British Journal of Pharmacology

DOI:10.1111/bph.13339
www.brjpharmacol.org

RESEARCH PAPER

Adenosine A_1 receptor activation attenuates cardiac hypertrophy and fibrosis in response to α_1-adrenoceptor stimulation *in vivo*

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Received
19 December 2014
Revised
25 August 2015
Accepted
27 August 2015

BACKGROUND AND PURPOSE

Adenosine has been proposed to exert anti-hypertrophic effects. However, the precise regulation and the role of the different adenosine receptor subtypes in the heart and their effects on hypertrophic signalling are largely unknown. We aimed to characterize expression and function of adenosine A_1 receptors following hypertrophic stimulation *in vitro* and *in vivo*.

EXPERIMENTAL APPROACH

Pro-hypertrophic stimuli and adenosine A_1 receptor stimulation of neonatal rat cardiomyocytes and male C57/Bl6 mice, sc. drug administration, real-time PCR, ^{3}[H]-leucine-incorporation assay, immunostaining, tissue staining, Western blots, gravimetric analyses and echocardiography were applied in this study.

KEY RESULTS

In neonatal rat cardiomyocyte cultures, phenylephrine, but not angiotensin II or insulin-like growth factor 1 (IGF1), up-regulated adenosine A_1 receptors concentration-dependently. The hypertrophic phenotype (cardiomyocyte size, sarcomeric organization, total protein synthesis, c-fos expression) mediated by phenylephrine (10 μM), but not that by angiotensinII (1 μM) or IGF1 (20 ng·mL^{-1}), was counteracted by the selective A_1 receptor agonist, N6-cyclopentyladenosine. In C57/BL6 mice, continuous N6-cyclopentyladenosine infusion (2 mg·kg^{-1}·day^{-1}; 21 days) blunted phenylephrine (120 mg·kg^{-1}·day^{-1}; 21 days) induced hypertrophy (heart weight, cardiomyocyte size and fetal genes), fibrosis, MMP 2 up-regulation and generation of oxidative stress – all hallmarks of maladaptive remodelling. Concurrently, phenylephrine administration increased expression of adenosine A_1 receptors.

CONCLUSIONS AND IMPLICATIONS

We have presented evidence for a negative feedback mechanism attenuating pathological myocardial hypertrophy following α_1-adrenoceptor stimulation. Our results suggest adenosine A_1 receptors as potential targets for therapeutic strategies to prevent transition from compensated myocardial hypertrophy to decompensated heart failure due to chronic cardiac pressure overload.

Abbreviations

ANF, atrial natriuretic factor; Ang II, angiotensin II; CPA, N6-cyclopentyladenosine; CTGF, connective tissue growth factor; EDD, end-diastolic diameter; ESD, end-systolic diameter; FS, fractional shortening; GSK3β, glycogen synthase kinase 3β; HW/BW, heart weight/body weight; IGF1, insulin-like growth factor 1; LV, left ventricular; MPTP, mitochondrial permeability transition pore; NOX, NADPH-oxidase; ROS, reactive oxygen species; β-MHC, myosin heavy chain isoform β; 8OHdG, 8-hydroxydiguanosine

Tables of Links

TARGETS
GPCRs[a]
Adenosine A_1 receptors
Adenosine A_{2B} receptors
α_1-Adrenoceptors
Enzymes[b]
GSK3β, glycogen synthase kinase 3β
MMP-2, matrix metalloproteinase 2
MMP-9, matrix metalloproteinase 9

LIGANDS
Adenosine
ANF
Ang II, angiotensin II
CPA, N6-cyclopentyladenosine
IGF1, insulin-like growth factor 1
Phenylephrine
Prazosin
TNF-α

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2013/14 ([a b c]Alexander et al., 2013a, b).

Introduction

Left ventricular (LV) hypertrophy occurs subsequent to myocardial infarction, arterial hypertension or valvular diseases and represents an independent risk factor for the development of heart failure. This maladaptive form of cardiac hypertrophy is mediated by neurohumoral activation with subsequent stimulation of myocardial GPCRs, and in particular, of adrenoceptors and angiotensin receptors. It is characterized by cardiomyocyte hypertrophy, quantitative and qualitative modulations in gene expression and protein expression, activation of MAPKs (Beisvag *et al.*, 2009, Ruwhof and van der Laarse, 2000), remodelling of the extracellular matrix (Gunasinghe et al., 2001, Spinale, 2007) and apoptotic, fibrotic and inflammatory events that lead to maladaptive LV remodelling, including ventricular dilatation and cardiac dysfunction (McMullen and Jennings, 2007, Opie *et al.*, 2006). Several inducers of maladaptive LV remodelling such as α_1-adrenoceptor stimulation have been described (Balakumar and Jagadeesh, 2010, Molkentin and Dorn, 2001). However, the number of factors identified to counteract pro-hypertrophic responses is limited.

Adenosine has been implicated in cardioprotective events, notably in the context of cardiac hypertrophy. Its levels are elevated in cardiac hypertrophy, experimental hypertension, acute and chronic phases of myocardial infarction and during the progression of heart failure (Funaya *et al.*, 1997). Adenosine exerts anti-hypertrophic and anti-adrenergic effects and is proposed to be cardioprotective in the setting of heart failure. This concept is supported by the observation that deletion of 5′ ectonucleotidase, one of the main regulators of adenosine metabolism, and the subsequent reduction in adenosine levels led to pronounced cardiac hypertrophy in a rat model of experimental hypertension (Xu *et al.*, 2008), while stimulation of adenosine receptors attenuated cardiomyocyte hypertrophy in this model (Liao *et al.*, 2003). Previous *in vitro* studies identified the adenosine A_1 receptor as the subtype responsible for adenosine-mediated anti-hypertrophic effects, at least following $G\alpha_q$-coupled α-adrenoceptor stimulation by phenylephrine (Liao et al., 2003, Gan *et al.*, 2005). Furthermore, the phenylephrine-induced hypertrophic response was accompanied by a compensatory up-regulation of adenosine receptors in an *in vitro* model (Pang *et al.*, 2010). However, it is presently unclear whether these *in vitro* effects are relevant in an *in vivo* model of cardiac hypertrophy. Furthermore, it remains to be determined whether activation of adenosine A_1 receptors antagonizes all $G\alpha_q$-mediated signalling or specifically signalling mediated by selected $G\alpha_q$-coupled receptors.

Besides its anti-hypertrophic effects, adenosine mediates anti-fibrotic effects through adenosine A_{2B} receptors *in vitro* (Epperson et al., 2009) and *in vivo* (Wakeno et al., 2006). Furthermore, we previously observed that adenosine prevents the expression of the pro-inflammatory cytokine TNF-α in the rat and failing human heart (Wagner *et al.*, 1999, Wagner *et al.*, 1998) and identified the autacoid as a negative regulator of MMP-9 (Ernens *et al.*, 2006). MMP-9 represents a risk marker for LV remodelling after myocardial infarction (Wagner *et al.*, 2006). Together with MMP-2, MMP-9 plays a crucial role in mediating LV dilatation and degradation of the fibrillar collagen-network subsequent to myocardial injury (Vanhoutte *et al.*, 2006).

Together, these studies indicate that adenosine is able to mitigate several characteristic features of maladaptive LV remodelling. Here, we characterized in more mechanistic detail the anti-hypertrophic and anti-fibrotic effects of adenosine *in vitro* and *in vivo*. We identified a potential negative feedback loop by which up-regulation and activation of adenosine A_1 receptors, but not other adenosine receptors, antagonize α-adrenoceptor, but not angiotensin AT_1 receptor-mediated pro-hypertrophic effects. Furthermore, activation of adenosine A_1 receptors also efficiently inhibited the development of moderate LV hypertrophy and fibrosis in response to α-adrenoceptor stimulation *in vivo*. These effects were associated with a substantial reduction in oxidative stress *in vivo*, a known mediator of cardiac hypertrophy and fibrosis. Our results highlight the potential therapeutic benefit provided

by activation of adenosine A_1 receptors, which may represent a novel target for the treatment of patients with cardiac hypertrophy and failure.

Methods

Cell culture

Neonatal Sprague Dawley rats, aged 3–4 days, were decapitated; hearts were excised under sterile conditions and cardiomyocytes isolated as described previously (Schnabel *et al.*, 2000) and seeded in F10-medium (10% horse serum, 5% fetal calf serum, 1% penicillin/streptomycin, Invitrogen, Basel, Switzerland) at 1.6×10^6 cells per 6 cm^2 plates or at 2.5×10^5 per well in 6-well plates. After incubation for 48 h, viability and contractility were evaluated microscopically, and cardiomyocytes were serum starved for 18 h before treated with 1–100 μM phenylephrine, 0.1–10 μM angiotensin II (Ang II), 0.2–20 $ng{\cdot}mL^{-1}$ insulin-like growth factor 1 (IGF1), 1 μM N6-cyclopentyladenosine (CPA) or 1 μM of the *α*-adrenoceptor antagonist prazosin for the indicated time periods (15 min to 48 h). All compounds were purchased from Sigma-Aldrich (Taufkirchen, Germany) unless indicated otherwise. Preliminary experiments showed that phenylephrine most effectively triggered cardiomyocyte hypertrophy when used at a concentration of 10 μM, whereas AngII triggered significant hypertrophy at a concentration of 1 μM and IGF1 at 20 $ng{\cdot}mL^{-1}$. Therefore, we decided to use for each agonist the lowest concentration shown to be effective to induce the hypertrophic response rather than using the highest concentration tested, to avoid masking the 'physiological' response and to avoid toxicity.

Animal studies

All animal care and experimental procedures complied with the European directive on laboratory animals (2010/63/EU) and the Guide for Care and Use of Laboratory Animals by the US National Institute of Health (8th edition, revised 2011) and were approved by the animal ethics committee of Saarland University. All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath et al., 2010). A total of 43 animals were used in the experiments described here.

Male C57/Bl6N mice (12 weeks, 24–25 g) were kept in groups of four under usual care (12 h dark/light regime, normal mouse chow and water *ad libitum*) and randomly assigned to treatment with continuous infusion of phenylephrine (120 $mg{\cdot}kg^{-1}{\cdot}day^{-1}$), CPA (2 $mg{\cdot}kg^{-1}{\cdot}day^{-1}$), phenylephrine + CPA or vehicle (0.9% NaCl). This treatment for 3 weeks was delivered by sc. implanted osmotic mini-pumps (Alzet Micro-Osmotic pump, model 1004, Alzet, Cupertino, CAN, supplied by Charles River, Sulzfeld, Germany). For implantation of mini-pumps, mice were anaesthetized with 2% isoflurane mixed with 98% O_2 (flow rate: 1 $L{\cdot}min^{-1}$).

Echocardiography

Echocardiography studies were performed using the VisualSonicsVevo® 770 imaging system (Visualsonics Inc., Toronto, CAN; Scanhead: RMV707B, 15–45 MHz, cardiac mouse). Mice were anaesthetized (3% isoflurane mixed with 97% O_2 at a flow rate of 1 $L{\cdot}min^{-1}$) in an induction chamber. After placing the mouse in a supine position on top of a pad with embedded ECG electrodes, anaesthesia was maintained via inhalation of 1.0–1.5% isoflurane and 98.5–99% O_2 at a flow rate of 1 $L{\cdot}min^{-1}$ using a nose mask. The ECG signal was monitored throughout the procedure. After immobilizing the mouse on the echocardiography stage with tape, chest hair was removed with hair removal cream, and a layer of preheated ultrasound gel was applied to the chest. Body temperature was monitored throughout the whole procedure by an inserted rectal probe and maintained within a narrow range (37.0°C ± 1.5°C) via the heated platform and a heat lamp. Two-dimensional (B-Mode) images were recorded in parasternal long-axis and short-axis projections with guided one-dimensional M-mode recordings at the midventricular level, apical of the posteriomedial papillary muscle in both views. Standard measurements of interventricular septum (IVS), LV posterior wall and LV internal diameter were performed in end-systole (LVESD) and end-diastole (LVEDD) in parasternal long-axis projection during at least three beats from this projection and averaged. ECG and respiration gating were used to suppress movement artefacts. Fractional shortening (FS) as a functional parameter was calculated based on calculated LV volumes (LV vol) as follows:

$$FS\,[\%] = ((\text{LVED vol} - \text{LVES vol})/\text{LVED vol})*100;\ \text{LVED vol}$$
$$\text{LVED vol}\ [\mu l] = ((7.0/2.4 + \text{LVEDD}))*\text{LVEDD}^3$$
$$\text{LVES vol}\ [\mu l] = ((7.0/2.4 + \text{LVESD}))*\text{LVESD}^3$$

MRI

MRI examinations were performed with a horizontal bore 9.4 T MRI animal scanner (BrukerBioSpin 94/20, Ettlingen, Germany) using a high-performance water-cooled gradient system as described previously (Puhl *et al.*, 2015). Bright blood cine sequences were acquired using an ECG-trigged fast low-angle shot sequence in short axis orientation covering the left ventricle from base to apex.

Tissue sampling and conservation

Mice were killed by an ip. injection of a mixture of ketamine hydrochloride (100 $mg{\cdot}kg^{-1}$; Pfizer, Karlsruhe, Germany) and xylazine hydrochloride (10 $mg{\cdot}kg^{-1}$; Bayer Healthcare, Berlin, Germany). After left thoracotomy, the hearts were explanted, the atria were removed, the myocardium was washed, weighed and split for biochemical and histological analyses (midventricular slice). LV tissue was frozen in liquid N_2 immediately after explantation for biochemical analyses and then stored at −80°C before RNA and protein isolation. Midventricular slices for histological analyses were fixed in 4% paraformaldehyde within 1 min after explantation of the heart and stored at 4°C.

Western blot analysis

For Western blot analysis of whole cell lysates, cultures of neonatal rat cardiomyocytes were harvested and washed, murine LV tissue was homogenised, respectively, lysed and prepared as described previously (Maack *et al.*, 2003). Primary

antibodies were purchased from Santa Cruz, CA, USA (adenosine A_{2A}-receptor, A_{2B}-receptor, A_3-receptor, p70S6K; 1:1000), Cell Signalling, Beverly, MA, USA (pAKT, AKT, pERK 1/2, ERK1/2, glycogen synthase kinase 3 β (pGSK3β), pp70S6K; 1:1000), Alpha Diagnostics, Brussel, Belgium (adenosine A_1 receptor; 1:500), Dianova, Hamburg, Germany (Calsequestrin; 1:2500), R&D, Wiesbaden, Germany (GSK3β, MMP-2,MMP-9; 1:500). Secondary antibodies were purchased from Sigma-Aldrich. Protein expression was quantified densitometrically using the Labworks 4.5 software (UVP, Upland, CA, USA).

Quantitative PCR-analysis

For PCR analysis of whole cell lysates, cultures of neonatal rat cardiomyocytes were harvested and washed, murine LV tissue was homogenised, respectively, lysed and prepared as described previously (Haas *et al.*, 2011). cDNA gained from 1 μg mRNA was diluted 10-fold for quantitative TaqMan (RT) PCR and fivefold for semi-quantitative PCR. PCR primers were obtained from MWG Eurofins (Ebersberg, Germany) and TIB Molbiol (Berlin, Germany), TaqMan probes were purchased from Applied Biosystems (Darmstadt, Germany). PCRs were run in triplicate. mRNA expression was normalized per sample against GAPDH. Relative quantitation of gene expression was calculated using the comparative ΔΔCT method, without knowledge of the treatments. All data based on expression analyses are expressed as fold over the mean value of the control group.

Histological analysis

For morphometric analyses, transverse midventricular tissue slices (5 μm) of formalin-fixed and paraffin-embedded murine heart tissue were stained with haematoxylin and eosin (H&E) or Sirius Red, as described previously (Muller *et al.*, 2008). LUCIA software (Nikon, Düsseldorf, Germany) was used for quantification of interstitial fibrosis in left ventricular tissue and for measurement of cardiomyocyte diameter and cross-sectional area in vertically oriented cardiomyocytes. At least 50 H&E-stained cardiomyocytes were measured per heart.

Immunostaining for 8-hydroxydiguanosine (8OHdG) was performed as described previously (Lenski *et al.*, 2011). Briefly, to detect 8OHdG-positive nuclei in murine cardiomyocytes and non-cardiomyocytes, transverse midventricular tissue slices (5 μm) were stained with goat polyclonal anti-8OHdG (1:300; Abcam, Cambridge, UK) at 4°C, overnight. Slides were incubated with secondary anti-goat IgG-TRITC (Tetramethylrhodamine isothiocyanate) antibody (Dianova, Hamburg, DE) at 37°C for 40 min. Cardiomyocytes were identified via co-immunostaining for α-sarcomeric actin (Sigma-Aldrich), followed by the incubation with the secondary anti-mouse IgM-FITC antibody (Dianova, Hamburg, Germany) as described previously. Nuclei were stained blue by DAPI. The percentage of 8OHdG positive cardiomyocyte and non-cardiomyocyte nuclei per heart was assessed by fluorescence microscopy using the LUCIA G software (Nikon, Düsseldorf, Germany). To assess sarcomeric organization by fluorescence microscopy, rat isolated cardiomyocytes, treated as indicated (48 h, 10 μM phenylephrine, 1 μM Ang II, 20 ng·mL^{-1} IGF1, 1 μM CPA), were stained with an FITC-conjugated antibody against α-sarcomeric actinin (anti-mouse-IgG-FITC, Dianova). Cross-sectional area was measured in at least 100 vertically oriented cardiomyocytes per condition.

Leucine incorporation assay

For more details, see Supporting Information Methods. Briefly, after applying medium containing 1 μCi [^{3}H]-leucine per well (Sigma), serum-starved isolated rat cardiomyocytes were incubated for 24 h with the indicated treatment (10 μM phenylephrine, 1 μM Ang II, 20 ng·mL^{-1} IGF1, 1 μm CPA). [^{3}H]-leucine incorporation in harvested and lysed cells was quantified by liquid scintillation counting.

Data analysis

Experimental animals and isolated cells were randomly assigned to the different treatment groups. Group sizes in *in vivo* experiments were estimated based on power analysis of heart weight/body weight ratios and atrial natriuretic factor (ANF) mRNA expression, as commonly used markers of hypertrophy with an α error of 5% and a power of 80%. To detect a 10% change in heart weight /body weight ratio with an expected SD of 5%, an experimental group size of $n = 5$ was estimated. To detect a 100% increase in ANF expression with a 60% SD $n = 7$ mice/group were required to fully power the present study. Some Western blot analyses and immunostaining analyses were performed in a subgroup of the study population due to tissue limitations. Here, $n = 4$ fulfilled the requirements to detect a 150% increase in 8OHdG signal with a 70% SD, and estimated group size of $n = 5$ was calculated to detect a 100% increase in abundance of the cleaved MMP-2 isoform with an SD of 50%.

Group sizes in *in vitro* experiments were estimated based on power analysis of [^{3}H]-incorporation as a commonly used hypertrophy marker with an α error of 5% and a power of 80%. To detect a 50% change with an expected SD of 20%, a group size of $n = 3$ was estimated to fully power the *in vitro* part of the present study.

All mRNA and echocardiographic analyses were performed without knowledge of the treatments.

Triplicates of one sample are treated as $n = 1$ independent experiment or individual in this study.

Continuous data are presented as means ± SEM. Normalized data are presented as fold over control mean. To determine differences between two groups, an unpaired Student's *t*-test was used. Differences between four groups were determined by using one-way ANOVA analysis and Bonferroni *post hoc*-test if *F*-test of variance had achieved the necessary level of statistical significance (i.e. $P < 0.05$). All calculated *P*-values are two-sided. Comparisons where $P < 0.05$ were considered to be statistically significant. All analyses were performed with Microsoft Excel 2007 and GraphPad Prism 5.0 software (GraphPad Software, Inc., CA, USA).

Results

Activation of adenosine A_1 receptors inhibits phenylephrine -, but not Ang II-, or IGF1 -induced hypertrophic response in vitro

To assess the role of adenosine A_1 receptors in the regulation of cardiomyocyte hypertrophy, neonatal rat cardiac myocytes

were incubated with the α_1-adrenoceptor agonist phenylephrine, Ang II or IGF1, in the absence and presence of the selective adenosine A_1 receptor agonist N6-cyclopentyladenosine (CPA). The hypertrophic phenotype was assessed by quantification of cardiomyocyte cross-sectional area (Figure 1A), protein-synthesis (Figure 1B), sarcomeric organization

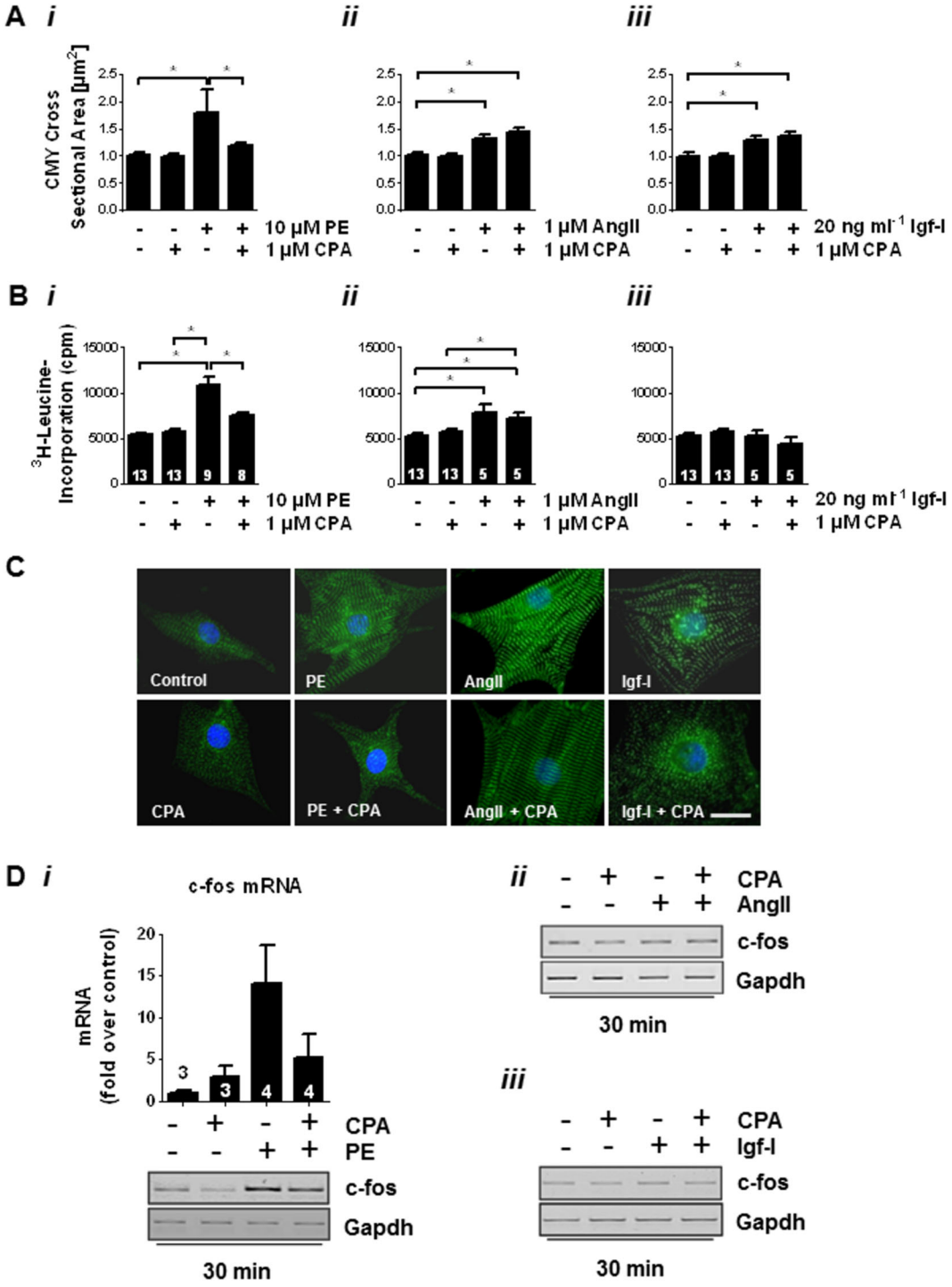

Figure 1

Activation of adenosine A_1 receptors inhibits phenylephrine -, but not Ang II-, or IGF1-induced hypertrophic response *in vitro*. Effect of the A_1 receptor agonist CPA (1 μM) on phenylephrine- (PE; *i*; 10 μM), Ang II- (*ii*;1 μM) and IGF1 (*iii*; 20 ng·mL^{-1}) induced hypertrophy in neonatal rat cardiomyocytes as assessed by cardiomyocyte cross-sectional area (CCSA; A; 48 h; ~100 cells per condition), total protein synthesis (B; [^{3}H]-incorporation; 24 h), sarcomeric organization (C; 48 h; ~50 cells per condition; scale bar, 10 μm) and c-fos expression [D; 30 min; inverted pictures of representative agarose gels; n = 3 and corresponding real-time PCR for (*i*)]. Untreated cells served as control. Values are shown as means or fold change over control mean ± SEM, *$P < 0.05$; significantly different as indicated.

(Figure 1C) and mRNA expression of the immediate early gene c-fos (Figure 1D). Incubation with phenylephrine induced a robust up-regulation of all hypertrophic markers studied, while Ang II had a slightly less pronounced effect on cardiomyocyte cross-sectional area (Figure 1A*ii*) and protein-synthesis, compared with phenylephrine (Figure 1B*ii*), a similar effect on sarcomere organization (Figure 1C) and no detectable effect on c-fos expression (Figure 1 D*ii*). IGF1 increased cardiomyocyte cross-sectional area (Figure 1A*iii*), augmented sarcomeric organization (Figure 1C, upper row), but did not increase [^{3}H]-leucine incorporation (Figure 1B*iii*) or c-fos expression (Figure 1D*iii*). The adenosine A_1 receptor agonist CPA blocked the pro-hypertrophic effects evoked by phenylephrine, but not the hypertrophic response mediated by Ang II or IGF1 (Figure 1). These observations suggest that adenosine specifically counteracts α_1-adrenoceptor-mediated cardiomyocyte hypertrophy by activating adenosine A_1 receptors.

Up-regulation of adenosine A_1 receptors by α_1-adrenoceptor stimulation

We next examined whether induction of the pro-hypertrophic response in cardiomyocytes is accompanied by modulation of the expression of adenosine A_1 receptors, using real-time PCR and Western blot analyses. Exposure to increasing phenylephrine concentrations (1–100 μM) up-regulated adenosine A_1 receptor mRNA and protein expression

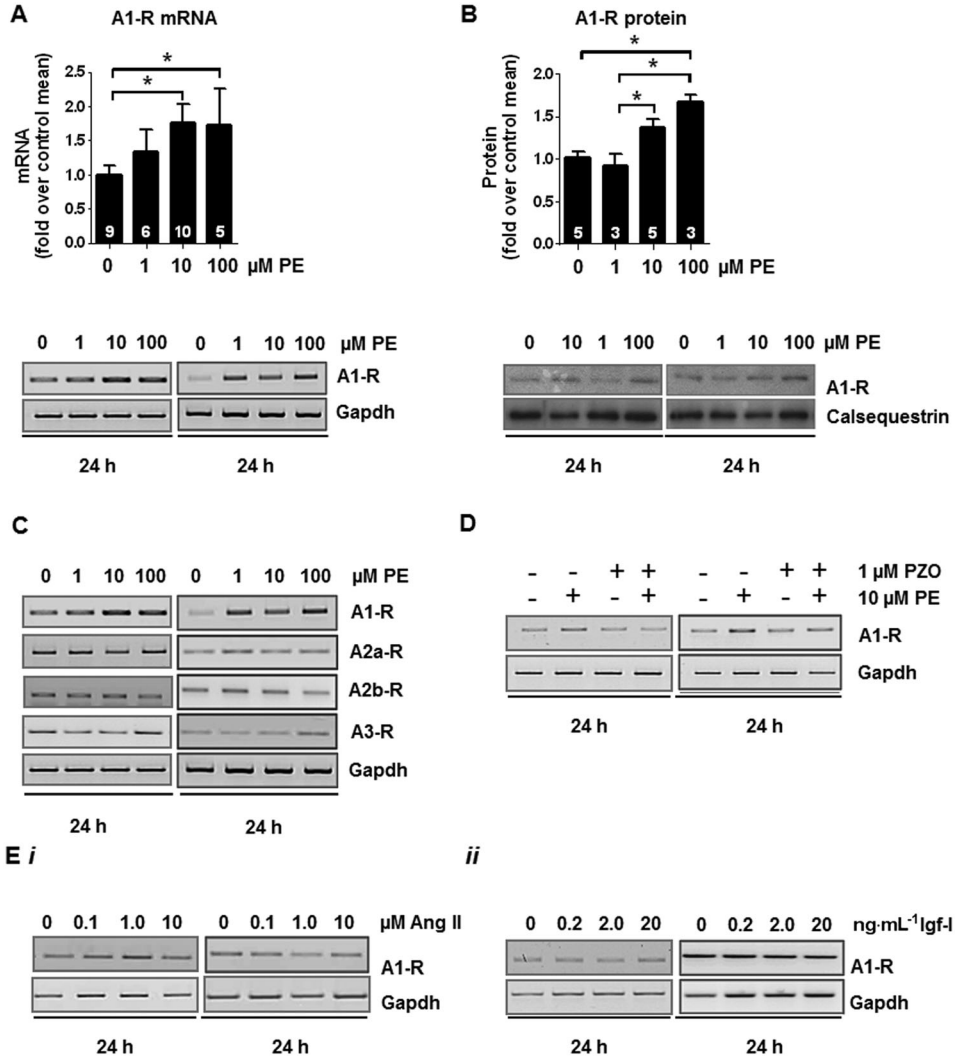

Figure 2

Adenosine A_1 receptors underlie a specific up-regulation by α_1-adrenoceptor stimulation. Effect of increasing phenylephrine (PE) concentrations (1–100 μM) on A_1 receptor (A1-R) mRNA (A; RT-PCR, representative agarose gels shown below) and protein expression (B; representative Western blots shown below) and on mRNA expression of adenosine A_{2A}, A_{2B} and A_3 receptors (C; representative agarose gels). Changes in A_1 receptor mRNA expression after co-stimulation with phenylephrine (10 μM) and prazosin (PZO; D; 1 μM; inverted pictures of representative agarose gels) and following stimulation with increasing concentrations of Ang II (E *i*; 0.1–10 μM; inverted pictures of representative agarose gels) and IGF1 (E *ii*; 0.2–20 ng·mL^{-1}; inverted pictures of representative agarose gels). Untreated cells served as control. $n = 3$ if not indicated otherwise; Values were determined 24 h after treatment. Values are shown as means or fold change over control mean ± SEM, $^*P < 0.05$; significantly different as indicated.

(Figure 2A,B). In contrast, no effects of phenylephrine treament was observed on the other adenosine receptor subtypes A_{2A}, A_{2B} and A_3 (Figure 2C). An addition of the α_1-adrenoceptor antagonist prazosin abolished the phenylephrine-induced increase in adenosine A_1 receptor mRNA expression (Figure 2D). In contrast to phenylephrine-induced α_1-adrenoceptor stimulation, neither Ang II (0.1–10 μM) nor IGF1 (0.2–20 ng·mL^{-1}), at concentrations that induced cardiomyocyte hypertrophy (Figure 1), affected adenosine A_1 receptor expression (Figure 2E). These data suggest that adenosine A_1 receptor up-regulation was specific for pro-hypertrophic α_1-adrenoceptor stimulation by phenylephrine.

α_1-Adrenoceptor activation up-regulates adenosine receptors in vivo

To confirm the pathophysiological relevance of these observations, made *in vitro*, in mice *in vivo* and to gain further insight into the underlying mechanisms, we continuously infused phenylephrine (120 mg·kg^{-1}·day^{-1}) in C57/Bl6N male mice for 3 weeks using an sc. implanted osmotic minipumps (ALZET). Because our *in vitro* experiments on neonatal rat cardiomyocytes showed up-regulated expression of adenosine A_1 receptors after α-adrenoceptor stimulation by phenylephrine, we determined adenosine A_1 receptor protein expression in LV tissue of phenylephrine-treated mice and found that α_1-adrenoceptor stimulation for 3 weeks resulted in pronounced augmentations of all adenosine receptor subtypes (Figure 3).

Activation of adenosine A_1 receptors attenuates phenylephrine-induced hypertrophic response in vivo

Continuous 3-week infusion of phenylephrine resulted in an increase of the heart weight/ body weight (HW/BW) ratio, cardiomyocyte diameter and cross-sectional area (Figure 4A–D). Echocardiographic and MRI analyses could not detect any evidence for phenylephrine-induced LV dilatation or dysfunction as features of progression into decompensated heart failure and thereby confirming the compensated state of the hypertrophied hearts in these mice treated with phenylephrine (Table 1).

However, phenylephrine did induce a characteristic reactivation of fetal genes, such as ANF and myosin heavy chain isoform β (β-MHC), with a consequent shift in the β-MHC/α-MHC ratio (Figure 4E–G). Interestingly, administration of the adenosine A_1 receptor agonist CPA (2 mg·kg^{-1}·day^{-1}) completely prevented the phenylephrine-induced increase in HW/BW ratio, cardiomyocyte size (Figure 4A–D) and the reactivation of fetal genes (Figures 4E–G). CPA alone had no effect on HW/BW ratio, cardiomyocyte size or ANF expression, but down-regulated baseline β-MHC expression and accordingly, the β-MHC/α-MHC ratio (Figure 4).

Activation of adenosine A_1 receptors counteracts the phenylephrine-induced pro-fibrotic response in vivo

To further investigate the mechanisms of adenosine A_1 receptor-mediated cardioprotective effects *in vivo*, we examined the influence of continuous CPA-infusion on phenylephrine-induced fibrosis, as another important characteristic of maladaptive cardiac remodelling. We found that, while phenylephrine alone led to a substantial increase in cardiac fibrosis, co-infusion of CPA completely blocked the phenylephrine-mediated pro-fibrotic phenotype, as measured by the LV collagen content in histological analyses (Figures 5A,B). In addition, phenylephrine evoked mRNA up-regulation of the pro-fibrotic cytokine TNF-α and a trend towards up-regulation of connective tissue growth factor (CTGF) and collagen Iα2 (Figures 5C–E). These changes were blunted by co-infusion with CPA while CPA *per se* had no significant effect on these markers (Figures 5C–E).

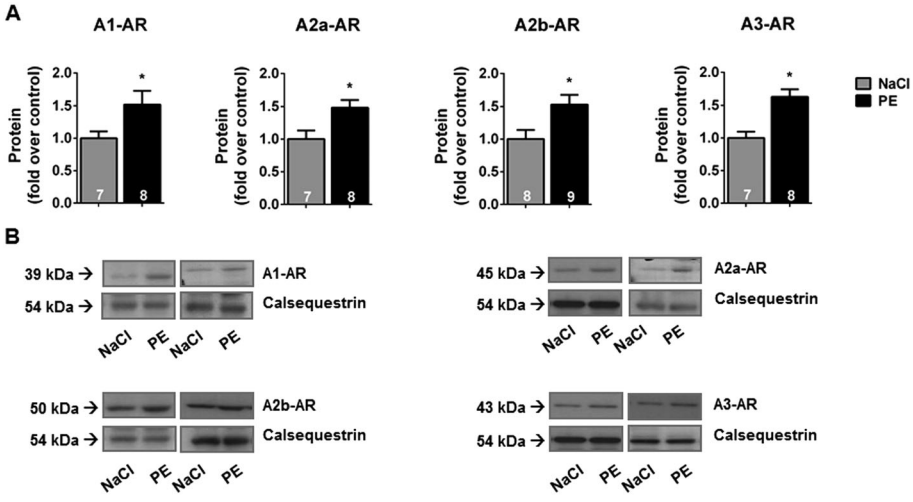

Figure 3

α_1-Adrenoceptor stimulation up-regulates adenosine receptors. Effect of 3 weeks continuous phenylephrine treatment (120 mg·kg^{-1}·day^{-1}) on protein expression of the adenosine receptors, A_1 (A1-R;39 kDa), A_{2A} (A2A-R; 45 kDa), A_{2B} (A2B-R;50 kDa) and A_3 (A3-R; 43 kDa) (A). Representative Western-blots for A_1 , A_{2B} and for A_{2A}-, A_3 receptors (B). Saline-treated mice served as control. Experimental numbers are indicated in bars; Values are shown as means or fold change over control mean ± SEM, *$P < 0.05$; significantly different as indicated.

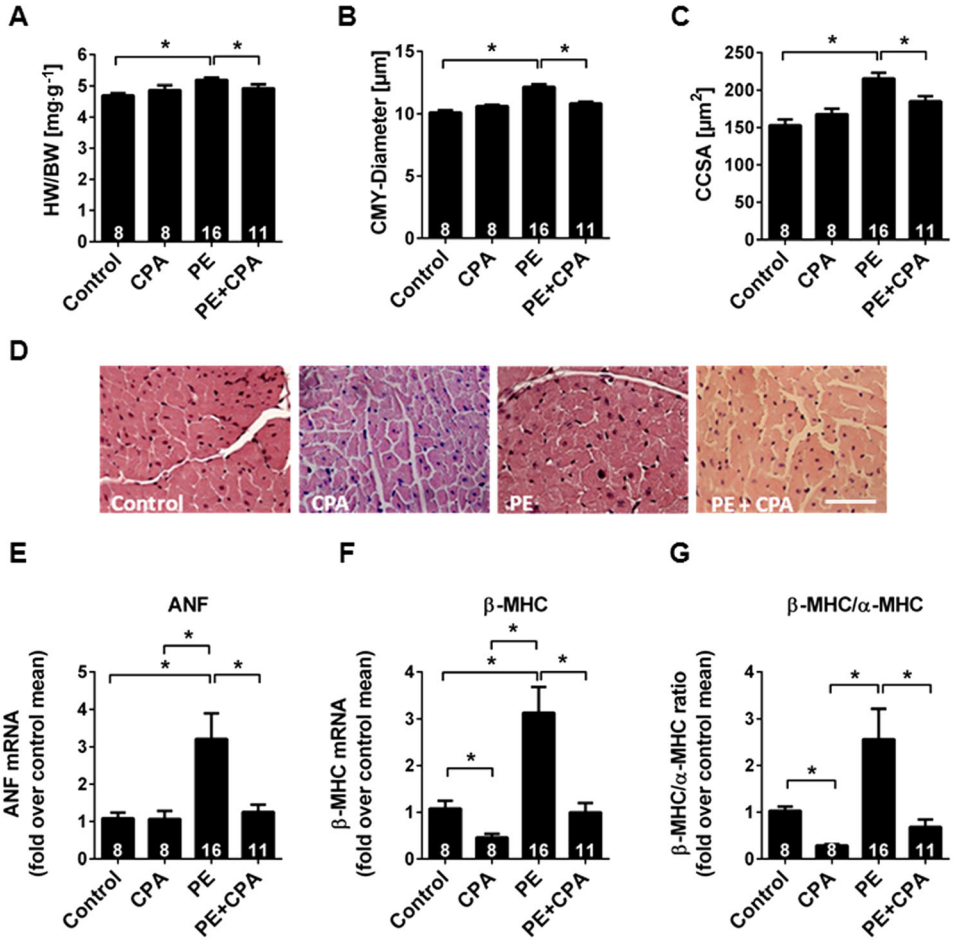

Figure 4

Activation of adenosine A_1 receptors attenuates phenylephrine-induced hypertrophic response *in vivo*. Effect of 3 weeks continuous A_1 receptor activation with CPA (2 mg·kg^{-1}·day^{-1}) on phenylephrine (PE; 120 mg·kg^{-1}·day^{-1}) induced hypertrophic phenotype in wild-type mice as assessed by heart weight/body weight ratio (A), cardiomyocyte (CMY) diameter and cross-sectional area (B; C; D representative histological sections of H&E-stained left ventricular myocardium) and by mRNA expression of the fetal genes ANF (E) and β-MHC (F) and β-MHC/α-MHC ratio (G). Saline-treated mice served as control. Experimental numbers are indicated in bars; Values are shown as means or fold change over control mean ± SEM, *$P < 0.05$; significantly different as indicated.

As an important downstream mechanism of α-adrenoceptor and TNF-α signalling that affects the extracellular matrix, phenylephrine up-regulated cleaved MMP-2 (also described as processed or mature MMP-2), while pro- (latent) MMP-2 or MMP-9 was unchanged (Figure 6A and Supporting Information Figure S1). This pro-fibrotic response was completely abolished by simultaneous activation of adenosine A_1 receptors (Figure 6A).

Activation of adenosine A_1 receptors reduces α_1-adrenoceptor-induced oxidative stress in vivo

To identify the intracellular mediators by which adenosine, acting via its A_1 receptors, antagonized phenylephrine-mediated pro-hypertrophic and/or pro-fibrotic signalling, we examined expression and phosphorylation patterns of canonical downstream targets of α_1-adrenoceptor and adenosine A_1 receptor signalling cascades. Western-blot analyses revealed neither a distinct role of the ERK1/2, nor a regulatory function of AKT, GSK3-β or P70S6k concerning the cross-talk between the two signalling pathways (Supporting Information Figures S2–5). Because activation of MAPKs is often only transient and has been described as a downstream target of NADPH-oxidase related formation of reactive oxygen species (ROS), we determined whether phenylephrine-induced activation of α-adrenoceptors to mediate myocardial oxidative stress, by staining LV myocardium for 8OHdG, a sensitive marker of the formation of H_2O_2 *in vivo*. As shown in Figure 6B, phenylephrine induced a substantial up-regulation of 8OHdG positive nuclei in both cardiomyocytes and non-cardiomyocyte ventricular cells, indicating an increase in oxidative stress by α-adrenoceptor stimulation. This α-adrenoceptor-mediated increase in oxidative stress *in vivo* was completely prevented by co-infusion of the adenosine A_1 receptor agonist CPA in both cardiomyocytes and non-cardiomyocytes. These data indicate that adenosine could efficiently counteract α-adrenoceptor-induced oxidative stress, an important trigger for cardiac hypertrophy and fibrosis, through activation of its A_1 receptors.

Table 1

Verification of compensated hypertrophy induced by α_1-adrenoceptor stimulation by phenylephrine (PE)

	Control (n = 7)	CPA (n = 5)	PE (n = 13/9)	PE + CPA (n = 11/7)
LVPW; d[a] [mm] ± SEM	0.63 ± 0.03	0.64 ± 0.04	0.62 ± 0.03	0.65 ± 0.02
IVS; d[b] [mm] ± SEM	0.55 ± 0.03	0.42 ± 0.03	0.53 ± 0.02	0.44 ± 0.21
LVEDD[c] [mm] ± SEM	4.52 ± 0.06	4.95 ± 0.16	4.60 ± 0.06	4.68 ± 0.08
LVESD[d] [mm] ± SEM	3.61 ± 0.09	4.14 ± 0.66	3.54 ± 0.07	3.80 ± 0.13
LVED vol[e] [μL] ± SEM	53.60 ± 3.88	59.00 ± 5.81	62.97 ± 4.06	50.28 ± 5.32
LVES vol[f] [μL] ± SEM	18.56 ± 2.02	23.82 ± 3.40	25.22 ± 2.59	17.00 ± 2.12
LV mass[g] [mg] ± SEM	79.59 ± 3.86	84.27 ± 3.53	87.69 ± 5.20	81.38 ± 3.20
LV mass[g] [μL] ± SEM	74.60 ± 4.82	80.26 ± 3.36	86.13 ± 4.76	77.50 ± 3.05
FS [%][h] ± SEM	20.12 ± 1.56	16.48 ± 1.14	22.98 ± 1.32	18.96 ±1.89
EF [%][i] ± SEM	65.00 ± 3.17	60.32 ± 2.19	60.38 ± 2.12	66.56 ±2.91
COj [mL·min^{-1}][j] ± SEM	18.50 ± 1.93	16.93 ± 1.60	17.71 ± 2.12	14.87 ±2.46

[a]End diastolic left ventricular posterior wall, determined via echocardiography,
[b]End diastolic intraventricular septum, determined via echocardiography.
[c]Left ventricular end diastolic diameter, determined via echocardiography.
[d]Left ventricular end systolic diameter, determined via echocardiography.
[e]Left ventricular end diastolic volume, determined via echocardiography; determined via cardiac MRI (Control: n = 5; CPA: n = 5; PE: n = 9; PE + CPA: n = 7). Values are shown as means ± SE.
[f]Left ventricular end systolic volume, determined via cardiac MRI (Control: n = 5; CPA: n = 5; PE: n = 9; PE + CPA: n = 7). Values are shown as means ± SEM.
[g]Left ventricular mass, determined via cardiac MRI (Control: n = 5; CPA: n = 5; PE: n = 9; PE + CPA: n = 7). Values are shown as means ± SEM.
[h]Fractional shortening.
[i]Ejection fraction, determined via cardiac MRI (Control: n = 5; CPA: n = 5; PE: n = 9; PE + CPA: n = 7). Values are shown as means ± SEM.
[j]Cardiac output, determined via cardiac MRI (Control: n = 5; CPA: n = 5; PE: n = 9; PE + CPA: n = 7). Values are shown as means ± SEM.

Discussion

The main finding of the present study is that activation of adenosine A_1 receptors prevented phenylephrine-mediated cardiomyocyte hypertrophy and cardiac fibrosis in a model of compensated hypertrophy and that this cardioprotection is accompanied by up-regulation of the A_1 receptors. These data suggest that full or partial agonists at adenosine A_1 receptors may also mitigate cardiac remodelling and thus potentially dysfunction in response to α_1-adrenoceptor stimulation.

In the present study, we observed that only phenylephrine-, but not Ang II- or IGF1-induced cardiomyocyte hypertrophy could be blocked by concurrent activation of adenosine A_1 receptors by CPA. This finding suggests that different signalling cascades are induced by Ang II and phenylephrine to evoke the maladaptive responses in cardiomyocytes, although both sets of receptors are $G\alpha_q$-coupled. The ability of adenosine A_1 receptor activation to counteract, in within the first 30 min, phenylephrine-induced up-regulation of c-fos, which is seen to play an important role during development of early hypertrophy, may reflect a definitive regulatory function of adenosine A_1 receptor-induced signalling in prevention of early hypertrophy. This observation agrees with the earlier study by Gan *et al.* (2005).

Another finding in our study was that α_1-adrenoceptor stimulation with phenylephrine evoked an up-regulation of adenosine A_1 receptor expression. This effect could be inhibited by the α_1- adrenoceptor antagonist prazosin and could not be mimicked by hypertrophic stimulation with Ang II or IGF1. Considering these findings, together with the observation made by Pang and colleagues, that endothelin-I and aldosterone did not affect adenosine A_1 receptor expression (Pang *et al.*, 2010), our findings suggest that up-regulation of these receptors was specific for pro-hypertrophic α_1- adrenoceptor stimulation. Contrary to the findings reported by Pang and colleagues (Pang *et al.*, 2010), we did not observe an increase in gene expression of the adenosine A_{2A} or A_3 receptors in isolated cardiomyocytes, neither by increasing phenylephrine concentrations nor in time response (data not shown). In addition, we determined transcription-state of the adenosine A2b receptor-subtype, which also remained unaffected by PE-treatment. By studying a mouse model with continuous phenylephrine infusion for 3 weeks, we could confirm for the first time α_1-adrenoceptor-dependent up-regulation of adenosine A_1 receptors *in vivo*. Furthermore, in this model, we cofirmed that continuous activation of adenosine A_1 receptors counteracted a pronounced phenylephrine-mediated cardiomyocyte hypertrophy not only in isolated cells but also *in vivo*. The latter was assessed by decrease in HW/BW ratios (full data set, see Supporting Information Table S2), cardiomyocyte size, expression pattern of the fetal genes ANF and β-MHC and a shift in β-MHC/α-MHC-ratio towards the adult isoform. These observations taken together with the lack of

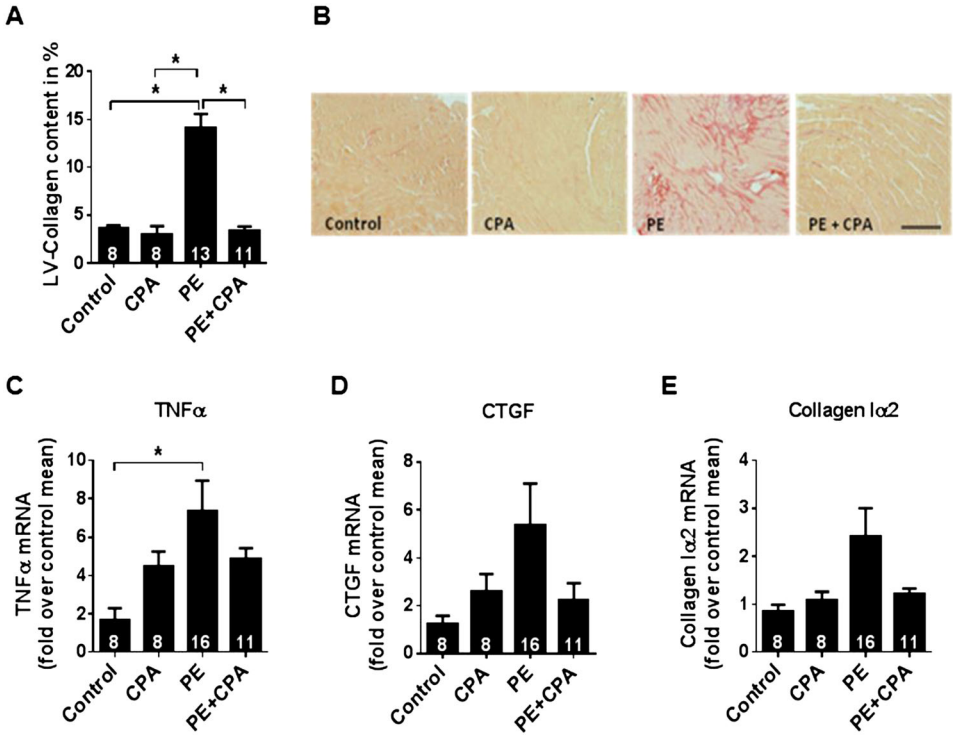

Figure 5

Activation of adenosine A_1 receptors counteracts phenylephrine-induced pro-fibrotic response *in vivo*. Effect of 3 weeks continuous phenylephrine treatment (PE; 120 mg·kg^{-1}·day^{-1}) in the presence and absence of CPA (2 mg·kg^{-1}·day^{-1}) on left ventricular collagen content (A; B representative histological sections of Sirius Red-stained left ventricular myocardium; scale bar 100 μm) and on mRNA expression of the fibrosis markers TNFα (C), CTGF (D) and Collagen Iα2 (E). Saline-treated mice served as control. Experimental numbers are indicated in bars; Values are shown as means or fold change over control mean ± SEM, *$P < 0.05$; significantly different as indicated.

obvious phenylephrine-induced increases in LV dimensions and wall thickness confirm a compensated state of the hypertrophied heart in this treatment group. The progressive hypertrophy process represents a continuum in time and in relation to the strength of the stimulus. In the present study, the treatment period and the dose of phenylephrine administered were sufficient to induce changes characteristic for hypertrophy but not strong and/or persistent enough to evoke alterations attributed to the maladaptive progression into decompensated hypertrophy. This experimental model allows a scope for (beneficial or detrimental) modifications by drug therapies. Our data obtained from this model suggest activation of adenosine A_1 receptors as a mechanism to interfere with the early stages of the remodelling process by attenuating cardiomyocyte hypertrophy before adverse cardiac remodelling (structural, geometrical and functional changes) establishes. Moreover, our study suggests a potential adenosine A_1 receptor-mediated mechanism attenuating the transition into a decompensated failing heart. However, at this stage, we have not shown a positive (or any) effects of adenosine A_1 receptor activation in the setting of decompensated hypertrophy. Thus, a potential (rescuing) anti-hypertrophic effect of activation of these receptors initiated at later remodelling stages, when LV dilatation and dysfunction are already established, remains to be elucidated.

It was surprising to note, that stimulation of adenosine A_1 receptors alone resulted in a significant decrease in β-MHC, compared with control mice. This observation could reflect a protective role of adenosine and A_1 receptors by maintaining cardiac contractility, as previously shown for adenosine in a rat model of myocardial infarction (Bousquenaud *et al.*, 2013).

The finding that adenosine A_1 receptors underlie a specific phenylephrine-mediated up-regulation, and in turn, that activation of these receptors counteracts phenylephrine-induced hypertrophic response *in vitro* and *in vivo*, may mirror a feedback mechanism, by which adenosine controls specifically pathological hypertrophy following α-adrenoceptor stimulation. It is well known that adenosine levels are elevated/altered in settings of post-ischaemic stress, hypertension, left ventricular hypertrophy and in the failing heart. This regulation is accompanied by alterations in adenosine handling and signalling via its receptors. We and others have shown that adenosine receptor expression can rapidly be increased in the presence of adenosine itself or stress induced by addition of lipopolysaccharide (Ernens *et al.*, 2006, Elson *et al.*, 2013, Murphree *et al.*, 2005, Velot *et al.*, 2008). However, it is not established yet if this reflects an adaptive or compensatory mechanism of the heart in the setting of cardiac stress and/or injury. At this stage, our data do not allow establishing a causal relationship between up-regulation of adenosine A_1 receptors and the attenuation of phenylephrine-induced hypertrophy by stimulation of these adenosine receptors. However, we suggest that the observed up-regulation of the

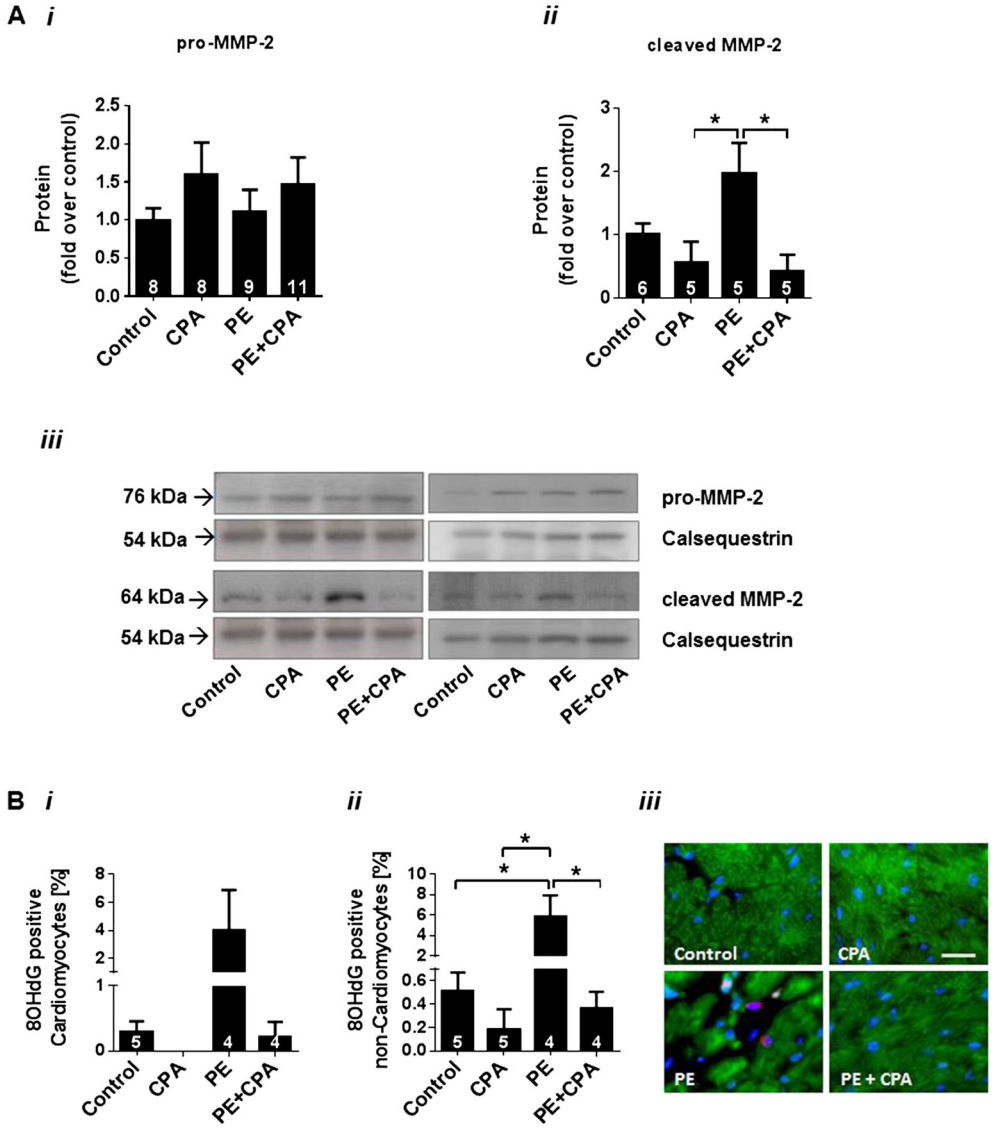

Figure 6

Activation of adenosine A_1 receptors inhibits phenylephrine-induced increase in abundance of cleaved MMP-2 and abolishes phenylephrine-evoked oxidative stress. Effect of 3 weeks continuous phenylephrine (PE) treatment (120 mg·kg^{-1}·day^{-1}) in the presence and absence of CPA (2 mg·kg^{-1}·day) on (A) protein expression of pro- (*i*) and cleaved MMP-2 isoforms (*ii*). Representative Western blots shown below (*iii*) and on (B) formation of oxidative stress as determined by 8OHdG positive nuclei in cardiomyocytes (*i*) and non-cardiomyocyte myocardial cells (*ii*; *iii* representative histological sections of left ventricular myocardium immunostained against 8OHdG (red) and α-sarcomeric actin (green); DAPI-stained nuclei (blue); scale bar 10 μm). Saline-treated mice served as control. Experimental numbers are indicated in bars; Values are shown as means or fold change over control mean ± SEM, *$P < 0.05$; significantly different as indicated.

adenosine receptor system by chronic adrenoceptor stimulation is at least partly triggered by the increase in its substrate abundance and facilitates adaptive changes in response to hypertrophic stimulation

However, mice overexpressing adenosine A_1 receptors are characterized by the development of (apparently reversible) cardiomyopathy, ventricular hypertrophy, fibrosis, cardiac dysfunction and dilatation, decreased heart rate and reduced calcium circulation (Funakoshi *et al.*, 2006). Besides, the PROTECT study has shown that antagonism of adenosine A_1 receptors is associated with beneficial effects on renal vasodilation, filtration, diuresis and natriuresis as well as with detrimental side effects as seizures and stroke (Lim, 2011, Teerlink *et al.*, 2012). Thus, mechanisms leading to moderate/partial selective activation and/or steady expression rate of adenosine A_1 receptors might represent new therapeutic targets preventing the transition from compensated hypertrophy to decompensated heart failure. We suggest that the observed CPA-mediated beneficial effects are predominantly due to adenosine A_1 receptor stimulation, although a role for other adenosine receptors cannot be fully ruled out. However, amongst the four adenosine receptors subtypes, the A_1 receptor shows the highest abundance in the mammalian heart as emphasized by an approximately 50-fold higher

mRNA expression, compared with other adenosine receptors, in the murine heart (Headrick *et al.*, 2013). Also, CPA is 200- to 1200-fold more selective for the A_1 receptor, compared with other adenosine receptors (Cordeaux *et al.*, 2000).

In our mouse model, we observed that continuous phenylephrine stimulation also provoked a pronounced fibrotic phenotype that was completely abolished by concurrent activation of adenosine A_1 receptors. Dubey and colleagues identified the A_{2B} receptor as the adenosine receptor subtype inhibiting fibrosis in rat cardiac fibroblasts (Dubey *et al.*, 1999, Dubey *et al.*, 2001), and Chen *et al.* demonstrated A_{2B} receptor-mediated anti-fibrotic effects by overexpression and knock-down of these receptors in rat cardiac fibroblasts (Chen *et al.*, 2004). Besides, *in vivo* studies of a rat model of myocardial infarction revealed that long-term activation of adenosine A_{2B} receptors has beneficial effects concerning fibrosis and ventricular remodelling (Wakeno *et al.*, 2006).

Consistent with the augmentation in connective tissue, phenylephrine also tended to up-regulate the CTGF- and collagen 1α2-coding genes, which regulate the synthesis of the extracellular matrix and activation of adenosine A_1 receptors tended to prevent this effect. However, the phenylephrine-evoked pro-fibrotic response seems to be mainly due to an increase in the cytokine TNFα. Selective activation of adenosine A_1 receptors tended to provoke also a slight augmentation in TNFα expression and to reduce the expression level of the pro-inflammatory factor in presence of the maladaptive agent. Taking into consideration, that only high TNFα levels evoke maladaptive effects, whereas moderate levels initiate adaptive responses (Mann, 2003), this observation could suggest an adaptive effect by which adenosine inhibits the transition from compensated hypertrophy to decompensated heart failure. Consistent with the phenylephrine-mediated up-regulation of TNFα, which can mediate the synthesis and activation of MMPs, we also observed a phenylephrine-mediated increased abundance of cleaved MMP-2, an important inducer of ventricular dilatation and matrix-degradation. This phenylephrine effect was abolished by concurrent adenosine A_1 receptor activation. Therefore, our observations could indicate that adenosine and in particular its A_1 receptor play a protective role in left ventricular remodelling. Wakeno *et al.* demonstrated only a contributory role of the A_{2B} receptor in the adenosine-mediated inhibition of MMP-2 in an infarction model (Wakeno *et al.*, 2006). However, their study was limited to expression analyses of the latent isoform of the enzyme. We focused on determining any potential effect of α_1-adrenoceptor stimulation on expression of the pro- and cleaved isoforms of MMP-2 and MMP-9 in the presence and absence of CPA. Extensive investigations over the last decades have identified these gelatinases to play major roles in the remodelling process. MMP-2 is known to process other pro-MMPs such as pro-MMP-1, −9, −13 (DeCoux *et al.*, 2014). Identifying potential inhibitors of abnormally enhanced MMP-2 expression/activity may therefore provide further benefits via interfering with processing of other MMPs. Expression analyses of pro-MMP-9 did not reveal any regulation following activation of adenosine A_1 receptors or α_1-adrenoceptors (Supporting Information Figure S1). Furthermore, the cleaved isoform of MMP-9 could not be detected in any group of mice. This could be due to the fact that MMP-9 is associated with the early remodelling process after myocardial infarction (Tao *et al.*, 2004, Ernens *et al.*, 2006, Wagner *et al.*, 2006). These findings suggested that MMP-9 plays a central role in the progressive remodelling process following tissue damage, where its activity underlies a negative regulation by adenosine, whereas it does not regulate the maladaptive response induced by long-term α-adrenoceptor stimulation.

Our data do not provide any evidence for a contribution of vasodilation or afterload reduction in the anti-hypertrophic effects mediated by the selective adenosine A_1 receptor agonist. We did not observe any CPA-mediated alterations in BP (Supporting Information Table S1), ejection fraction, FS or CO as (Cardiac output) determined via cardiac MRI nor did echocardiographic analyses hint towards any structural abnormalities of the aortic valve in any of the treatment groups. Furthermore, activation of adenosine A_1 receptors by CPA attenuates cAMP production subsequent to adenylate cyclase inhibition and would rather trigger vasoconstriction as reported in the aortic and coronary vasculature (Tawfik *et al.*, 2005, Tawfik *et al.*, 2006). Besides, our observation that CPA counteracts phenylephrine-induced hypertrophy by attenuating the increase in cardiomyocyte size, protein synthesis, sarcomeric organization and c-fos expression in isolated cardiomyocytes in the absence of any vasculature would indicate an anti-hypertrophic effect of CPA, independent of vasodilation and afterload reduction.

The next aim in our *in vivo* study was to investigate the mechanism by which activation of adenosine A_1 receptors antagonized the phenylephrine-provoked maladaptive response. Components of the MAP-kinase-pathway are well-known downstream targets of GPCR stimulation but our Western blot analyses did not reveal any key role of ERK1/2 in the cross-talk between the two signalling pathways (Supporting Information Figure S2) in spite of several reports of its contribution to regulating maladaptive hypertrophy (Dorn and Force, 2005, Bueno and Molkentin, 2002). However, our finding agrees with the work of Gan and colleagues (Gan *et al.*, 2005). Furthermore, expression patterns of inactive and active forms of GSK3-ß, AKT and p70S6K, already identified as targets of pathways induced by adaptive and maladaptive hypertrophic stimuli, did not reveal a regulatory role in our study (Supporting Information Figures S3–5).

Next, we addressed another mediator of maladaptive response – the formation of oxidative stress, which mediates cardiac remodelling processes including interstitial fibrosis following α_1-adrenoceptor stimulation (Ide *et al.*, 1999, Maack *et al.*, 2003). The up-regulation of 8OHdG, a marker of oxidative stress, (Valavanidis *et al.*, 2009) in cardiomyocytes as well as in non-cardiomyocyte myocardial cells by phenylephrine treatment was abolished by simultaneous activation of adenosine A_1 receptors. Increased ROS production is known to induce electromechanical dysfunction (Flesch et al., 1999), activation of adaptive and maladaptive MAP kinases (Giordano, 2005), induction of apoptosis (von Harsdorf *et al.*, 1999), pro-fibrotic events, cardiomyocyte hypertrophy (Sawyer *et al.*, 2002) and activation of MMP-2 and −9 (Gardner *et al.*, 2010), all of which are directly linked to LV remodelling. Therefore, it is possible that adenosine counteracts hypertrophy and fibrosis and thereby attenuates the transition from compensated hypertrophy to heart failure, by inhibiting ROS production. This hypothesis is

supported by previous studies showing that adenosine A_1 receptor-induced anti-hypertrophic effects in neonatal cardiomyocytes following phenylephrine stimulation are mediated by activation/opening of K_{ATP} channels (Xia *et al.*, 2004, Xia *et al.*, 2007, Hoque *et al.*, 2000), which in turn regulates ROS generation. In addition, it could be shown that selective adenosine A1 receptor activation inhibits opening of the Mitochondrial permeability transition pore (MPTP) and reduces ROS production following opening of K_{ATP}-channels under hypoxic conditions (Narayan *et al.*, 2001). Furthermore, Narayan and colleagues observed that adenosine A_1 receptor stimulation reduces ROS production and attenuates myocardial stunning following re-oxygenation after hypoxia/ischaemia by opening mitochondrial K_{ATP} channels (Narayan *et al.*, 2001). Inhibition of maladaptive ROS production under hypoxic and normoxic hypertrophic conditions would add to the effects of adenosine A_1 receptors in mediating cardioprotection. The molecular mechanisms underlying this effect and the origin of phenylephrine-induced ROS production deserve further investigations. It is likely that the pro-hypertrophic and pro-fibrotic formation of oxidative stress, following $G\alpha_q$-coupled α-adrenoceptor stimulation, represents the consequence of mitochondrial ROS generation. This hypothesis is based on the observation, made by Kimura and colleagues, that Ang II-mediated NOX (NADPH-oxidase) activation results in a short-time preconditioning via opening of mitochondrial K_{ATP} channels and triggering of mitochondrial ROS formation (Kimura *et al.*, 2005). Furthermore, Rabinovitch *et al*. reported that in transgenic mice, cardiac remodelling, evoked by persistent angiotensin AT_1-receptor (also coupled to $G_{\alpha q}$) stimulation, can be prevented by specific SS-31-mediated inhibition of mitochondrial ROS production, as well as by mitochondrial-targeted catalase expression (Dai *et al.*, 2011a). It has also been documented that the Ang II-mediated ROS-formation can be abrogated by inhibition of channels of the inner mitochondrium membrane, such as the MPTP and the IMAC (Inner membrane anion channel) (Dai *et al.*, 2011b). These findings suggest that a $G\alpha_q$-induced, NOX-dependent, ROS production entails an augmented mitochondrial radical production. Hence, mitochondrial ROS production would be predominantly responsible for induction of cardiac remodelling. This assumption is supported by the observation that activation of mitochondrial K_{ATP} channels reduces ROS formation. In turn, activation of these K_{ATP} channels has already been described as an adenosine-mediated cardioprotective mechanism (Narayan *et al.*, 2001, Xiang *et al.*, 2010).

In conclusion, the present study demonstrated that stimulation of adenosine A_1 receptors efficiently counteracted α_1-adrenoceptor-induced cardiomyocyte hypertrophy, fibrosis, processing of MMP-2 and the generation of oxidative stress – all characteristic of pathological hypertrophy. Because stimulation of the α_1-adrenoceptor is accompanied by an up-regulation of adenosine A_1 receptors, while stimulation of the receptors for Ang II- or IGF1 has no such effect, this may resemble a negative feedback mechanism by which adenosine specifically counteracts α_1-adrenoceptor-induced pathological hypertrophy. Moderate activation of adenosine A_1 receptors could represent a novel therapeutic target to prevent the transition from compensated to decompensated myocardial hypertrophy leading to heart failure.

Acknowledgements

We thank Dr Heiko Kilter for his contribution to the study. S.-L. P. and the work were supported by grants from the Ministry of Culture, Research and Higher Education and the National Research Fund of Luxembourg (AFR/BFR 04/20), by Intramural funding of the Universitaet des Saarlandes (HOMFOR) and the Deutsche Bundesministerium für Bildung und Forschung (BMBF 0314101). C. M. was supported by the Deutsche Forschungsgemeinschaft (Heisenberg Programm, SFB-894).

Author contributions

S.-L.P. designed, performed, analysed and interpreted the experiments, and wrote, submitted and revised the manuscript. A.M. performed and analysed MRI examinations. A.K. performed and analysed immunostaining experiments. P.F. contributed to the design and application of MRI examinations. D.W. contributed to the study design. M.B. supervised the work. C.M. supervised the work, contributed to experimental design and reviewed the manuscript. Y.D. contributed to the study design and revised the manuscript. All authors approved the final version for submission.

Conflict of Interest

None declared.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

http://dx.doi.org/10.1111/bph.13339

Figure S1 MMP-9 expression does not underlie a distinct regulation by α_1-AR or A1-R.
Figure S2 α_1-AR and A1-R stimulation, both tend to inactivate ERK42/44.
Figure S3 AKTexpression/activation does not underlie a distinct regulation by α_1-AR or A1-R.
Figure S4 Co-stimulation of α1-AR and A1-R leads to a shift towards the activated P70S6K.
Figure S5 Co-stimulation of α_1-AR and A1-R leads to activation of GSK3β.