



Beta₂-adrenergic receptor regulates cardiac fibroblast autophagy and collagen degradation

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ARTICLE INFO

Article history:

Received 28 January 2010

Received in revised form 12 June 2010

Accepted 6 July 2010

Available online 15 July 2010

Keywords:

Catecholamine isoproterenol norepinephrine

propranolol salbutamol ICI-118,551

dihydroalprenolol

Beta adrenergic receptor

LC3 macroautophagy lysosome autophagy

LAMP1

Heart failure cardiac fibrosis remodeling

ABSTRACT

Autophagy is a physiological degradative process key to cell survival during nutrient deprivation, cell differentiation and development. It plays a major role in the turnover of damaged macromolecules and organelles, and it has been involved in the pathogenesis of different cardiovascular diseases. Activation of the adrenergic system is commonly associated with cardiac fibrosis and remodeling, and cardiac fibroblasts are key players in these processes. Whether adrenergic stimulation modulates cardiac fibroblast autophagy remains unexplored. In the present study, we aimed at this question and evaluated the effects of β₂-adrenergic stimulation upon autophagy. Cultured adult rat cardiac fibroblasts were treated with agonists or antagonists of beta-adrenergic receptors (β-AR), and autophagy was assessed by electron microscopy, GFP-LC3 subcellular distribution, and immunoblot of endogenous LC3. The predominant expression of β₂-ARs was determined and characterized by radioligand binding assays using [³H]dihydroalprenolol. Both, isoproterenol and norepinephrine (non-selective β-AR agonists), as well as salbutamol (selective β₂-AR agonist) increased autophagic flux, and these effects were blocked by propranolol (β-AR antagonist), ICI-118,551 (selective β₂-AR antagonist), 3-methyladenine but not by atenolol (selective β₁-AR antagonist). The increase in autophagy was correlated with an enhanced degradation of collagen, and this effect was abrogated by the inhibition of autophagic flux. Overall, our data suggest that β₂-adrenergic stimulation triggers autophagy in cardiac fibroblasts, and that this response could contribute to reduce the deleterious effects of high adrenergic stimulation upon cardiac fibrosis.

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1. Introduction

Macroautophagy (herein referred to as autophagy) represents a highly conserved cellular process among eukaryotes, which consists of the degradation of cytoplasmic elements such as long-lived, damaged, or aggregated proteins, as well as complete organelles. The engulfment of these elements within double-membraned structures called autophagosomes is followed by its fusion with the lysosome, where it ends with the enzymatic degradation of the autophagosomal inner mem-

brane and its cargo. Broken-down elements return to the cytosol as macromolecules that serve for the synthesis of new structures [1]. Although autophagy primarily represents an essential homeostatic mechanism activated in face of different stress conditions, it can also contribute to execute an infrequent form of programmed cell death (named type-II or autophagic cell death), which is characterized by abundant vacuolization and is defined by its inhibition when autophagic *Atg* genes are inactivated [2,3]. In the heart, basal autophagy contributes to the maintenance of cellular energy by the physiological turnover of essential metabolic organelles such as mitochondria or peroxisomes, but it becomes strongly upregulated in response to nutritional challenges, a condition frequently associated to pathological states such as cardiac remodeling/hypertrophy [4], or imposed by others such as ischemic disease [5] or myocardial infarction [6]. Current theories support a protective role of autophagy in the heart rather than a detrimental one [7], however, most of the evidence gathered thus far has been obtained from the study of cardiac myocytes, whereas to date there exist very few studies focused on fibroblasts. Cardiac fibroblasts represent 2/3 of the cardiac cellular population, and they play important roles by regulating structural, biochemical, mechanical and electrical

Abbreviations: 3-MA, 3-methyladenine; ACFs, adult cardiac fibroblasts; ATE, atenolol; β-AR, beta-adrenergic receptor; DHA, dihydroalprenolol; E/P, E64d + pepstatin A; FBS, fetal bovine serum; GFP, green fluorescent protein; ISO, isoproterenol; LAMP1, lysosomal associated membrane protein 1; LC3, microtubule-associated protein 1 light chain 3; MAPK, mitogen-activated protein kinase; MOL, multiplicity of infection; NE, norepinephrine; NS, nutritional stress; PBS, phosphate-buffered saline; PI3K-III, class-III phosphatidylinositol-3-kinase; PROP, propranolol; RAP, rapamycin; TEM, transmission electron microscopy

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properties of the heart [8]. A major one among these functions is the maintenance of the heart tissue, which is achieved by regulating the homeostasis of the extracellular matrix, as well as by the production of factors involved in maintaining the balance between synthesis and degradation of connective tissue components such as collagen, cytokines, growth factors and matrix metalloproteinases [9].

The beta-adrenergic receptor (b-AR) signaling pathway plays a key role in regulating cardiac function; their stimulation by catecholamines provides the most important regulatory mechanism for cardiovascular performance [10]. The mammalian heart expresses primarily β_1 -AR (75–85%), although a substantial number of β_2 -AR and β_3 -AR can be detected in cardiac tissue [11,12]. The three subtypes are present in cardiac myocytes, in which β_1 -AR is the predominant subtype. However, in cardiac fibroblasts, the most expressed b-AR subtype is the β_2 -AR [13]. Indeed, b-ARs have been identified on both neonatal and adult rat cardiac fibroblasts, and their stimulation by b-adrenergic agonists promotes DNA synthesis [14–16] and modulates collagen secretion [17]. Whether adrenergic stimulation regulates cardiac fibroblast autophagy remains unexplored. In the present study, we aim at this question and evaluated the effect of β_2 -AR stimulation upon autophagy, by using primary cultured adult rat cardiac fibroblasts. The results presented here indicate that specific β_2 -AR activation leads to a significant increase in autophagic flux, which increases collagen degradation, thus playing a crucial role in the maintenance of the cardiac extracellular matrix homeostasis.

2. Materials and methods

2.1. Cardiac fibroblast isolation

Animal handlings were performed conforming to the Guide for the Care and Use of Laboratory Animals (NIH publication No. 85-23). Male Sprague–Dawley rats (250 g) were anesthetized with ketamine-xylazine (66 mg/kg and 1.6 mg/kg i.p., respectively). Adult rat cardiac fibroblasts (ACFs) were isolated by retrograde aortic perfusion as described previously [18], with a few modifications. Briefly, the hearts were digested with a collagenase-hyaluronidase (1:1) containing solution and cells centrifuged at 500 rpm for 1 min. The supernatant, containing mainly ACFs, was centrifuged at 1,000 rpm for 10 min and then resuspended in DMEM F-12 plus 10% FBS and seeded in non-treated culture dishes during 2 h. The cells were washed with PBS in order to eliminate debris and non-adherent cells. ACFs were used at passage 1, and seeded on plastic dishes at density of 2×10^4 cell/cm².

2.2. Collagen assays

For collagen imaging, ACFs were seeded in coverslips, subjected to different treatments and fixed with 4% paraformaldehyde. The fluorescence imaging of cells was conducted in a Zeiss LSM-5, Pascal 5 Axiovert 200 confocal microscope. For collagen quantification via immunowesternblot, ACFs were seeded in 60 mm culture dishes, lysed in 10 mM Tris–HCl pH 7.5, 10 mM EDTA, 0.4% deoxycholate, 1% NP-40, 1 mM phenylmethylsulfonyl fluoride and 0.1% sodium dodecyl sulphate, subjected to SDS–PAGE, and electro-transferred onto PVDF membranes.

2.3. Evaluation of autophagy by GFP-LC3 redistribution

After 24 h of culture, ACFs were transduced with an adenovirus encoding the fusion protein GFP-LC3, with a multiplicity of infection (MOI) of 100. This adenovirus was kindly donated by Dr. Sharon Tooze (London Research Institute, Cancer Research UK, England) [19]. Subcellular distribution of GFP-LC3 was monitored by standard fluorescence microscopy. ACFs evidencing autophagic vacuolization were counted using a Zeiss AxioScope 20 fluorescence microscope at

40 \times magnification. Vacuolization index was calculated by counting the number of cells with GFP-LC3 translocation as a percentage of the total number of fluorescent cells (counted independently by P.A-U. and R.T.) [20].

2.4. Evaluation of autophagy by electron microscopy

Preparation of samples for electron microscopy and morphometric measurements were performed as described [21]. The number of autophagic vacuoles was counted under the electron microscope (EM-109, Zeiss) by systematically screening the sections at 12,000 \times using grid squares as sampling units as previously described [22]. Five grid squares were screened for each sample. The number of vacuoles per cell profile was then counted for each grid square separately.

2.5. Evaluation of autophagy by endogenous levels of LC3-II by immunowesternblot

ACFs were exposed to the different agonists for the indicated times. Cell lysates were prepared using 10 mM Tris–HCl pH 7.5, 10 mM EDTA, 0.4% deoxycholate, 1% NP-40, 1 mM phenylmethylsulfonyl fluoride and 0.1% sodium dodecyl sulphate. Twenty micrograms of protein from total cell lysates were analyzed via SDS–PAGE (15%). Proteins were transferred onto a PVDF membrane and immunoblotted with commercially available specific antibodies.

2.6. Radio-ligand binding assay

[³H]-Dihydroalprenolol (DHA) binding assays were performed on membranes of ACFs as described in [23]. For competitive binding experiments, [³H]-DHA (15 nM) was used as labeled compound. Non-labeled b-adrenergic antagonists: nonspecific b-AR propanolol (1 mM), β_1 -AR atenolol (1 mM) and β_2 -AR ICI-118,551 (1 mM) were used to confirm the subtype of adrenergic receptor.

2.7. Reagents and treatments

All culture mediums were purchased from GIBCO. Ketamine, xylazine, collagenase, hyaluronidase, E64d, pepstatin, rapamycin, and secondary antibodies coupled to horseradish peroxidase were purchased from Sigma Chemicals Co (St. Louis, MO). Fetal Bovine Serum (FBS) was from Hyclone. Antibodies against LC3, b-tubulin and collagen-I were purchased from Cell Signaling Technology (Beverly, MA). Antibodies against GFP and vimentin were from Abcam (Cambridge, UK). Nutritional stress was induced by incubation of ACFs in serum/amino acids-free Earle's balanced salt solution. The concentration of the different treatments, unless otherwise indicated, was: Norepinephrine [10 μ M], Isoproterenol [10 μ M], Atenolol [1 mM], Propanolol [1 mM], ICI-118,551 [10 μ M], Rapamycin [100 nM], E64d [10 μ g/ml], pepstatin A [10 μ g/ml].

2.8. Statistical analysis

Data are presented as mean \pm SD of at least, 3 independent experiments. Student *t* test for comparisons between 2 groups, and one-way ANOVA followed by a Tukey's *post hoc* test, for multigroup comparisons, were used. Significance was accepted at $p < 0.05$.

3. Results

3.1. Catecholamines induce positive autophagic flux in cultured cardiac fibroblasts

Transmission electron microscopy (TEM) provides a *sine qua non* methodology for the certain assessment of autophagosome formation [24]. Additionally, the type-II microtubule-associated protein 1 light

chain 3 (LC3-II) is widely used as a marker of autophagy, because its lipidation and specific recruitment to autophagosomal membranes provides a shift from diffuse to punctate staining, and increases its electrophoretic mobility on gels compared to the terminally-unprocessed form LC3-I [25,26]. Here we used three alternative approaches to determine whether catecholamine stimulation induces autophagy; first, we monitored autophagic puncta by means of exogenously introduced GFP-LC3 with an adenoviral transduction, we also evaluated endogenous LC3 processing via immunoblotting, and we further confirmed autophagosome formation by TEM. Subcellular redistribution of GFP-LC3 into autophagic puncta was evaluated in ACFs treated for 2–48 h with catecholamines, rapamycin and nutritional stress. Under these different conditions, a marked increase in punctate fluorescent dots per cell (white arrows) was evidenced, whereas untreated cells (maintained in complete medium) displayed a diffusely distributed GFP-LC3 (Fig. 1A). Quantification of the percentage of GFP-LC3 vacuolized cells revealed a significant increase in autophagic puncta after already at 2 h of catecholamine treatment, reaching levels comparable to rapamycin- or starvation-treated cells at 24 h (Fig. 1B). Given that the expression of exogenous GFP-LC3 could lead to false positives of LC3 aggregation, we evaluated the processing of endogenous LC3-I in catecholamine-treated cells by immunoblotting. Both, the β_1/β_2 agonist isoproterenol (ISO) and norepinephrine (NE) led to a significant increase in LC3 lipidation at 24 and 48 h (Fig. 1C). Quantification of the LC3-II/LC3-I ratio can lead to misinterpretations of LC3 processing. This results from the fact that at initial stages of autophagosome formation LC3-II increases and LC3-I is subject to *de novo* synthesis; but during later stages of autophagy, LC3-II decreases due to lysosomal degradation and LC3-I decreases due to an arrest of its synthesis, as well as by its own processing/lipidation [27]. For this reason, we quantified the LC3-II ratio over tubulin, whose levels are not affected during autophagic degradation. ACFs treated with ISO and NE displayed a significant increase in the LC3-II/tubulin ratio when compared to control cells, in a similar way to rapamycin-treated cells (Fig. 1D). In order to further corroborate autophagosome formation, we performed TEM analysis of catecholamine-treated ACFs. Both ISO and NE led to the formation of double-membraned early autophagosomes (marked with white arrows), as well as single-membraned degradative autolysosomes (marked with black arrows), whereas in control cells these structures were almost undetectable (Fig. 1E). Quantification of the number of autophagic vacuoles per cell revealed a significant increase in catecholamine- and rapamycin-treated cells, as compared to untreated cells (Fig. 1F). These combined results strongly suggest that catecholamines induce the formation of autophagosomes in ACFs. However, an increase in the number of autophagosomes can result from an induction of the autophagic process (known as on-rate autophagic flux), as well as from an arrest of their lysosomal fusion/degradation (known as off-rate autophagosome accumulation) [24]. In order to discriminate between these two possibilities, we subjected ACFs to treatment with catecholamines in the presence of E64d and Pepstatin A (E/P), both of which are known to inhibit lysosomal acidic proteases, thereby arresting the degradation of LC3-II. Under these conditions, an increase in LC3-II levels can only account for its accumulation resulting from the generation and impaired-degradation of autophagosomes, and therefore fits into “on-rate” autophagic flux [24,27,28]. Immunofluorescence studies with an antibody that recognizes preferentially the lipidated form of endogenous LC3, revealed an increased accumulation of immunostained LC3-II puncta in ACFs treated with ISO and E/P, in comparison to cells treated with ISO alone (Fig. 2A). In a similar approach, we measured LC3 processing via immunoblotting after ISO and NE treatment, which accordingly demonstrated a higher accumulation of LC3-II when lysosomal degradation is impaired by E/P treatment (Fig. 2B). Under these conditions, the quantification of the LC3-II/tubulin ratio after ISO treatment revealed a 6-fold increase in LC3-II accumulated levels

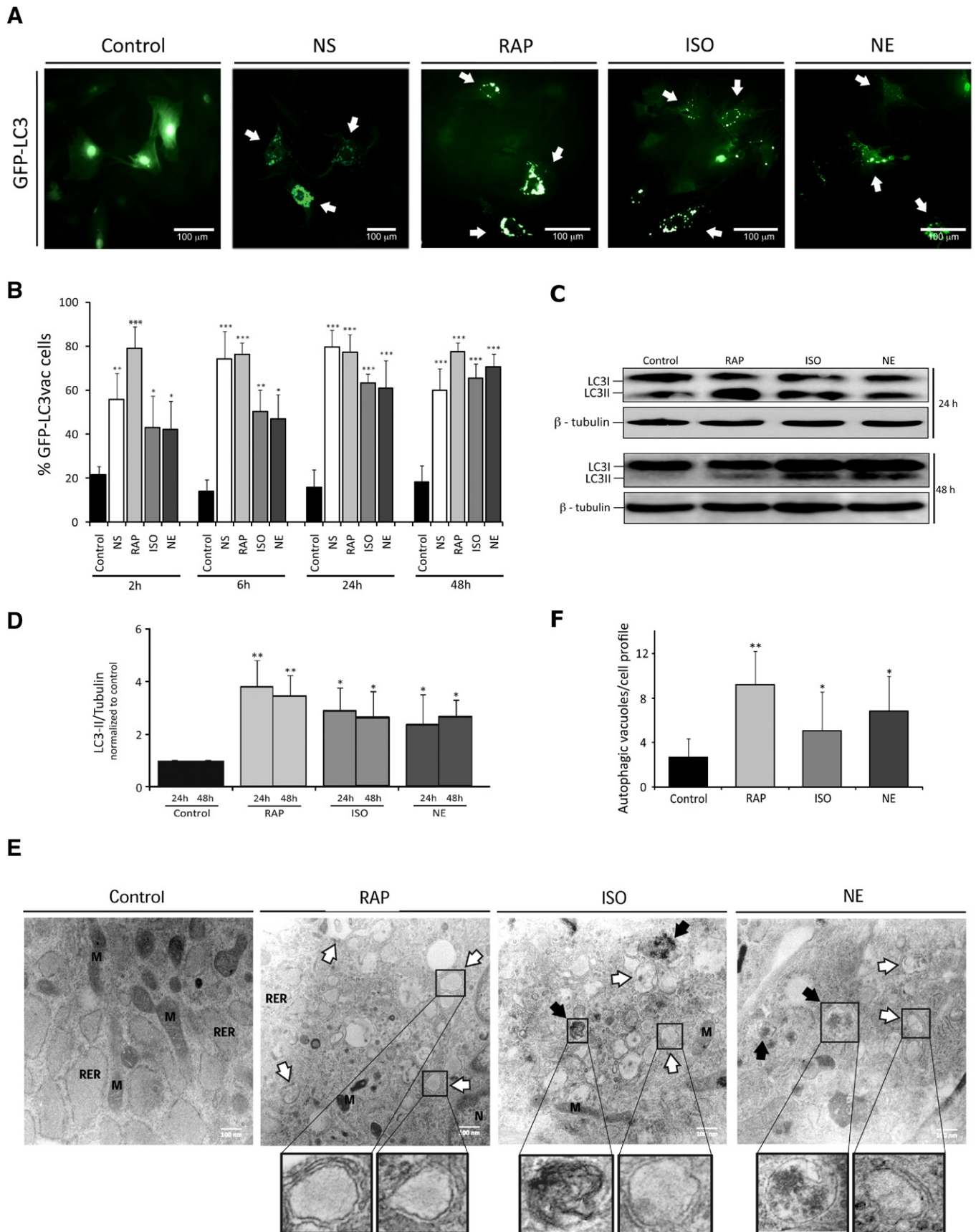
(Fig. 2C), compared to the 3-fold increase in the absence of E/P (Fig. 1D), which is indicative of an enhanced formation of autophagosomes induced by catecholamines, rather than just an impairment of autophagosome degradation. Finally, in order to corroborate that catecholamines induce autophagosome formation, we subjected ACFs to treatment with ISO in combination with 3-methyladenine (3-MA), which impairs autophagosome formation by inhibiting the class-III phosphatidylinositol-3-kinase (PI3K-III) that catalyzes phagophore nucleation and elongation [29]. ACFs treated with ISO and 3-MA displayed an LC3 processing equivalent to untreated cells (Fig. 2D and F), further confirming the pro-autophagic effects of catecholamines in these cells. Altogether, these results demonstrate that catecholamines strongly induce “on-rate” autophagic flux via canonical PI3K-III-mediated mechanisms of autophagosome formation.

3.2. Specific activation of β_2 -AR leads to cardiac fibroblast autophagy

The presence of β -AR in cardiac fibroblasts has been proposed before [16,30]. In order to validate these premises, we evaluated the expression of β -AR in our ACFs setting by performing radio-ligand studies. Binding assays demonstrated that the radioactivity of [3 H]-dihydroalprenolol (DHA), which binds to β -AR, was displaced by the non-selective β -AR antagonist propranolol (Fig. 3A). Accordingly, GFP-LC3 processing induced by ISO or NE was completely blocked by propranolol (Fig. 3B and C). Next, we evaluated the expression of β_2 -ARs in ACFs, which so far have been proposed as the predominant β -AR isoform expressed in cardiac fibroblasts [14,31]. Binding of radiolabelled [3 H]DHA was displaced by propranolol and by the β_2 -AR specific antagonist ICI-118,551, but not by atenolol, which specifically blocks β_1 -ARs (Fig. 4A). The dissociation constant for [3 H]DHA binding sites was 23 ± 9 nM; whereas its density was 32 ± 4 fmol/mg protein. Collectively, these results confirm the predominant expression of β_2 -AR in ACFs. Therefore, in order to correlate the autophagic effects of catecholamines to the specific activation of β_2 -AR, we monitored LC3 processing and GFP-LC3 redistribution. The effects of ISO upon endogenous LC3 processing were abolished by the β_2 -AR antagonist ICI-118,551, but were unaffected by the β_1 -AR antagonist atenolol (Fig. 4B and C). In a similar way, ICI-118,551 but not atenolol, was able to abrogate GFP-LC3 redistribution into autophagic puncta induced by ISO (Fig. 4D and E). Given that the β_2 -AR antagonist ICI-118,551 completely blocked the effects of ISO (unspecific β -AR agonist), and that atenolol showed no effect, these results validate the predominant expression and functionality of β_2 -ARs in ACFs. In order to irrevocably establish that the β_2 -AR isoform mediates ACF autophagy, we treated ACFs with the specific β_2 -AR agonist salbutamol, and assayed endogenous LC3 processing via immunoblotting (Fig. 4F). Salbutamol treatment led to a significant increase in the LC3-II/tubulin ratio in a dose-responsive fashion, with a maximal effective response at the micromolar range (Fig. 4G). Altogether, these results confirm that ACFs express predominantly the β_2 -AR isoform, and that its activation by catecholamines, or pharmacological agonists positively regulates autophagy.

3.3. β_2 -adrenergic receptor promotes collagen degradation via autophagy

Reportedly, a physiological outcome of β_2 -AR activation in cardiac fibroblasts is the regulation of collagen secretion [17]. In order to evaluate the physiological effects of β_2 -AR-mediated autophagy, we monitored the degradation of collagen type I, which is the principal type of collagen fiber present in inner organs. Immunofluorescence studies with antibodies against lysosomal associated membrane protein 1 (LAMP1) and collagen-I revealed that treatment with ISO strongly increased the colocalization of collagen with lysosomes (Fig. 5A), which suggests that catecholamines regulate collagen-I degradation via autophagy.



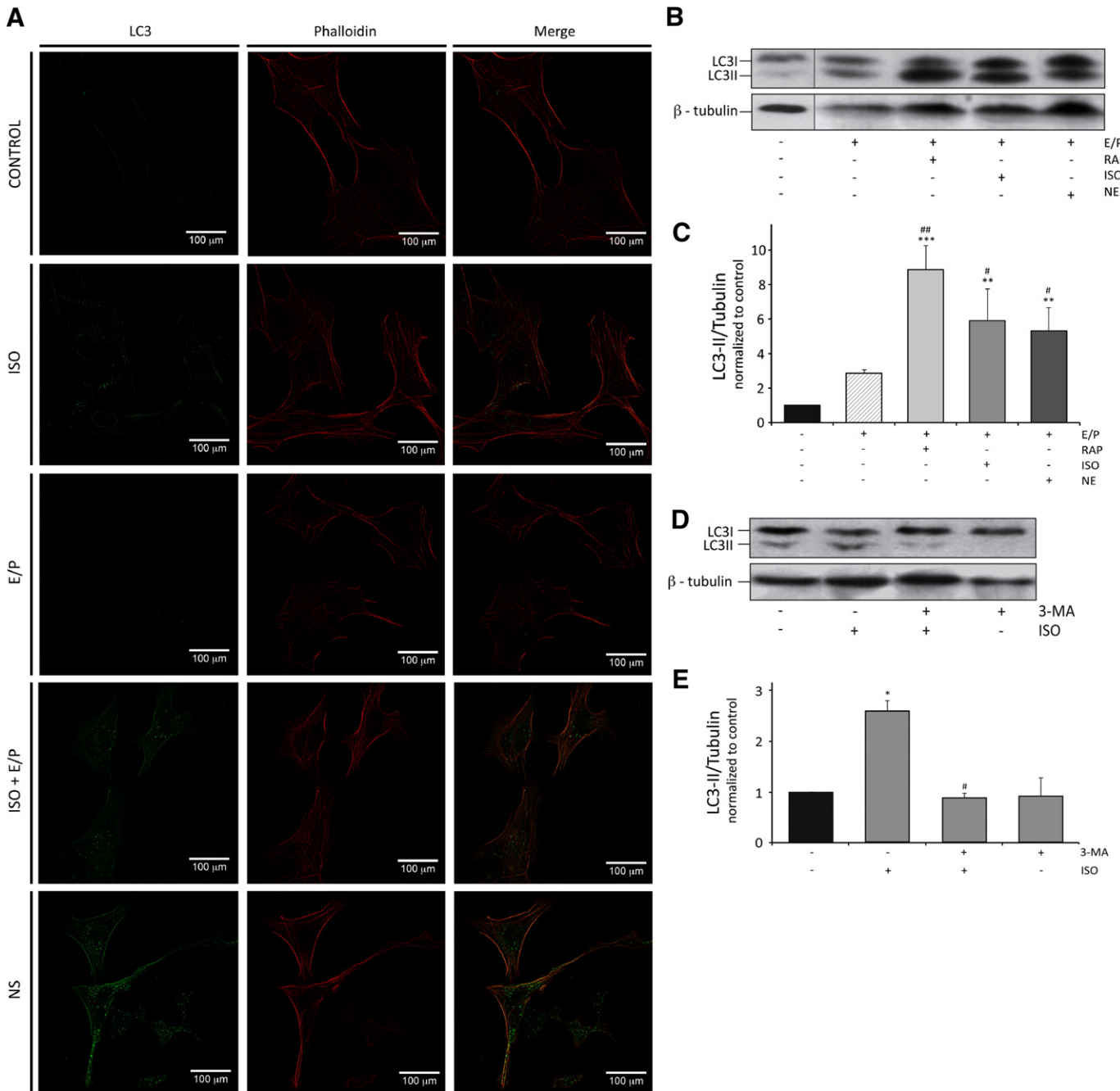


Fig. 2. Catecholamines induce “on-rate” autophagic flux. Panel A: Adult cardiac fibroblasts (ACFs) were either maintained in complete medium, subjected to nutritional stress, or treated with isoproterenol (ISO) for 24 h in the absence or presence of E64d+Pepstatin A (E/P). Confocal images of immunostained LC3-II and phalloidin are shown. High LC3-II staining is indicative of increased autophagosome formation. Panel B: Immunowesternblot analysis of ACFs maintained in complete medium and treated with ISO, rapamycin (RAP) or norepinephrine (NE), either in the absence or presence of E/P. A representative of 3 independent experiments is shown. Panel C: Quantification of the LC3-II/tubulin ratio, normalized as fold over control. Panel D: Immunowesternblot analysis of ACFs maintained in complete medium and treated with ISO, in the absence or presence of the autophagy inhibitor 3-methyladenine (3-MA). Panel E: Quantification of the LC3-II/tubulin ratio, normalized as fold over control. Results shown are mean \pm S.D. of three independent experiments ($***p < 0.001$ vs. control, $**p < 0.01$ vs. control, $*p < 0.05$ vs. control, $##p < 0.01$ vs. E/P, $#p < 0.05$ vs. E/P in C, $#p < 0.05$ vs. ISO in E).

In order to evaluate this possibility, we quantified protein levels of collagen-I normalized to vimentin, an intermediate filament that is a fundamental component of the cytoskeleton. Treatment with ISO

led to a significant reduction of collagen-I levels (Fig. 5B), and the inhibition of autophagic flux with E/P rescued collagen degradation to control levels (Fig. 5B and C). Overall, our data highlights the

Fig. 1. Catecholamines induce autophagy in cultured cardiac fibroblasts. Panel A: Adult rat cardiac fibroblasts (ACFs) expressing GFP-LC3 were treated for 2–48 h with isoproterenol (ISO), norepinephrine (NE), rapamycin (RAP) or subjected to nutritional stress (NS). Fluorescent images (40 \times) of 24 h-treated cells are shown, and are representative of three independent experiments; white arrows indicate autophagic cells. Panel B: ISO, NE, RAP and NS increased the frequency of GFP-LC3 vacuolated cells in a time-dependent manner. Panel C: Immunowesternblot analysis of endogenous LC3-II conversion in ACFs. Cells were treated with ISO, NE, Rap or NS for 24–48 h. Total protein extracts were analyzed by using anti-LC3 and anti- β -tubulin antibodies. Panel D: Ratio of LC3-II/tubulin normalized as fold over control is shown. Panel E: Detection of autophagosomes by TEM in ACFs. Cells were stimulated with ISO, NE or RAP for 24 h and analyzed by TEM. White and black arrows are indicative of autophagosomes and autolysosomes, respectively. Images are representative of three experiments. Panel F: Quantification of autophagic vacuoles per TEM cell profile. Results shown are mean \pm S.D. of three independent experiments ($***p < 0.001$; $**p < 0.01$ and $*p < 0.05$ vs. control).

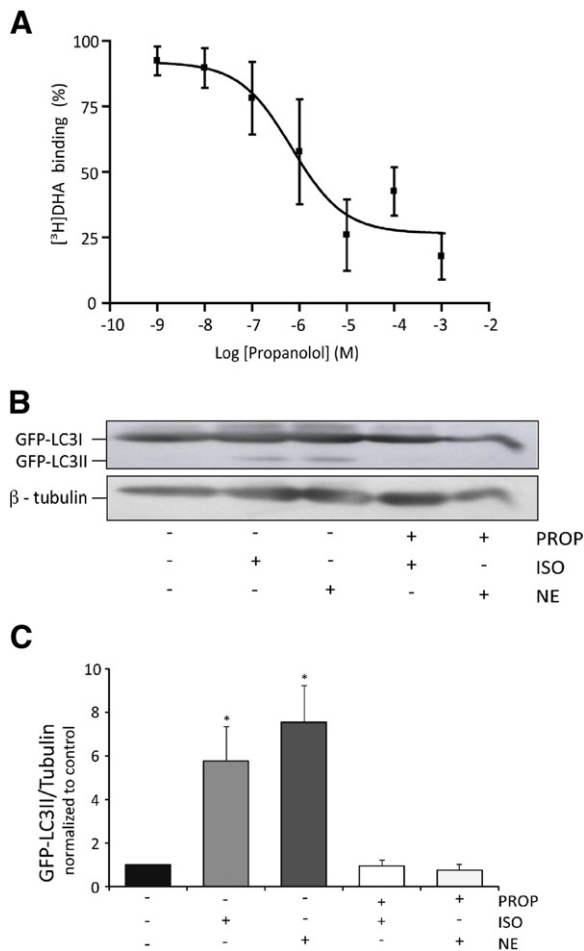


Fig. 3. [³H]-Dihydroalprenolol (DHA) competition curves and displacement assays in cultured cardiac fibroblasts. Panel A: Displacement curve for b-adrenergic receptors (b-AR) using 15 nM radiolabelled [³H]-DHA for binding, and propranolol (ranging from 1 nM to 1 mM) for displacement. Panel B: Immunoblot analysis of total lysates from GFP-LC3 expressing adult cardiac fibroblasts (ACFs) maintained in complete medium and treated with isoproterenol (ISO) or norepinephrine (NE), either in the absence or presence of 1 mM propranolol (PROP). Representative blots of 3 independent experiments using GFP and b-tubulin antibodies are shown. Panel C: Quantification of the GFP-LC3-II/tubulin ratio, normalized as fold over control. Results shown are mean ± S.D. of three independent experiments (**p* < 0.05 vs. control).

pro-autophagic effects of catecholamines and b₂-AR activation, which contribute to the degradation of collagen in the cardiac fibroblast.

4. Discussion

The results from this and previous reports [14,16,17,30–32] indicate that adult rat cardiac fibroblasts (ACF) predominantly express the b₂-AR isoform. Here in particular, we demonstrate that b₂-AR activation leads to a strong autophagic response, which increases autophagosome formation and degradation, therefore fitting into the classification of “on-rate” or degradative autophagic flux. These effects were observed via transmission electron microscopy, fluorescent microscopy studies of GFP-LC3 subcellular redistribution, and endogenous LC3 processing/lipidation assayed by immunoblot. Importantly, the pro-autophagic effects of b₂-AR activation led to an increased degradation of collagen-I, an effect previously unexplored in the contribution of catecholamines to the regulation of cardiac collagen homeostasis.

Despite the relevant role that fibroblasts play in heart remodeling, nothing is known concerning the effects of b-adrenergic

stimulation on cardiac fibroblast autophagy. The most fundamental question for autophagy in heart disease is whether its role is harmful or protective, although severity and duration of the autophagic response, or the nature of the autophagic substrate may determine the patho-physiological outcome [4]. This dose- and context-dependent role of autophagy in heart disease poses special challenges. At present, it is unknown how long one can activate autophagy without detrimental consequences. Experimental studies concerning autophagy in the heart have been primarily focused in cardiomyocytes, using ischemia/reperfusion models associated to nutritional stress [33]. In this study, we demonstrate that serum withdrawal, in a concentration dependent manner, triggers cardiac fibroblast autophagy (Supplementary Fig. 1), however we did not focus our work in studying the adaptive or detrimental effects of cardiac fibroblast autophagy induced by nutritional insults. Here we evidence the induction of autophagy as a novel effect of adrenergic stimulation in cardiac fibroblasts, as demonstrated by the punctate GFP-LC3 distribution in the cytoplasm and the corresponding increase in GFP-LC3 vacuolated cells (Fig. 1B). This response was achieved rapidly and was sustained, ranging from 2 h until 48 h, although it was weaker compared to rapamycin or nutritional stress. We additionally monitored LC3 processing/lipidation via immunoblot. Cardiac fibroblasts treated with adrenergic agonists displayed increased LC3-II levels, which correlates with enhanced autophagosome formation. In this response, rapamycin was again more effective as inducer of LC3 conversion (Fig. 1D). Finally, we corroborated autophagosome formation via TEM, and we observed that catecholamines and rapamycin increased the number of early double-membraned autophagy vacuoles, but importantly, the increase in the number of single-membraned late autophagic vacuoles was more distinguishable in catecholamines- than in rapamycin-treated ACFs (Fig. 1E), which evidences that catecholamines trigger positive autophagic flux, as we further proved by different methodological approaches including confocal microscopy and immunoblot (Fig. 2).

The present study shows that adult rat cardiac fibroblast predominantly express b₂-AR. Accordingly, previous studies provide values of K_d and B_{max} that agree with the data from our radioactively labeled binding studies [34,35]. However, an effect of b₂-AR upon cardiac fibroblast autophagy has not been established until now. The increase in LC3-II levels, produced by b₂-adrenergic stimulation, was determined to be a critical component for autophagy induction. By itself, isoproterenol caused a significant increase in LC3-II levels compared with non-treated controls, as well as an increase in autophagic puncta, in a concentration dependent manner (Supplementary Fig. 2). The same effect was observed with salbutamol, a specific b₂-adrenergic agonist (Fig. 4G). However, using three methods to evaluate autophagy, namely GFP-LC3 subcellular redistribution (Fig. 4D and E), endogenous LC3/tubulin ratio (Fig. 4B and C), and GFP-LC3 levels by immunoblot (Fig. 3B and C), we show that only in cells that were pretreated with propranolol (unspecific b-AR antagonist), or ICI-118,551 (specific b₂-AR antagonist), but not with atenolol (specific b₁-AR antagonist), autophagy was blocked, which is indicative that b₂-AR activation triggers autophagy.

In cardiac myocytes, b-adrenergic stimulation has been reported to inhibit autophagy [36,37]. Although these results are different to our findings, they have been made in whole heart where b₁-adrenergic is the main receptor on cardiac myocytes, therefore, the autophagic response depends primarily on the cellular type involved. Isolated cardiomyocytes from Atg5 deficient mouse heart display increased sensitivity to the b-AR agonist isoproterenol compared to wild type cells [38]. Moreover, isoproterenol treatment for 7 days leads to left ventricular dilation and cardiac dysfunction in autophagy deficient mice but not in wild type mice, suggesting that autophagy protects cells against excessive b-

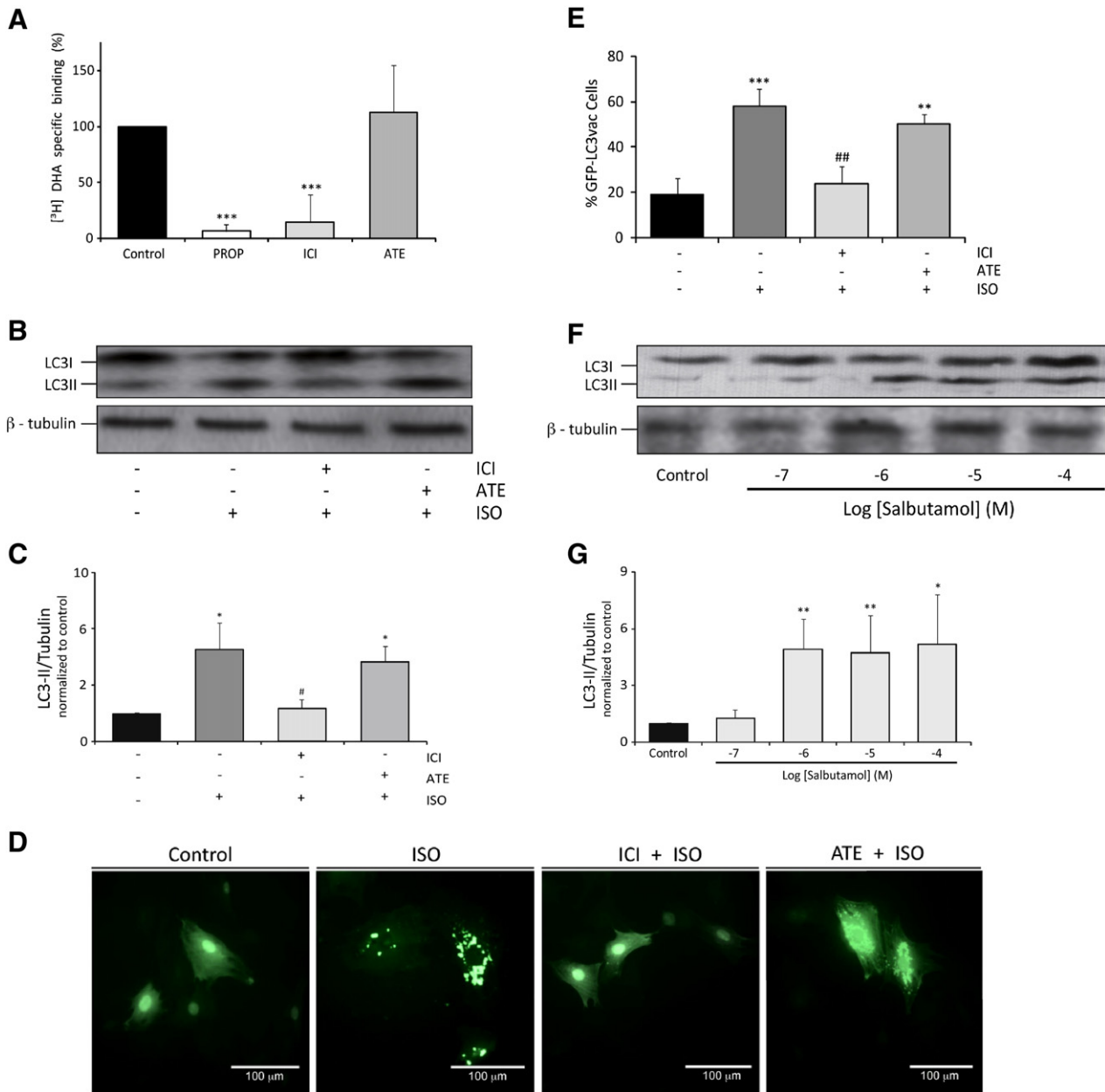


Fig. 4. Specific activation of beta₂-adrenergic receptors leads to cardiac fibroblast autophagy. Panel A: Displacement assays for the beta₂-adrenergic receptor using [³H]-DHA (15 nM, black bar), and displaced with propranolol (1 mM, white bar), ICI-118,511 (10 μM, light gray bar) or atenolol (1 mM, gray bar), respectively. Panel B: Immunoblot analysis of total lysates from adult cardiac fibroblasts (ACFs) maintained in complete medium and treated with isoproterenol (ISO), either in the absence or presence of 1 mM atenolol (ATE) or 10 μM ICI-118,511 (ICI). Representative blots of 3 independent experiments using LC3 and b-tubulin antibodies are shown. Panel C: Quantification of the LC3-II/tubulin ratio, normalized as fold over control. Panel D: ACFs expressing GFP-LC3 were treated for 24 h with complete medium, or isoproterenol (ISO) combined with 1 mM ATE or 10 μM ICI, respectively. Representative fluorescent images (40×) of three independent experiments are shown. Panel E: Quantification of the percentage of GFP-LC3 vacuolated cells, from the experiments performed in C. Panel F: Immunoblot analysis of total lysates from ACFs treated with increasing concentrations of salbutamol, as indicated. Panel G: Quantification of the LC3-II/tubulin ratio, normalized as fold over control. Results shown are mean ± S.D. of three independent experiments (***p<0.001 vs. control, **p<0.01 vs. control, *p<0.05 vs. control, ##p<0.01 vs. ISO alone, #p<0.05 vs. ISO alone).

adrenergic stimulation. In cardiac fibroblasts, autophagy may function as a defensive mechanism against high adrenergic stimulation. To date, very few studies have evaluated the physiological outcome of specific beta₂-AR activation in cardiac fibroblasts *in vitro*. Adrenergic stimulation in cardiac fibroblast triggers cell proliferation [16], activate MAPKs [32,39], increases the production of nitric oxide (NO) [31], increases DNA synthesis [14], and notably, reduces collagen secretion [17]. Although we did not evaluate extracellular collagen secretion here, we observed that the specific activation of beta₂-AR in cardiac fibroblasts strongly promotes the

autophagic degradation of intracellular collagen. Thus, we speculate that autophagy induced by overactive adrenergic stimulation might additionally contribute to reduce fibrosis by the regulation of the extracellular matrix. Looking beyond, modulation of autophagy by adrenergic stimulus could be considered as a pharmacological target, a notion that represents an interesting subject for further investigation.

In summary, the results presented here evidence the autophagic effects of beta₂-adrenergic receptor, which is the predominant isoform present in adult rat cardiac fibroblasts. By degrading collagen,

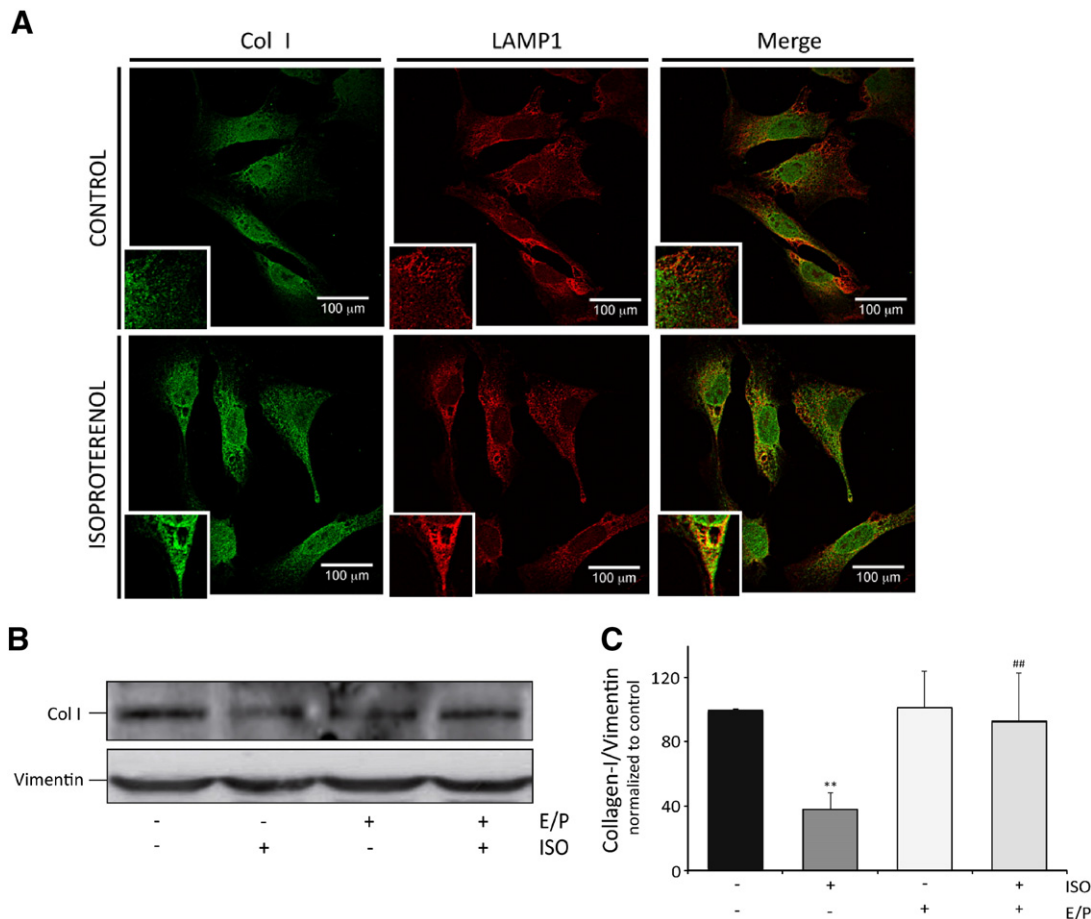


Fig. 5. Beta₂-adrenergic receptor stimulation induces autophagy of collagen-I. Panel A: Adult cardiac fibroblasts (ACFs) were treated or not with isoproterenol (ISO) for 24 h and fixed. Confocal images of immunostained collagen-I and lysosomal associated protein 1 (LAMP1) are shown, indicative of lysosomal localization of collagen-I. Panel B: Immunoblot analysis of collagen-I and vimentin of total lysates from ACFs treated with ISO for 24 h in the absence or presence of EG64d/Pepstatin A. Panel C: Quantification of the collagen-I/vimentin ratio, normalized to control. Results shown are mean \pm S.D. of three independent experiments ** p < 0.01 vs control, ## p < 0.01 vs. ISO alone).

autophagy might contribute to prevent fibrosis induced by pathological adrenergic stimulation.

Acknowledgements

P.A-U holds a doctoral fellowship from CONICYT, Chile. J.M.V. and R.T. are FONDAF post-doctoral fellows. This work was supported by CONICYT grant 24080143 to P.A-U, FONDECYT grant 1061059 to GDA, FONDECYT grant 1100443 to GDA, as well as by FONDAF grant 15010006 to SL.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbadis.2010.07.003.

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