

Beta 3 adrenoceptors protect from hypertrophic remodelling through AMP-activated protein kinase and autophagy

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Abstract

Aims The abundance of beta 3-adrenergic receptors (β 3-ARs) is upregulated in diseased human myocardium. We previously showed that cardiac-specific expression of β 3-AR inhibits the hypertrophic response to neurohormonal stimulation. Here, we further analysed signalling pathways involved in the anti-hypertrophic effect of β 3-AR.

Methods and results *In vitro* hypertrophic responses to phenylephrine (PE) were analysed in neonatal rat ventricular myocytes (NRVM) infected with a recombinant adenovirus expressing the human β 3-AR (AdVh β 3). We confirmed results in mice with cardiomyocyte-specific moderate expression of human β 3-AR (β 3-TG) and wild-type (WT) littermates submitted to thoracic transverse aortic constriction (TAC) for 9 weeks. We observed a colocalization of β 3-AR with the AMP-activated protein kinase (AMPK) both in neonatal rat and in adult mouse cardiomyocytes. Treatment of NRVM with PE induced hypertrophy and a decrease in phosphorylation of Thr172-AMPK ($/2$, $P = 0.0487$) and phosphorylation of Ser79-acetyl-CoA carboxylase (ACC) ($/2.6$, $P = 0.0317$), inducing an increase in phosphorylated Ser235/236 S6 protein ($\times 2.5$, $P = 0.0367$) known to be involved in protein synthesis. These effects were reproduced by TAC in WT mice but restored to basal levels in β 3-AR expressing cells/mice. siRNA targeting of AMPK partly abrogated the anti-hypertrophic effect of β 3-AR in response to PE in NRVM. Concomitant with hypertrophy, autophagy was decreased by PE, as measured by microtubule-associated protein 1 light chain 3 (LC3)-II/LC3-I ratio ($/2.6$, $P = 0.0010$) and p62 abundance ($\times 3$, $P = 0.0016$) in NRVM or by TAC in WT mice (LC3-II/LC3-I ratio: $/5.4$, $P = 0.0159$), but preserved in human β 3-AR expressing cells and mice, together with reduced hypertrophy.

Conclusions Cardiac-specific moderate expression of β 3-AR inhibits the hypertrophic response in part through AMPK activation followed by inhibition of protein synthesis and preservation of autophagy. Activation of the cardiac β 3-AR pathway may provide future therapeutic avenues for the modulation of hypertrophic remodelling.

Keywords Adrenergic receptor; AMP-activated kinase (AMPK); Autophagy; Heart failure; Cardiac hypertrophy

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Introduction

Haemodynamic overload, ischaemic, or oxidative stress promotes adverse cardiac remodelling, a leading cause of worsening heart failure¹. Most of these pathophysiologic conditions are associated with adrenergic stimulation,

resulting in adrenoceptor (AR) activation on different cell types within the myocardium. Among these, β 1-ARs are classically considered to mediate short-term positive effects on all aspects of myocardial contractility; however, long-term stimulation produces adverse effects on myocardial remodelling, in part through activation of calcium-dependent

pro-hypertrophic effects, ultimately associated with cardiomyocyte loss.² Such 'maladaptive' remodelling is usually accompanied with left ventricular geometry disruption and interstitial fibrosis leading to progressive diastolic and systolic heart failure. Conversely, β 3-AR mediates effects on cardiac myocytes that are antipathetic to β 1-AR, that is, protection from hypertrophic³ or fibrotic⁴ remodelling. Deciphering the underlying signalling pathways should yield new therapeutic strategies to favourably modulate remodelling.

AMP-activated protein kinase (AMPK) is a heterotrimeric serine/threonine protein kinase composed of one catalytic (AMPK α) and two regulatory subunits (AMPK β and AMPK γ) present under several isoforms (two for α and β and three for γ).⁵ AMPK is an important cellular sensor of energy level. Its activation derives from an increase in the intracellular AMP-to-ATP ratio that typically occurs during metabolic stresses, such as myocardial ischaemia.⁶ AMP stimulates AMPK allosterically via its binding to the AMPK γ -subunit and protects AMPK from de-phosphorylation on Thr172, a residue located in the activation loop of the AMPK α catalytic subunit. Once activated, AMPK participates in the maintenance of energy homeostasis by switching off anabolic pathways that consume ATP and by switching on alternative catabolic pathways that generate ATP.⁷ It is currently recognized that AMPK effects extend beyond its metabolic role.⁷ In agreement, it has been shown that AMPK activation prevents cardiac hypertrophy development.⁷ Accordingly, AMPK deletion leads to enhanced hypertrophy.⁸ At the molecular level, AMPK is known to inhibit the mechanistic target of rapamycin (mTOR) pathway via a complex mechanism.⁷ The mTOR pathway promotes protein synthesis and inhibits autophagy. Consequently, AMPK activation leads to a net balance in favour of protein degradation.

Autophagy is a conserved process from yeast to mammals for the bulk degradation and recycling of long-lived proteins and organelles.⁹ Intracellular components are surrounded by double membrane-bound autophagic vesicles which then fuse with lysosomes to form autolysosomes for degradation. Controlled by autophagy-related proteins (Atgs) such as Atg5 and 7, beclin 1, and microtubule-associated protein 1 light chain 3 (LC3), autophagy plays an essential role in maintaining cellular homeostasis and was initially believed to be a non-selective process. In eukaryotic cells, autophagy occurs constitutively at low levels to facilitate homeostatic functions such as protein and organelle turnover.¹⁰ It is rapidly upregulated when cells need to generate intracellular nutrients and energy or to remove damaging components resulting from oxidative stress or protein aggregates accumulation. Increasing evidence demonstrates that autophagy plays an essential role in cardiac remodelling to maintain cardiac function and cellular homeostasis in the heart. In certain circumstances, when excessive, the pro-survival functions of autophagy may paradoxically become deleterious.^{10,11}

In this study, we dissected the signalling pathway involved in the anti-hypertrophic effect of the human β 3-AR expressed in rodent cardiac myocytes, as previously used by us.^{3,4} We identified β 3-AR coupling to AMPK and downstream autophagy as key components for this protection.

Methods

Animals

The investigation conforms to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1985). All experimental protocols were approved by the local Ethics Committee.

Male mice harbouring an α -MHC-driven human β 3-AR transgene (β 3-TG), generated as described previously,¹² were used between 12–16 weeks. Ascending aorta constriction was performed as described.⁴ Briefly, after anesthetizing, a constrictive band was placed and tightened around the aorta constricted by a cannula with a width of 27 G. The ligature was not tightened in sham-operated mice. Doppler measurements of trans-stenotic gradients were systematically performed at Day 1, Weeks 3 and 9 post-surgery. Only mice with a velocity higher than 2.5 m/s were kept into the experiment. Mice were also submitted to the protease inhibitor leupeptin treatment to inhibit autophagic degradation (Leup, 40 mg/kg, intraperitoneal, 1 h).

In vitro cardiac myocytes preparations

Adult mouse ventricular myocytes (AMVM) were isolated from the hearts of 8-week-old β 3-TG mice. Mice were killed by an intraperitoneal injection of sodium pentobarbital overdose (300 mg/kg) with heparin (8000 units/kg), and the heart was rapidly excised. The ascending aorta was cannulated with a needle, and the heart was retrogradely perfused in a Langendorff perfusion system at 37 °C for 5 min with perfusion buffer. This was followed by 8 min of perfusion with digestion buffer [4 mg/mL trypsin, 5 mg/mL liberase (Roche), and 0.3 mM CaCl₂]. The ventricles were removed, chopped into small pieces in stop buffer (BSA 50 mg/mL, 0.12 mM CaCl₂), and gently agitated for 3 min. The supernatant containing isolated myocytes was centrifuged (1000 rpm for 1 min), and the myocytes were resuspended in stop buffer and subjected to a step-wise recalcification protocol (5 × 4 min stepwise increase in CaCl₂ concentration from 62 to 112 to 212 to 500 μ M to 1 mM). The myocytes were plated on laminin-coated Labtek culture slides. After 1 h, stop buffer was replaced by plating medium (MEM with GBS 5%, BDM 10 mM, penicillin 100 U/mL, and L-glutamine 2 mM).

Ventricular myocytes from 1–2 days old neonatal rats were isolated by collagenase/pancreatin digestion as previously described.³ Approximately 20 h post-isolation, myocytes were transferred to serum-free media and infected with a recombinant adenovirus encoding a polycistronic construct encoding the human *ADRB3* (β 3-AR) cDNA (form C) and GFP at a multiplicity of infection of 1.0 plaque forming units per cell; an adenovirus encoding GFP only was used as control. Twenty-four hours after infection, myocytes were treated with either phenylephrine (PE, 20 μ M) to induce hypertrophy or the protease inhibitor leupeptin to inhibit autophagic degradation (Leup, 150 μ M) in serum-free conditions.

AMPK knockdown by small interfering RNA transfection

Neonatal rat ventricular myocytes (NRVMs) at a confluence of 50% were transfected with either control non-targeting siRNA or pooled siRNA targeting both AMPK α 1 and AMPK α 2 catalytic isoforms (AMPK α 1/ α 2 siRNA) (from GE Healthcare 50 nM) using lipofectamine RNAimax transfection reagent (Invitrogen) according to the manufacturer's protocol. PE treatment was started 66 h post-siRNA transfection.

Subcellular fractionation

Left ventricular extracts were prepared in 1 mL of 0.5 M Na₂CO₃ (pH 11) and added to 1 mL of 80% sucrose (in 0.15 M NaCl; 25 mM MES, pH 6.5; final sucrose concentration, 40%). A two-step gradient was loaded on the top of the sample (4 mL of 35% sucrose overlaid with 4 mL of 5% sucrose). After ultracentrifugation at 38 000 rpm (18 h, 4 °C), fractions were collected from top to bottom, precipitated with trichloroacetic acid (7.2%), washed, and resuspended in Laemmli for discontinuous (8% to 12% SDS-polyacrylamide) electrophoresis and immunoblotting with antibodies (Supporting Information, *Table S1*).

Western blotting

Denatured proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred on nitrocellulose membrane, then blocked 1 h in 5% non-fat dry milk in TBS-Tween and incubated overnight in 1% milk Tween-TBS with primary antibodies. Following washing (3 \times 10 min in Tween-TBS) membranes were incubated with relevant secondary antibodies in 1% milk Tween-TBS. After a final washing step (3 \times 10 min Tween-TBS), membranes were revealed by chemiluminescence. Further details on primary and secondary antibodies are provided in Supporting Information, *Table S1*.

Radiolabelled aminoacids incorporation

Neonatal rat ventricular myocyte (NRVM) were plated at 1 000 000 per plate in six-wells plates. After infection, myocytes were treated at the same time with PE (20 μ M) and [¹³C]-phenylalanine (5 μ Ci/mL) in serum-free conditions for 24 h. Cells were then lysed in 50 mM HEPES, 50 mM KF, 1 mM Kpi, 5 mM EDTA, 5 mM EGTA, 15 mM β -mercapto-ethanol, and 5% triton and incubated with 10% trichloroacetic acid for 20 min at 4 °C to precipitate proteins. Precipitated material was neutralized with 100 mM NaOH. The samples were precipitated a second time with 10% trichloroacetic for 20 min at 4 °C. After re-suspension of protein pellets in formic acid, incorporated radioactivity was measured by liquid scintillation counting.

Histology and immunostaining

All morphometric and histologic measurements were obtained from hearts arrested in diastole (intracardiac KCl), washed, and fixed. Transversal cryo-sections were defrosted slowly in acetone, before washing (PBS; 3 \times 5 min). Sections were then incubated with wheat germ agglutinin (1/150; 2 h, in the dark). After additional washing, sections were mounted with Vectashield and observed with Axioimager Z1 Apotome system with MRm Rev3 AxioVision-Deconvolution 3D camera. Data from multiple sections per heart were analysed using AxioVision software 4.8.2.0 (Carl Zeiss Microimaging GmbH, Germany).

Immunocytochemistry and proximity ligation assay

Neonatal rat ventricular myocyte (NRVM) were fixed with 4% paraformaldehyde in PBS for 20 min. After washes (PBS, 3 \times 5 min), cells were permeabilized with 0.1% triton X-100 in PBS for 10 min. After three washes, cells were incubated with 5% BSA in PBS for 30 min and then incubated with primary antibodies as needed (Supporting Information, *Table S1*) overnight at 4 °C. After three washes, cells were incubated with secondary antibodies diluted at 1/300 for 1 h, then with Dapi 1/50 000 in PBS for 5 min and mounted.

A PLA kit II (Eurogentec, Seraing, BE) was used according to the manufacturer's instructions with slight modifications. Briefly, cardiomyocytes were fixed and transiently permeabilized (with 4% paraformaldehyde in PBS; 10 min), washed (PBS; 2 \times 2 min), quenched (50 mM NH₄Cl; 2 \times 5 min), washed, and blocked (blocking buffer; 30 min, 37 °C) before being incubated overnight (4 °C) with primary antibodies (Supporting Information, *Table S1*). The following day, cardiomyocytes were washed (Buffer A; 2 \times 5 min) and

incubated with the PLA probe secondary antibodies [plus = anti-rat (1/150 v/v); minus = anti-mouse (1/8 v/v); 60 min, 37 °C]. After this step, cardiomyocytes were washed (Buffer A), and the ligation and amplification steps were carried out by addition of ligase (1/40 v/v in ligation buffer; 30 min, 37 °C) followed by washing (Buffer A), and addition of polymerase (1/80 v/v in amplification buffer; 100 min, 37 °C). After a final washing step (2 × 10 min 1 × Buffer B, 1 min 0.1× Buffer B), Labteks were mounted with Duolink mounting medium with DAPI and observed with Axioimager Z1 Apotome system with MRm Rev3 AxioVision-Deconvolution 3D camera (Zeiss, Zaventem, BE). The data analysis was performed with Axiovision software (Zeiss).

Statistical analysis

Results are expressed as mean ± SEM calculated from the measurements obtained from each group of cells and mice. When normal distribution (Kolmogorov–Smirnov test) was confirmed, raw data were analysed using two-way ANOVA followed by Bonferroni *post hoc* test for multi-group comparisons. In the absence of normal distribution, data were compared using nonparametric tests (Kruskal–Wallis followed by Dunn correction for multiple comparisons or Mann–Whitney). Statistical significance was accepted at the level of $P < 0.05$

Results

β3-AR and AMPK are co-localized in caveolae

We used a heterozygous transgenic mouse line with cardiac myocyte-specific, moderate level expression of the human β3-AR (β3-TG) to examine the subcellular distribution of the β3-AR and AMPK. Cardiac extracts were resolved by isopycnic ultracentrifugation. As shown in *Figure 1A*, immunoblotting revealed that the β3-AR protein was mainly distributed in light fractions, together with a majority of caveolin-3 (Cav-3), the main caveolar coat protein expressed in muscle cells.³ Moreover, in the same extracts, a part of AMPK (using antibody recognizing both AMPKα1/2 catalytic subunits) co-segregated with β3-AR and Cav-3 signals (*Figure 1A*). To verify that this corresponds to the location in intact cells, we used proximity ligation assay to examine the co-localization of AMPK and Cav-3 in AMVM; as illustrated in *Figure 1B*, positive fluorescence signals indicated co-localization of the two proteins in adult cardiomyocytes.

Effect of neurohormonal stimuli on AMPK signalling pathway in neonatal rat cardiac myocytes

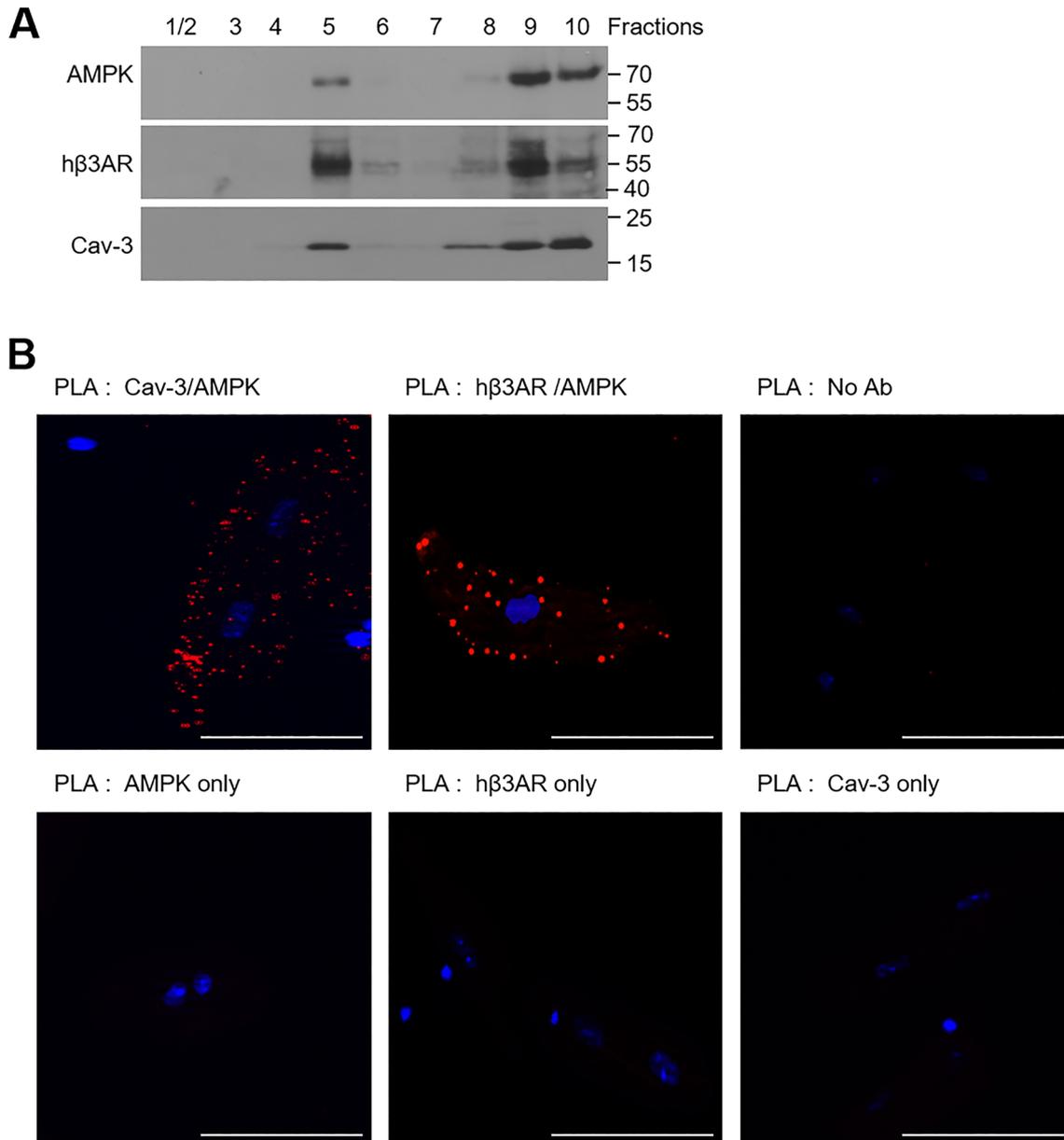
To examine how expression of the β3-AR could attenuate hypertrophy in response to a neurohormonal stimulus, we used an *in vitro* model of homotypic cultures of neonatal rat cardiac myocytes (NRVM) with adenoviral expression of the human β3-AR (AdV-β3-AR) or GFP alone used as control (AdV-GFP). AdV-β3-AR-infected NRVM expressed human β3-AR transcripts in proportion to the titer of recombinant viruses, as measured by RT-qPCR (*Figure 2A*). Immunocytochemistry confirmed the expression of the human β3-AR protein at the membrane of infected NRVMs (*Figure 2B*). We also used proximity ligation assay to verify the co-localization of AMPK and β3-AR in NRVM; as illustrated in *Figure 2C*, positive fluorescence signals indicated co-localization of the two proteins both in AdV-GFP (with low endogenous β3-AR expression) and in AdV-β3-AR-infected neonatal cardiac myocytes, both in absence and after stimulation with phenylephrine. No PLA signal was observed in negative controls (i.e. in the absence of anti-AMPK primary antibody, Supporting Information, *Figure S1*).

Control, GFP-infected myocytes developed hypertrophy in response to phenylephrine (PE).³ This was associated with a decrease in AMPK signalling pathway, identified by a decrease in Thr172 phosphorylated-AMPK (*Figure 2D*) and a decrease in phosphorylation of its substrate acetyl-CoA carboxylase (ACC) on Ser79 (*Figure 2E*). The inhibition of AMPK pathway correlated with the activation of mTOR pathway, assessed from the phosphorylation state of its indirect downstream target, the ribosomal S6 protein (S6) on Ser235/236 (*Figure 2F*). This was associated with an increase in radiolabelled amino acids integration into proteins (*Figure 2G*). In contrast, AdV-β3-AR-infected myocytes retained basal level of AMPK, ACC, and S6 phosphorylation and unchanged protein synthesis rate despite PE stimulation (*Figures 2D–2G*).

Effect of haemodynamic overload on AMPK signalling pathway in mice

Adult β3-TG mice were subjected to transaortic constriction (TAC) and their phenotype analysed at 9 weeks post TAC. Morphometric data are illustrated in *Figure 3A*. All parameters were identical between genotypes in sham-operated mice. All mice included in the TAC study [β3-TG and wild type (WT)] developed a trans-stenotic gradient with maximum velocity (by Doppler echo) of at least 2.5 m/s, and gradients were comparable between genotypes. WT mice developed myocardial hypertrophy, as evidenced by an increase in left ventricular weight normalized to tibial length (*Figure 3A*) and increased myocyte transverse area measured

Figure 1 β 3-adrenoceptor and AMPK subcellular localization in β 3-transgenic mouse hearts. (A) Subcellular fractionation of cardiac extracts of TG mice separated by ultracentrifugation on sucrose gradients; each fraction (1 to 10 fractions of increasing densities) was analysed by immunoblotting for AMPK, β 3-AR, and myocyte-specific caveolin-3 (Cav-3) proteins. (B) Colocalization of Cav-3 or β 3-AR and AMPK in transgenic adult mouse ventricular myocytes by proximity ligation assay; red dots indicate co-localization of both proteins in the presence of the two respective primary antibodies (upper row, left, and center); negative controls are illustrated in the presence of only one primary Ab or no Ab. Scale bar is 100 μ m.



histologically (Figure 3B). However, increases in both parameters were significantly milder in β 3-TG mice.

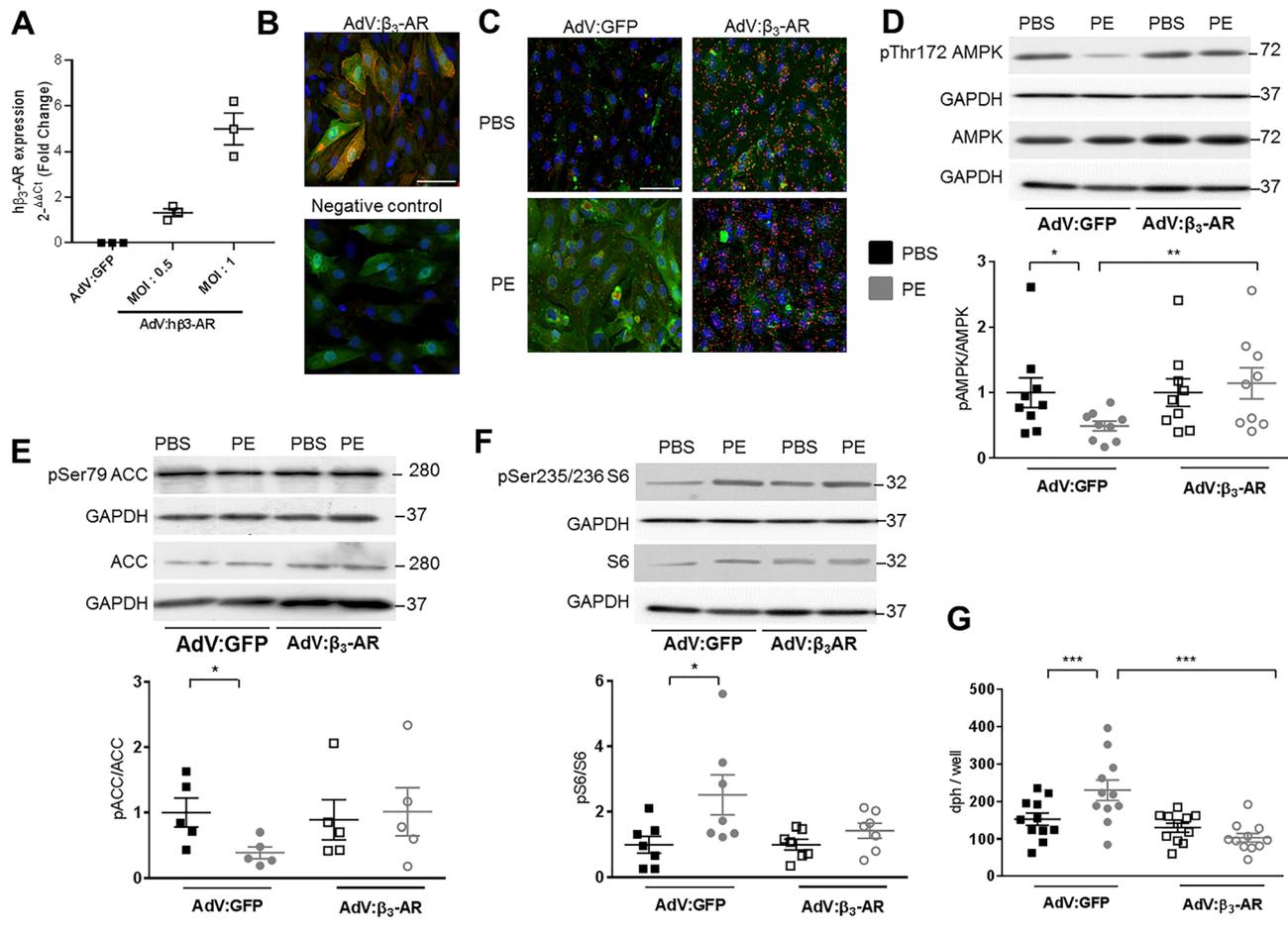
At the molecular level, 9 weeks of TAC resulted in a significant decrease in AMPK signalling, as identified by a decrease in AMPK phosphorylation on Thr172 (Figure 3C), a decrease in ACC phosphorylation on Ser79 (Figure 3D), and an increase in S6 phosphorylation on Thr389 (Figure 3E) in WT mice. However, basal levels of phospho-Thr172-AMPK (Figure 3C), phospho-Ser79-ACC (Figure 3D), and phospho-Thr389-S6

(Figure 3E) appeared unchanged, albeit with some variability, from sham-operated controls in β 3-TG mice.

Role of AMPK in the anti-hypertrophic effect of β 3-AR

These data and our observation of co-localization of β 3-AR and (at least part of) AMPK in caveolar fractions (see previous

Figure 2 Effect of β_3 -adrenoceptor expression on AMPK signalling pathway after phenylephrine treatment in neonatal cardiac myocytes. (A) RT-qPCR of β_3 -AR mRNA in AdV-GFP (control) and AdV- β_3 -AR-infected neonatal rat ventricular myocytes (NRVM). Data expressed as fold change compared with the condition M.O.I.: 0.5. (B) (Upper) Immunocytochemical detection of β_3 -AR in AdV- β_3 -AR-infected NRVM and (lower) negative control in the absence of primary antibody. (C) Colocalization of AMPK with endogenous β_3 -AR in AdV-GFP (control) and with β_3 -AR in AdV- β_3 -AR-infected NRVM by proximity ligation assay; red dots indicate co-localization of both proteins in the presence of the two respective primary antibodies. Scale bar is 50 μ m. (Negative controls in the absence of primary antibody are presented in Supporting Information, Figure S1). (D, E, and F) Western blot of phosphorylated AMPK (D), ACC (E), and S6 (F) in response to phenylephrine (PE) in AdV-GFP (control) and AdV- β_3 -AR-infected NRVM (immunoblotted signals normalized to respective control values in PBS). (G) Quantification of radiolabelled phenylalanine incorporated into proteins in response to PE in AdV-GFP (control) and AdV- β_3 -AR-infected NRVMs. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ from at least five experiments.



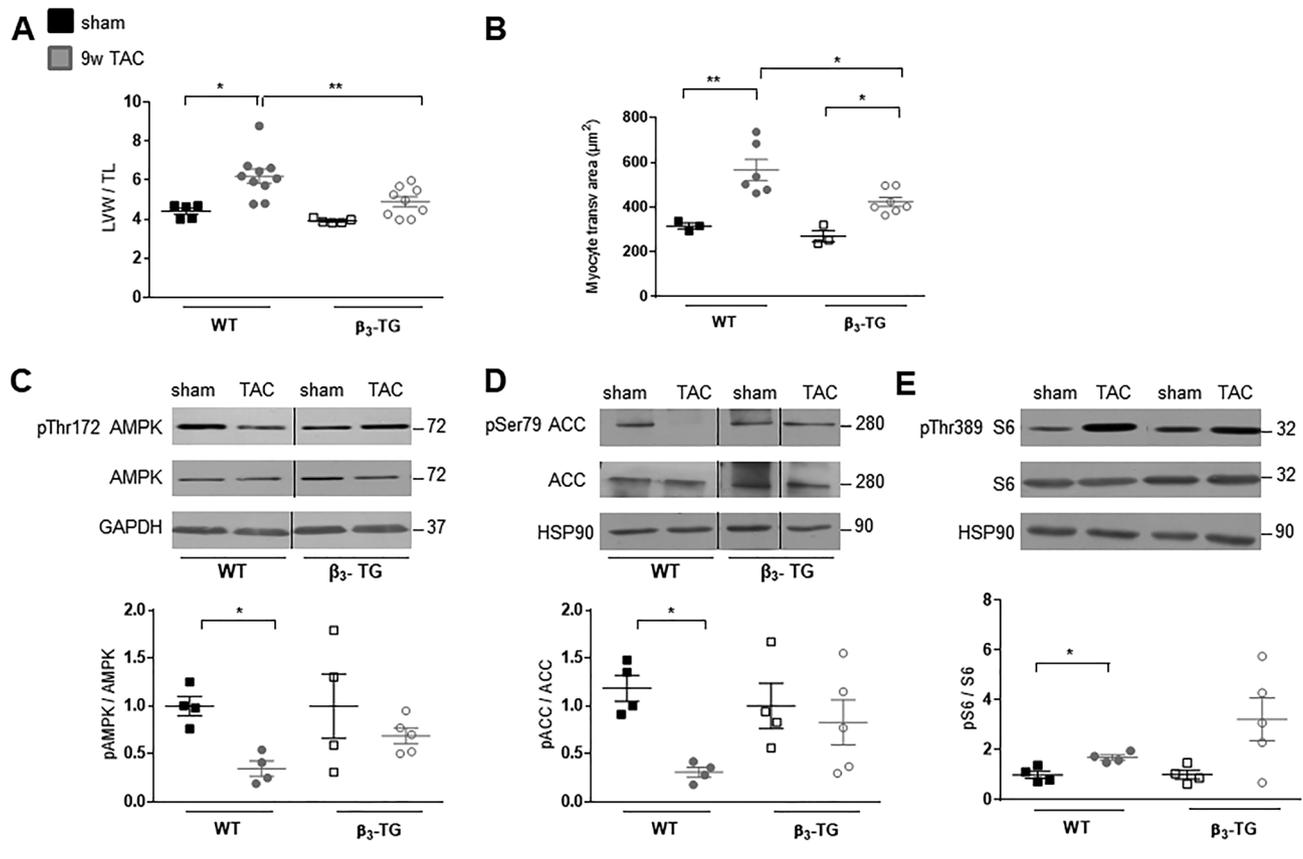
texts) prompted us to examine the causality of AMPK in the modulation of the hypertrophic response by β_3 -AR. To do this, we tested the effect of AMPK downregulation on the hypertrophic response to PE in NRVM. Cultured NRVMs with adenoviral expression of the human β_3 -AR (or GFP control) were transfected with siRNAs targeting both AMPK α 1/2 catalytic subunits (or scramble control) and then treated with PE. High efficiency of AMPK α 1/2 knockdown was validated by immunoblot showing the complete disappearance of AMPK α signal (Figure 4). As expected, β_3 -AR expression almost completely attenuated the hypertrophic response to PE compared with GFP control in si-scramble-transfected cells (Figure 4, bottom left). AMPK silencing potentiated the

hypertrophic response to PE in GFP-expressing myocytes; although β_3 -AR expression still attenuated the hypertrophic response to PE, it was only reduced by half, in si-AMPK-transfected cells compared with GFP control (Figure 4, bottom right).

Effect of neurohormonal stimuli on autophagy signalling pathway in cardiac myocytes

Control AdV-GFP-infected myocytes developed hypertrophy in response to PE together with a decrease in autophagy identified by a decrease in LC3 II/I ratio (Figure 5A), an

Figure 3 Effect of β_3 -adrenoceptor expression on AMPK signalling pathway after 9 weeks of TAC in mice. (A and B) Changes in (A) left ventricular mass assessed by gravimetry, (B) myocyte transverse section areas at 9 weeks post TAC in WT and β_3 -TG mice. (C, D, and E) Western blot of phosphorylated AMPK (C), ACC (D), and S6 (E) at 9 weeks post TAC in WT and β_3 -TG mice (signal normalized to respective control values in sham). * $P < 0.05$ and ** $P < 0.01$, from at least three sham mice and six TAC mice.



increase in p62 expression (Figure 5B), and a 24.5% decrease of autophagic cells (by LC3 immunostaining quantification) (Figure 5C). By contrast, AdV- β_3 -AR-infected myocytes maintained their basal level of autophagy (monitored with the same three criteria) despite PE stimulation. Of note, a similar decrease in LC3 II/I ratio was observed after TAC in myocardial extracts from WT mice; however, basal levels were again maintained in β_3 -TG mice after TAC (Figure 5D).

We then analysed autophagic flux by inhibiting autophagic degradation with the protease inhibitor leupeptin (Figure 6). As expected, leupeptin treatment increased the level of LC3 II/I ratio and percentage of LC3-marked cells in AdV-GFP-infected myocytes, unravelling a basal autophagic flux (Figures 6A and 6C). Leupeptin also reversed the decrease of these indexes induced by PE treatment (Figures 6A and 6C). However, leupeptin had no effect in AdV-h β_3 -infected myocytes, suggesting that autophagy is already maximal in these cells, with or without PE (Figure 6B). Interestingly, these effects of leupeptin were reproduced in hearts of WT and β_3 -TG mice (Figure 6D) after intraperitoneal injection.

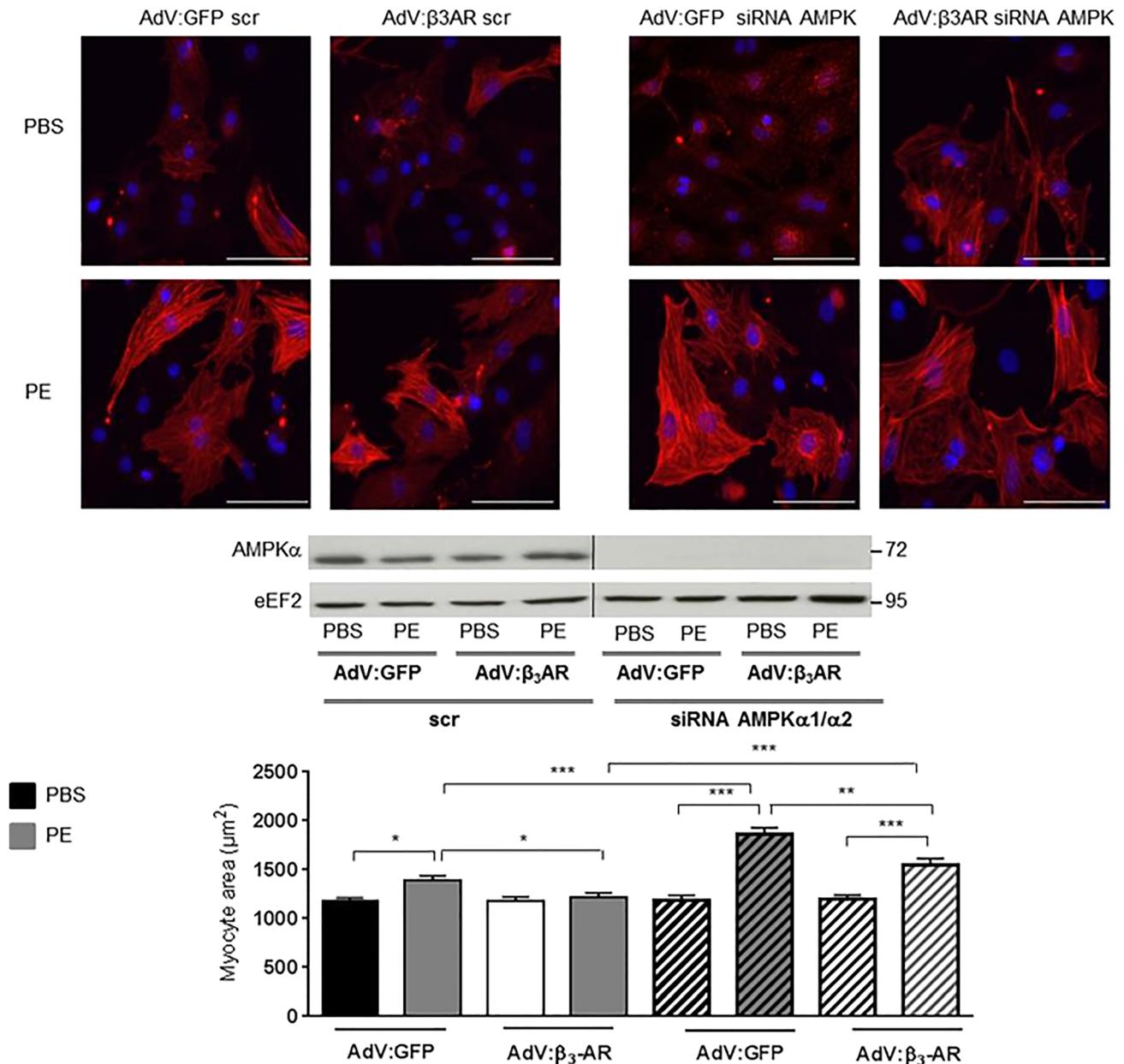
Similar effects were reproduced with chloroquine that inhibits the autophagosome-lysosome fusion and degradation (not shown).

Discussion

The main findings of this study are the following: (i) the β_3 -AR co-localizes with (part of) AMPK in caveolae-enriched membrane fractions of AMVM; (ii) β_3 -AR expression restores basal level of AMPK signalling and autophagy in response to neurohormonal (PE) and haemodynamic (TAC) stress; and (iii) AMPK is, at least partially, involved in the anti-hypertrophic effect of β_3 -AR.

The β_3 -AR was first cloned from a human adipocyte cDNA library and is expressed mainly in brown/beige adipocytes, where it mediates non-shivering thermogenesis.¹³ It was later found to be expressed in cardiac myocytes from several species, including human ventricular cardiac biopsies in which,

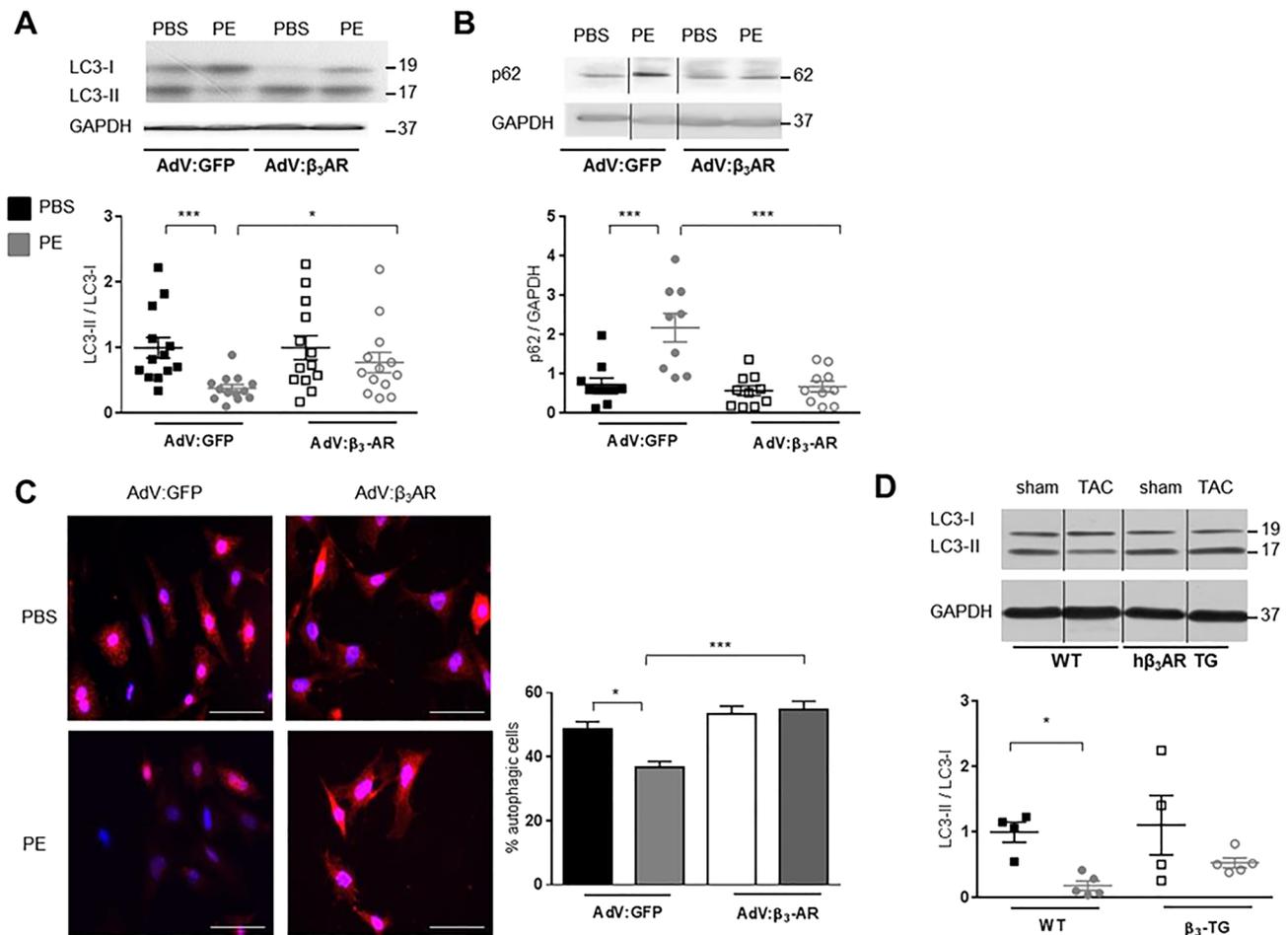
Figure 4 Effect of AMPK inhibition on β_3 -adrenoceptor regulation of hypertrophy in cardiac myocytes. Changes in cell size in AdV-GFP and AdV- β_3 -AR infected NRVMs after PE alone or with concurrent administration of siRNA targeting AMPK- $\alpha_1/2$ (or scramble control). Upper: representative images of NRVM stained for alpha-actinin (red) and nuclei (DAPI: blue). Scale bar is 100 μm . Lower: representative western blot showing the inhibitory effect of the siRNA construct on AMPK α expression. Bottom: bar graphs illustrating the quantification of myocyte sizes under the different treatment conditions * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$, from six experiments (at least 185 cells analysed).



contrary to β_1 -2ARs, β_3 -AR stimulation decreases contractile tone *in vitro*.¹⁴ As β_3 -AR expression increases in cardiomyopathic hearts,¹⁵ this led to the hypothesis that β_3 -AR may confer protection from the deleterious overstimulation of β_1 -2AR as observed in the course of heart failure progression.¹⁶ In this study, we used a heterozygous transgenic mouse with cardiac-specific, moderate expression of

the human β_3 -AR that replicated the expression level of the receptor and the functional response to β_3 -AR agonists observed in human biopsies.^{4,12} Contrary to β_1 -2 AR transgenics, these β_3 -AR transgenic mice are protected from neurohormone and TAC-induced hypertrophic remodelling.³ The present study further analysed the mechanisms underlying this protection.

Figure 5 Effect of β_3 -adrenoceptor expression on autophagy after PE treatment in cardiac myocytes. (A and B) Western blot of LC3-II/LC3-I (A) and p62 (B) in AdV-GFP and AdV- β_3 -AR-infected NRVMs with/without PE stimulation (signal normalized to respective control values in PBS). (C) Quantification of percentage of autophagic cells in AdV-GFP and AdV- β_3 -AR-infected NRVMs with/without PE stimulation (at least 70 images analysed/condition). (D) Western blot of LC3-II/LC3-I in cardiac extracts from WT and β_3 -TG mice after 9 weeks of TAC or sham operation (signal normalized to respective control values in sham). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$, from at least nine experiments *In vitro* and four sham mice and five TAC mice.

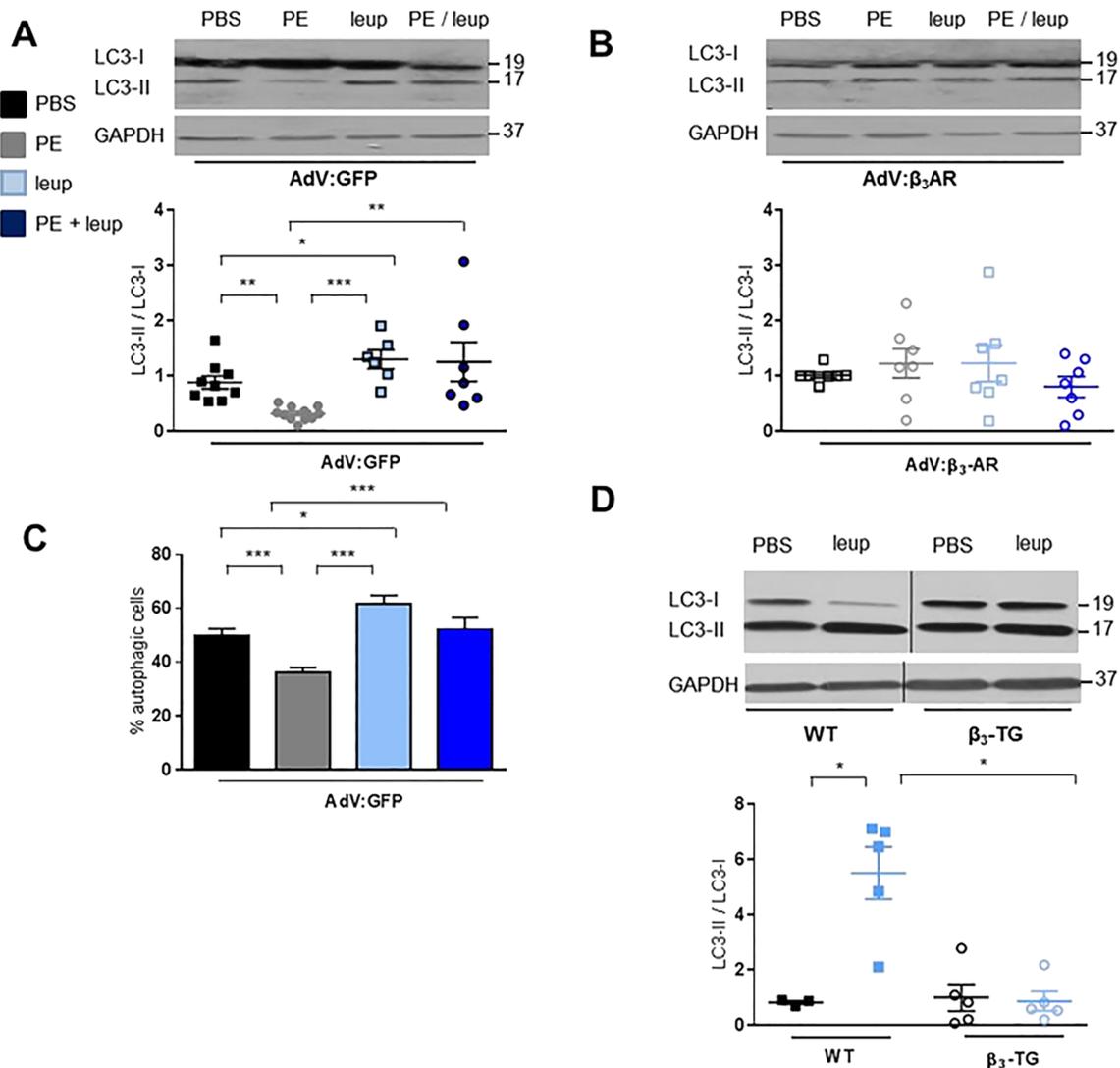


We previously reported that the recombinant β_3 -AR colocalized with Cav-3 in lipid rafts/caveolae,³ together with the endothelial nitric oxide synthase (eNOS).¹⁷ Here, we show that AMPK is also partially confined with the recombinant β_3 -AR in caveolae in adult mouse cardiac myocytes, as previously observed with caveolar NOX in rat cardiac myocytes.¹⁸ Moreover, we confirmed a similar colocalization of AMPK with endogenously expressed β_3 -AR by PLA in NRVM, suggesting interaction of the two proteins at natural expression levels of the receptor.

Because adult cardiomyocytes have little capacity for cellular proliferation, their only means of growth is hypertrophy. Cardiac hypertrophy is considered to be an initially adaptive response, counteracting increased wall tension and helping to maintain cardiac output. Cardiac mass is determined by the balance between protein synthesis and degradation, known to be tightly regulated during the development of

cardiac hypertrophy.¹⁹ Accordingly, we observed that hypertrophy induced a decrease in phosphorylation of AMPK on its Thr172 activation site, resulting in AMPK signalling pathway inhibition. Modulation of AMPK activity during cardiac hypertrophy is controversial in the literature. Indeed, in one study, no modification in AMPK activity or phosphorylation was found during the first 8 weeks of TAC-induced hypertrophy²⁰ whereas another study reported an activation after 17 to 19 weeks post-surgery.²¹ Such late AMPK activation can be hypothesized as a negative feedback mechanism to reduce hypertrophy progression. In both *in vitro* and *in vivo* protocol settings used in our study, hypertrophic stimuli clearly induced AMPK inhibition. Hypertrophy-mediated AMPK downregulation was associated with a decrease in S6 phosphorylation and an increase in radiolabelled aminoacid incorporation into proteins. Expression of the human β_3 -AR clearly restored AMPK signalling pathway and attenuated

Figure 6 Autophagic flux in β_3 -adrenoceptor-expressing cardiac myocytes *in vitro* and *in vivo*. (A and B) Western blot of LC3-II and LC3-I in response to PE with/without leupeptin (leup) in AdV-GFP and AdV- β_3 -AR-infected NRVMs (signal normalized to respective values in PBS control). (C) Quantification of percentage of autophagic cells in response to PE with/without leupeptin in AdV-GFP infected NRVMs (at least 33 images analysed/condition). (D) Western blot of LC3-II and LC3-I in response to leupeptin in cardiac extracts from WT and β_3 -TG mice (signal normalized to respective values in PBS control). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$, from at least six experiments *in vitro* and three mice in each group.

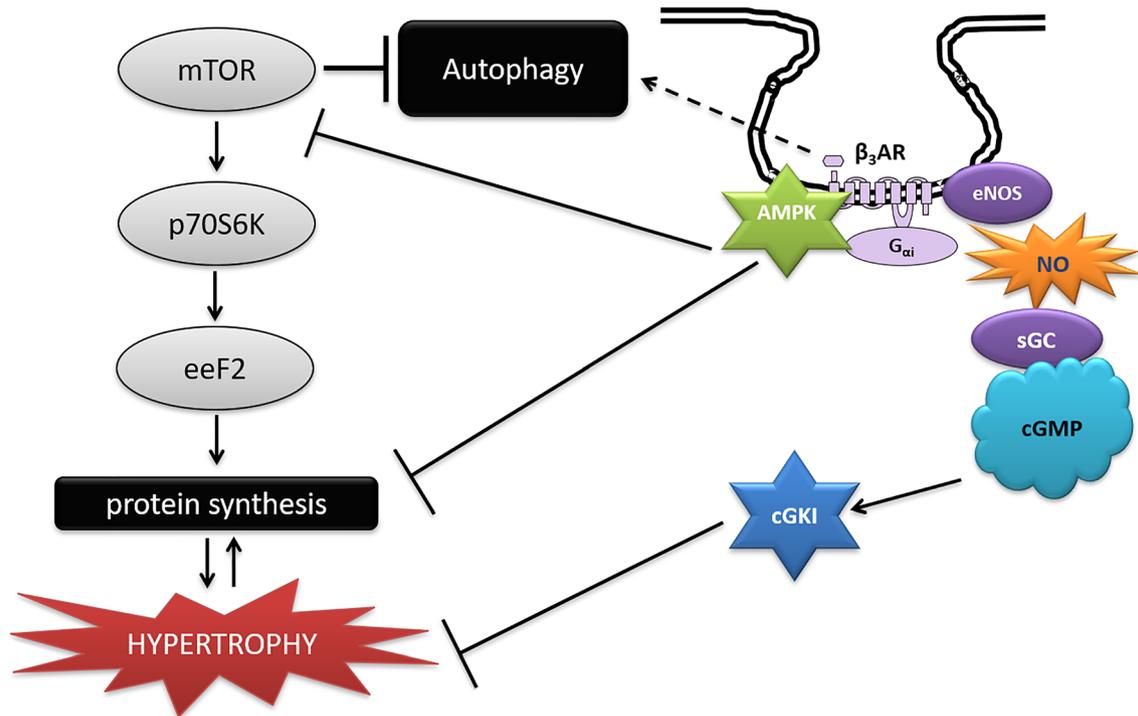


hypertrophy in PE-stimulated NRVMs and hearts with TAC-induced hypertrophic remodelling. The implication of AMPK downstream β_3 -AR activation was clearly demonstrated *in vitro* with the use of specific siRNA against AMPK catalytic subunits, which partly restored the hypertrophic response in β_3 -AR overexpressing cells. This is in line with recent data showing that exercise training protects the heart against ischaemia/reperfusion injury via the stimulation of a β_3 -AR-AMPK signalling pathway.²² Note that we previously demonstrated that the heterologously expressed β_3 -AR is constitutively active,³ explaining its tonic effect in the absence of stimulation with specific agonists. Nevertheless, despite

AMPK silencing, the β_3 -AR retained a diminished but still present anti-hypertrophic effect that could be explained by alternative coupling to pathways downstream endothelial (e-) and neuronal (n-) nitric oxide synthases, (Figure 7) as previously reported by us.³

mTOR integrates upstream signals from various stresses such as cellular energy levels and inhibits autophagy. Reciprocally, AMPK could activate autophagy by inhibiting mTOR, and both AMPK and mTOR can themselves be phosphorylated by Unc-51-like autophagy activating kinase 1, providing an additional level of regulatory feedback to modulate autophagy.²⁴ Autophagy is an evolutionarily conserved

Figure 7 Schematic representation of β_3 -adrenoceptor inhibitory signalling to hypertrophic pathways. β_3 -AR colocalizes with several effectors in caveolar membranes of cardiac myocytes; (right) previously identified coupling to nitric oxide synthase (eNOS) activates NO/sGC/cGMP/cGKI pathway known to attenuate stress-induced hypertrophy (for review, see Farah *et al.*,²³); (left) the presently identified interaction with AMPK recruits this pathway to inhibit protein synthesis and hypertrophy under stress as well as the upstream pro-hypertrophic mTOR complex; inhibition of mTOR would also relieve its negative effect on autophagy, thereby increasing autophagic flux, although the latter may also be independently activated by β_3 -AR coupling to alternative effectors.



mechanism that targets damaged proteins or organelles for degradation through lysosomes. Autophagy can be non-selective or organelle specific, such as mitochondria-specific autophagy, and plays an essential role in maintaining cellular homeostasis in the heart at baseline and in response to stress. Indeed, reduced autophagy gradually accumulates harmful proteins and generates reactive oxygen species from damaged mitochondria in the hypertrophied hearts. In line with this, we observed that TAC-induced or PE-induced hypertrophy was accompanied with a decrease in autophagy both *in vitro* and *in vivo* and expression of the human β_3 -AR restores autophagy, consistent with its protective effect against hypertrophy and remodelling. We used leupeptin, an inhibitor of autophagolysosomal proteases (as well as chloroquine, an inhibitor autophagosome-lysosome fusion and degradation), to monitor autophagic flux in these models. Leupeptin has already been reported to induce LC3II accumulation in adult rat cardiomyocytes.²⁵ As expected, we observed an accumulation of LC3II in NRVMs and hearts treated with these inhibitors. Interestingly, leupeptin (or chloroquine) did not further increase LC3II in β_3 -AR-expressing myocytes *in vitro* or *in vivo*, possibly because autophagy flux may already have been maximally stimulated.

These results are consistent with previous observations on autophagy in cardiac hypertrophy. Indeed, autophagy was shown to be suppressed in TAC-induced hypertrophied hearts at 1 week post-surgery compared with sham-operated hearts²⁶ or diminished in response to supravalvular aortic constriction²⁷ or β -adrenergic stimulation.²⁸ Interestingly, autophagy has been shown to participate in the anti-hypertrophic action of AMPK.²⁹ Nevertheless, we cannot exclude that β_3 -AR may activate autophagy independently of AMPK at this point. More generally, autophagy is also decreased in the aging heart whereas cardiovascular diseases progressively increase with age.³⁰ Increasing evidence suggests that autophagy is involved in the regulation of lifespan and aging. Autophagy may be especially important in non-proliferating cells, such as differentiated cardiac myocytes, in which there is no dilution of accumulated toxic material with cell division.

Limitations and perspectives

Admittedly, our observations are based on models with heterologous expression of a recombinant human β_3 -AR in

cardiac myocytes *in vitro* and *in vivo*, with the caveat that genetically reconstituted signalling may not faithfully replicate the coupling of the endogenous receptor *in situ*. However, we used a line of transgenic animals selected on the basis of levels of β 3-AR expression that replicates the abundance observed in human myocardial biopsies, as demonstrated previously.⁴ We also verified the co-confinement of AMPK and low endogenously expressed β 3-AR in NRVM. Given the ubiquitous expression and widespread role of AMPK, it would be of interest to verify similar coupling to β 3-AR in other tissues. β 3-AR transcripts³¹ and proteins³² have clearly been identified in human white and brown adipocytes, where they can be activated by selective β 3-AR agonists, such as mirabegron.³¹ Given the emerging importance of brown adipose tissue in regulating metabolism and cardiovascular function^{33,34} and the role of AMPK in promoting mitochondrial biogenesis,³⁵ critical for brown fat lipolytic activity, we can speculate that the β 3-AR coupling mechanism identified here could be exploited therapeutically to prevent or treat cardiovascular diseases associated with obesity and disordered metabolism. Such systemic effects are currently being studied in the European beta 3-LVH trial testing the re-purposing of mirabegron in patients with structural cardiac remodelling with or at risk of developing heart failure with preserved ejection fraction.³⁶ Further, β 3-AR modulation of visceral adipose tissue may have remote effects on cardiac aging by regulating fibroblast senescence,³⁷ although this has not been directly demonstrated so far.

In conclusion, moderate expression of β 3-AR in cardiac myocytes protects from myocardial remodelling by modulation of AMPK signalling pathway and autophagy. These observations may open new therapeutic perspectives with specific β 3-AR agonists for the treatment of hypertrophic cardiomyopathies.

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Conflict of interest

None declared.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1. Negative control in absence of anti-AMPK primary antibody (h β 3 AR Ab only) for colocalization of h β 3-AR and AMPK in AdV-GFP (control) and AdV- β 3-AR-infected NRVM by Proximity Ligation Assay. Note different magnification, scale bar is 50 μ m.

Table S1. List of antibodies used.

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