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Loss of myocardial LIF receptor in experimental heart failure reduces cardiotrophin-1 cytoprotection. A role for neurohumoral agonists?

Natalia López [a], Nerea Varo [a,b], Javier Díez [a,c], María Antonia Fortuño [a,*]

[a] *Division of Cardiovascular Sciences, Centre for Applied Medical Research, University of Navarra, Spain*
[b] *Department of Clinical Biochemistry, Spain*
[c] *Department of Cardiology and Cardiovascular Surgery, Universitary Clinic, University of Navarra, Pamplona, Spain*

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Abstract

Objectives: Cardiomyocyte loss is involved in the transition from compensatory left ventricular hypertrophy (LVH) to heart failure (HF). Our aim was to investigate the status of the leukaemia inhibitory factor receptor (LIFR)/gp130 survival pathway and its cytoprotective activity in intact cardiac tissue and in cardiomyocytes obtained from adult spontaneously hypertensive rats (SHR) with LVH (non-failing SHR) and from aged SHR with overt HF (failing SHR).
Methods: Cardiac morphometry was assayed by planimetry in an image analysis system. mRNA and protein expression were quantified by real time RT-PCR and Western blotting. Receptors were localized by immunocytochemistry. Trypan blue staining, TUNEL, and MTT cell viability assays were employed to study the cytoprotective activity of cardiotrophin-1 (CT-1) in isolated caridomyocytes.
Results: Compared to non-failing SHR, failing SHR exhibited enhanced myocardial cell death ($p<0.01$) demonstrated by the increase in Bax/Bcl-2 ratio, caspase-3 activation and poly (ADP-ribose) polymerase (PARP) fragmentation. Failing SHR had a 7-fold diminished expression ($p<0.01$) of LIFR, no changes in gp130, and 1.6-fold increased myocardial expression ($p<0.01$) of CT-1. In cardiomyocytes isolated from non-failing SHR, recombinant CT-1 inhibited apoptotic and non-apoptotic cell death induced by angiotensin II or hydrogen peroxide. LIFR protein was entirely absent in cardiomyocytes isolated from failing SHR, which were resistant to the cytoprotective effects of CT-1. Finally, stimulation of non-failing SHR cardiomyocytes with angiotensin II, aldosterone, norepinephrine or endothelin-1 significantly decreased ($p<0.01$) LIFR expression.
Conclusions: These data suggest that loss of CT-1-dependent survival mechanisms may contribute to the increase of cell death associated with HF in SHR. Neurohumoral activation may contribute to this alteration via suppression of LIFR.
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1. Introduction

Accompanying hypertensive heart disease (HHD), the myocardium initially develops adaptative left ventricular hypertrophy (LVH) which ultimately leads to cardiac dysfunction and development of congestive heart failure (HF). During this transition, cardiomyocyte loss facilitates both contractility deterioration and side-to-side slippage of the remaining cardiomyocytes, leading to ventricular dilation in HF [1]. Several mechanical and humoral factors present in pressure-overloaded hearts induce cardiomyocyte death [2,3]. Alternatively, the loss of contractile cells may reflect deterioration of intrinsic survival pathways that protect cardiomyocytes from external death factors.

The heterodimer constituted by the leukaemia inhibitory factor receptor (LIFR) and the glycoprotein 130 (gp130) is a signaling transducer that triggers the best characterized

* Corresponding author. Centre for Applied Medical Research (CIMA). Avda. Pio XII, 55. 31008. Pamplona. Spain. Tel.: +34 948 19 47 00x3023; fax: +34 948 19 47 16.
E-mail address: fortuto@unav.es (M.A. Fortuño).

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survival pathway operating in cardiac tissue [4–7]. Ligand binding to the low affinity 190 kDa LIFR induces heterodimerization with gp130 resulting in the high affinity receptor. JAKs constitutively associated with the cytoplasmic domains of the two receptors are activated by trans-or autophosphorylation and in turn phosphorylate tyrosine residues in both LIFR and gp130, creating approaching sites for downstream signaling molecules [8,9]. Cardiotrophin-1 (CT-1) is the most important ligand of this receptor in the heart [10,11]. In terminally differentiated cardiomyocytes this cytokine induces cell survival against ischemic and non-ischemic death stimuli via ERK1/2 and PI3K/Akt pathways [12,13] and promotes hypertrophy through ERK5 and STAT3 signaling pathways [14].

Previous studies have shown that mice with cardiac-specific inactivated gp130 displayed massive induction of cardiomyocyte apoptosis, chamber dilation and fulminant HF in response to acute pressure overload, suggesting the importance of this survival mechanism in situations of mechanical overload [15]. The spontaneously hypertensive rat (SHR) is an animal model of genetic hypertension which, similar to humans, develops HF with aging after a long period of compensatory hypertrophy [16,17]. The enhanced cardiomyocyte cell death exhibited by SHR at different ages is a pathogenic factor that contributes to cardiac deterioration [18–22]. We have previously shown that cardiomyocytes isolated from adult SHR are more susceptible to apoptosis than cardiomyocytes isolated from normal rats, although the specific origin of this predisposition remains unknown [23].

We hypothesize here that alterations in the LIFR/gp130 survival pathway leading to diminish cytoprotective action of CT-1 may facilitate cell death and contribute to the transition from compensated LVH to HF in SHR. To test this hypothesis we have analyzed the expression of this pathway and its association with cardiomyocyte death in adult SHR with LVH (non-failing SHR) and old SHR with overt HF (failing SHR). In addition, experiments assaying the functionality of the CT-1 activated LIFR/gp130 pathway were performed on cardiomyocytes isolated from the two groups of animals.

2. Methods

2.1. Animals

The investigation was performed in accordance with the Guide for Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication no. 82–23, revised in 1996). Animals were provided by Harlam UK Limited (Bicester, England). All rats were housed in individual cages and fed a standard rat chow and tap water *ad libitum*. Animals were maintained in a quiet room at constant temperature (20 to 22 °C) and humidity (50 to 60%). For *ex vivo* determinations, we employed 30-week-old SHR ($N=20$, non-failing group), and 76-to 80-week-old SHR with manifest HF ($N=20$, failing group). HF diagnosis was performed by clinical criteria previously reported for this animal model [16,17]. Animals over 72 weeks of age were observed daily by two independent experts, to achieve a description of normal activity, responsiveness to manipulation, weight, respiration, and general aspect. All SHR included in failing group exhibited 3 of the following symptoms for at least 2 weeks: weight loss, lethargy, subcutaneous edema, increased respiratory rate and effort. Age-matched normotensive Wistar Kyoto rats (WKY, $N=10$ per group) were employed to evaluate aging-induced cardiac cell death in the absence of hypertension. Systolic blood pressure was measured by the standard tail-cuff method using a LE 5007 Pressure Computer (Letica Scientific Instruments). Animals were sacrificed by decapitation under anesthesia i.p. with Ketamine 75 mg/kg (Imalgene 1000, Merial) and Xilacine 5 mg/kg (Rompun, Bayer). After sacrifice, HF diagnosis was confirmed in the failing group by the presence of ascites, internal congestion and other signs such us hepatomegaly or pleural effusion. Hearts were extracted, weighed to obtain the cardiac index (CI, cardiac weight in milligrams/body weight in grams) and immediately frozen in liquid nitrogen for molecular studies, or fixed by immersion in 10% buffered formalin during 48 hours and embedded in paraffin for morphometric determinations. For the study employing isolated cardiomyocytes, SHR were sacrificed as described above and the hearts were excised for cardiomyocyte isolation.

2.2. Morphometry

Myocardial dimensions were determined in 5 μm-thick sections stained with haematoxylin as recently described [14]. Left ventricular wall thickness (LVWT) was measured in three different regions of left ventricle free wall in quadruplicate. Chamber diameter (ChD) and chamber area (ChA) were measured in triplicate. All measurements were performed in an automated image analysis system (Soft Imaging System Analysis) by two blinded observers. Images were calibrated with known standards.

2.3. Western blotting

Left ventricles or cultured cardiomyocytes were homogenized in lysis buffer containing 7 mol/L urea; 2 mol/L thiourea; 65 mmol/L CHAPS; 65 mmol/L DTT. Protein concentration was determined by the Bradford method (Bio-Rad). Proteins (20 μg) were separated by 12% (for CT-1, Bax and Bcl-2), 10% (for Akt-P, Akt, Bad-P, Bad and Caspase-3) and 7.5% (for PARP, gp130 and LIFR) SDS polyacrylamide gel electrophoresis and transferred to PVDF membranes (Amersham Biosciences). The following primary polyclonal antibodies were employed: CT-1 (a kind gift from Dr Martínez, Area of Genetic Therapy, CIMA), Bax, Bcl-2, gp130 and LIFR (Santa Cruz Biotechnology), Akt, Akt-P (Ser^{473}), Bad, Bad-P (Ser^{112}), caspase-3, and PARP (Cell Signaling). Blots were visualized using ECL-Plus chemiluminiscence detection system (Amersham Biosciences). Optical density values were normalized with β-actin.

2.4. Reverse transcription and real-time PCR

Total RNA was extracted from hearts or cardiomyocytes using Trizol (Invitrogen) and subsequently purified using QIAGEN's RNeasy Total RNA Isolation kit. Reverse transcription was performed with 250 ng of total RNA. Real-time PCR was performed with an ABI PRISM 7000 Sequence Detection System by using specific TaqMan MGB fluorescent probes (Applied BioSystems). Constitutive 18S ribosomal RNA was used as endogenous control. For the relative quantitative analysis of unknown samples two calibration curves were prepared for both the target and the endogenous reference, representing Ct values as a function of the log amount of starting material. For each myocardial sample or experimental condition, the mean quantity of target gene and endogenous control was obtained from the appropriate standard curve in triplicate. The SD among these triplicates was always <0.2. The mean value of the target gene was divided by the mean value of the endogenous control to obtain a normalized mean quantity per sample or experimental condition.

2.5. Isolation of cardiomyocytes

Primary cultures of adult cardiomyocytes were obtained as recently described [13,14]. The average number of cardiomyocytes obtained was 2.5×10^6 in non-failing SHR and 5.0×10^5 in failing SHR. Cells were plated in laminin-precoated (0.5 μg/cm^2) culture plates at a density of 10^4 cells/cm^2 in serum-free Medium 199 with Hanks′salts supplemented with 26 mmol/L $NaHCO_3$, 10^{-4} mmol/L insulin, 5 mmol/L creatinine, 2 mmol/L l-carnitine, 0,2% BSA, 10^{-5} mmol/L Ara-C, 5 mmol/L taurine, 100 IU/mL penicillin, 0.1 mg/mL streptomycin, 10 mmol/L N-(2-hydroxyethyl) piperazine-N′2-ethanolsulfonic acid (HEPES), pH 7.4. After 4 h of pre-incubation, attached cells were ready for experiments. In cell death assessments, cardiomyocytes were incubated overnight with angiotensin II (10^{-7} mol/L) (Sigma) or H_2O_2 (10^{-8} mol/L) (Sigma). Recombinant murine CT-1 (R&D Systems) was added at 10^{-9} mol/L 1 h prior to the addition of death stimuli. To investigate the regulation of LIFR expression, cells isolated from non-failing SHR were incubated with angiotensin II (10^{-7} mol/L), aldosterone (10^{-6} mol/L) (Fluka), norepinephrine (10^{-7} mol/L) (Sigma), endothelin-1 (10^{-8} mol/L) (Sigma) or CT-1 (10^{-9} mol/L) for 3 h for mRNA analysis and 24 h for protein determination.

2.6. Cell death assessment in isolated cells

After treatment, trypan blue (Sigma) was directly added to culture medium at a final dilution of 0.04% (v/v). At least 350 cells per well were counted under a phase contrast microscope, and total cell death was expressed as the percentage of dead (blue) cells per total cells. Measurement of cell death in this methodology is based on the incapacity of dead cells to exclude trypan blue, and thus includes both apoptotic and oncotic cell death. *In situ* detection of cardiomyocyte apoptosis was performed by means of fluorescent Annexin V staining and TUNEL labeling as recently described [13]. The analysis was performed with an epifluorescence-inverted microscope equipped with three filters that allowed detection of 4′-6-diamino-2 phenylindole (DAPI), FITC and Alexa 568-stained cells. For quantitation, only cells exhibiting positive staining for Annexin V and TUNEL were recorded as apoptotic. A minimum of 500 nuclei was counted in each preparation and the apoptotic index was expressed as the percentage of apoptotic nuclei per total nuclei. Cell viability was determined in cells plated in 96 well plates using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell respiration assay (Roche), which assesses mitochondrial activity in living cells, according to the manufacturer's instructions.

2.7. Immunostaining

Cardiomyocytes plated in glass-covered 2 cm^2 wells were fixed in 3% formaldehyde in PBS, during 15 min at 37 °C. After blockade with 3% bovine serum albumin (BSA) in PBS, rabbit anti-gp130 or rabbit anti-LIFR antibodies (Santa Cruz Biotechnology) were incubated during 1 h at 1:500 dilution in PBS and fluorescein-conjugated goat anti-rabbit secondary antibody (Sigma) was added for 30 min at 1:1000. After washing, cells were mounted in DAPI-containing medium (Vectashield), proteins were viewed with an epifluorescence-inverted microscope and images captured using a digital camera. Negative controls excluding primary and secondary antibodies were included in each experiment.

2.8. Statistical analysis

Results are presented as mean±standard error, computed from the average measurements obtained from each experimental condition or from each group of animals. Normal distribution of data was checked by the Shapiro Wilks test and the homogeneity of variances was tested by a Levene statistical test. The unpaired Student's *t* test or the Mann Whitney *U* test was used to assess statistical differences between the two groups of animals or two experimental conditions. *p* values lower than 0.05 were considered significant.

Table 1
General parameters and myocardial morphometric analysis of non-failing (NF) and failing (F) SHR

Parameter	NF-SHR ($n=10$)	F-SHR ($n=10$)	*p*
SBP (mmHg)	233±3	206±2	<0.01
Body weight (g)	432±1	393±7	Ns
Heart weight (g)	1.91±0.01	2.16±0.03	<0.05
CI (mg/g)	4.43±0.02	5.14±0.08	<0.05
LVWT (mm)	41.6±0.6	33.2±1.2	<0.01
LVChD (mm)	25.5±0.4	30.5±0.9	<0.01
Ratio (LVWT/LWChD)	1.63±0.03	1.09±0.04	<0.01
LVChA (μm^2)	393±18	490±13	<0.01

CI: cardiac index; LVWT: left ventricle wall thickness; LVChD: left ventricle chamber diameter; LVChA: left ventricle chamber area.

3. Results

3.1. Blood pressure and left ventricular dimensions

General animal data and parameters describing left ventricle morphometry are shown in Table 1. Compared with non-failing SHR, failing rats exhibited decreased ($p<0.01$) systolic blood pressure, and increased heart weight and left ventricle ventricular dimensions (both, $p<0.05$).

3.2. Cell death and LIFR/gp130 pathway in the intact hearts

As shown in Fig. 1, failing SHR exhibited higher myocardial Bax expression (8.3±0.9 vs 4.1±0.5 arbitrary units, $p<0.01$) and lower Bcl-2 expression (1.1±0.8 vs 7.6±0.3 arbitrary units, $p<0.01$) than non-failing SHR. Thus the index of apoptosis susceptibility, Bax/Bcl2, was higher in failing SHR than in non-failing SHR (7.0±0.7 vs 0.6±0.2, $p<0.01$). Myocardial activation of caspase-3, assayed by the ratio caspase-3/procaspase 3, was higher in failing than in non-failing SHR (10.4±0.8 vs 1.3±0.3, $p<0.01$). Finally, both the large (85 KDa) and the small (<50 KDa) fragments of PARP were more abundant in failing SHR than in non-failing SHR (large, 11.3±0.21 vs 5.3±0.25 arbitrary units, and small, 2.8±0.25 vs 1.1±0.27 arbitrary units, both $p<0.01$) (Fig. 1). To ensure that the higher apoptosis observed in failing SHR was associated with HF rather than with aging, the three parameters were determined in age-

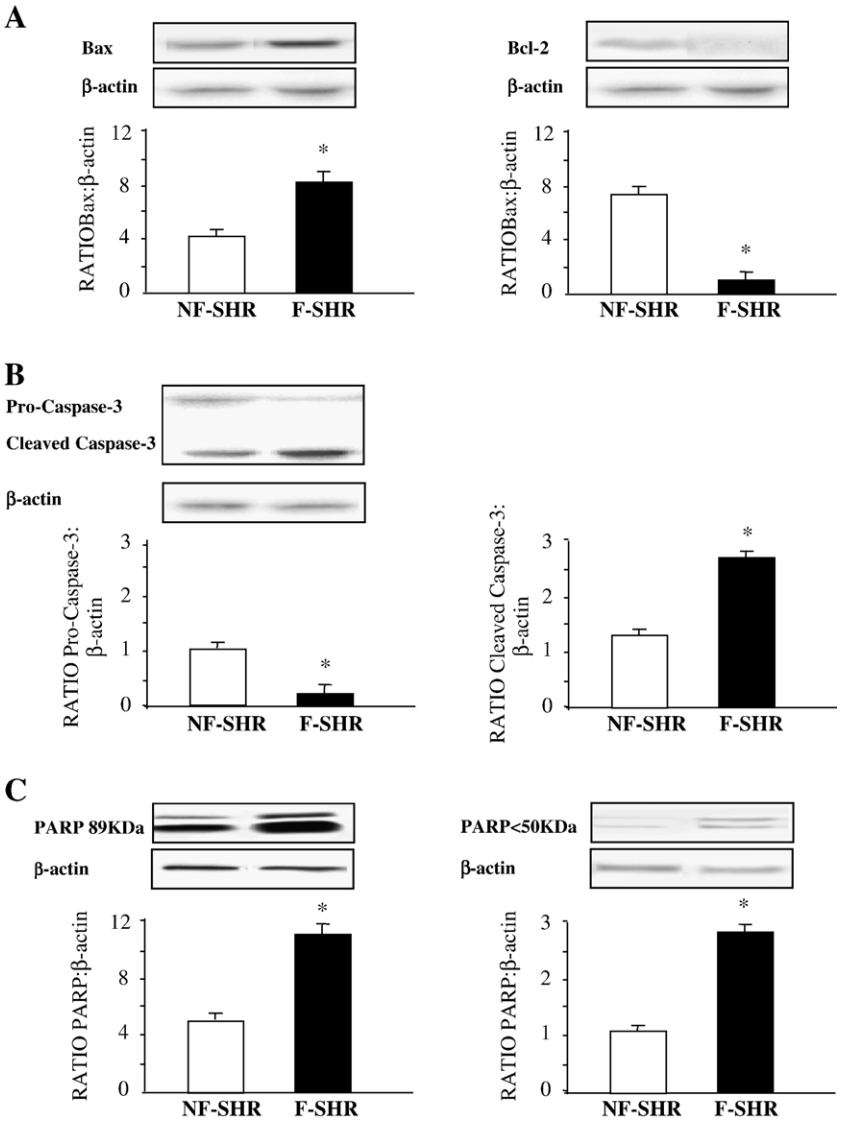

Fig. 1. Myocardial apoptosis in non-failing and failing SHR. Three approaches were employed to assay apoptosis: determination of Bax and Bcl-2 expression (panel A) to obtain the ratio Bax/Bcl-2 as an index of apoptosis susceptibility; measurement of procaspase-3 and caspase-3 to estimate the activation of this protease (panel B); and quantification of PARP fragmentation (panel C) by measuring large (85 kDa, left) and small fragments (<50 kDa, right). A representative Western blot and the histogram with bars representing the mean+standard error ($N=10$) of the ratio protein to β-actin obtained in triplicate are shown for each protein. *, $p<0.01$ vs NF-SHR. NF-SHR: non-failing SHR; F-SHR: failing SHR.

matched normotensive WKY rats. The index of apoptosis susceptibility Bax/Bcl-2 was 0.69±0.07 in 30 weeks-old WKY and 1.56±0.70 arbitrary units in 75 weeks-old WKY. The ratio caspase 3/procaspase 3 was 0.71±0.22 in 30 weeks-old WKY and 0.93±0.24 arbitrary units in 75 weeks-old WKY. Whereas PARP small fragments were very scarce in the two groups WKY, large fragments were 1.67±0.69 arbitrary units in 30-week-old WKY and 2.56±0.86 arbitrary units in 75-week-old WKY. Thus, age-matched WKY had lower ($p<0.01$) apoptotic parameters than failing SHR, indicating that HF rather than aging is the main inductor of apoptosis in this model.

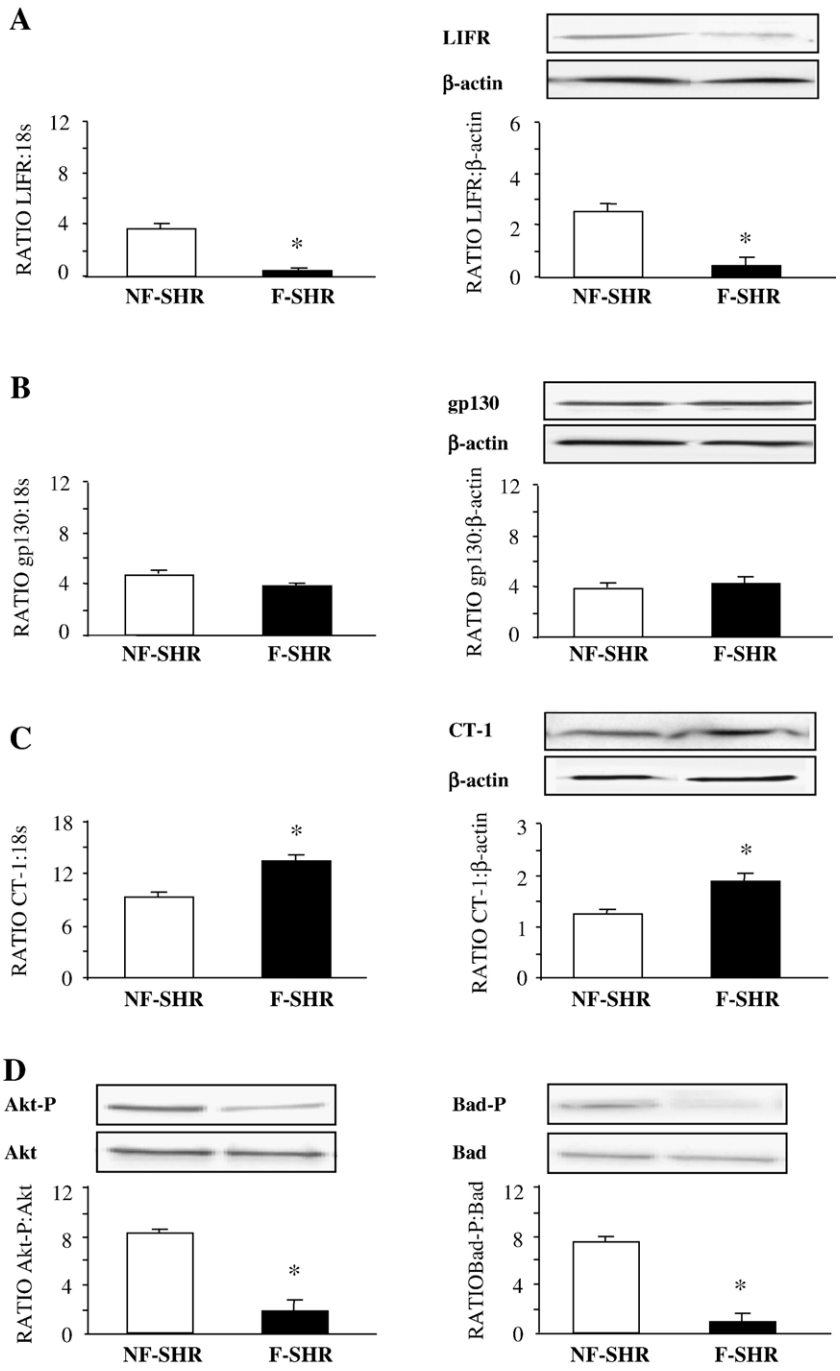

Fig. 2. Myocardial LIFR/gp130 survival pathway expression. LIFR (panel A), gp130 (panel B) and CT-1 (panel C) were quantified by real time RT-PCR and Western blotting. For mRNA analysis in the left part of each panel, the mean + standard error ($N=10$) values of the target genes normalized with the internal control 18S are plotted. For proteins, a representative Western blot and the histogram with bars representing the mean + standard error ($N=10$) of the ratio protein to β-actin obtained in triplicate in the two groups of animals are shown (right). The degree of Akt and Bad phosphorylation (panel D) in the myocardium of the two groups of rats was assayed by Western blotting and the mean + standard error ($N=10$) are plotted. *, $p<0.01$ vs NF-SHR. NF-SHR: non-failing SHR; F-SHR: failing SHR.

Fig. 2 (panels A–C) illustrates myocardial mRNA and protein expression of LIFR, gp130 and CT-1, determined in the two groups of rats. Compared to non-failing SHR, failing SHR exhibited decreased ($p<0.01$) LIFR expression (mRNA: 0.54±0.25 vs 3.8±0.6 arbitrary units; protein: 0.51±0.07 vs 2.52±0.22 arbitrary units) and similar expression of gp130. Additionally, myocardial expression of CT-1 was higher ($p<0.01$) in failing SHR than in non-failing SHR (mRNA: 13.5±1.2 vs 8.9±1.4 arbitrary units; protein: 1.9±0.1 vs 1.2±0.1 arbitrary units). We have previously reported that binding of CT-1 to LIFR/gp130 induces survival in adult cardiomyocytes via PI3K-Akt and Bad phosphorylation [13]. Thus, we next assayed the degree of phosphorylation of these signaling molecules in the myocardium of non-failing and failing rats. As shown in Fig. 2 (panel D), the phosphorylation of both Akt and Bad proteins was lower ($p<0.01$) in the myocardium of failing SHR than in the myocardium of non-failing SHR.

3.3. Studies in isolated cardiomyocytes

To clarify whether the differences observed in myocardial LIFR expression specifically affected contractile cells, both gp130 and LIFR subunits were assayed in cardiomyocytes isolated from the two groups of rats. Whereas no changes were found in the expression of gp130 in cells from the two groups of rats, mRNA and protein expression of LIFR was lower ($p<0.01$) in cardiomyocytes from failing SHR than in

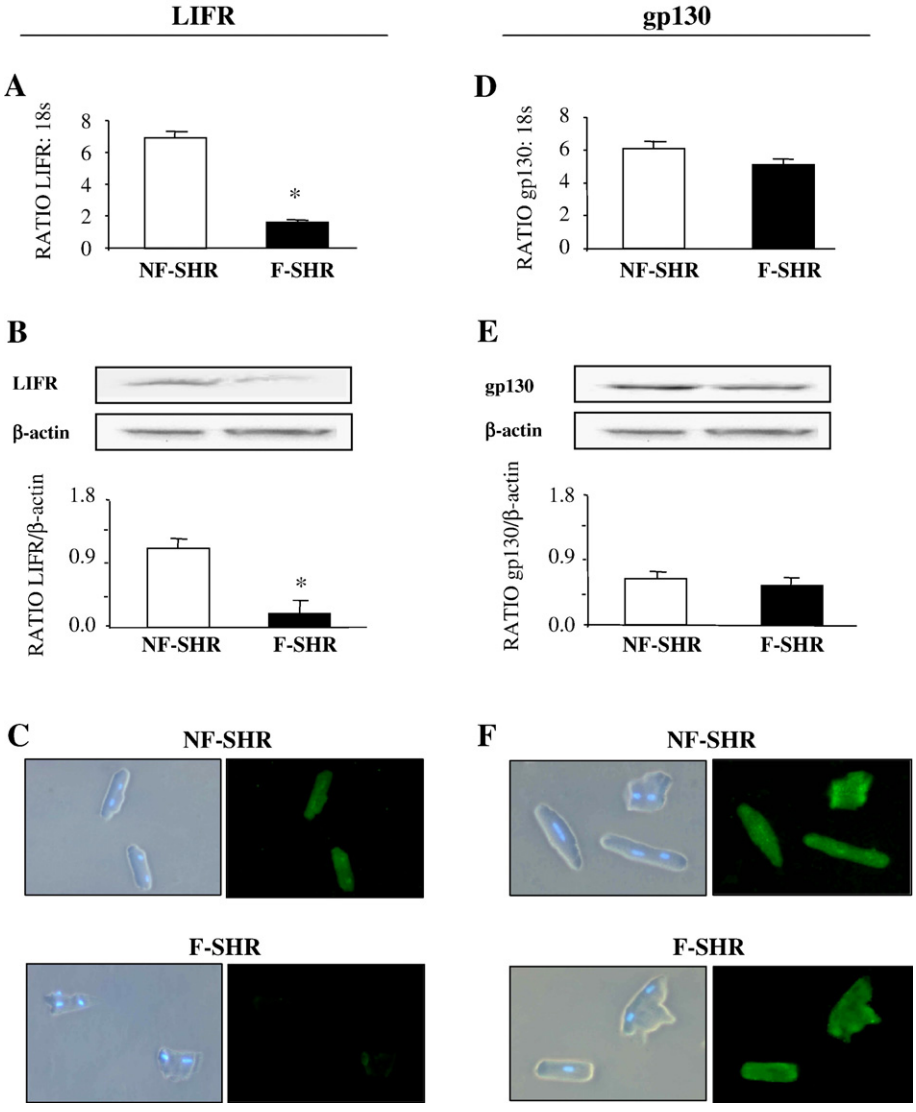

Fig. 3. LIFR and gp130 expression in cardiomyocytes isolated from non-failing and failing SHR. For mRNA quantification, real-time PCR (panels A and D) of each target gene was performed in triplicate and normalized with the internal control 18S. Proteins were quantified by Western blotting in triplicate (panels B and E). Values represent the mean+standard error of three independent cell isolations. *, $p<0.01$ vs NF-SHR. Fluorescent immunocytochemistry was employed to visualized the two proteins (panels C and F). Representative phase-contrast photomicrographs of each culture are shown beside the corresponding fluorescein staining for LIFR (panel C) or gp130 (panel F). Experiments in triplicate yielded similar results. Magnification 400×. NF-SHR: non-failing SHR; F-SHR: failing SHR.

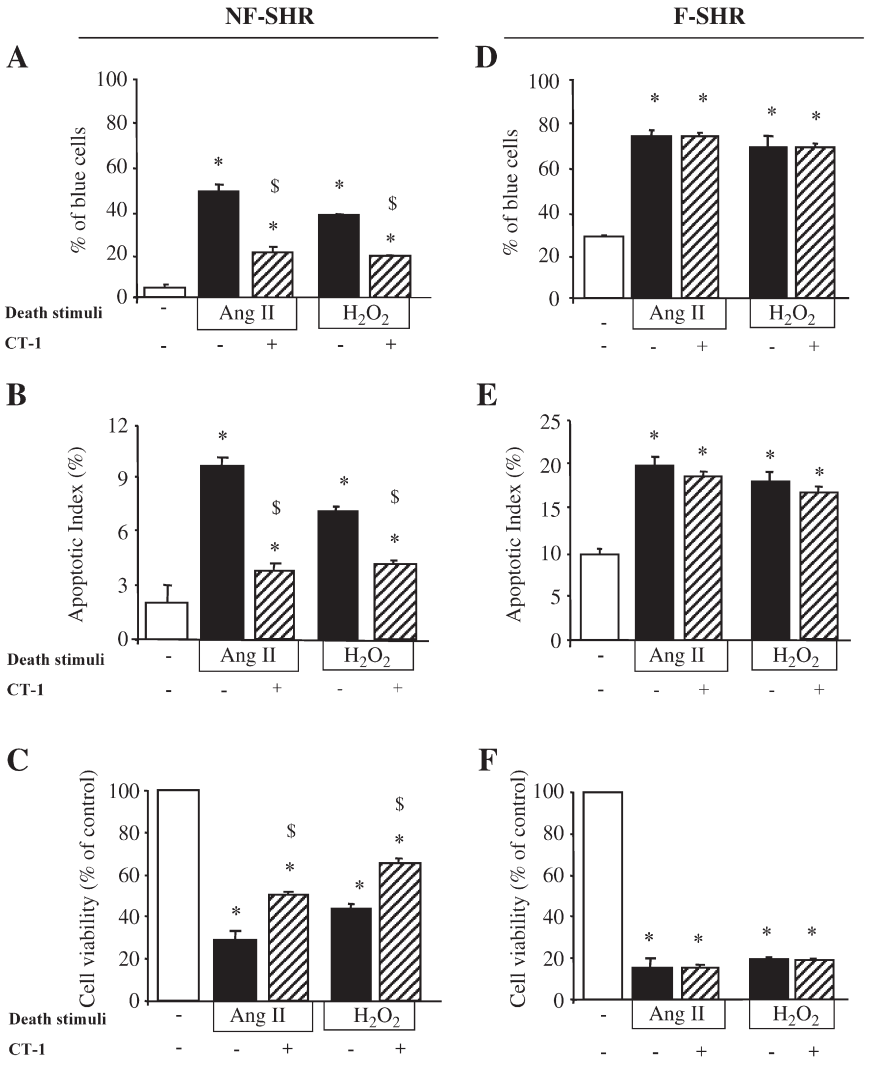

Fig. 4. CT-1 survival effects in isolated cardiomyocytes. Cytoprotective effects of CT-1 (10^{-9} M) in cardiomyocytes isolated from non-failing and failing SHR were assayed by trypan blue exclusion (panel A), TUNEL (panel B) and MTT (panel C). Values represent the mean + standard error of at least 350 cells in trypan blue assay, at least 500 nuclei in TUNEL and 8 wells from 96 plates in MTT. Experiments performed in cell isolations yielded similar results. *, $p<0.01$ vs baseline; $, $p<0.01$ vs death stimulus; $p<0.01$ vs NF-SHR. NF-SHR: non-failing SHR; F-SHR: failing SHR. ANG II: angiotensin II; CT-1: cardiotrophin-1.

cardiomyocytes from non-failing SHR (Fig. 3, panels A and B). Moreover, LIFR was not detected by immunocytochemistry in failing cardiomyocytes (Fig. 3, panel C).

We next explored whether the decrease in LIFR had any functional effect in cardiomyocytes from failing SHR by analysing the responsiveness to the ligand CT-1. As illustrated in Fig. 4 (Panels A and B), recombinant CT-1 inhibited ($p<0.01$) total and apoptotic cell death induced by angiotensin II or H_2O_2 in cardiomyocytes from non-failing SHR. In addition, CT-1 increased cell viability when cells were exposed to the same stimuli (Fig. 4, panel C). As previously reported [13], the protective effects of CT-1 were blocked in the presence of specific antibodies against LIFR (90% and 94% for angiotensin II and H_2O_2 respectively) or gp130 (88% and 95% for angiotensin II and H_2O_2 respectively) and CT-1 alone exerted no effect on cardiomyocytes (data not shown). Recombinant CT-1 exerted no cytoprotective effect in cells from failing SHR exposed to either angiotensin II or H_2O_2 (Fig. 4, panels D to F). Cardiomyocytes isolated from failing SHR exhibited higher (both $p<0.01$) total and apoptotic cell death in baseline conditions and after incubation with any of the two death stimuli (Fig. 4).

3.4. Neurohumoral regulation of LIFR expression in isolated cardiomyocytes

To explore potential repressors of LIFR expression, cardiomyocytes from non-failing SHR were incubated with neurohumoral factors known to be elevated in HF. As shown in Fig. 5, angiotensin II, aldosterone, norepinephrine and endothelin-1, as well as the ligand CT-1, decreased ($p<0.01$) LIFR mRNA and protein levels in this cell type. These results together with the observation noted above that CT-1 protects non-failing cardiomyocytes against angiotensin II-induced cell

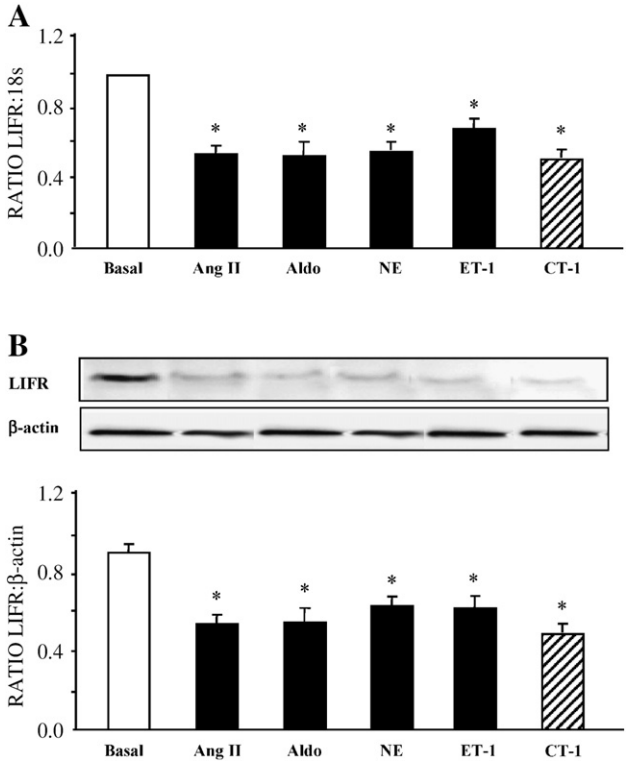

Fig. 5. Regulation of LIFR expression in isolated cardiomyocytes. Cardiomyocytes isolated from non-failing SHR were incubated during 3 (for mRNA assay) or 24 h (for protein assay) with the shown stimuli as described in Methods. Changes in LIFR mRNA expression (panel A) were assayed by real-time PCR and normalized with the internal control 18S in triplicate. The histograms with bars represented the mean+standard error (N=3). Results were referred to baseline conditions considered 1. Changes in LIFR protein were assayed by Western blotting (panel B) in triplicate. A representative Western blot and the histogram with bars represented the mean+standard error of the ratio protein to β-actin (N=3). ANG II: angiotensin II; CT-1: cardiotrophin-1; ALD: aldosterone; NE: norepinephrine; ET-1: endothelin-1.

death (Fig. 4), led us to assume that the decrease in LIFR protein induced by these two ligands (Fig. 5) is not high enough to completely blunt CT-1 survival actions in these cells.

4. Discussion

The major findings reported here are the following: 1) compared with non-failing SHR, failing SHR exhibit diminished myocardial LIFR expression in association with impaired activation of the intracellular LIFR/gp130-dependent cytoprotective pathway (Akt and Bad phosphorylation), and with higher apoptotic cell death, 2) cardiomyocytes isolated from failing SHR do not express LIFR and are resistant to the cytoprotective actions of CT-1, and, 3) neurohumoral agonists that are increased in HF down-regulate LIFR expression in cardiomyocytes isolated from non-failing SHR.

A critical role of the LIFR/gp130 survival pathway in the maintenance of left ventricle morphometry and function under pressure overloading conditions has been previously demonstrated in mice with cardiac-specific inactivation of gp130. Whereas hearts from wild-type mice exhibited compensatory myocardial hypertrophy after transverse aortic constriction, the conditional mutants with the LIFR/gp130 survival pathway inactive developed massive cardiomyocyte apoptosis, chamber dilation and HF [15]. Here, we report that cardiomyocytes from failing SHR are resistant to the protective actions of CT-1, probably because of the low expression of LIFR. In addition, we show that hearts from failing SHR do not preserve the cytoprotection conferred by the LIFR/gp130 pathway, as shown by the low activation of the Akt-Bad intermediates and by the high apoptotic cell death observed. Taken together, these data suggest that down-regulation of LIFR and subsequent loss of cardiomyocyte survival mechanisms may contribute to the increase in myocardial cell death present in SHR with overt HF.

An emerging question to address is the mechanism that leads to LIFR down-regulation in the failing hearts. One possibility points to the mechanisms of protein degradation. Besides the proteosomal degradation shared with its homologous gp130 and oncostatin M receptor (OSMR), only LIFR is susceptible to lysosomal degradation via ERK1/2 mediated phosphorylation in its cytoplasmic domain [24]. We here observed that the major components of neurohumoral reflex characteristically activated in HF decrease LIFR protein expression. Interestingly, all the agonists tested here, including CT-1, are able to activate the ERK1/2 signaling pathway in adult cardiomyocytes [13,25,26] and they thus might induce LIFR degradation by this pathway. A second possibility points to the mechanism of LIFR turnover regulation in response to CT-1, LIF or oncostatin M (OSM) binding, which has been reported to be different from its two homologous. Whereas the initial ligand-induced degradation of the three receptors is followed by a compensatory increase in gp130 and OSMR synthesis, LIFR expression remains low [27,28]. Our data showing that CT-1 reduces LIFR mRNA and protein in vitro, add the terminally differentiated cardiomyocyte to the list of cells employing this turnover regulation, and lead us to speculate that the induction of LIFR gene transcription, if present, is not efficient enough to compensate protein degradation. Furthermore, the high CT-1 expression found in the hearts of failing SHR may suggest a role for the excessive cytokine in LIFR down-regulation. Finally, changes in histone 3 acetylation in the promoter region of LIFR might also be involved in the low mRNA expression observed, since inhibition of deacetylases has been reported to induce LIFR transcription associated with the promoter acetylation in various cell lines [29]. Although the existence of an inverse correlation between the two phenomena has not been demonstrated, the possibility that LIFR down-regulation observed in cardiac tissue and in cardiomyocytes from failing SHR is associated with histone 3 deacetylation in the promoter region should be investigated.

The finding that gp130 expression is unchanged in failing SHR, confirms previous findings in Dahl-sensitive rats with and without HF [30]. Moreover, several types of *in vitro* evidence support the notion that gp130 is more stable than

LIFR in cell lines [27,28,31]. Despite of extensive functional similarities shared by the two subunits, specific differences have been reported regarding their signaling properties [32] and the molecular mechanisms that regulate their turnover [27]. Although it is established that gp130 is necessary for LIFR recruitment and signal triggering [10,11], the loss of LIFR is enough to inhibit LIFR/gp130 signaling [28]. Thus, further studies are required to clarify the real significance that changes in each subunit expression may have in the pathological myocardium.

The enhanced myocardial expression of CT-1 in the transition from LVH to HF found in SHR is in accordance with results obtained in other experimental models of HF such as Dahl salt-sensitive rats [30] and paced dogs [33]. Previous studies have shown that mechanical stretch, angiotensin II and norepinephrine induce CT-1 expression in cardiac cells [34–37]. Our data support the hypothesis that the two major mechanisms potentially involved in the increase of myocardial CT-1 in HF are, on the one hand, the higher mechanical stress present in the failing myocardium, and on the other hand, the enhanced neurohumoral reflex present in HF. However, future studies will clarify this aspect.

In summary, data here presented suggest a role for depressed LIFR/gp130 mediated cytoprotective mechanisms in the increase of cardiomyocyte death characteristic of the transition from LVH to HF in SHR. Our findings suggest that neurohumoral agonists may be involved in such alteration thus adding information about the mechanisms through which activation of neurohumoral reflex may be deleterious in overt HF.

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