Alpha-linolenic acid protects against cardiac injury and remodelling induced by beta-adrenergic overstimulation

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Abstract

We investigated the effect of α-linolenic acid (ALA) in protecting the heart from injury caused by β-adrenergic overstimulation.

ALA role was studied either on isoproterenol (ISO)-treated isolated rat cardiomyocytes (H9c2 cells) or in *in vivo* rat hearts. On isolated cardiomyocytes *in vitro* the involvement of kinases (Src and PI3K) in protection was tested using the specific inhibitors (PP2 or LY294002 respectively), while the role of caveolae was assessed by their disruption with methyl-β-cyclodextrin.

The rats underwent either a normal chow diet or, alternatively, an ALA-enriched diet before, during and throughout 60 days after 5 days of isoproterenol administration. Before sacrifice, the hemodynamic changes were measured with echocardiography. In the explanted hearts, histological changes together with molecular markers of cardiac fibrosis and hypertrophy were evaluated.

On H9c2 cell ALA abolished the ISO-induced reduction of viability. This effect was suppressed by both PP2 or LY294002 inhibitors and methyl-β-cyclodextrin caveolae disrupter. In the rats ALA prevented ISO-induced myocardial fibrosis and hypertrophy and kept cardiac mechanical function as in the control. It also counteracted the increased expressions of transforming growth factor-β (TGF-β) and β-myosin (β-MHC), the decreased expression of tissue inhibitor metalloproteinase-1 (TIMP-1) and the enhanced activity of matrix metalloproteinase-2 (MMP-2).

In conclusion, ALA-induced protection requires the integrity of caveolae where β_2-adrenergic receptors (β_2ARs) are restricted and mediate the activation of Src-PI3K protective pathway. By preserving this β_2ARs pro-survival pathway, ALA-enriched diet protects the heart against ISO-induced fibrosis and hypertrophy.

***Keywords**:* α-linolenic acid; caveolae; isoproterenol; myocardial fibrosis; myocardial hypertrophy; PI3K.

Introduction

An increased sympathetic stimulation may occur in either cardiovascular or non-cardiovascular diseases [1]. While the formers include hypertension, heart failure and ischaemic heart disease, the latters consist in diseases of the autonomic nervous system, metabolism and kidney [2-5]. The prognosis of patients suffering from sympathetic hyperactivity is sometimes very poor, mainly if associated with ageing, heart failure and severe kidney disease [1].

A feedback seems to exist between sympathetic overactivity and the severity of pre-existent cardiovascular diseases. In fact, a positive correlation has been demonstrated between infarct size and the subsequent increase in norepinephrine plasma level [6] as well as between elevated cardiac norepinephrine concentration and worsening of heart failure, occurrence of arrhythmias and sudden death [7]. Actually, an increased β-adrenergic activity accelerates the progression of myocardial damage and is capable to produce necrosis even in non-ischemic hearts [8].

The most common morphological changes induced by sympathetic overstimulation consist in myocardial fibrosis and hypertrophy [9-11]. Indeed, fibrosis is generally characterized by excessive production, deposition and shrinking of extracellular matrix (ECM) caused by unbalance between synthesis and degradation [12] and it is usually associated with ventricular hypertrophy [13]. Since this process is not remissible, the only possibility to avoid cardiac remodeling is to prevent the death of cardiomyocytes and the onset of fibrosis.

A large body of evidence is in favour of a potential protective effect of seafood-derived omega-3-polyunsaturated fatty acids (PUFAs), e.g. eicosapentaenoic acid (EPA) and docosahexanoic acid (DHA), against coronary heart disease and sudden cardiac death [14, 15]. In contrast, only a few studies have been devoted to clarify the effects mediated by plant-derived PUFAs, such as α-linolenic acid (ALA), on various cardiomyopathies. These studies gave uncertain results [16-19] inasmuch as ALA supplementation has been reported to be associated only with a slight reduction of cardiovascular risk [19]. Since PUFAs cannot be synthesized by humans, they should be better supplemented with an appropriated diet. Compared to EPA and DHA, ALA is more affordable and widely available.

Recently, it has been reported that ALA treatment restores caveolin-3 expression in neonatal cardiomyocytes [20] reverting the tumor necrosis factor-α receptor response from pro-apoptotic to anti-apoptotic.

The present investigation was designed to ascertain whether ALA can avoid the myocardial injuries triggered by β-adrenergic overstimulation. Firstly, we assessed the triggering of pro-survival pathways in an *in vitro* model (H9c2 rat cardiomyocytes). Then, we studied the effects of an ALA-enriched diet [18, 20, 21] in adult Wistar rats, which were repeatedly injected with isoproterenol (ISO). This treatment can mimic the effect of β-adrenergic overstimulation and cause infarct-like myocardial injuries [22, 23] through β_1-adrenergic receptor (AR) binding [24].

Materials and Methods

Data mining

ProteinQuest™ literature mining tool (BioDigitalValley S.r.l., Pont-Saint-Martin, Aosta, Italy) and Reactome FI app [25] for Cytoscape [26] version 3.2.0 (http://www.cytoscape.org) were used to detect and analyse correlations and interactions in the β-adrenergic receptor downstream signaling pathways (see Supplementary data). Among them, we selected for investigation the molecular targets involved in cell survival, fibrosis and hypertrophy onset (Fig. S1).

Chemicals

All chemicals were purchased from Sigma-Aldrich (Saint Louis, MO, USA) except where otherwise indicated.

In vitro Studies

H9c2 cell treatments and viability assay

The embryo rat cardiomyocyte cell line H9c2 was a generous gift from Dr. Claudia Penna (University of Turin). Cells were standardly cultured in 75 cm^2 TPP® flasks (TPP Techno Plastic

Products AG, Trasadingen, Switzerland) in RPMI-1640 medium (PAA Laboratories GmbH, Cölbe, Germany) supplemented with 10% fetal calf serum (FCS) (PAA Laboratories GmbH), 100 U/ml penicillin G, 40 μg/ml gentamicin sulfate and 2.5 μg/ml amphotericin B at 37 °C in a humidified 5% CO_2 atmosphere.

To assess the protective effect of ALA against a severe isoproterenol-induced cell damage, serial ISO concentrations ranging from 50 μM to 1 mM were tested. This procedure allowed us to identify the ISO concentration able to induce a reduction of cell viability by about 50% after 24 h of incubation (not shown).

H9c2 cells were plated at the density of 3×10^4/well in 96-well plates (Becton Dickinson Labware, BD Italia, Buccinasco, Milano, Italy). The day after, cells were treated in accord with one of the following protocols: (i) twenty four hours of incubation in medium added with ISO (250 μM); (ii) twenty four hours of incubation in medium added with ALA (20 μM) [20], followed by other 24 hours of incubation in medium added with ISO and ALA at the same concentrations; (iii) one hour of pretreatment with the Src kinase inhibitor 3-(4-chlorophenyl)-1-(1,1-dimethylethyl)-1H-pyrazolo[3,4-d]pyrimidin-4-amine alone (PP2, 10 μM)**;** (iv) one hour of pretreatment of with the PI3K inhibitor LY294002 alone (100 nM); (v) one hour of pretreatment with the caveolae disrupter methyl-β-cyclodestrin (MβCD, 2.5 mM). For these last three conditions, pre-incubation was followed by the same protocol of (ii) group, but in the presence of the inhibitor; and, vi) untreated H9c2 cells were used as control.

Cell viability was then assessed after the injury by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) [27]. Briefly, MTT was added to the medium (final concentration 0.5 mg/ml) and cells were further cultured for 3 h. Then, medium was replaced with an equal volume of dimethyl sulfoxide. The MTT absorbance was read at 560 nm with an Asys UVM-340 microplate reader (Biochrom, Cambridge Science Park, Cambridge, UK). MTT data were expressed as percentage of the control. Experiments were done in triplicate.

In vivo Studies

Animals

Two month-old male Wistar rats (Harlan-Italy, S. Pietro al Natisone, Italy) received humane care in compliance with Italian law (DL-116, January ST-segment 27, 1992) and in accordance with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). The purposes and the protocols of the studies have been approved by the Ministero della Salute, Rome, Italy and by the Ethical Committee of the University of Turin, Turin, Italy.

Experimental model

Forty-two rats were randomly divided into three groups (n=14 each): Control group (CTRL), ISO group and ALA-ISO group (Fig. 1). The experimental period included 7 days of acclimatization, 5 days of treatment and further 60 days of observation for a total of 72 days. In CTRL, the rats received a daily subcutaneous injection of 0.5 ml of saline solution during the treatment period, while in ISO group saline solution was replaced by 100 mg/kg of isoproterenol [23, 28]. Both these groups were fed with a standard chow diet (Harlan Laboratories S.p.A., Udine, Italy) *ad libitum* for the entire experimental period. The animals of the ALA-ISO group were treated as those of the ISO group, but they were fed *ad libitum* with an ALA-enriched diet proposed and validated by Fiaccavento et al [18] from the first day of the acclimatization period to the sacrifice. The diet, consisting of carrots, apples and flaxseed (20:50:30 w/w), provides macro- and micronutrients needed for maintenance of animal health as well as the standard chow [18]. On the basis of the content in ALA of flaxseed as reported by Rodriguez-Leyva et al.[29], we estimate that each animal took 2.85g/day of ALA.

The weight of the animals was checked every 7 days. At the end of the experimental period, the rats were anaesthetized and the hearts were rapidly excised and weighed. Heart weights were normalized for the corresponding body weight of each animal to calculate heart/body weight ratio.

Then, the hearts were either fixed for histological examination or immediately snap frozen in liquid nitrogen and stored at -80°C until they were processed for biomolecular analysis.

Heart function assessment

Left ventricular ejection fraction (EF) and fractional shortening (FS) were assessed in all animals at the beginning of the acclimatization period as well as 30 and 60 days after the last subcutaneous injection. EF and FS were evaluated using an echocardiographic system equipped with a 7.5-MHz transducer (ESAOTE-MEGAS, Esaote, Genova, Italy). Briefly, the rats were anesthetized with ketamine (100 mg/kg) and xylazine (5 mg/kg). Then, the animals were tied in supine position on a pad and the chest was shaved to avoid the presence of air between the skin and the probe. At each time point, EF and FS were measured 5 times in each animal and the values were averaged.

Histological examination

Hearts were fixed with 4% paraformaldehyde, embedded in paraffin and then sliced to obtain 4 μm thick serial ventricular sections. Slices were stained with the Masson's trichrome (Bio-Optica, Milan, Italy) in accordance with the manufacturer's instructions and blindly examined.

To evaluate the extension of fibrosis, photomicrographs of Masson's trichrome stained ventricular sections were taken (100× magnification) using a Leica DM4000B microscope equipped with DFC-320 Leica digital camera (Leica Microsystems, Wetzlar, Germany). Blue areas, which correspond to collagen deposition, were "color-range" selected with PhotoShop CS4 (Adobe Systems Inc., San Jose, CA, USA) and quantified with ImageJ Software (U.S. National Institutes of Health, Bethesda, MD, USA, http://rsb.info.nih.gov/ij/, 1997-2012). Quantification was performed on 3-5 randomly selected ventricular sections from 4 animals each group and was expressed as fold changes with respect to the control normalized value.

To study the occurrence of cardiomyocyte hypertrophy, photomicrographs of Masson's trichrome stained ventricular sections were taken (400× magnification). Thus, the cross-sectional area of cardiomyocytes was measured with IM50 software (Leica Microsystem) by tracing the outlines of those positioned perpendicular to the plane of the section and with a clear nucleus image, in accord with Kelly's method [30]. Typically, about 200 cardiomyocyte cross-sectional areas were measured and averaged for each group.

Metalloproteinase activity

Latent (72 kD) and active (66 kD) forms of MMP-2 as well as latent (92 kD) and active (82 kD) forms of MMP-9 were detected by gelatin zymography [31]. Briefly, samples of frozen heart were powdered and resuspended in RIPA buffer for protein extraction. One hundred micrograms of total proteins were separated by SDS–PAGE on a 10% acrylamide gel, containing 0.1% gelatin under non-reducing conditions. Gels were washed in renaturing buffer (2.5% Triton X-100 in distilled water) for 30 min at room temperature with gentle agitation, and then in distilled water. Afterword, they were equilibrated and incubated in developing buffer (50 mM Tris-HCl, pH 7.8, 0.2M NaCl, 5mM $CaCl_2$, and 0.02% v/v Tween-20) overnight at 37°C. Gels were stained with 0.05% w/v Coomassie Brilliant Blue, 5% v/v methanol and 10% v/v acetic acid in distilled water for 1 h at room temperature and then destained with a 10% v/v methanol and 5% v/v acetic acid solution. Bands quantification was performed with ImageJ software.

Protein level expression

Western blot analysis was performed as previously described [32]. After protein extraction and quantification (Bradford protein assay), 100 μg of total proteins were separated on 10% SDS–PAGE, and electrotransferred to a PVDF membrane (Macherey-Nagel, Düren, Germany). Membranes were incubates with specific primary antibodies against TGF-β1, TIMP-1 and β-myosin

heavy chain (β-MHC) and thus with species-specific horseradish peroxidase-conjugate secondary antibodies (Table 1). The expression of β-actin was evaluated in the same blot as loading control. Immunolabeled bands were detected using enhanced chemiluminescence (ECL) system (BioVision, Mountain View, CA, USA). Image acquisition and quantification were made with a Kodak Image station 440CF (Kodak, Rochester, NY, USA). Each experiment was done in triplicate.

Statistical analysis

The experimental data are expressed as the mean of technical and biological replicates ± SD. Statistical significance was assessed by one-way ANOVA followed by Newman-Keuls Multiple Comparison Test, and significant differences were accepted with $p<0.05$ as the significant cut-off. All analyses were carried out with GraphPad Prism version 6.0c (GraphPad Software, San Diego California, USA).

Results

In vitro Studies

ALA prevents ISO-induced H9c2 cell viability impairment

MTT assays (fig. 2) demonstrated that the viability of H9c2 cells was significantly reduced to 59.9±8.6% ($p<0.001$) after 24h of incubation with ISO at 250 μM concentration. When ISO treatment was preceded by 24 h incubation with ALA, H9c2 cell viability was reduced only to 89.2±12.8% with respect to controls ($p<0.05$), strongly counteracting the impairments provoked by ISO ($p<0.001$).

The pretreatment with the PI3K inhibitor LY294002, the Src inhibitor PP2 or with the caveolae disrupter MβCD suppressed the ALA-mediated protection from ISO damage on H9c2 cell viability (59.4±14.9%, 63.6±11.4% and 64.4±15.5%, respectively). None of these groups was significantly different from ISO group, but they were significantly reduced in cell viability if compared to ALA-ISO group (each $p<0.001$).

In vivo Studies

ALA-enriched diet abolishes animal mortality during ISO treatment

Repeated subcutaneous injection of ISO provoked an high acute mortality rate (50%) amongst rats of ISO group, which were fed with standard diet. Fatal occurrences took place in the period of ISO treatment (2 animals after the first day, 3 animals after the second day, and 2 more after the fourth day) while no other rats died until the end of the experimentation. No deaths were observed after ISO injection in rats fed with the ALA-enriched diet, as well as in those of CTRL group, which were injected with saline solution.

ALA-enriched diet prevents the reduction of heart mechanical function

After the treatment with either ISO or saline, rats were followed for 60 days in which EF and FS were monitored. In CTRL group both parameters did not change during the observation period. On the other hand, EF and FS decreased significantly in ISO group by about 10% and 15% respectively ($p<0.01$). The decrease was almost the same 30 and 60 day after the end of treatment. The ISO-induced reduction of cardiac function did not occur when the animals were fed with ALA-enriched diet (Fig. 3a and 3b).

ALA-enriched diet prevents ISO-induced cardiac fibrosis

The extension of cardiac fibrosis was assessed with Masson's trichrome staining. Hearts from control group showed weak expression of extracellular matrix (Fig. 4a). On the contrary, a remarkable production and deposition of ECM occurred in the hearts of ISO group, where newly formed collagen mainly surrounded cardiomyocytes throughout entire ventricular sections (Fig. 4b). In hearts from ALA-ISO group, the expression and localization of the ECM were similar to those observed in control hearts (Fig. 4c). In the Masson's trichrome staining, the fibrotic area increased

about 8 fold in ISO group with respect to CTRL, while it did not show any appreciable change in ALA-ISO group (Fig. 4d).

These data were supported by the increased activity of both latent and active forms of MMP-2 ($p<0.05$ and $p<0.01$, respectively) by about 50% in the ISO group with respect to CTRL (Fig. 5a). Such increases were prevented when the ISO-treated animals were fed with ALA-enriched diet. Unlike MMP-2, the activity of MMP-9 was not detectable in our experiments. In addition, the expression of the inhibitor of metalloproteinase TIMP-1 was reduced by about 25% in response to ISO treatment, while no changes were observed when the treated animals were fed with the ALA-enriched diet (Fig. 5b). Also a four-fold increase in expression ($p<0.001$) of TGF-β1 took place in the ISO group, while no change occurred in ALA-ISO group (Fig. 5c).

ALA-enriched diet prevents ISO-induced cardiac hypertrophy

During the observation period, the weight of the animals increased with time from 144±8 to 334±20 g in CTRL, from 145±5 to 334±17 g in ISO group and from 144±4 to 320±9 g in ALA-ISO group, without any significant difference among the groups.

At the post-mortem examination, the heart weights were significantly ($p<0.01$) higher in ISO group (1.36±0.06 g) with respect to CTRL (1.08±0.16 g) and ALA-ISO group (1.07±0.15 g). No significant difference was found between CTRL and ALA-ISO groups.

The heart/body weight ratio was 3.31±0.40‰ in CTRL. It was slightly but significantly increased in ISO group (4.04±0.16‰; $p<0.01$), while it remained unchanged when animals were fed with ALA-enriched diet (3.23±0.44‰) (Fig. 6a). The above data on cardiac hypertrophy were supported by the histological examination, which consider 200 cardiomyocytes for each experimental group. An increase in cardiomyocyte cross-sectional area was detected in ISO group (250±10 μm^2; $p<0.001$) with respect to the CTRL (199±3 μm^2), while no significant differences were found between CTRL and ALA-ISO (198±2 μm^2) groups (Fig. 6b). In addition to TGF-β1 overexpression (Fig. 5c) that is

also associated with myocardial hypertrophy, a 3-fold increased expression ($p<0.001$) in β-MHC, a marker of pathological cardiac hypertrophy, was observed in ISO group. This increase did not occur if the animals were fed with an ALA-enriched diet (Fig. 6c).

Discussion

The present investigation demonstrated that ALA prevents cardiac damage induced by β-adrenergic overstimulation.

In cardiovascular diseases, β-adrenergic receptors (βAR) trigger a broad range of pathways, which lead to the progressive degeneration of myocardium. The mining analysis of βAR-triggered pathways reveals the involvement of more than one hundred molecules (Fig S1), which are accountable for about five hundred interactions. These pathways could be however responsible for either tissue remodeling (fibrosis and hypertrophy) or cell survival, on the basis of the different location of βAR subtypes.

In fact, β_1ARs are present on the cell membrane where they are exclusively coupled with G_s protein, which triggers the increase of cAMP levels [33] and induces myocyte apoptosis and necrosis [34], cardiac fibrosis [10] and hypertrophy [11]. Unlike these receptors, β_2ARs can be confined into the caveolae, where they are coupled with G_i protein, thus impairing cAMP production and activating a pro-survival pathway [33, 35-37]. Chronic βAR over-stimulation also induces the downregulation of caveolin-3 [38], the structural component of caveolae, allowing β_2AR to move out of caveolae where they mediate the same effects of β_1AR [33, 35, 39].

In order to validate the hypothesis that ALA can exert protective effect against βAR over-stimulation, we performed an *in vitro* study, which showed that ALA treatment limited the isoproterenol-induced H9c2 impairment of viability. This effect was due to the activation of a pathway involving Src kinase and PI3K, as demonstrated by its suppression after the cells were pre-incubated with PP2 and LY294002. These specific inhibitors abolished the pathway elicited by G_i protein coupled β_2AR stimulation [37] and the resulting myocardial protection, but were ineffective

when βARs interacted with G_s protein [40]. A similar pro-survival suppression occurred after caveolae disruption with MβCD and demonstrated that the integrity of caveolae is essential to allow ALA to exert the protective effect. These results are consistent with the reported capability of ALA to enhance caveolin-3 level and preserve the integrity of caveolae [20].

ISO administration was reported to reduce caveolin-3 expression and caveolae density [33, 38, 41-43]. The dislocation of β_2AR out of caveolae and its coupling with G_s protein may contribute to β_1AR-induced TGF-β overexpression leading to fibrosis and hypertrophy. It is likely that if ALA preserves the integrity of caveolae [20], the pro-survival pathway can be triggered [37, 44], since the caveolar membrane is characterised by a hight content of G_i protein-coupled β_2AR [36, 45]. Hence, ALA protective effect was studied in rats using a well-known experimental model of infarct-like myocardial damage obtained by repeated injections of ISO [22, 23]. The enrichment of ALA was achieved employing an already validated diet, which was used to address the role of ALA mediated protection in cardiomyocytes and hamsters with genetic cardiomyopaties [18, 20, 21]. Here, we estimate that 2.85 g/day of ALA was assumed by each rat. Interestingly, treatment with similar amount of ALA (2.9 g/day) was also reported in human where it significantly lower the risk to undergo both cardiac death and nonfatal myocardial infarction [46].

In the present investigation we demonstrate that an ALA-enriched diet preserves the heart from the injuries caused by adrenergic overstimulation. During ISO treatment, 50% of the animals died, presumably as a result of arrhythmias caused by spatial dispersion of action potential duration as previously demonstrated [47]. ALA-enriched diet may have suppressed this mortality because of an anti-arrhythmic effect mediated by the reduction of myocardial excitability [48] and the shortening of action potential duration [49].

As expected, 60 days after the end of the treatment, the histological examination of hearts from ISO group revealed the presence of fibrosis and hypertrophy, *i.e.* an abundant deposition of ECM among cardiomyocytes and an increase in cell size. These morphological alterations were accompanied by an increased expression of TGF-β and β-MHC, a decrease of TIMP-1 expression and an increase in

the activity of both latent and active forms of MMP-2, as it was found in the late remodeling phase of myocardial infarction [50, 51].

The increased expression of TGF-β by β-adrenergic overstimulation [52] is responsible for both fibrosis and hypertrophy. In the case of fibrosis, TGF-β causes the differentiation of fibroblasts into myofibroblasts, thus leading to an excess of ECM production via a complex regulation of MMP and TIMP interaction [53]. Actually, the regulatory role of TGF-β on MMP/TIMP system is rather complex because MMP-2 can in turn promote the activity of TGF-β through the proteolytical cleavage of its latent form [54]. Because of this positive feedback, the role of TGF-β and MMP/TIMP system in inducing myocardial fibrosis is strengthened. Although the active form of MMP-2 is generally accompanies by MMP-9 activity during the fibrosis onset [55], in our experiments MMP-9 activity was not present. The absence was in line with the short persistence of this enzyme after the induction of a myocardial damage [50, 56] and was also supported by the mining analysis.

In the case of hypertrophy, TGF-β mediates the upregulation of β-MHC RNA expression, the increase of cardiomyocyte protein content, the sarcomeric organization and cell size [57]. It has been suggested that in hypertensive rats an increased MMP activity may be involved in hypertrophy, and that this enhanced activity is accompanied by an increase in TGF-β expression [58], so that it is difficult to deal with these two processes separately.

In the myocardium of animals fed with ALA-enriched diet, ISO was not able to provoke any of the above mentioned histological and molecular alterations, leaving the various markers in the range of the healthy controls. The present findings provide evidence for the first time that ALA counteracts TGF-β overexpression and the increase in MMP activity in the rat heart, thus preventing the ISO-induced fibrosis and hypertrophy. The abolishment of the protection after disruption of the caveolae suggests the possibility that a shift in the β_2AR signaling from G_i to G_s by ISO administration is prevented when ALA preserves the integrity of caveolae.

ISO-treated animals displayed a decrement in contractility, as revealed by reduced EF and FS. Since it has been demonstrated that MMP-2 can *per se* impair ventricular function in the absence of

superimposed injury, it is possible that MMP-2 contributed to the reduction of EF and FS even independently of the fibrosis [59, 60]. It may then be argued that flaxseed supplementation maintained a physiological contractility because ALA prevented both structure derangement and functional impairment of myocardium.

In conclusion, the present study demonstrated that ALA effectiveness is due to the preservation of caveolae integrity that are essential for the activation of the Src-PI3K pro-survival pathway. Moreover, ALA-enriched diet reduces mortality and prevents cardiac fibrosis, hypertrophy as well as mechanical dysfunction induced by the β-adrenergic overstimulation in these experimental models.

Our data provide the basis for the development of future appropriate strategies for the prevention of various pathophysiological conditions characterized by a sympathetic overactivity. In particular, further studies are required to fully unravel the mechanism underlying β-adrenergic over-stimulation and to clarify whether ALA is protective also in other types of cardiac remodeling.

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Conflict of interest

The authors declare that they have no competing interests.

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Figure legends

Figure 1 In vivo experimental protocols. Each bar summarizes the treatments and the diets followed by the animals of the three experimental groups. Control (CTRL), isoproterenol (ISO) and α- linolenic acid (ALA).

Figure 2 α-linolenic acid (ALA) prevented ISO-induced impairment of H9c2 cell viability. The addition of specific inhibitors (LY294002 or PP2) and of caveolae disrupter (MβCD) completely reverted the ALA mediated protection. Values are means ± SD. * $p<0.05$ and *** $p<0.001$ vs. control (CTRL); ### $p<0.001$ vs. ALA-ISO.

Figure 3 α-linolenic acid (ALA)-enriched diet counteracted isoproterenol (ISO)-induced alterations of heart mechanical function. Reduction of left ventricular ejection fraction (EF) (a) and fractional shortening (FS) (b) in ISO group 30 and 60 days after the end of ISO-treatment. No change was observed in control (CTRL) and ALA-ISO groups. Values are means ± SD. * $p<0.05$ and **

p<0.01 *vs* CTRL; ## p<0.01 and ### p<0.001 *vs* ALA-ISO group; § p<0.05 and §§ p<0.05 *vs* basal condition (0 day).

Figure 4 α-linolenic acid (ALA)-enriched diet prevented isoproterenol (ISO)-induced fibrosis in ventricular myocardium. Masson's trichrome stained ventricular section showed a basal deposition of extracellular matrix in control (CTRL) (a) which heavily increased after ISO treatment (b). The increase was prevented by ALA-enriched diet (c). The images are representative of randomly selected slices (scale bar=500 μm). Quantification of collagen deposition (d) was expressed as fold changes with respect to the normalized control value. ***p<0.001 *vs* CTRL; ###p<0.001 *vs* ALA-ISO group.

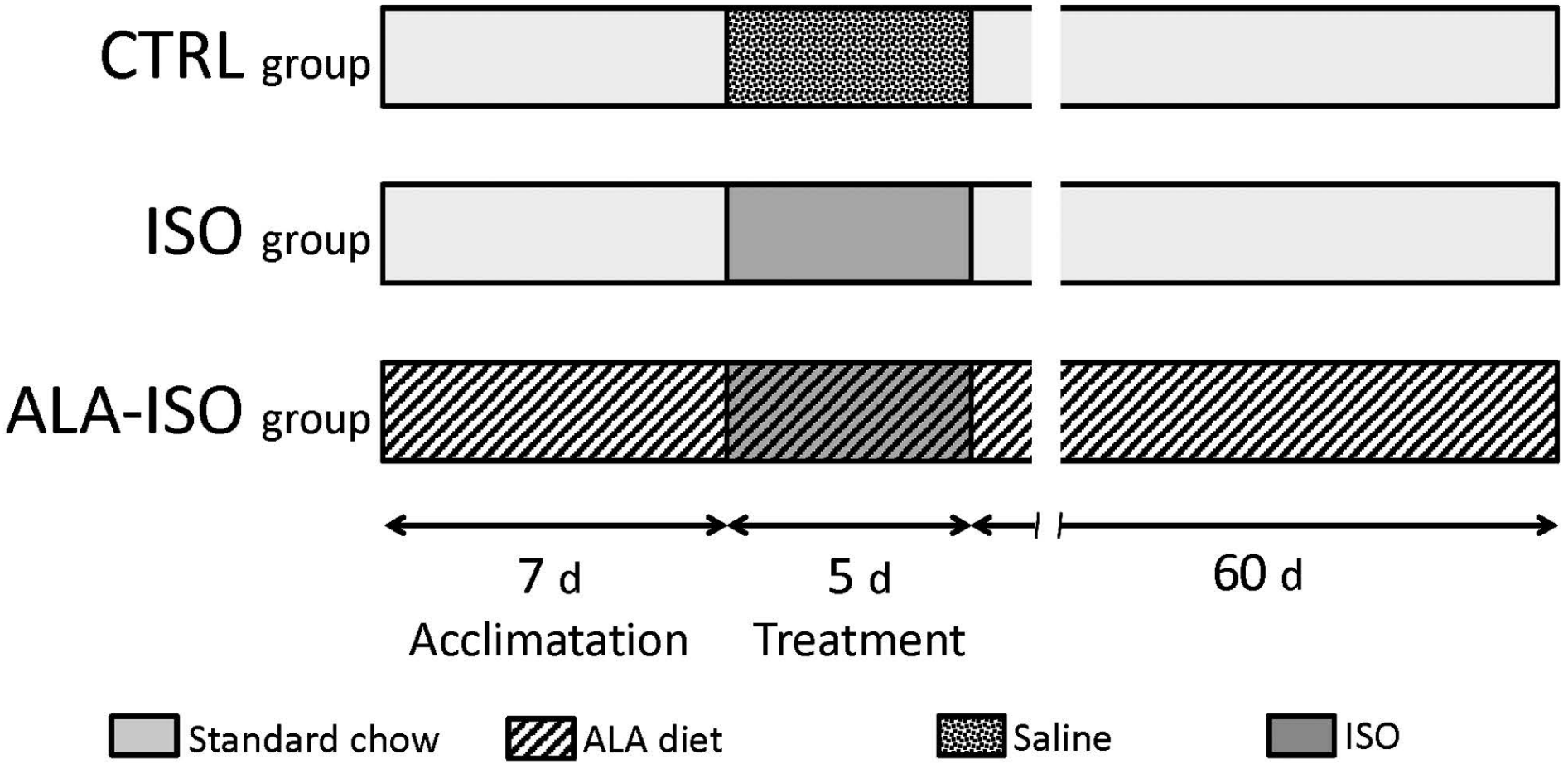
Figure 5 α-linolenic acid (ALA)-enriched diet effect on isoproterenol (ISO)-induced alterations of molecular markers of fibrosis. ALA-enriched diet prevented the increase in active and latent MMP-2 after ISO treatment (a); ALA-enriched diet prevented the reduction in TIMP-1 and the increase in TGF-β protein expressions. Band intensities were normalized to β-actin expression Values are means ± SD. * p<0.05 and ** p<0.01 *vs*. control (CTRL); # p<0.05, ## p<0.01 and ### p<0.001 *vs*. ALA-ISO group.

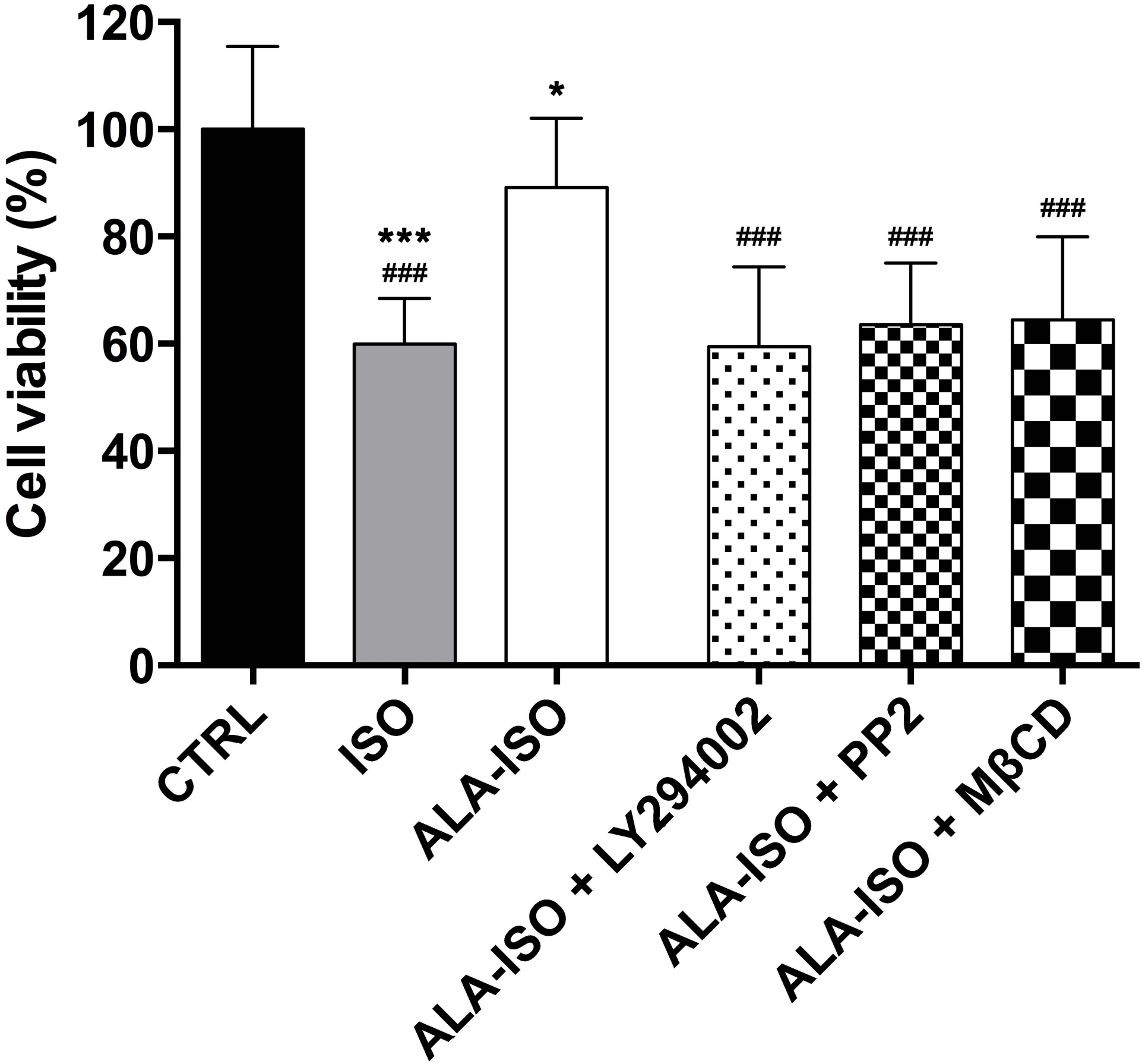
Figure 6 α-linolenic acid (ALA)-enriched diet protected ventricular myocardium against isoproterenol (ISO)-induced hypertrophy by preventing increase of HW/BW ratio (a); increase of cardiomyocyte cross-sectional (CSA) with representative images of randomly selected slices (scale bar=50 μm) (b); increase of β- myosin heavy chain (β-MHC) expressions (c);. Values are means ± SD. ** p<0.01 and *** p<0.001 *vs*. control (CTRL); ## p<0.01 and ### p<0.001 *vs*. ALA-ISO.

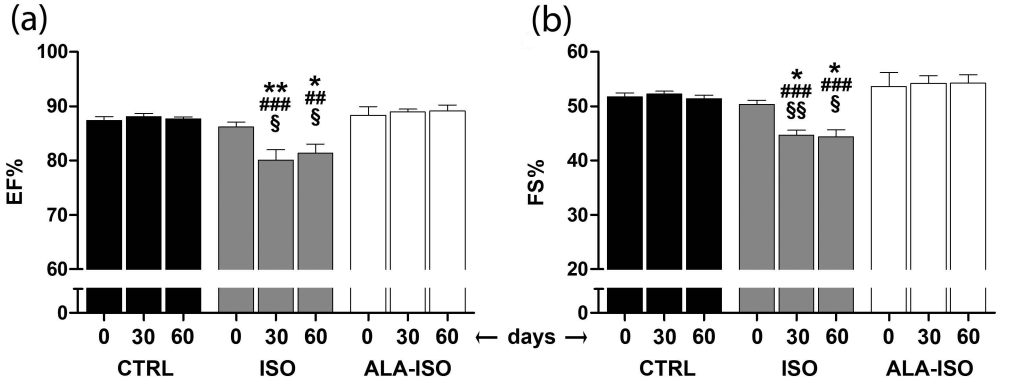
Table 1. Antibodies employed for Western Blot analysis

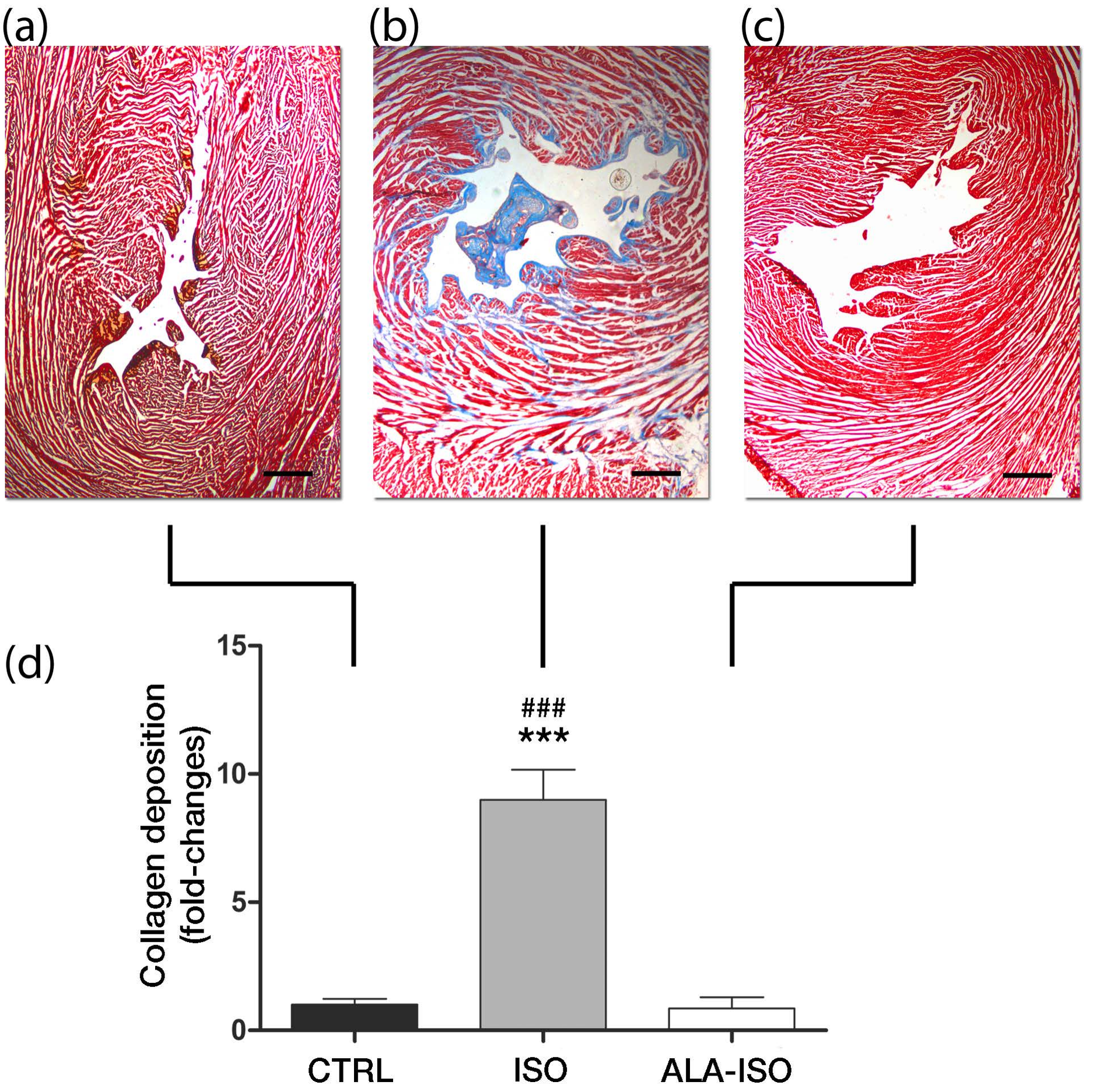
Primary Ab	**Clone**	**Host**	**Dilution**	**Supplier**
TGF-β1	V	Rabbit	1:200	SantaCruz Biotechnology, Inc
TIMP-1	H150	Rabbit	1:200	SantaCruz Biotechnology, Inc
β-MHC	NOQ7.5.4D	Mouse	1:1000	Sigma-Aldrich
β-actin	C11	Rabbit	1:1000	Sigma-Aldrich
Secondary Ab	**Reactivity**	**Host**	**Dilution**	**Supplier**
IgG-HRP	Rabbit	Goat	1:2000	SantaCruz Biotechnology, Inc
IgG-HRP	Mouse	Goat	1:2000	SantaCruz Biotechnology, Inc

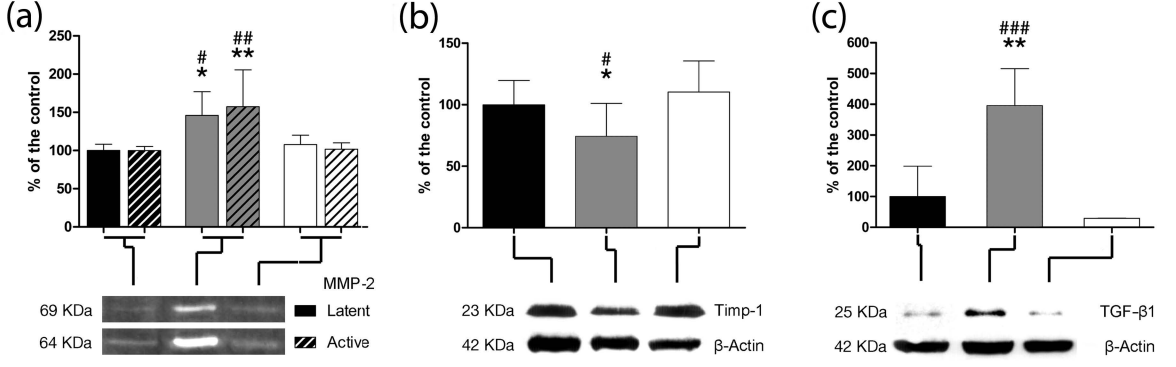
Abbreviations: TGF, transforming growth factor; TIMP, tissue inhibitor of metalloproteinase; MHC, myosin heavy chain; HRP, horseradish peroxidase.

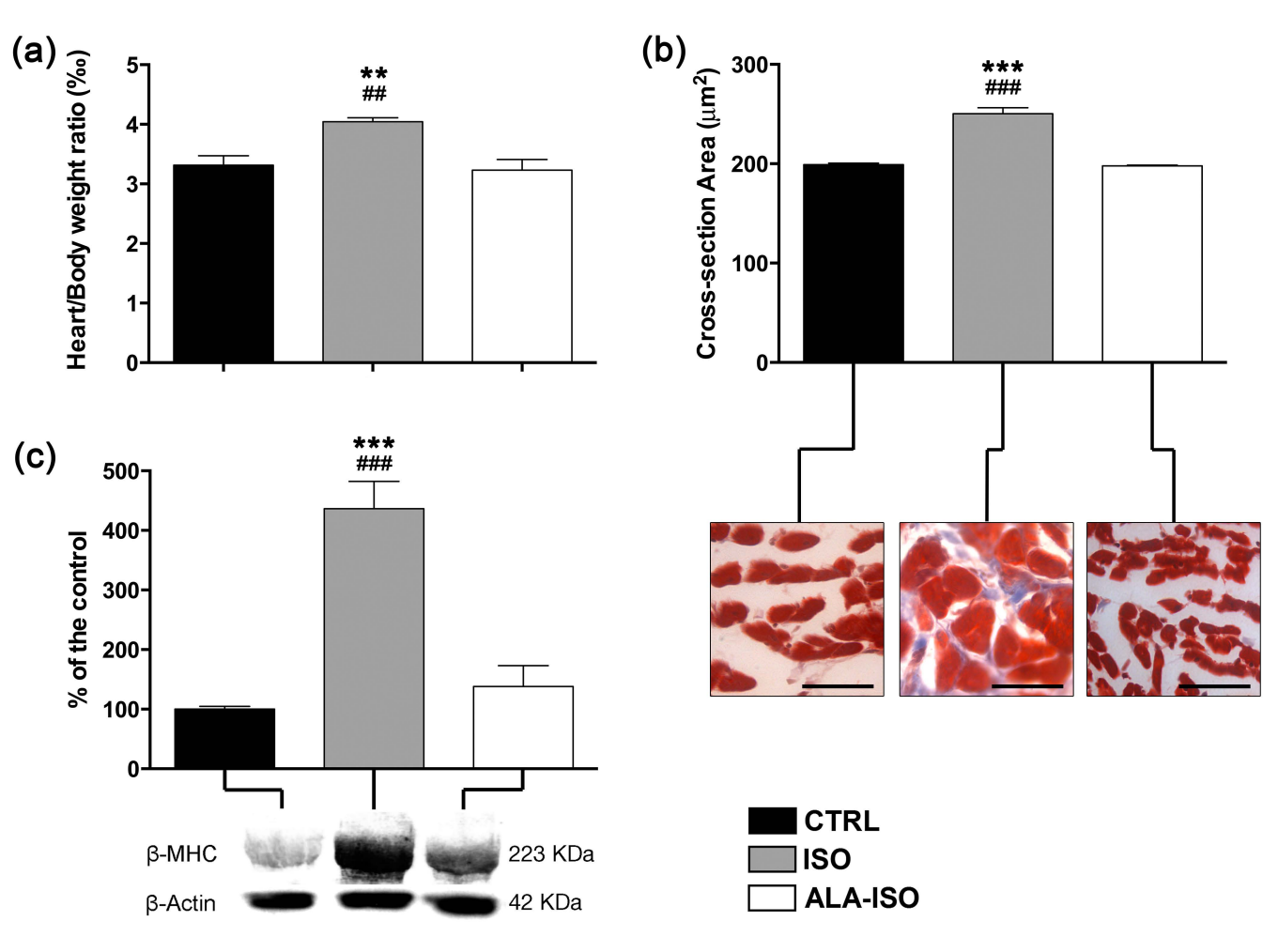

CTRL group
ISO group
ALA-ISO group
7 d
Acclimatation
5 d
Treatment
60 d
Standard chow
ALA diet
Saline
ISO

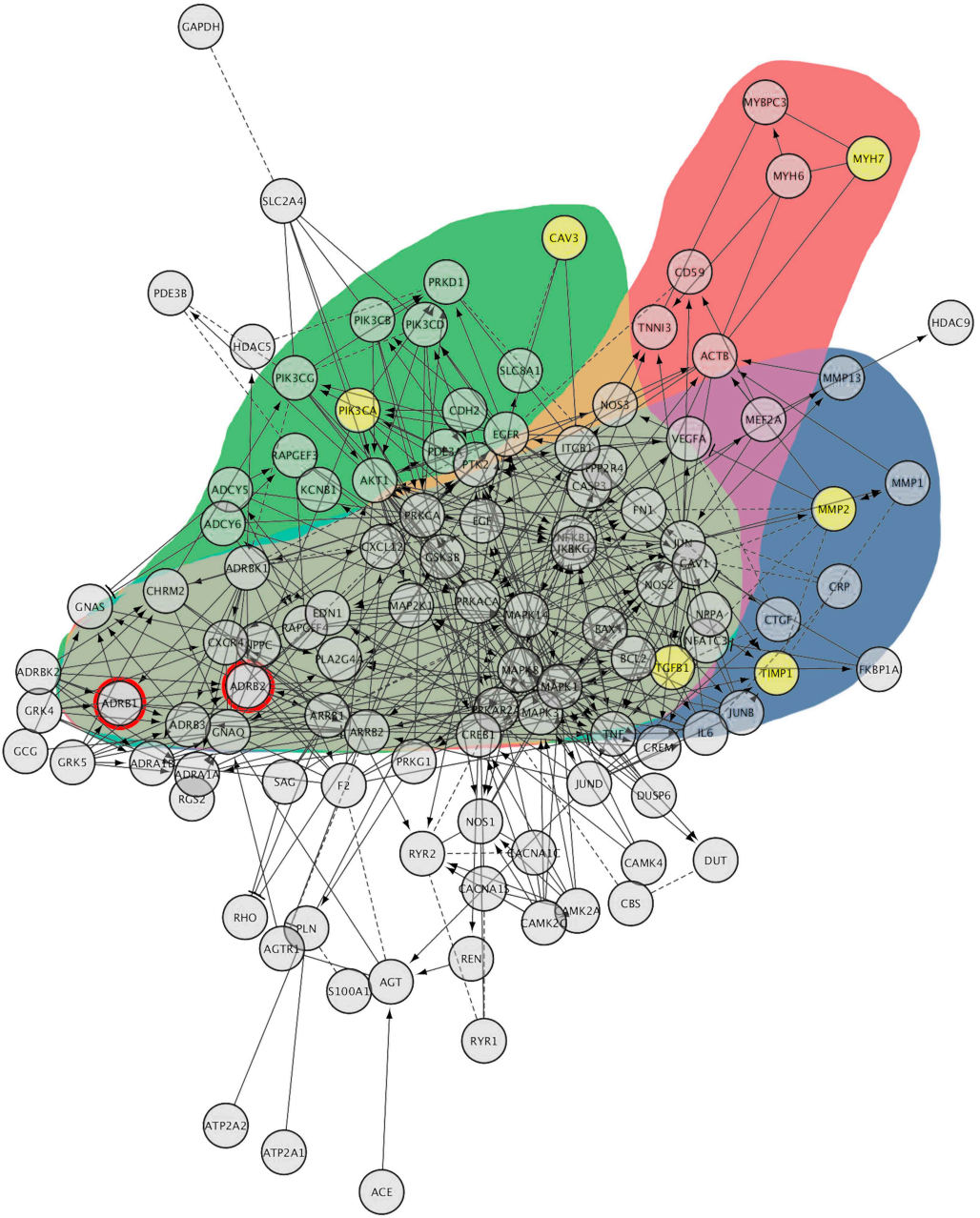

Cell viability (%)
120
100
80
60
40
20
0
***
###
*
###
###
###
CTRL
ISO
ALA-ISO
ALA-ISO + LY294002
ALA-ISO + PP2
ALA-ISO + MβCD


(a)
EF%
100
90
80
70
60
0
**
###
§
*
##
§
0 30 60 0 30 60 0 30 60
CTRL
ISO
ALA-ISO
(b)
FS%
60
50
40
30
20
0
*
###
§§
*
###
§
0 30 60 0 30 60 0 30 60
CTRL
ISO
ALA-ISO
← days →


(a)
(b)
(c)
(d)
Collagen deposition
(fold-changes)
15
10
5
0
###
***
CTRL
ISO
ALA-ISO


(a)
% of the control
250
200
150
100
50
0
#
*
##
**
MMP-2
69 KDa
64 KDa
Latent
Active
(b)
% of the control
150
100
50
0
#
*
23 KDa
42 KDa
Timp-1
β-Actin
(c)
% of the control
600
500
400
300
200
100
0
###
**
25 KDa
42 KDa
TGF-β1
β-Actin
CTRL
ISO
ALA-ISO


(a)
Heart/Body weight ratio (‰)
5
4
3
2
1
0
**
##
(b)
Cross-section Area (μm²)
300
200
100
0
***
###
(c)
% of the control
500
400
300
200
100
0
***
###
β-MHC
β-Actin
223 KDa
42 KDa
CTRL
ISO
ALA-ISO


GAPDH
SLC2A4
PDE3B
HDAC5
PRKD1
PIK3CB
PIK3CD
CAV3
PIK3CG
PIK3CA
SLC8A1
CDH2
EGFR
NOS3
RAPGEF3
PDE3A
PTK2
ITGB1
PPP2R4
CAV3
AKT1
ADCY5
KCNB1
ADCY6
PRKCA
EGF
FN1
CXCL12
GSK3B
NFKB1
IKBKG
JUN
CAV1
ADRBK1
CHRM2
GNAS
EDN1
MAP2K1
PRKACA
MAPK14
NOS2
NRPA
CTGF
CXCR4
GPCR
PLA2G4A
BAX
NFATC3
BCL2
TGFB1
TIMP1
FKBP1A
ADRBK2
GRK4
ADRB1
ADRB2
MAPK8
MAPK1
ARRB1
PRKAR2A
MAPK3
JUNB
ADRB3
GNAQ
ARRB2
CREB1
TNF
IL6
GCG
GRK5
ADRA1B
ADRA1A
PRKG1
CREM
RGS2
SAG
F2
JUND
DUSP6
NOS1
RYR2
CACNA1C
CAMK4
DUT
CACNA1S
CAMK2A
CAMK2D
CBS
RHO
PLN
AGTR1
REN
S100A1
AGT
RYR1
ATP2A2
ATP2A1
ACE
MYBPC3
MYH7
MYH6
CD59
TNNI3
ACTB
HDAC9
MMP13
MEF2A
VEGFA
MMP1
MMP2
CRP
FBXO32

Supplementary data

Materials and Methods

Data mining

ProteinQuest™ literature mining tool (BioDigitalValley S.r.l., Pont-Saint-Martin, Aosta, Italy) was used to detect correlations and interactions in the β-adrenergic receptor downstream signaling pathways. In particular, the query ["Adrenoceptor beta proteins" AND Myocardium AND ("Heart Failure" Or "Myocardial Ischemia")] was investigated. The analysis of literature obtained by the ProteinQuest™ mining tool detected 344 papers, from which 114 network nodes (proteins) and 499 edges (interactions) were retrieved. Reactome FI app [1] for Cytoscape [2] version 3.2.0 (http://www.cytoscape.org) was employed to analyse pathways and networks (Fig. S1). Among these, Caveolin-3 (CAV3) and PI3K, as well as Src kinase (retrievable as linked to PI3K in further analysis refinements), were assessed for their involvement in cell survival. The role of TIMP1 and MMP2 were investigated to address the fibrosis onset. β-myosin heavy chain (MYH7, β-MHC) was employed as marker of hypertrophy, while TGF-β1 was recruited as marker of both fibrosis and hypertrophy.

Supplementary references

1. G. Wu, E. Dawson, A. Duong, R. Haw and L. Stein, *F1000Research*, 2014, **3**, 146.
2. P. Shannon, A. Markiel, O. Ozier, N. S. Baliga, J. T. Wang, D. Ramage, N. Amin, B. Schwikowski and T. Ideker, *Genome research*, 2003, **13**, 2498-2504.

Supplementary figure legends

Figure S1 Data mining. Reactome FI representation of the analysis performed by Protein Quest™. β_1AR and β_2AR where circled in red while the main actors and object of our investigation were highlighted in yellow. Coloured areas represent cell survival (green), fibrosis (blue), hypertrophy (red), or their ovelapping.