this finding is questionable.

3.4 Down-regulation of adrenergic NFAT activation and incomplete NFAT antagonism by DYRK1A in vivo

To further evaluate the discrepancy between the substantial inhibition of NFAT and hypertrophy in cultured cardiomyocytes and the lack of substantial improvement in the mouse model, we examined NFAT signalling in vivo using transgenic mice expressing luciferase in cardiomyocytes under control of an NFAT-sensitive promoter (NFAT-Luc). These mice were cross-bred with mice expressing DYRK1A and/or tTA, and hypertrophic signalling was stimulated with continuous infusion of PE via an osmotic minipump. In this model, there was a significant reduction of transcutaneous light emission 12 h after implantation of the minipump in the NFAT-Luc/Dyrk1a/tTA mice compared with the NFAT-Luc/tTA TG mice, suggesting that chronically enhanced expression of DYRK1A may blunt acute activation of NFAT-dependent transcription (Figure 4A). However, after 7 days of continuous PE infusion, light emission was reduced to the levels prior to implantation and was equal in both genotypes. This suggests that NFAT activation by chronic PE infusion may not be fully sustained. Moreover, in mice subjected to TAC, enhanced DYRK1A expression did not reduce NFAT-sensitive mRNA expression of regulator of calcineurin 1, splice variant 1–4 isoform (Rcan1–4), suggesting that enhanced DYRK1A activity alone is insufficient to chronically antagonize NFAT activity in this model (Figure 4B). In the
Dyrk1a/CnA/tTA mice, in which alternative NFAT regulators may be less central, Rcan1–4 expression was reduced by DYRK1A, which might explain the minimal improvement in myocyte diameter observed in this model.

4. Discussion

Consistent with a previous study showing reduced PE-dependent cardiomyocyte growth,16 our data demonstrate significant inhibition of NFAT-dependent hypertrophy and nuclear localization of NFAT upon acute DYRK1A overexpression in vitro. Therefore, we continued to examine the role of DYRK1A in vivo by evaluating the ability of enhanced DYRK1A activity to suppress myocardial hypertrophy in two different mouse models. Earlier, enhanced DYRK1A expression during embryogenesis was demonstrated to evoke cardiovascular abnormalities, including blunted heart valve development and heart failure.11,12 Therefore, we generated transgenic mice with tetracycline-controlled expression of DYRK1A to exclude the possibility of developmental defects. Despite the acute anti-hypertrophic effects seen in isolated cardiomyocytes, enhanced DYRK1A expression in transgenic mice did not appreciably reduce the hypertrophic growth induced either by TAC or by co-expression of constitutively active calcineurin in triple transgenic animals. Our data suggest that this apparent discrepancy can be explained by several mechanisms. Firstly, our mouse models reveal activation of maladaptive genes not previously described to directly depend on NFAT activation. This is demonstrated by the increase in Bnp expression and the myosin heavy chain isoform expression shift, which were insensitive to enhanced expression of DYRK1A. Secondly, data from the in vivo luciferase imaging suggest that PE-induced activation of NFAT transcriptional activity may not be fully sustained. Therefore, the activation of alternative pro-hypertrophic pathways and the down-regulation of NFAT activation might limit the anti-hypertrophic effect of DYRK1A in the mouse models. Rcan1–4 (formerly MCIP or DSCR1) expression is a good indicator of NFAT transcriptional activity due to the presence of several NFAT binding sites in its promoter region.23 In our TAC experiment, the sustained elevation of Rcan1–4 suggests insufficient repression of NFAT activity by DYRK1A. This may be another explanation for the lack of an anti-hypertrophic effect for DYRK1A. In addition, AngII-induced hypertrophic growth occurs via activation of NFAT24 and is completely resistant to DYRK1A in cultured cardiomyocytes, further supporting insufficient
inhibition of NFAT by DYRK1A. Indeed, experimental data and mathematical modelling of DYRK1A-dependent signalling imply that NFAT activation levels can be largely insensitive to enhanced expression of DYRK1A due to complex feedback regulation in some, but not all tissues.\textsuperscript{12} Unlike certain regions of the brain as in Down syndrome, our observations suggest that adult cardiomyocytes exhibit a strong capacity for compensation of both enhanced DYRK1A expression and upstream stimuli, such as PE-induced activation of NFAT. Finally, a minor pro-hypertrophic effect that was observed in cultured cells might potentially counteract the anti-hypertrophic effects. Notably, in addition to NFAT, multiple substrates of DYRK1A have been identified\textsuperscript{25–30} suggesting that DYRK1A may be a highly pleiotropic kinase. Thus, this effect in cultured cardiomyocytes could arise from the impact on signal transduction pathways other than calcineurin/NFAT.

In conclusion, our data suggest that the elevation of DYRK1A activity beyond physiological levels is unlikely to promote chronic repressive effects on hypertrophic growth in cardiomyocytes. Nevertheless, we observed clear inhibitory effects of DYRK1A on NFAT in cultured cardiomyocytes and transient repression of PE-induced NFAT activity in the NFAT-luciferase reporter mouse. Thus, our findings suggest that a likely role for DYRK1A in adult cardiomyocytes...
may be to add impedance or resistance to an acute elevation of NFAT activity.

**Supplementary material**

Supplementary material is available at *Cardiovascular Research* online.

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