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Energy-preserving effects of IGF-1 antagonize starvation-induced cardiac autophagy

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Aims Insulin-like growth factor 1 (IGF-1) is known to exert cardioprotective actions. However, it remains unknown if autophagy, a major adaptive response to nutritional stress, contributes to IGF-1-mediated cardioprotection.

Methods and results We subjected cultured neonatal rat cardiomyocytes, as well as live mice, to nutritional stress and assessed cell death and autophagic rates. Nutritional stress induced by serum/glucose deprivation strongly induced autophagy and cell death, and both responses were inhibited by IGF-1. The Akt/mammalian target of rapamycin (mTOR) pathway mediated the effects of IGF-1 upon autophagy. Importantly, starvation also decreased intracellular ATP levels and oxygen consumption leading to AMP-activated protein kinase (AMPK) activation; IGF-1 increased mitochondrial Ca^{2+} uptake and mitochondrial respiration in nutrient-starved cells. IGF-1 also rescued ATP levels, reduced AMPK phosphorylation and increased $p70^{S6K}$ phosphorylation, which indicates that in addition to Akt/mTOR, IGF-1 inhibits autophagy by the AMPK/mTOR axis. In mice harbouring a liver-specific *igf1* deletion, which dramatically reduces IGF-1 plasma levels, AMPK activity and autophagy were increased, and significant heart weight loss was observed in comparison with wild-type starved animals, revealing the importance of IGF-1 in maintaining cardiac adaptability to nutritional insults *in vivo*.

Conclusion Our data support the cardioprotective actions of IGF-1, which, by rescuing the mitochondrial metabolism and the energetic state of cells, reduces cell death and controls the potentially harmful autophagic response to nutritional challenges. IGF-1, therefore, may prove beneficial to mitigate damage induced by excessive nutrient-related stress, including ischaemic disease in multiple tissues.

Keywords IGF-1 • Macroautophagy • Heart • ATP • Akt • mTOR

1. Introduction

Autophagy is a highly conserved process among eukaryotes, which involves protein degradation, organelle turnover, and the breakdown of cytoplasmic components during nutrient starvation or stress.[1] Macroautophagy (herein referred to as autophagy) begins with the formation of an autophagosome, a double-membrane structure that engulfs cytoplasmic contents and fuses with lysosomes for cargo delivery and degradation.[2,3] Materials degraded within autophagolysosomes are recruited to anabolic reactions in order to maintain energy levels and to provide macromolecules for the synthesis of more complex structures (nucleic acids, proteins, or organelles), thereby promoting cell metabolism, homeostasis, and survival. Despite this vital role, autophagy also contributes to cell death when it is executed excessively or inefficiently, as occurs during tissue and organ development, or pathological states.[4–6] Indeed, autophagic cell death has

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been coined as a morphological term derived from electron microscopic observations that denote a cell death phenotype characterized by abundant autophagic vacuoles in the cytoplasm.[7,8] In the failing human heart, autophagic features have been observed in cardiomyocytes. However, it remains unclear whether the abnormal increase in the number of autophagic vacuoles is a primary cause of heart failure due to cardiomyocyte death, or if it only reflects hyperfunction of the autophagic process in response to stress conditions.[9–12]

Insulin-like growth factor-1 (IGF-1) plays a key role in the regulation of cell survival, proliferation, differentiation, and metabolism.[13,14] The biological effects of IGF-1 are mediated predominantly by the IGF-1 receptor (IGF-1R), although in cardiomyocytes there is significant cross-talk with insulin receptors.[15] The IGF-1R belongs to the superfamily of tyrosine kinase receptors. Upon ligand binding and receptor auto-phosphorylation, the IGF-1R activates two main signalling pathways, the mitogen-activated protein kinase (MAPK)/extracellularly regulated kinase (ERK), and the phosphatidylinositol 3-kinase (PI3K) pathways.[16,17] These pathways elicit different biological actions in the heart, of which the most important are the stimulation of physiological cellular hypertrophy and positive inotropic effects. Additionally, our group and others have shown that IGF-1 protects cardiomyocytes from apoptosis induced by different types of stress.[18–20] Despite the increasing understanding on the effects of IGF-1, diverging evidence exists concerning the effects of IGF-1 on autophagy, and the mechanistic pathways involved in these effects. An inhibitory role for IGF-1 on autophagy has been observed in different cell types, including human osteocarcinoma cells,[21] vascular cells from patients with atherosclerotic lesions stimulated with TNF-α[22], and cells present in mammary gland involution.[23] Conversely, IGF-1 has been demonstrated to promote autophagy in the H9c2 cell line[24] and in Purkinje neurons.[25] Importantly, it remains unknown whether IGF-1 promotes or prevents autophagy in cardiomyocytes.

In this study, we investigated the effects of IGF-1 on cardiac autophagy by using cultured neonatal rat cardiomyocytes, as well as mice bearing a genetic modification that leads to a substantial decrement in circulating IGF-1 levels. Results presented here show that IGF-1 negatively regulates nutrient deprivation-induced cardiac autophagy both *in vitro* and *in vivo*, hence protecting cardiomyocytes from the adverse consequences of hyperactivated autophagy induced by prolonged and severe nutrient depletion.

2. Methods

2.1 Culture of cardiomyocytes

Rats were obtained from the Animal Breeding Facility of the Faculty of Chemistry and Pharmaceutical Sciences, University of Chile. All experiments were performed in agreement with National Institutes of Health guidelines (NIH publication N°85-23, revised 1996), and were approved by the Institutional Bioethical Committee. Cardiomyocytes were prepared from hearts of 1–3 day old Sprague-Dawley rats as described previously.[26] In brief, rat ventricles were minced and cells dissociated in a solution of collagenase and pancreatin. After enzymatic digestion, the cells were plated in gelatin-coated plastic dishes and cultured in DMEM/ M199 (4:1) containing 10% FBS, 5% FCS, and antibiotics (streptomycin and penicillin, 100 U/mL). To prevent the overgrowth of fibroblasts and smooth muscle cells, bromodeoxyuridine (10 μM) was used in the cell culture media. Cardiomyocytes were 96% pure after a 24 h plating period (evaluated with an anti-β-myosin heavy chain antibody).

2.2 Mice

Animal care and maintenance were provided by the Mount Sinai School of Medicine and University of Texas Southwestern Medical Center. All experiments were performed in agreement with National Institutes of Health guidelines (NIH publication N°85-23, revised 1996) and with the approval of the Animal Care and Use Committee. For heart and liver isolation, mice were euthanized by CO_2 inhalation.

2.3 Stress conditions

Nutritional stress (NS) *in vivo* was induced by subjecting mice to starvation for 48 h (mice had free access to water at all times).[27] NS *in vitro* was induced by serum and glucose deprivation.[28] Cells were incubated in RPMI 1640 medium without glucose for different lengths of time in the presence or the absence of inhibitors before induction of stress. IGF-1 (10 nM) was added in the culture medium for 1 h before the induction of stress and was present throughout the treatments.

2.4 Evaluation of autophagic GFP-LC3 puncta and degradation

Adenovirus encoding GFP-LC3 (light chain 3) was transduced at a multiplicity of infection of 30 for 24 h.[29] GFP-LC3 transduced cells were fixed with PBS containing 4% paraformaldehyde. Samples were evaluated using a scanning confocal microscope. For FACS analysis of autophagic flux, the level of GFP fluorescence intensity in each treatment was normalized to the control.

2.5 Western blotting

The primary antibodies used were anti-tubulin (Sigma-Aldrich), anti-LC3 (Cell Signaling), anti-phosphorylated, and total form of Akt, $p70^{S6K}$, and AMPK (Cell Signaling). Then the blots were incubated with a horseradish peroxidase-coupled secondary antibody (Pierce). ImageJ software (NIH) was used for image densitometry.

2.6 Cell viability

Cell viability was assessed by a MTT assay, as directed by the manufacturer (Invitrogen, Carlsbad, CA, USA). The integrity of the plasma membrane was assessed by determining the ability of cells to exclude propidium iodide (PI). The level of PI incorporation was quantified in a FACScan flow cytometer.

2.7 Intracellular ATP content determination

Intracellular ATP content was determined using a CellTiter-Glo® Luminescent Cell Viability Assay following the manufacturer's instructions (Promega, Madison, WI, USA). Final luminescence was measured in a Top-Count NXT microplate luminescence counter (Perkin-Elmer, Waltham, MA, USA).

2.8 Mitochondrial Ca^{2+} uptake

To determine mitochondrial Ca^{2+} levels, images were obtained from cultured cardiomyocytes preloaded with Rhod2-AM using an inverted confocal microscope (Carl Zeiss LSM-5, Pascal 5 Axiovert 200 microscope).[30]

2.9 Oxygen consumption

Cells were plated on 60 mm Petri dishes and treated according each experiment. Cells were then trypsinized, and the suspension (in PBS) was placed in a chamber at 25°C, coupled to a Clark electrode 5331 (Yellow Springs Instruments) where the oxygen uptake was measured polarographically.[30]

2.10 Body composition

Body composition was measured in non-anaesthetized mice using the Bruker minispec body composition analyzer (Bruker Optics, Woodlands, TX, USA).[31,32]

2.11 Serum levels of IGF-1, insulin, and glucose

Blood was collected from the retro-orbital sinus of 8-week-old male mice. Serum IGF-1 levels were determined using RIA kits (National Hormone and Pituitary Program, Harbor, UCLA Medical Center, Torrance, CA, USA). Serum insulin was measured using an SRI-13K kit (Linco Research, Inc., St Charles, MO, USA) and glucose was measured using a Glucometer Elite (Bayer, Elkhart, IN, USA).

2.12 Echocardiography

Echocardiograms were performed on conscious, gently restrained mice using a Vevo 2100 system with a MS400C scanhead.[33]

2.13 Expression of results and statistical analysis

Values are represented as mean ± SEM of the number of independent experiments indicated (3–5) or as examples of representative experiments performed on at least three separate occasions. Data were analysed by ANOVA and comparisons between groups were performed

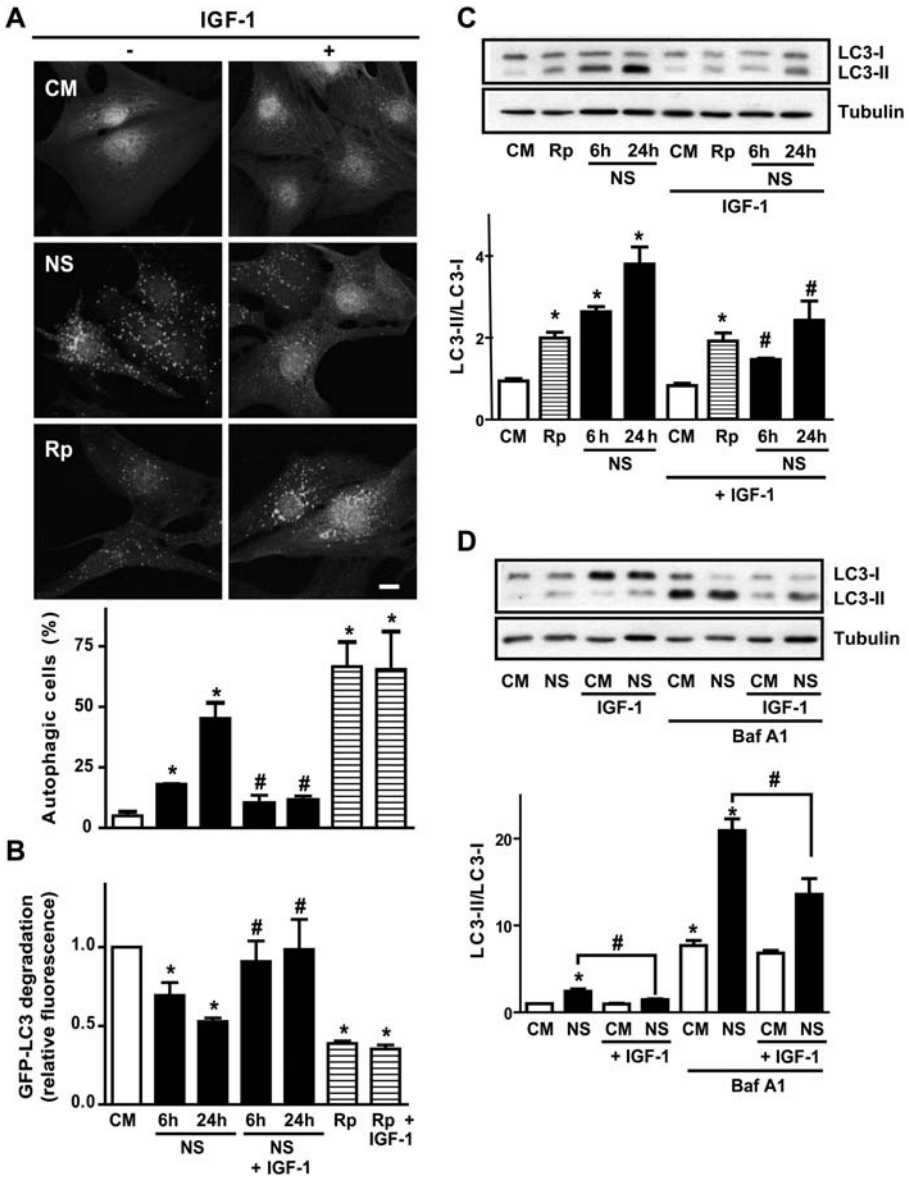

Figure 1 IGF-1 inhibits starvation-induced cardiac autophagy. Cardiomyocytes transiently expressing adenovirally encoded GFP-LC3 were subjected to nutritional stress (NS) for 6 and 24 h and the effect of IGF-1 (10 nM) was evaluated. (*A*) Representative fluorescence confocal images are displayed. The bar corresponds to 10 μm (top) and quantification of cells containing autophagic features (bottom). For each condition, 150 cells were analysed. (*B*) GFP-LC3 degradative autophagic flux was measured using fluorocytometry and normalized to cells incubated in complete medium (CM). (*C*) Processing of LC3-I into LC3-II. Cells were exposed to NS for 6 and 24 h and LC3 protein levels were determined by immunoblotting. Rapamycin (Rp, 100 nM) was used as positive control. The figure corresponds to a representative blot from three independent experiments (top) and quantification of LC3 processing (bottom). (*D*) LC3 processing was measured in the presence of Bafilomycin A1 (50 nM) (top) and quantification of LC3 processing. Data are mean ± SEM of three independent experiments. $^{*}P < 0.05$ vs. CM; $^{\#}P < 0.05$ vs. NS.

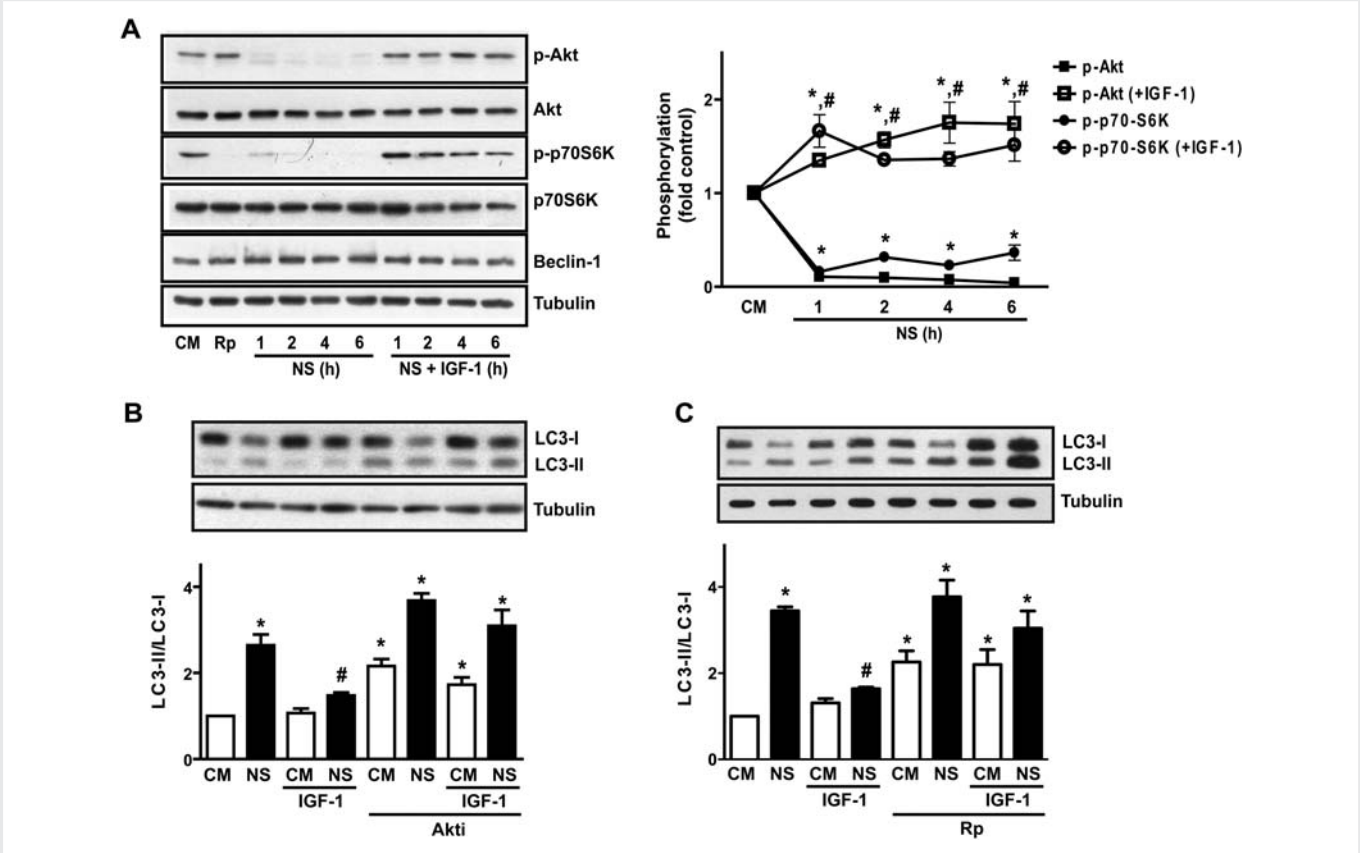

Figure 2 Participation of Akt/mTOR/p70^{S6K} in the anti-autophagic response to IGF-1. Cardiomyocytes were exposed to nutritional stress (NS) or maintained in complete medium (CM). Cell lysates were subjected to western blot against Akt, p70^{S6K}, beclin-1, and tubulin, where p indicates phosphorylated forms. (*A*) Representative blots (left panel) and quantitative analysis of protein expression (right panel). LC3 processing was measured in the presence of Akti (10 μM) (*C*) or rapamycin (100 nM). Data are mean ± SEM of three independent experiments. *$P < 0.05$ vs. CM; #$P < 0.05$ vs. NS.

using a protected Tukey's *t*-test. A value of $P < 0.05$ was set as the limit of statistical significance.

3. Results

3.1 IGF-1 inhibits starvation-induced cardiac autophagy via mTOR signalling

The microtubule-associated protein 1 LC3 undergoes post-translational modifications before binding to autophagosomal membranes.[34,35] The conjugation of soluble LC3 to phosphatidylethanolamine (PE) by the Atg12–Atg5 complex leads to the formation of the autophagosome-associated form (LC3-II). LC3-II is used as a specific marker of autophagy because the addition of PE also accelerates its electrophoretic mobility on gels compared with LC3-I and change its subcellular distribution (punctate staining formation).[36,37] Moreover, GFP-LC3 fusion proteins can be used to visualize autophagosome dynamics by fluorescence microscopy,[38,39] and to quantify autophagic flux by fluorocytometry.[40,41] Here, we used cultured neonatal rat cardiomyocytes transiently expressing GFP-LC3 in order to study the autophagic effects of NS stimulated by serum/glucose deprivation. When compared with cells maintained in complete medium (CM), NS led to a significant increase in autophagic *puncta* at 6 h, and it was sustained until 24 h of treatment; this response was also evident in rapamycin-treated cells (positive control) (*Figure 1A*). Treatment of cardiomyocytes with IGF-1 significantly inhibited the pro-autophagic effects of NS, as observed by autophagic puncta (*Figure 1A*), autophagic flux quantified by fluorocytometry (*Figure 1B*), and endogenous LC3-I processing (*Figure 1C*). NS induces a further increase in steady-state LC3-I levels in the presence of bafilomycin A1 (which inhibits the final stage of autophagic degradation), confirming that NS increases autophagic flux (*Figure 1D*). Although IGF-1 treatment decreased the steady-state of LC3II levels slightly, a dramatic reduction in LC3 II levels triggered by IGF-1 treatment was observed under conditions of lysosomal inhibition. Thus IGF-1 significantly reduced autophagic flux (*Figure 1D*).

The effects of NS on autophagy are canonically regulated by the protein kinase Akt/mammalian target of rapamycin (mTOR).[42,43] As demonstrated in *Figure 1A* and *B*, the inhibitory effects of IGF-1 on autophagy were not observed in rapamycin-treated cells. This indicates that inhibition of mTOR by rapamycin impairs the anti-autophagic cascade initiated by IGF-1. These results agree with previous reports in which the effects of IGF-1 upon autophagy are inhibited by rapamycin,[23,44] and are consistent with the fact that the IGF-1R activates the PI3K/Akt/mTOR pathway.[16]

To corroborate involvement of the Akt/mTOR pathway, we monitored the phosphorylation of Akt, and phosphorylation of the mTOR kinase substrate, p70^{S6K}. NS led to the

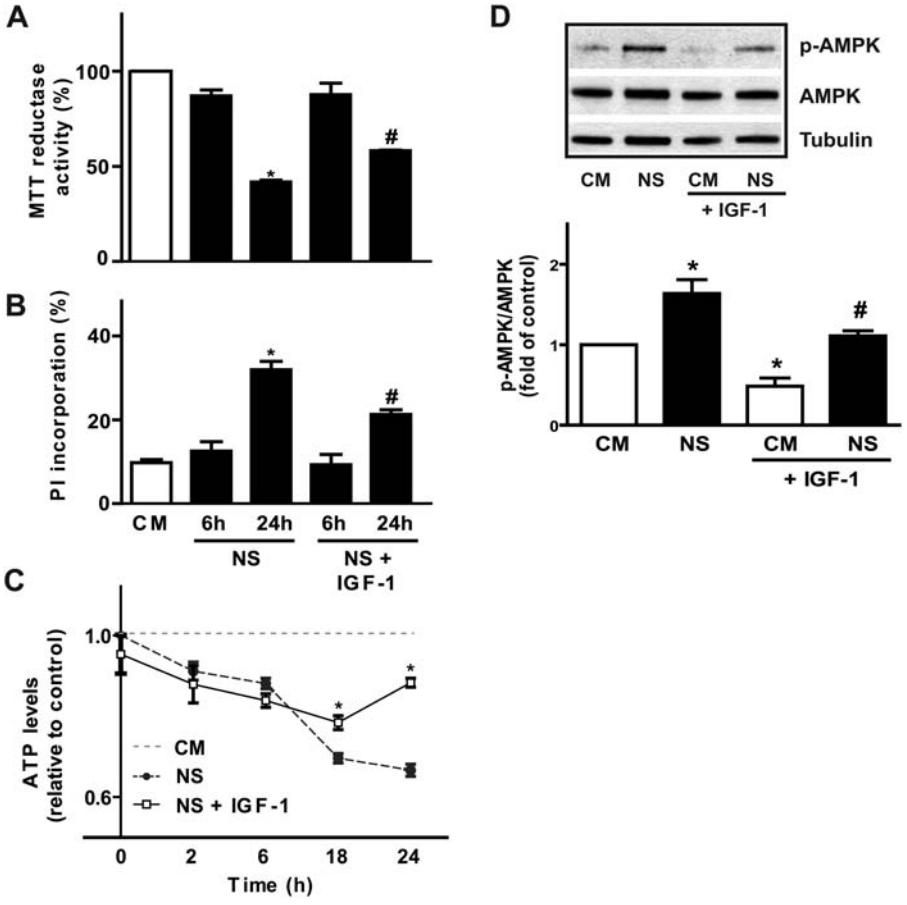

Figure 3 IGF-1 protects cardiomyocytes from nutrient starvation-induced cell death. Cardiomyocytes were pre-incubated with or without IGF-1 and exposed to NS between 2 and 24 h. (*A*) Cell viability was determined by MTT colorimetric assay and compared with complete medium (CM) controls. (*B*) Cells were labelled with PI and analysed by fluorocytometry. (*C*) Intracellular ATP levels were determined by luminescence using the luciferin/luciferase assay; dash line represents the cells in complete medium. (*D*) Cardiomyocyte lysates were treated for 6 h and analysed by western blot (top) and quantitative analysis of protein expression (bottom). Data are mean ± SEM or representative of three to five independent experiments. $^{*}P < 0.05$ vs. CM; $^{\#}P < 0.05$ vs. NS.

de-phosphorylation of Akt, and this effect was reversed by IGF-1. Additionally, $p70^{S6K}$ (which is phosphorylated by mTOR) displayed the same pattern (*Figure 2A*), thus indicating that IGF-1 inhibits starvation-induced autophagy by means of mTOR activation without modifying beclin-1 levels. To further evaluate participation of the Akt/mTOR pathway, we employed inhibitors of Akt (Akti) and mTOR (rapamycin). These results showed that inhibition of Akt or mTOR abolished the effects of IGF-1 on autophagy determined by the processing of LC3-I (*Figure 2B* and *C*). Collectively, these data suggest that the anti-autophagic effects of IGF-1 require the Akt/mTOR pathway activation.

3.2 IGF-1 protects cardiomyocytes from starvation-induced cell death

NS is a strong stimulus that leads to cell death in pathological conditions such as ischaemia/reperfusion, stroke, and myocardial infarction.[45,46] To assess the protective role of IGF-1 against starvation-induced cell death, we subjected cultured cardiomyocytes to NS in the presence or absence of IGF-1 and measured cell viability. We observed that 24 h of NS decreased cell viability significantly as measured by MTT reductase activity (*Figure 3A*) and PI incorporation (*Figure 3B*), in agreement with our recent report in which starvation activates non-apoptotic cell death.[28] Treatment with IGF-1 significantly inhibited the deleterious effects of NS (*Figure 3A* and *B*), whereas the established autophagy inductor rapamycin did not induce cell death after 24 h of treatment (data not shown).

To unveil the effects of IGF-1 on the energetic state of cardiomyocytes under NS, we quantified intracellular ATP levels. We observed that NS significantly decreased intracellular ATP content, and treatment with IGF-1 rescued ATP levels significantly (*Figure 3C*). To evaluate AMPK activity, we performed western blot analysis, which revealed that NS effectively increased phospho-AMPK (active form) and this response was significantly decreased in starved cells exposed to IGF-1 (*Figure 3D*). Moreover, the autophagy induction by NS was AMPK dependent (see Supplementary material online, *Figure S1*).[47]

To directly evaluate whether IGF-1 regulates mitochondrial function, mitochondrial Ca^{2+} uptake and oxygen consumption were measured. IGF-1 increased mitochondrial Ca^{2+} uptake in cardiomyocytes exposed to CM or NS (*Figure 4A–C*). NS dramatically

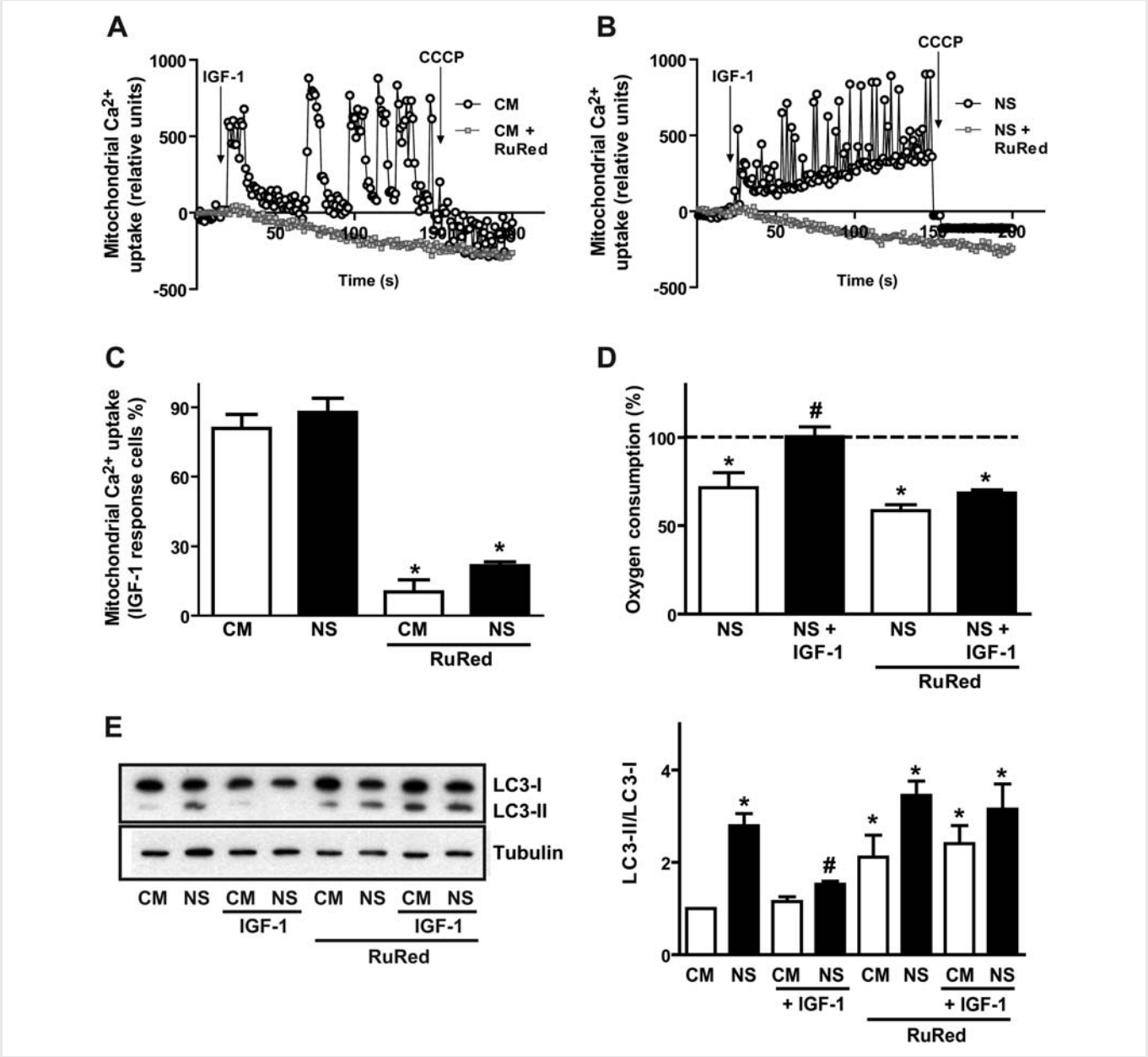

Figure 4 Increase of bioenergetics by IGF-1 regulates autophagy. Representative traces of mitochondrial Ca^{2+} uptake in cardiomyocytes control (*A*) or nutrient starved (*B*) exposed to IGF-1 in the presence or absence or Ruthenium Red (10 μM). (*C*) Quantification of cells that increased the mitochondrial Ca^{2+} uptake in response to IGF. (*D*) Oxygen consumption was measured in cardiomyocytes control or nutrient starved exposed to IGF-1 in the presence or absence or Ruthenium Red, dash line represents the cells in complete medium. (*E*) Cardiomyocyte lysates were treated for 6 h and analysed by western blot (left panel) and quantitative analysis of protein expression (right panel). Data are mean ± SEM or representative of three to five independent experiments. $^{*}P < 0.05$ vs. CM; $^{\#}P < 0.05$ vs. NS.

reduced oxygen consumption, whereas IGF-1 restored it to basal levels (*Figure 4D*). Inhibition of mitochondrial Ca^{2+} uptake by the specific Ca^{2+} uniporter blocker Rutenium Red[48] significantly reduced the effects of IGF-1 on oxygen consumption (*Figure 4D*) and LC3 processing (*Figure 4E*). These results suggest that IGF-1 inhibits NS-induced autophagy increasing mitochondrial metabolism. Altogether, these results indicate that IGF-1 negatively regulates autophagy *in vitro* by acting on mTOR via the Akt pathway, as well as by promoting elevation of ATP levels by an increase in mitochondrial bioenergetics.

3.3 IGF-1 regulates cardiac autophagy *in vivo*

To evaluate the effects of IGF-1 on starvation-induced cardiac autophagy *in vivo*, we studied mice with chronic deficiency of circulating IGF-1 produced by a liver-specific *igf1* genetic deletion (LID). This deletion results in 75% lower IGF-1 plasma levels than in wild-type (WT) mice. LID or WT mice were either fed *ad libitum*, or starved. After 48 h, we measured cardiac function in LID and WT mice by echocardiography. No differences in cardiac function were observed between the different experimental groups

Table 1 Influence of starvation on body weight and cardiac function in WT and LID mice

	WT		LID	
	Control	Starvation	Control	Starvation
N	6	6	6	6
BW, g	21.6 ± 0.3	17.3 ± 0.3*	21.4 ± 0.7	17.2 ± 0.5$^{\#}$
Fat, g	4.7 ± 0.3	2.8 ± 0.1*	5.5 ± 0.3	3.6 ± 0.1$^{\#,\&}$
Lean, g	16.9 ± 0.3	14.6 ± 0.3*	15.5 ± 0.5	13.2 ± 0.4$^{\#,\&}$
HW, g	0.61 ± 0.01	0.70 ± 0.10*	0.65 ± 0.10	0.65 ± 0.10
HW/BW, %	2.8 ± 0.1	4.5 ± 0.4*	2.8 ± 0.2	3.7 ± 0.1$^{\#,\&}$
LVFS, %	70.3 ± 1.7	60.9 ± 7.7	72.8 ± 2.5	58.0 ± 7.2
LVESD, mm	0.8 ± 0.1	1.3 ± 0.4	0.8 ± 0.1	1.2 ± 0.4
LVEDD, mm	2.8 ± 0.1	3.2 ± 2.3	3.1 ± 0.2	2.7 ± 0.4
LVPWD, mm	0.8 ± 0.1	0.8 ± 0.1	0.9 ± 0.1	0.9 ± 0.1

*$P < 0.05$ vs. WT fed; $^{\#}P < 0.05$ vs. LID fed; $^{\&}P < 0.05$ vs. WT starved.
BW, body weight; HW, heart weight; HW/BW, heart to body weight ratio; LVFS, left ventricular fractional shortening; LVESD and LVEDD, left ventricular end-systolic and end-diastolic diameter; LVPWD, left ventricular posterior wall diameter. Data are mean ± SEM.

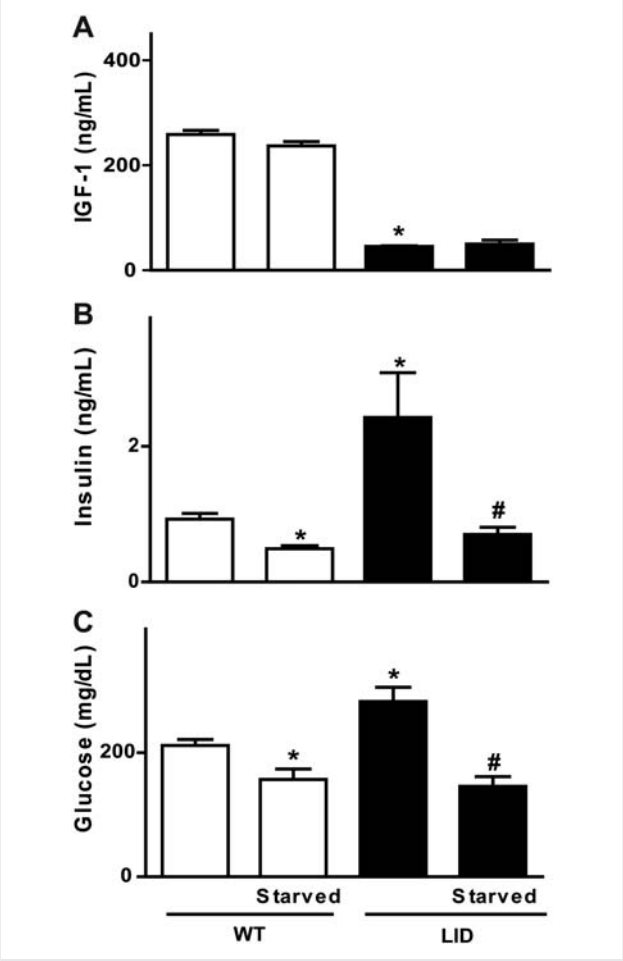

Figure 5 Comparison of serum levels of IGF-1, insulin and glucose in WT and LID mice subjected to nutritional stress. Serum samples of WT and LID mice fed *ad libitum* or starved for 48 h were analysed. (*A*) Total IGF-1, (*B*) Insulin and (*C*) Glucose levels are shown. Data are mean ± SEM. *$P < 0.05$ vs. WT fed; $^{\#}P < 0.05$ vs. knockout fed.

(*Table 1*). Next, we characterized the animals by heart weight, total body weight, as well as total amount of lean mass and fat mass. Total body weight was significantly lower in the starved group independent of strain (*Table 1*). LID mice had lower lean mass and higher fat mass than WT mice, and this tendency was maintained after starvation (*Table 1*). Importantly, a significant loss in heart weight was detected only in LID mice subjected to starvation and not in WT mice (*Table 1*). This response, however, was not observed in the liver of studied animals (see Supplementary material online, *Figure S2A*). Together, these results reveal the importance of IGF-1 in maintaining cardiac adaptability to NS.

After evaluating physical parameters, serum levels of IGF-1, insulin, and glucose were determined in both strains. As expected, due to liver-specific genetic deletion, LID mice displayed lower circulating IGF-1 concentrations than WT mice under fed conditions, and these levels were not significantly modified after starvation (*Figure 5A*). However, independent of the strain, serum insulin concentrations were considerably lower in the starved group compared with the fed mice (*Figure 5B*). Although basal insulin levels in LID mice fed *ad libitum* are increased due to elevated levels of growth hormone,[49] this increase does not occur in starved LID mice, probably because there is no need to activate IGF-1 or insulin signalling pathways. Despite the higher levels of insulin observed in LID mice, serum glucose concentrations were higher in LID mice in the basal state compared with WT mice (*Figure 5C*), likely due to insulin resistance. However, glucose levels were decreased in starved mice as expected, independent of the genetic strain.

To measure the autophagic state of LID mouse hearts, we analysed cardiac lysates by western blot. As expected, starvation led to an increase in cardiac LC3-II levels in WT mice. IGF-1 deficiency, however, markedly enhanced this increase (*Figure 6A*). We also detected an increase in AMPK phosphorylation (*Figure 6B*), which is consistent with our *in vitro* data. Importantly, no differences in LC3-II were found in the liver of starved LID mice compared with WT mice (see Supplementary material online, *Figure S2B*). Altogether, these results suggest that IGF-1 negatively regulates cardiac autophagy and AMPK activity *in vivo*.

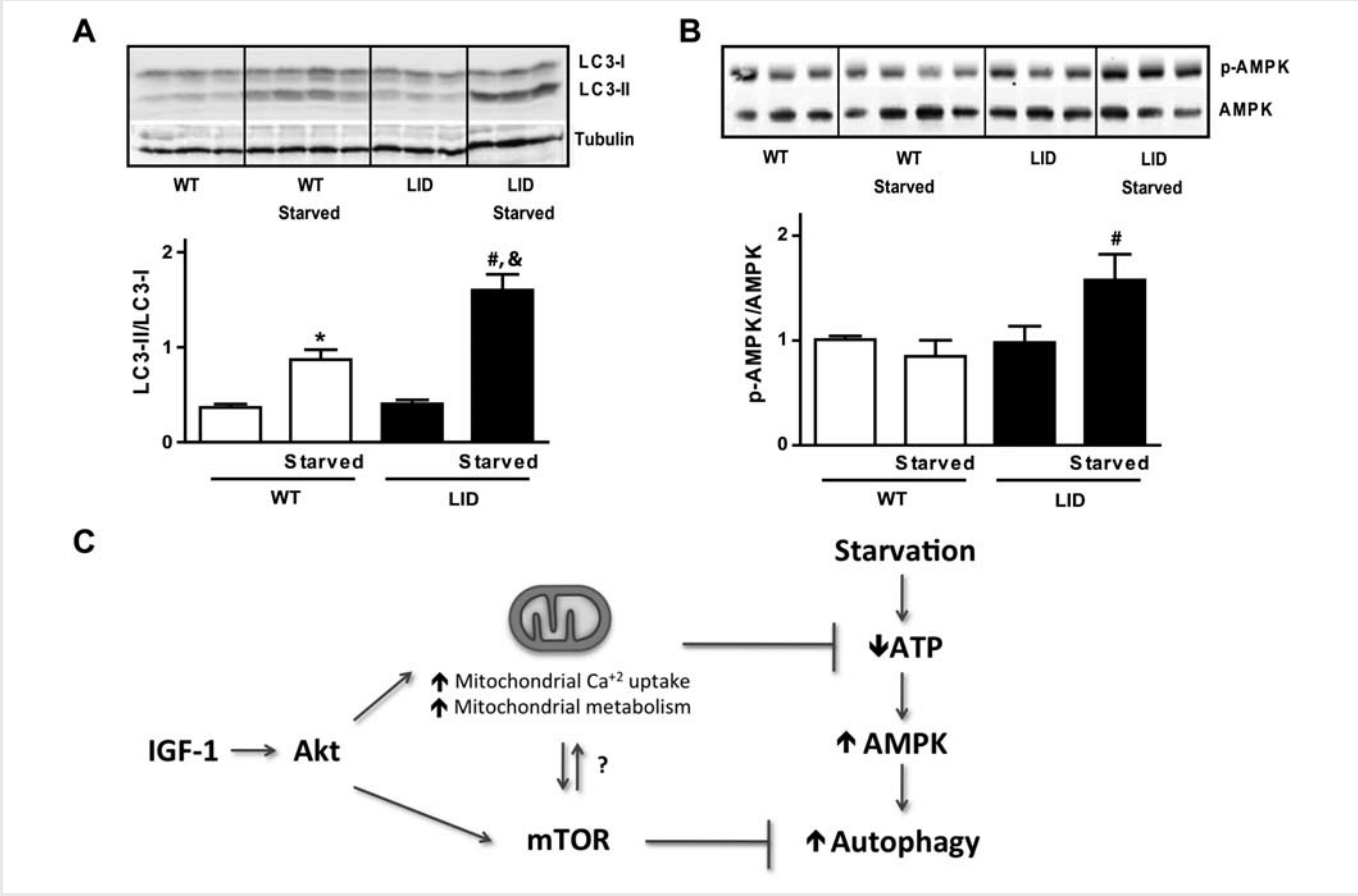

Figure 6 Effects of nutritional stress *in vivo* upon cardiac LC3 and AMPK. (*A*) Heart lysates from mice fed *ad libitum* or starved during 48 h were subjected to western blot for the detection of (*A*) endogenous LC3 processing (top) and quantitative analysis of protein expression (bottom), and (*B*) p-AMPK and AMPK total (top) and quantitative analysis of protein expression (bottom). Data are mean ± SEM. $^{*}P < 0.05$ vs. WT fed; $^{\#}P < 0.05$ vs. knockout fed; $^{\&}P < 0.05$ vs. WT starved. (*C*) Model for the proposed mechanism mediating the protective effect of IGF-1. Nutritional stress leads to a decay of intracellular ATP levels, activating AMPK and autophagy to restore the energetic state of cardiomyocytes and to prevent cell death. IGF-1 rescues ATP levels and activated the Akt/mTOR pathways inhibiting the activation of autophagy.

4. Discussion

Results from this and previous studies indicate that IGF-1 exerts an inhibitory effect upon cellular autophagy.[21–23] Further, we show here that IGF-1 negatively regulates cardiac autophagy *in vivo*, protects from NS-induced cell death, and increases ATP levels and mitochondrial metabolism in cultured cardiomyocytes. The inhibitory effects of IGF-1 upon autophagy can be explained by the stimulation of the IGF-1R/Akt/mTOR axis and by increases in mitochondrial Ca^{2+} levels, oxygen consumption, and ATP levels that lead to AMPK inhibition in cultured cardiomyocytes. Additionally, we also observed that IGF-1 deficiency increases cardiac AMPK activity during starvation conditions, suggesting a distinct role for IGF-1 in preserving cardiac energetics under these conditions. Thus, by maintaining ATP levels, IGF-1 additionally regulates autophagy by inhibiting the AMPK/mTOR axis during NS (*Figure 6C*).

There is a growing body of evidence highlighting the cardioprotective role of IGF-1.[50] These effects have been attributed to the regulation of metabolism,[51] inhibition of apoptosis,[52–54] increases in cardiomyocyte size,[55] intracellular Ca^{2+} regulation,[18,56,57] and direct effects on cardiomyocyte contractility.[58] Although it seems clear that IGF-1 is cardioprotective, very little is known regarding its effects on autophagy, a major adaptive mechanism. Contrary to data reported here, it has been previously documented that IGF-1 increases autophagy and cell death during starvation;[24] and moreover, that these responses were enhanced by over-expression of the class-I PI3K, which is largely considered as an inhibitor of autophagy and cell death.[44,59] A possible explanation for these differences is the different experimental models. Those effects were observed in the cardiac-derived cell line H9c2, and not in primary cultured cardiomyocytes or intact animals as we studied here. H9c2 is a subclone of the original clonal cell line derived from embryonic BD1X rat heart tissue by Kimes and Brandt and exhibits many of the properties of skeletal muscle.[60] Thus the response of the cell line could be limited by transformation/alteration of the cardiac phenotype. To our knowledge, there are no other studies of the role of IGF-1 in cardiac autophagy. Thus, by considering our study, as well as many others in which IGF-1 exerts cardioprotective functions, we propose that IGF-1 primarily protects during nutritional insults in the heart.

Although autophagy could contribute to cell death under certain experimental settings, most of the evidence gathered thus far indicates that autophagy, more than a cell death modality on its own, represents a pro-survival mechanism.[61] Our findings indicate that NS induces death of cultured cardiomyocytes,[28] and IGF-1 inhibits

autophagy and cell death. From these results, excessive autophagy could lead to cell death and consequently IGF-1 inhibits autophagy and prevents cardiomyocyte death. However, we prefer the idea that starvation-induced autophagy becomes inhibited because it is no longer needed as a source of ATP. We observed that IGF-1 prevented declines in ATP levels and activation of AMPK both in cultured cardiomyocytes and *in vivo*. In accordance with this notion, we observed that inhibition of autophagy using 3-methyladenine enhanced cell death, rather than diminished it (see Supplementary material online, *Figure S3*),[47] which further suggests that autophagy is a pro-survival mechanism to cope with NS.

We observed that IGF-1 promotes the generation of ATP and thereby leads to a balance of the autophagic rates during starvation. This is the first report showing that IGF-1 increases mitochondrial Ca^{2+} uptake and mitochondrial respiration in cultured cardiomyocytes, increasing the mitochondrial metabolism and ATP levels in nutrient-starved cells. Accordingly, IGF-1 increases mitochondrial respiration in serum-deprived prostate cancer cells in an Akt-dependent manner.[51] Hahn–Windgassen *et al.*[62] showed that Akt activity increases ATP levels and reduces AMPK activity leading to mTOR activation. In addition, growth factor deprivation causes loss of several cell-surface transporters, including those of glucose, amino acids, low-density lipoproteins, and iron, thus triggering a catabolic state in which activation of the Akt/mTOR axis acts to maintain these transporters at the membrane.[63] Moreover, IGF-1 induces an inositol 1,4,5-trisphosphate (InsP3)-dependent calcium increase in cardiomyocytes,[56] probably due to positive regulation of the inositol 1,4,5-triphosphate receptor (InsP3R) that increases its calcium-releasing activity.[64,65] Mitochondrial Ca^{2+} uptake is required to provide optimal bioenergetics by providing reducing equivalents to support mitochondrial ATP production. The activities of three dehydrogenases of the Krebs cycle (isocitrate dehydrogenase, α-ketoglutarate dehydrogenase, and pyruvate dehydrogenase) are regulated by Ca^{2+}.[66,67] Moreover, Cardenas *et al.*[68] showed that basal IP3R activity is required to provide a physiological Ca^{2+} signal sensed by the mitochondria, thereby controlling mitochondrial bioenergetics. Absence of this Ca^{2+} transfer results in enhanced phosphorylation of pyruvate dehydrogenase and AMPK activation, which triggers autophagy.[68] Thus, constitutive InsP3R Ca^{2+} release to mitochondria is critical for mitochondrial respiration and maintenance of normal cell bioenergetics.

On the other hand, AMPK, a sensor of nutritional energy *in vivo*,[69–71] stimulates both glucose uptake and fatty acid oxidation during NS, thus restoring intracellular ATP levels.[48,72] Cardiac autophagy during NS was observed previously as an adaptive mechanism dependent on AMPK and mTOR.[64] Altogether, these studies (including ours) clearly point out that autophagy is activated in the setting of metabolic challenge, and the pathways activated by IGF-1 help to regulate the energetic state of cells and to restore autophagy to basal levels, beyond which it can be deleterious.

In conclusion, this is the first study that evaluates the effects of IGF-1 in regulation of starvation-induced cardiac autophagy. These results agree with previous findings in which IGF-1 exerts cardioprotective actions via the activation of the Akt/mTOR, as well as the AMPK/mTOR, pathways and by stimulating mitochondrial activity. Here, we observed that autophagy becomes activated during nutritional challenge, and that IGF-1 primarily up-regulates the energetic state of cardiac cells, thus obviating the need for autophagy to provide nutrient supply. Although autophagy is protective, prolonged activation of the autophagic response can be maladaptive. The effect of IGF-1 therefore might reduce the permanent damage to cardiomyocytes produced during NS-related pathological states, such as ischaemic heart disease or myocardial infarction.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

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