Synergistic and antagonistic effects of TNF-α and IGF-I in heart failure: *in vitro* and *in vivo* study of cardiac and skeletal muscle

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To my family and friends for all the caring and support

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ABBREVIATIONS

ACE	angiotensin converting enzyme
Ang II	angiotensin II
AT_1R	angiotensin II receptor type 1
AT_2R	angiotensin II receptor type 2
CHF	congestive heart failure
DABCO	1,4-diazabicyclo [2.2.2] octane
DAPI	4',6-diamidino-2-phenylindole
Des-IGF-I	Des-(1-3) insulin-like growth factor-I
DMEM	Dulbecco's modified Eagle's medium
ERK 1/2	extracellular signaling-regulated kinase 1/2
IGF-I	insulin-like growth factor-I
IGF-II	insulin-like growth factor-II
IGFBP	insulin-like growth factor binding protein
MuRF1	muscle RING finger 1
MAFbx	muscle atrophy F-box
MAPK	mitogen-activated protein kinase
MEK 1/2	mitogen-activated ERK activating kinase
PBS	phosphate buffered saline
PI3K	phosphoinositide 3-kinase
RAS	renin-angiotensin system
RIPA	radioimmunoprecipitation
RT-PCR	reverse transcriptase polymerase chain reaction
S.E.	standard error
TNF-α	tumor necrosis factor-alpha
TNFR-1	tumor necrosis factor receptor 1
TNFR-2	tumor necrosis factor receptor 2
UPP	ubiquitin-proteasome pathway
vARC	ventricular adult rat cardiomyocytes

I. SUMMARY

Cardiovascular diseases are a major cause of morbidity and mortality in industrialized countries. All forms of myocardial injury first lead to compensatory hypertrophy, which eventually progresses to heart failure. The pathophysiologic mechanisms underlying this process are not fully understood. Nevertheless, cellular remodeling is considered as a prime contributor to the pathogenesis of heart failure. The remodeling process involves cardiomyocyte hypertrophy, alterations in gene expression and myocyte shape as well as changes in the extracellular matrix. The same factors that induce cardiac hypertrophy during early compensatory changes, can also lead to apoptosis and secondary detrimental events associated with the development of heart failure. In the present thesis I will focus on a three of these factors, namely tumor necrosis factor-alpha (TNF-α), insulin-like growth factor (IGF)-I and angiotensin II (Ang II).

TNF-α is a pro-inflammatory cytokine produced in the myocardium in response to various types of injury. Studies using experimental animals demonstrated the important role of TNF-α in the development of heart failure, however the use of TNF-α blockers in clinical trials did not demonstrate beneficial effects. A frequent consequence of catabolic conditions, including chronic heart failure, is muscle mass loss. TNF-α is considered to play a major role in muscle catabolism. With evidence of beneficial next to detrimental effects in both cardiac and skeletal muscle, the role of TNF-α remains controversial.

IGF-I is involved in maintaining cardiac function in post-infarct events. This growth factor has also been shown to induce survival and hypertrophy in many cells, including skeletal and cardiac muscle cells. Important modulators of IGF-I activity are the IGF-binding proteins (IGFBPs). Interactions between TNF-α and IGF-I have been reported. Most of the studies were undertaken in skeletal muscle and showed essentially an inhibitory effect of TNF-α either on IGF-I-induced responses or on IGF-I and/or IGFBPs expression.

The neurohormone Ang II plays a central role in hypertension and cardiovascular diseases, and is also involved in the myocardial remodeling process. Functional crosstalk between Ang II and TNF-α exists in cardiac hypertrophy, and is believed to promote tissue damage.

The present work was undertaken in order to gain more insight into the mechanisms of regulation involved in cardiac remodeling and muscle atrophy through multiple factor interactions. To this end, we used two cell culture models of cardiac and skeletal muscle cells, as well as animal models.

In primary cultures of adult rat cardiomyocytes, we show that TNF-α acts on the IGF-I system by downregulating mRNA expression of IGFBP-4, by interfering with IGF-I-induced Akt signaling, and by potentiating IGF-I-induced activation of the ERK1/2 signaling pathway. The latter effect may present a synergistic role for TNF-α and IGF-I in cardiomyocyte hypertrophy. In this model we also show that TNF-α has immediate positive effects by increasing cardiomyocyte viability, however longer-term incubation resulted in decreased viability and enhanced expression of apoptotic markers.

To determine the *in vivo* relevance of the IGF-I system regulation by factors involved in cardiac remodeling, we analyzed the expression pattern of cardiac IGFBPs in two animal models of hypertension. We show up-regulation of IGFBP-4 mRNA expression in both models, increased IGFBP-5 in salt-fed Dahl salt sensitive rats, and decreased IGFBP-3 in Ang II-infused rats. Specific down-regulation of IGFBP-3 by Ang II may play an important role in pressor-independent cardiac effects of this neurohormone.

We also analyzed protein content regulation in the skeletal muscle cellular model. Using C_2C_{12} mouse myotubes, we show that TNF-α and IGF-I both enhance protein synthesis by activating different signaling pathways. TNF-α acts mainly via PI3K-Akt and to a lesser extent via MEK-ERK1/2, while IGF-I acts independently of PI3K. Mechanisms which activate protein degradation through the ubiquitin proteasome pathway were analyzed by measuring Atrogin-1 mRNA expression. Levels of this marker of atrophy were transiently increased by TNF-α via the p38 MAPK signaling pathway, and this effect was inhibited by IGF-I. However, longer-term incubations with TNF-α decreased Atrogin-1 mRNA levels suggesting inhibition of protein breakdown.

To conclude, this work demonstrates regulation of cardiac IGFBPs expression by TNF-α and Ang II at the cellular and tissue level, respectively. In the models studied here, we show that factors involved in the remodeling process can modulate IGF-I,

which is important for cardiac function maintenance, through regulation of the IGFBPs. These mechanisms highlight the important role of multiple factor interactions in the development of heart failure. Furthermore, by studying the regulation of skeletal muscle protein content, TNF-α proved to increase protein synthesis and to inhibit protein degradation mechanisms by decreasing Atrogin-1 expression. These results propose a novel beneficial role for TNF-α in the prevention of muscle wasting.

II. INTRODUCTION

II. A. Background

From hypertrophy to heart failure: the remodeling process

Congestive heart failure (CHF) is a common clinical problem resulting in significant morbidity and mortality. Considerable progress has been made in understanding the pathophysiologic mechanisms leading to heart failure, however much remains unknown [1]. Cellular remodeling is a prime contributor to the pathogenesis of various clinical disorders including hypertension and heart failure. This process is a complex set of events involving cardiomyocyte hypertrophy, alteration of gene expression, of myocyte shape, and extracellular matrix. These changes result in thickening of the cardiac wall, followed by chamber dilation and myocardial dysfunction [2].

Myocardial hypertrophy is a common hallmark of the remodeling process and is an initial adaptive process to a variety of physiological and pathological conditions associated with increased cardiac work. The hypertrophic response initially normalizes wall stress and maintains ventricular function. However, decompensated CHF occurs when the adaptive process fails. The process of ventricular hypertrophy is mediated by a variety of systems including sustained neurohormonal activation, mechanical load (stretch or distension), and/or growth factor [2] as well as cytokine [1] release. It has recently been established that the same factors that induce cardiac hypertrophy can also lead to apoptosis, thus establishing a direct link between early compensatory changes and detrimental secondary effects during the development of heart failure (reviewed in [1]).

Fig 1 summarizes three factors involved in the cardiac remodeling process, which will be the focus of the present work: tumor necrosis factor-alpha (TNF-α), insulin-like growth factor-I (IGF-I) and angiotensin II (Ang II).

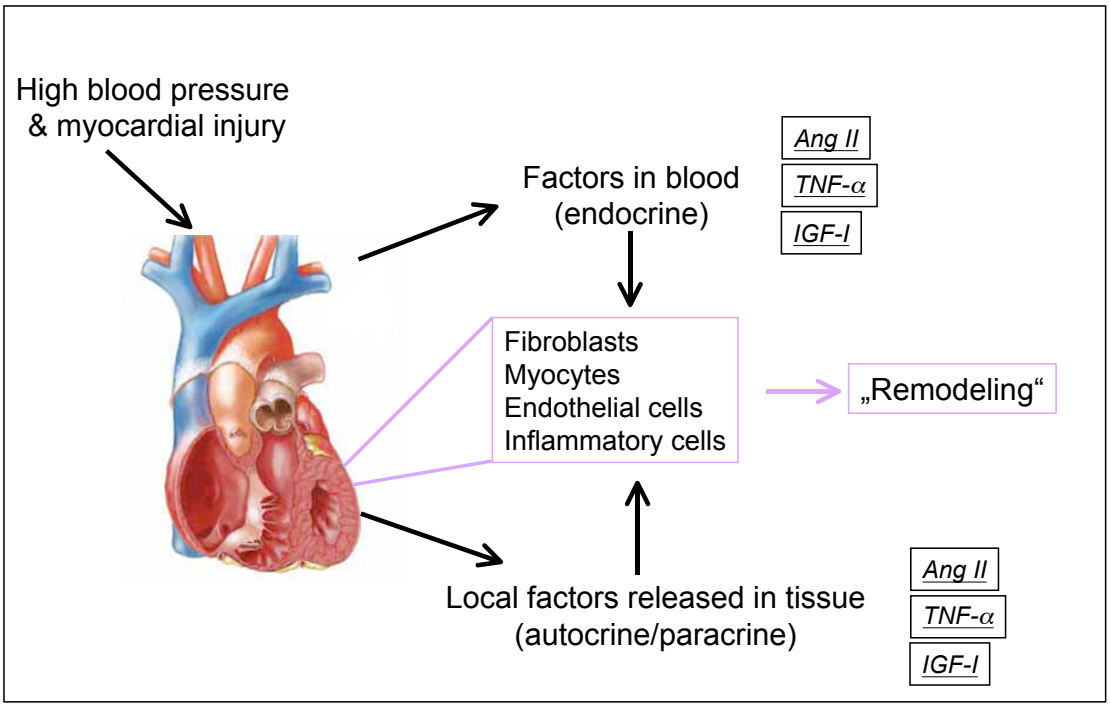

Fig 1. Summary of endocrine and autocrine factors involved in cardiac remodeling

Various hormones and cytokines including insulin-like growth factor-I (IGF-I), angiotensin II (Ang II), and tumor necrosis factor-alpha (TNF-α) are produced systemically and locally upon cardiac injury or pressure overload. These factors act on the myocardium to induce the remodeling process.

II. B. Tumor necrosis factor- alpha (TNF-α)

II. B. 1. TNF-α in the progression of heart failure

TNF-α is a pro-inflammatory cytokine expressed locally in cardiac tissue in response to various types of injury and plays an important role in initiating adaptive myocardial responses. However, it has the potential to produce cardiac decompensation when expressed at high concentration or for long duration (reviewed in [3]). TNF-α has pleiotropic biological effects, its plasma levels are elevated in a variety of cardiovascular diseases, such as myocardial infarction and cardiopulmonary bypass (reviewed in [4]). Moreover, TNF-α can be produced by cardiac myocytes and non-myocytes in response to hemodynamic pressure overload [5].

A large number of studies in experimental animals such as administration of TNF-α and transgenic overexpression of TNF-α in the heart have shown to replicate the heart failure phenotype, including progressive left ventricular dysfunction, fetal gene expression and cardiomyopathy [6]. Furthermore, attenuation of TNF-α biological

activity abrogates the development of heart failure in model systems. This demonstrated the important role of TNF-α in the development of heart failure and suggested that anti-cytokine therapy could prove beneficial in the treatment of patients with heart failure [7]. However, the use of TNF-α blockers (Etanercept and Infliximab) in large multicenter, randomized and placebo-controlled clinical trials in patients with CHF did not demonstrate beneficial effects. These studies showed a trend toward increase mortality and hospitalization [8].
Effects induced by TNF-α in cardiomyocytes are controversial and the mechanisms involved remain poorly defined.

II. B. 2. The biology of TNF

TNF was first identified as a substance with profound antitumor effects *in vitro* and *in vivo*. It is now clear that TNF has a variety of different biological capacities, such as influencing growth, differentiation and/or function of virtually every cell type investigated, including cardiac myocytes. TNF is initially synthesized as a transmembrane protein of approximately 26 kD. A 17-kD fragment is proteolytically cleaved off the plasma membrane by a membrane-bound enzyme, TNF-α convertase, to produce the secreted form of TNF. It then rapidly assembles as a homotrimer to form the biologically active 51-kD peptide capable of binding TNF receptors (Fig 2) (reviewed in [6]). Currently, two isoforms of TNF have been identified and share similar inflammatory activies. TNF-α is the smaller and more abundant [9].
TNF receptors (TNFRs) signal as homotrimers and exist either as membrane-bound or as truncated soluble forms. Two distinct cell surface receptors mediate the effects of TNF, TNFR-1 (p55) and TNFR-2 (p75) (Fig 2). Despite conserved extracellular domains, the cytoplasmic domains of the two receptors lack homology, suggesting activation of different downstream transduction pathways. Both receptors are found in human and rat cardiac myocytes. TNFR-1 is the main receptor subtype in most cells, including the heart and its downstream signaling system has been extensively studied (reviewed in [9]).

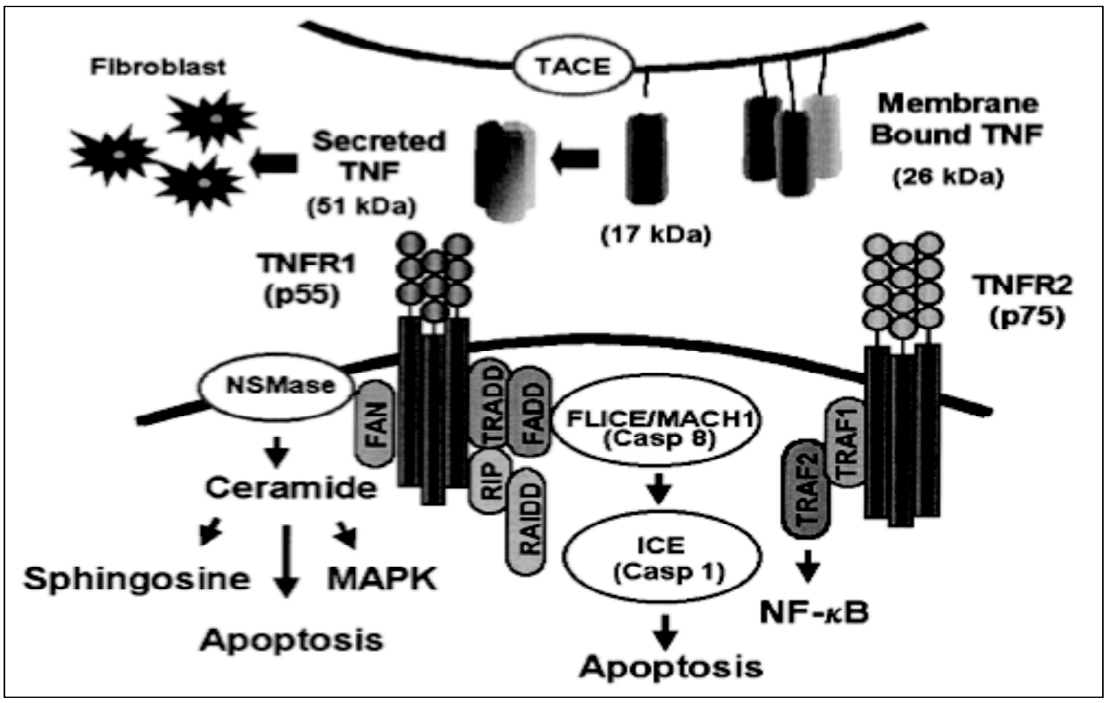

Fig 2. Proposed schematic of TNF-induced signaling in the adult heart

Secreted TNF binds TNF receptor on different cell types, whereas membrane-bound TNF is confined to binding to TNF receptors on adjacent cells. Binding of TNF induces trimerization of the receptors with subsequent recruitment of several signaling proteins to the cytoplasmic membrane. TNFR1-induced apoptosis occurs via 2 different pathways coupled to distinct domains of TNFR1. On the one hand the Fas-associated death domain protein (FADD)-caspase 8 (casp 8) cascade and on the other hand the neutral sphingomyelinase (NSMase) pathway. TNFR2-mediate activation of TRAF2 is closely linked to protection against apoptosis via activation of nuclear factor-kappa B (NF-κB). TRAF2 also binds the TNFR1 through FADD (not shown). [6]

II. B. 3. Effects of TNF-α in the heart

Several recent studies provided evidence for beneficial next to detrimental roles of TNF-α. Biological responses exerted by TNF-α in the adult mammalian heart through both receptor subtypes have been reported as follows. TNF-α mediates negative inotropic effects, apoptosis of cardiac myocytes and cytoprotection via the TNFR-1, while only cytoprotective effects are mediated through the TNFR-2 (reviewed in [6]).

Transgenic mice over-expressing TNF-α in the heart developed ventricular hypertrophy and dilation, fibrosis, and overt CHF. In this model, TNF-α induced both pro- and anti-apoptotic pathways resulting in an increase of apoptosis primarily in non-myocytes. The study concluded that, *in vivo*, TNF-α by itself does not induce myocyte apoptosis [10]. Consistently, a recent study undertaken on samples from human infarcted myocardial tissue showed that cardiomyocyte loss in the acute stage of myocardial infarction was due to apoptosis but this was not directly triggered

by TNF-α[11]. Several lines of evidence among which the direct activation by TNF-α of the transcription factor nuclear factor-kappa B (NF-κB), a mediator of anti-apoptotic pathways, support that TNF-α can activate rapid protective pathways in cardiomyocytes [3, 10].

The emerging idea from these accounts is that TNF-α expression in the acute stage in response to stress may be beneficial whereas persistent expression in chronic stages, or exposure to excessive amounts of the cytokine, may be harmful to the myocardium [12].

II. B. 4. Effects of TNF-α in skeletal muscle

Muscle atrophy is a frequent consequence of many catabolic conditions such as diabetes, cancer, sepsis and chronic heart failure. TNF-α is considered to play a major role in muscle catabolism because circulating levels of TNF-α are markedly increased in catabolic states [13-15]. Moreover, enhanced protein degradation and muscle loss have been observed in TNF-α-transgenic animals [16, 17] or after chronic administration of the cytokine [18]. Apoptosis represents a potential pathway by which wasting can occur in chronic diseases [14], however, a large part of the protein breakdown process has been attributed to the activation of the ubiquitin-proteasome pathway (UPP). The latter requires three distinct enzymatic components amongst which the muscle specific ubiquitin ligases MuRF1 and Atrogin-1 (reviewed in [19]).

On the other hand, TNF-α was also shown to promote cell growth in primary adult human skeletal muscle cells [20] and to transiently induce proliferation and survival in C_2C_{12} myoblasts [21]. In this view, the role of TNF-α in muscle cells remains controversial. Depending on the conditions (time of exposure, concentrations used and stage of differentiation), TNF-α can be a survival factor, promoting proliferation and differentiation, or a cytotoxic factor, inducing apoptosis and necrosis [22, 23].

II. C. Insulin-like growth factor-I (IGF-I)

II. C. 1. IGF-I in the heart and skeletal muscle

IGF-I is one of the other factors that increase with the onset of left ventricular pressure overload. The IGF axis is directly involved in post-infarct events by maintaining cardiac function via induction of adaptive hypertrophy and decreasing

apoptotic cell death. Both these effects have been demonstrated in various cell types (reviewed in [24]). Administration of IGF-I in patients with CHF showed acutely improved cardiac performances and lipid profile as well as increased insulin sensitivity [25].

The important role of IGF-I in cardiac muscle biology also includes stimulation of the formation of cardiac myofibrils. This effect has been described *in vitro* and *in vivo* [26-28]. Besides, in cultured cardiac myocytes IGF-I modulates the expression of various genes including those involved in the regulation of intracellular signaling, mitochondrial function, cell survival and calcium signaling [29].

Autocrine IGF-I production has been shown to play a crucial role in muscle growth [30]. Indeed, IGF-I is sufficient to induce skeletal muscle hypertrophy [31] as was demonstrated by transgenic mice overexpressing IGF-I in skeletal muscle [32, 33]. The signaling pathways activated by IGF-I and involved in this process have been well described (reviewed in [19]).

II. C. 2. The biology of the IGF-system

IGF-I and IGF-II are single-chain polypeptides (70 and 67 amino acids, respectively) that share homology with each other and with proinsulin. Systemic IGF-I and IGF-II levels are determined mainly by production in the liver, but many other cells synthesize these growth factors. Among the broad range of physiological actions, the IGFs regulate metabolic functions and cell cycle; they induce protein synthesis and function as survival factors. Actions of the IGFs are mediated by specific membrane receptors with tyrosine kinase activity responsible for intracellular signaling (reviewed in [34]).

IGFs activity is modulated by the IGF binding proteins (IGFBPs). The IGFBPs are a family of six proteins, which interact with high affinity with the IGFs. Their affinity for the IGFs is 2- to 50-fold greater than the affinity of the IGFs for their receptor. The traditional view of the IGFBPs is that they function as carrier proteins for the IGFs in the circulation, regulating their turnover and transport towards the tissue. At the tissue level, IGFBPs compete with IGF receptors for IGF binding. The significant difference in the biochemical characteristics of these proteins accounts for the differences in their biological actions. While some members of the IGFBP family have been shown to inhibit IGF actions, others potentiate IGF actions [35-39]. Specific IGFBP proteases have been identified. These enzymes play a critical role in modulating IGF availability at the cellular level [34].

II. D. Interactions between TNF-α and the IGF-I axis

Several studies have reported that cytokines impair the IGF-I axis and have analyzed the mechanisms in various *in vitro* models. For example, TNF-α promotes IGF-I receptor resistance in neurons and suppresses the ability of IGF-I to induce survival [40]. In rat aortic smooth muscle cells, TNF-α markedly suppresses IGF-I mRNA expression and dramatically upregulates IGFBP-3 mRNA levels as wells as secretion of the protein [41]. In human skeletal myoblasts, TNF-α blocks basal and IGF-I-stimulated differentiation [20], and in differentiating C_2C_{12} cells, TNF-α blocks IGFBP-5 secretion [42]. The *in vivo* relevance of the latter effect was established in the gastrocnemius of rats continuously infused with TNF-α [43]. In the same *in vivo* model, TNF-α decreases IGF-I plasma concentrations and IGF-I mRNA expression mainly in muscle tissue including the heart [44].

These TNF-α-induced mechanisms, which act on the IGF-I system, may play a fundamental role in the development of catabolic states such as neurodegeneration, cancer-related muscle wasting, or, as assessed in this thesis, in cardiac remodeling and cardiac cachexia associated with CHF.

II. E. Angiotensin II (Ang II)

II. E. 1. The renin-angiotensin system (RAS) in hypertension and heart failure

Ang II plays a central role in hypertension and cardiovascular diseases [2], and has been implicated in the development of heart failure in humans after myocardial infarction [1]. Ang II is involved in the myocardial remodeling process either through mechanical load release or via neurohormonal stimulation. In cultured cardiac myocytes, Ang II has been shown to induce hypertrophic responses [1].

Production of Ang II is the result of a series of enzymatic activities referred to as the renin-angiotensin system (RAS). The RAS plays a central role in cardiovascular homeostasis. The generation of renin is the rate limiting step of the entire cascade. Angiotensinogen, the renin substrate, may be found either locally (at the endothelial level) or systemically. After conversion of angiotensinogen to angiotensin I, the angiotensin converting enzyme (ACE) generates Ang II, an octapeptide. Ang II is a potent vasoconstrictor with growth-promoting properties. Numerous studies have demonstrated that pharmacological inhibition of the ACE improves the outcome in

patients with several cardiovascular disorders (hypertension, heart failure, ischemic heart disease) [45].

Ang II signals through two types of G-coupled transmembrane receptors, the AT_1R and the AT_2R. Both receptors are expressed in human heart [1]. Data from *in vivo* studies show that AT_1R-blockers reverse myocyte remodeling back toward normal and improve outcome [46].

II. E. 2. Interactions between Ang II and TNF-α

The importance of interactions between pro-inflammatory cytokines and the RAS in the heart has recently become apparent. There is indeed evidence of functionally significant crosstalk between the RAS and inflammatory mediators in cardiac hypertrophy and failure. Ang II provokes inflammatory responses in the heart through NF-κB dependent pathways, whereas TNF-α provokes activation of the RAS in the heart through increased ACE activity. Both of these pathways converge on overlapping mitogen activated protein kinase (MAPK) signal transduction pathways. This type of crosstalk leads to deleterious self-amplifying positive feedback loops that promote tissue damage, particularly in the setting of chronic activation (reviewed in [47]).

III. OBJECTIVES

The previous chapter has highlighted the involvement of TNF-α, a pro-inflammatory cytokine, in the cardiac remodeling process and the role of this cytokine in skeletal muscle atrophy associated with heart failure. To date, the actions of TNF-α in both cardiac myocytes and skeletal muscle cells remain controversial. Beneficial as well as detrimental effects have been described, and the mechanisms involved are not well defined.

In my introduction, I also emphasized that interactions exist at various levels between TNF-α and IGF-I, an important growth factor whose survival and hypertrophic effects in both cardiac and skeletal muscle cells have been extensively studied. Numerous reports, mainly on skeletal muscle cells, show that TNF-α interferes negatively with the IGF-I system.

Another important player implicated in the development of heart failure is the neurohormone Ang II. Functional crosstalk at the signaling pathway level exists between Ang II and TNF-α in cardiac hypertrophy, and is believed to promote tissue damage.

The aim of this thesis is to analyze different levels of interplay between IGF-I and TNF-α on the one hand, and IGF-I and Ang II on the other hand, in cardiac and skeletal muscle.

The objectives are:

1. To analyze the effects of TNF-α on the expression of IGFBPs and to determine if this cytokine modulates specific intracellular signaling pathways induced by IGF-I in adult rat cardiomyocytes. Focus is placed on two IGF-I-induced signaling cascades, the Akt and the ERK1/2 pathways.

2. To analyze the expression pattern of cardiac IGFBPs in two *in vivo* models of hypertension, namely Ang II- and salt-induced hypertensive rats.

3. To examine the effects of TNF-α on protein synthesis and protein degradation in comparison to IGF-I-mediated actions. The model used for this purpose is the mouse C_2C_{12} myoblast cell line. Our analysis of protein breakdown focuses on the UPP, more specifically on the regulation of Atrogin-1 expression.

IV. EXPERIMENTAL PROCEDURES

Detailed experimental procedures are described hereafter for the cell culture models as well as for the complementary results, which are presented in addition to the manuscripts. For all other procedures, the reader is referred to each of the three manuscripts.

IV. A. Primary cell culture of ventricular adult rat cardiomyocytes

The protocol was performed in accordance with institutional guidelines for the care of experimental animals. Ventricular cardiomyocytes were isolated from 6-week-old Sprague-Dawley rats (IFFA Credo, L'Arbresle, France) as follows. Animals were anesthetized with 2.5 μl/g sodium pentobarbital (ABBOTT AG, Switzerland), the heart rapidly excised and mounted on a Langendorff apparatus. The heart was rinsed free of blood with chilled basic buffer (126 mM NaCl, 4.4 mM KCl, 1.0 mM $MgCl_2$, 4.0 mM $NaHCO_3$, 10 mM Hepes, 30 mM 2,3-Butanedione Monoxime, 5.5 mM glucose, 1.8 mM pyruvate, pH 7.3, bubbled with oxygen) containing 200 μM calcium then perfused with basic buffer at 37 °C. After 5 min the perfusate was switched to basic buffer containing 1 mg/ml type II collagenase (Worthington, Lakewood, NJ) for another 20 min. The ventricules were minced and passed through a 100 μm Nylon cell strainer (BD Falcon, Franklin Lakes, NJ) to remove undigested cells and connective tissue. This was followed by three consecutive washes with basic buffer containing 100 μM, 200 μM and 600 μM calcium, plus 0.4, 0.2 and 0.2 g of bovine serum albumin (BSA) respectively. The cardiomyocytes were then seeded at a density of 4 x 10^5 cells per 10 cm diameter dishes (Sarstedt AG, Switzerland) previously coated with 20 μg/ml laminin (Sigma-Aldrich, Germany). The culture medium consisted of Dulbecco's modified Eagle's medium (DMEM) containing 1000 mg/L glucose (Invitrogen AG, Basel, Switzerland). Cells were treated after isolation as detailed in the results section with recombinant mouse TNF-α(□D systems, Minneapolis, MN, Des-IGF-I (IBT GmbH, Reutlingen, Germany) or both together. In one experiment, cardiomoycytes were cultured for 7 days in DMEM medium supplemented with 20% heat-inactivated fetal calf serum (Amimed AG, Basel, Switzerland).

IV. B. Murine C_2C_{12} myoblast cell line culture

Myoblasts from the muscle derived C_2C_{12} cell line were obtained from American Type Culture Collection (Manassas, VA). The seeding density used throughout the experiments was 5 x 10^5 cells per 10 cm diameter dish. Undifferentiated cells were grown in DMEM supplemented with 10% inactivated foetal calf serum at 37°C in the presence of 5% CO_2. This medium will be referred to as growth medium (GM). The myoblasts were fused into myotubes by shifting the GM to differentiation medium (DM, DMEM supplemented with 2% heat inactivated horse serum). The time point at which the differentiation is induced is referred to as day 0 (D0). The medium was changed after 48 h and the differentiation was allowed to continue for 96 h. All the experiments were performed at Day 6 (D6). Muscle cells were examined for evidence of myotube formation by phase contrast microscopy (Axiovert 40C microscope, ZEISS). To preserve the characteristics of the C_2C_{12} cell line, the splitting of the cells was done up to a maximum of seven times. Cells were treated as detailed in the results section with recombinant mouse TNF-α(□D systems, Minneapolis, MN, IGF-I (IBT GmbH, Reutlingen, Germany) or both together, in the presence or absence of specific inhibitors (Calbiochem, Merck Bioscience; Darmstadt, Germany) LY294002 (20 μM, 30 min pretreatment), PD98059 (20 μM, 1 h pretreatment) or SB203580 (10 μM, 1 h pretreatment).

IV. C. Immunocytochemistry

C_2C_{12} Cells were grown on laminin coated (20 μg/ml) glass coverslips in 24-well plates (Sarstedt AG, Switzerland). Cardiomyocytes were used right after isolation. The cells were fixed in 4% formaldehyde (Polysciences Inc., Warrington, PA) and permeabilised with 0.1% NP-40 (Fluka, Switzerland). After 10 min incubation with 0.1 M PBS-glycine, followed either (1) an overnight incubation at room temperature in a humid chamber with primary antibody against α-sarcomeric-actin clone 5C5 (Sigma, Saint-Louis, Missouri), α-sarcomeric-actinin clone EA-53 (Sigma), muscle actin clone HHF35 (DAKO), troponin I (Santa Cruz Biotechnologies, CA) or troponin T (Sigma) diluted 1/100 in PBG (PBS containing 0.5% BSA and 0.2% gelatin); or (2) a 5 min incubation with rhodamine phalloidin (Molecular Probes) diluted 1/300 in PBG. After the reaction with FITC-conjugated (diluted 1/200 in PBG) or Cy3-conjugated (diluted 1/400 in PBG) secondary antibodies (Jackson ImmunoResearch, West Grove, PA),

followed a 5 min incubation with DAPI diluted 1/1000 in PBS. Coverslips were mounted on slides with mounting medium (2.4 g Mowiol, 6 g glycerol, 12 ml 0.2 M Tris/HCl pH 8.5) containing 2.5% DABCO (Sigma), and observed by fluorescence microscopy (Axiophot microscope, ZEISS) or confocal microscopy (Axiovert 100M microscope, ZEISS). For fluorescence pictures, images were acquired using the AnalySIS software (Soft Imaging System GmbH, Germany) and for confocal pictures, using the LSM 510 software (ZEISS).

IV. D. Western blotting

After treatment with growth factors and/or cytokines, cells were lysed in RIPA buffer (50 mM Tris-HCl, pH 7.4; 1% NP-40; 150 mM NaCl; 0.25% sodium deoxycholate; 1 mM Na_3V0_4; 1 mM NaF; 1 mM PMSF plus, Roche Diagnostics; 1 μg/μl pepstatin; "Mini-Complete" protease inhibitor cocktail, Roche Diagnostics). Protein concentrations were measured with the Micro BCA protein assay kit (PIERCE, Switzerland) and equal amounts, 20 μg, were resolved by SDS-PAGE and transferred to PVDF membranes (Millipore AG, Switzerland). The membranes were blocked with TBST (25 mM Tris, 150 mM NaCl, pH 7.4 containing 0.1% Tween)- milk (with 5% nonfat dry milk) and probed either overnight at 4°C with primary antibodies against total and phosphorylated Akt, ERK1/2 or p38 MAPK (Cell Signaling Technology Inc, Danvers, MA) diluted $1/10^3$ in TBST-5% BSA, or 1 h at room temperature with primary antibody against myogenin (Santa Cruz Biotechnologies, CA) diluted 1/200 in TBST-milk. After reaction with secondary antibodies (diluted $1/10^4$ in TBST-milk) conjugated with horseradish peroxidase (Jackson ImmunoResearch, West Grove, PA), the bands were visualized using enhanced chemiluminescence reagents (Supersignal West Pico, PIERCE, Switzerland) and exposure to autoradiographic film. For quantification, the intensity of each phosphorylated protein band was normalized to the corresponding total protein band by image analysis using the NIH Image 1.62 software.

IV. E. Real-time PCR

Total RNA was isolated from treated and non treated C_2C_{12} myotubes using Tri-Reagent (Molecular Research Centre, Cincinnati, OH) following the manufacturer's protocol. RNA concentrations and purity were determined by spectrophotometric

analyses, then subjected to cDNA synthesis with the reverse transcriptase OMNISCRIPT RT kit (Qiagen), using 0.5 μg/μg RNA of random hexamers (Promega) and 20 U/tube RNase inhibitor (Promega), according to the manufacturer's instructions. A total RNA of 1 μg was reverse transcribed into cDNA and 2.5 ng cDNA was added to each reaction of the real-time PCR. The primers used were designed with assistance of the computer program Primer Express (Applied Biosystems, Foster City, CA) and synthesized by Microsynth AG (Switzerland). Primer sequences for Atrogin-1 and β-tubulin genes are given in Table 1. The real-time PCR analysis was performed on a Light-Cycler apparatus (Applied Biosystems) using a ITaQ Syber Green kit (BioRad, Reinach, Switzerland). The thermocycling conditions were as follows: 95°C 2 min, 95°C 15 sec and 60°C 45 sec. Each reaction was performed in a final volume of 25 μl containing 5 μl of cDNA sample and 300 nM of each primer, in addition to ready-to-use ITaQ Syber Green mix. After the PCR reaction, GeneAmp 5700 SDS software (Applied Biosystems) plotted a profile of fluorescence against the cycle number. An arbitrary threshold of fluorescence was set within the exponential phase of amplification. The cycle at which the amplification of the product exceeded this threshold was determined and used as Ct value for calculation. The expression of Atrogin-1 within each sample was quantified relative to the β-tubulin gene as a reference. Calculations were performed using the Pfaffl method ratio formula [48] displayed in Table 2.

Table 1. Primers sequences for Atrogin-1 and β-tubulin genes

	Atrogin1 gene	β-tubulin gene
Primers: • forward • reverse	5'-CCATCAGGAGAAGTGGATCTATGTT-3' 5'-GCTTCCCCCAAAGTGCAGTA-3'	5'-CCGGACAGTGTGGCAACCAGATCGG-3' 5'-TGGCCAAAAGGACCTGAGCGAACGG-3'
Species	Atrogin1/MAFbx rat cDNA	β-tubulin mouse cDNA

Table 2. Pfaffl method formula and real-time PCR efficiencies of studied genes

ratio formula	$\frac{(E_{target})^{\Delta Ct\ target\ (control-sample)}}{(E_{ref})^{\Delta Ct\ ref\ (control-sample)}}$
E_{target}: Atrogin-1	1.9661
E_{ref}: β-tubulin	1.9257

IV. F. Statistical analysis

The data presented are the mean ± standard error (S.E.). Statistical analysis was performed by t-test and values of $P < 0.05$ were considered significant.

V. CHARACTERIZATION OF THE CELL CULTURE MODELS

The following section presents a morphological description of the two *in vitro* models used in this work.

V. A. Ventricular adult rat cardiomyocytes

The ventricular adult cardiomyocytes (vARCs) are terminally differentiated cells. Immunostainings of different contractile structures were performed to characterize the model and images were visualized by fluorescence or confocal microscopy.

Freshly isolated vARCs are shown in Fig 3A with their characteristic rod shape and cross striations. DAPI nuclear staining shows the presence of two distinct nuclei in each cell (Fig 3C). One of the major characteristics of contractile cells is the repeated arrangment of contractile proteins in the myofibrils. Numerous proteins are associated with these structures, but the predominant ones are actin and myosin. Rhodamine phalloidin was used to stain actin filaments in the vARCs, as shown in Fig 3B. Further stainings with specific antibodies against troponin I (Fig 3D), muscle actin (Fig 3E) and α-sarcomeric-actin (Fig 3F) were performed which allowed to clearly distinguish the repeated contractile structures associated with the myofibrils.

In long-term culture, vARCs undergo a de-differentiation and re-differentiation process. Upon attachment to the substratum, the originally rod-shaped cells flatten and spread out. During the first 2 days the preexisting myofibrils are degraded, and then new myofibrils start being assembled and grow out into the expanding cell periphery. After 4-6 days the vARCs resume rhythmic contractility. In addition, long-term cultured vARCs reexpress isoforms of proteins normally occurring during fetal cardiac development [49]. Fig 4 shows phase contrast images of vARCs cultured during 7 days.

The results presented in this work were performed on freshly isolated vARCs treated for a maximum of 24 h.

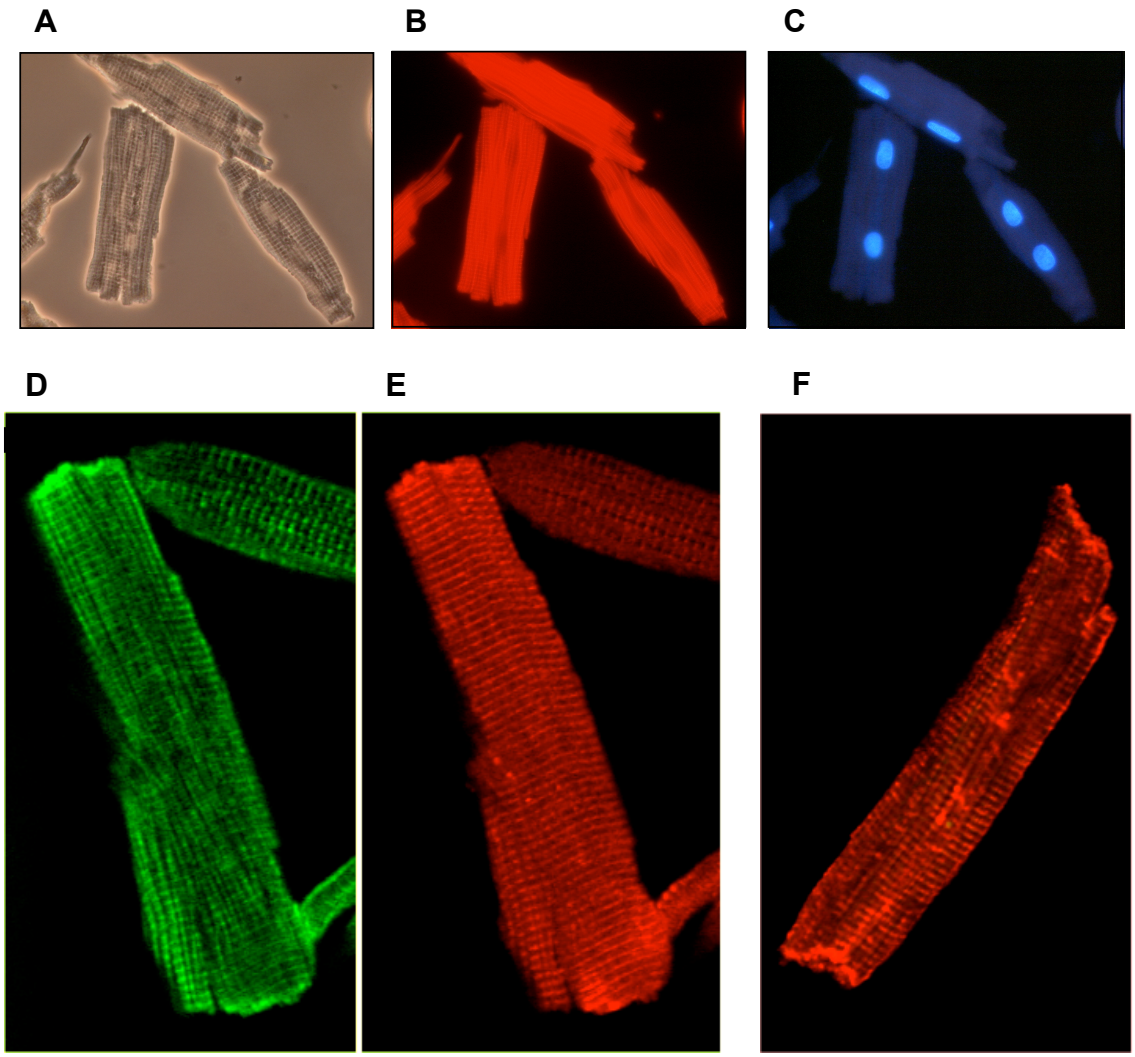

Fig 3. Morphological characterization of ventricular adult rat cardiomyocytes

Phase contrast microscopy of freshly isolated vARCs show rod-shaped cells and cross striations (**A**), actin filaments are stained by rhodamine phalloidin (**B**) and DAPI staining shows two nuclei per cell (**C**); *A-C: magnification x40*. Confocal microscopy of troponin I (**D**), muscle actin (**E**) and α-sarcomeric-actin (**F**) immunostainings shown sarcomeric structures in one vARC; *D-F: magnification x63*.

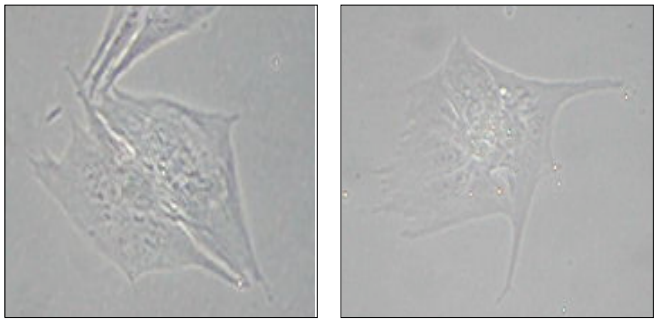

Fig 4. Ventricular adult rat cardiomyocytes in long-term culture

Phase contrast microscopy of vARCs cultured for 7 days in DMEM containing 20% FCS. The cells resumed contractility and changed shape along time. *Magnification x40*

V. B. C_2C_{12} murine myoblast cell line

The C_2C_{12} myoblasts can fuse and form myotubes in specific culture conditions (see details in section IV.B). In Fig 5B we show that myotubes are formed after myoblasts (Fig 5A) were set to differentiate for 6 days. Morphological and biochemical characterization of C_2C_{12} differentiation were realized by performing myogenin immunoblots and various contractile protein stainings.

The expression of myogenin is correlated with induction of the differentiation process. Fig 5C shows elevated myogenin protein levels in differentiated myotubes at D6. DAPI staining shows that many nuclei can be observed in the myotubes, providing evidence of cell fusion during the differentiation process (Fig 5E,G). In the differentiated C_2C_{12} cells, α-sarcomeric-actin staining is stronger than in proliferating cells (Fig 5F). Immunostaining of other contractile proteins of the myotubes, namely troponin T (Fig 5H,I) and α-sarcomeric-actinin (Fig 5J,K), shows distinctive cross striations. These structures are specific of terminally differentiated striated muscle cells.

In the present work C_2C_{12} analyses were performed solely on differentiated myotubes.

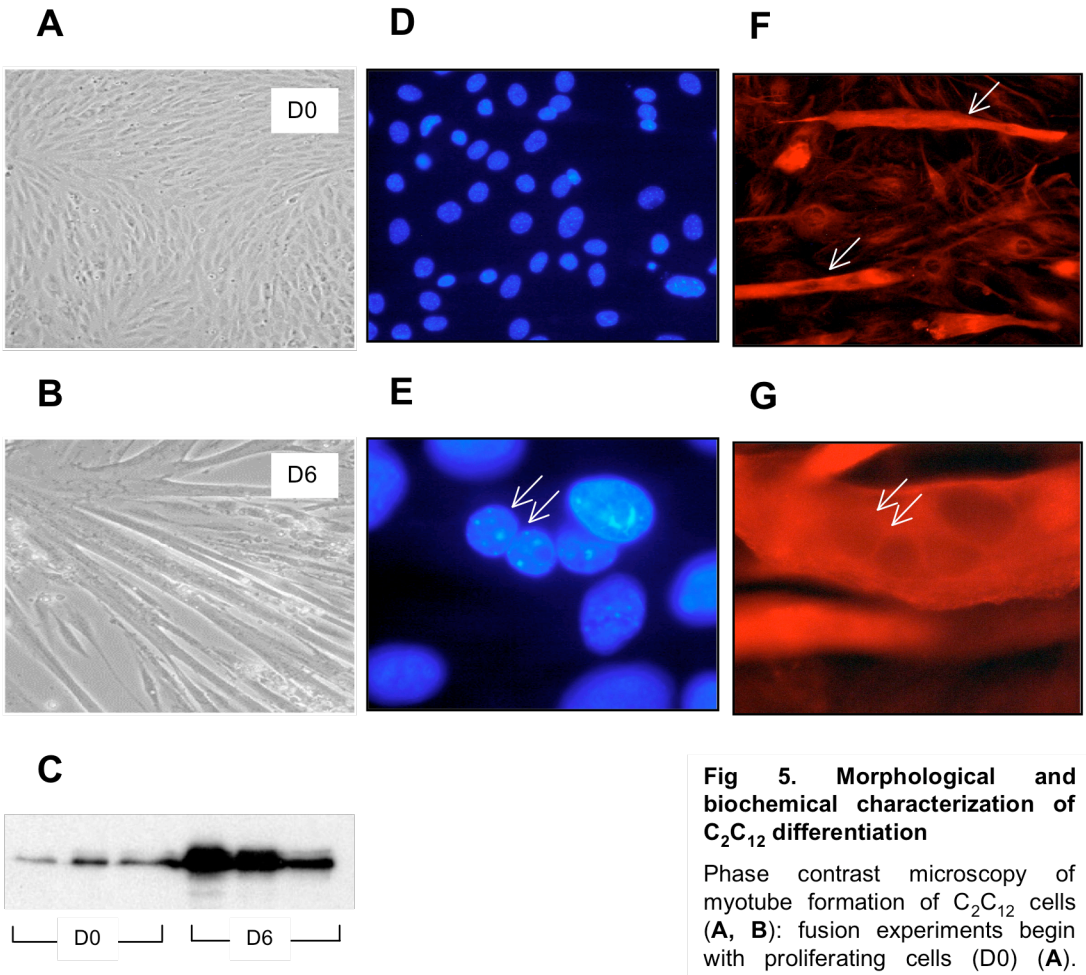

Fig 5. Morphological and biochemical characterization of C_2C_{12} differentiation

Phase contrast microscopy of myotube formation of C_2C_{12} cells (**A, B**): fusion experiments begin with proliferating cells (D0) (**A**). After 24 h, cultures are incubated in differentiation medium. After 6 days of differentiation (D6) the majority of cells have fused to myotubes (**B**); *A, B: magnification x40*. Immunoblot analysis of myofibrillar protein expression (**C**): myogenin expression is correlated with induction of the differentiation process. Immunostaining of C_2C_{12} cells nuclei with DAPI (**D, E**) or α-sarcomeric-actin (**F, G**), troponin T (**H, I**), α-sarcomeric-actinin (**J, K**): actin staining is strong in differentiated cells (arrows) compared to the undifferentiated cells (**F**). During differentiation, multiple nuclei can be seen per differentiated myotube after the fusion process (arrows, **E, G**). In the differentiated myotubes, cross-striated structures can be seen (arrows, **I, J**). *D, F, H, J: magnification x40; E, G, I, K: magnification x63.*

VI. RESULTS

The results section of this thesis is built in three parts consisting of two manuscripts submitted for publication (VI.A and VI.B) and one in preparation (VI.C). Each article is preceded by a brief summary and followed by a concise conclusion highlighting the major findings. The results from complementary experiments are presented and discussed after the first and the third article (section VI.A and VI.C).

VI. A. Effects of TNF-α on the IGF-I axis in adult cardiomyocytes

In the first part, we analyze the effects of TNF-α on the IGF-I system, namely regulation of the IGFBPs and modulation IGF-I-induced signaling pathways in cardiac myocytes.

VI. A. 1. TNF-α downregulates IGFBP-4 mRNA expression

The article presented hereafter is a study based on the hypothesis that TNF-α interferes with the IGF-I axis by regulating the IGFBPs in adult rat cardiomyocytes. To verify this, we analyzed the effects of TNF-α on IGFBP-4 and IGFBP-5 mRNA. We show that TNF-α decreases IGFBP-4 mRNA levels in a concentration- and time-dependent manner, but has no effect on IGFBP-5 mRNA expression. We also analyzed cell viability as well as protein levels of apoptotic markers, and we show that TNF-α has immediate positive effects on cardiomyocyte viability. However, longer-term (24 h) incubation with TNF-α resulted in decreased cell viability and was accompanied by enhanced expression of apoptotic markers.

TNF-α reduces IGFBP-4 mRNA expression and enhances viability of adult rat cardiomyocytes

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Thomas Dieterle, and Marijke Brink

Abstract

Tumor necrosis factor-alpha (TNF-α) plays an important role in adaptive myocardial responses to injury but also has the potential to produce cardiac decompensation. Insulin-like growth factor (IGF)-I on the other hand is fundamental to cardiac function because it positively regulates a range of cellular mechanisms such as inotropy, myofibrillogenesis and cell survival. The mechanisms behind the dual responses to TNF-α in the heart are poorly understood and therefore subject of the present study. We hypothesized that TNF-α acts on cardiomyocytes by interfering with the IGF-axis, in particular with the IGF binding proteins (IGFBPs), proteins that bind the IGFs with high affinity and thereby modulate their activity. After 3 and 6 h, TNF-α treatment decreased IGFBP-4 mRNA levels, but not those of IGFBP-5, and this was associated with increased cell viability. After 24 h, IGFBP-4 was still significantly lowered, viability and expression of the anti-apoptotic protein Bcl-2 were markedly reduced, whereas the pro-apoptotic Bax was increased. IGFBP-4 is described to consistently inhibit IGF-I, thus our results suggest enhanced IGF-I activity in our model. The down-regulation of IGFBP-4 may represent an important novel mechanism by which TNF-α exerts its beneficial effects, and could contribute to limiting cardiac damage after myocardial injury.

Key words: TNF-α, cardiomyocyte, IGFBP, viability, apoptosis

Introduction

Understanding the development of cardiovascular diseases, one of the major causes of morbidity and mortality in industrialized countries, can lead to better prevention and therapeutic strategies. All forms of myocardial injury or stress initially lead to compensatory cardiac myocyte hypertrophy, accompanied by alterations in gene expression, as well as apoptosis and fibrosis. These events mediate the decline in myocardial function that occurs with the transition from hypertrophy to failure. A multitude of stressors and humoral factors have been associated with development of cardiac failure in animal models and humans, amongst which pro-inflammatory cytokines, such as tumor necrosis factor-alpha (TNF-α), interleukin-1, and interleukin-6 (reviewed in [1]).

These cytokines are expressed locally in cardiac tissue in response to various types of injury and play an important role in initiating adaptive myocardial responses. However, they also have the potential to produce cardiac decompensation when expressed at high concentration or for long duration (reviewed in [2]). TNF-α, the factor that we focus on in our study, has pleiotropic biological effects, its plasma levels are elevated in a variety of cardiovascular diseases (reviewed in [3]) and moreover, TNF-α can be produced by cardiac myocytes and non-myocytes in response to hemodynamic pressure overload [4]. Controversy exists with respect to the effects induced by TNF-α in cardiomyocytes, and the mechanisms involved remain poorly defined.

Insulin-like growth factor-I (IGF-I) is one of the other factors that increase with the onset of left ventricular pressure overload. The IGF axis is directly involved in post-infarct events by maintaining cardiac function via induction of adaptive hypertrophy and decreasing apoptotic cell death. Both effects have been demonstrated in many other cell types (reviewed in [5]). IGF-I activity is modulated by the IGF binding proteins (IGFBPs). The IGFBPs are a family of six proteins, which interact with high affinity with the IGFs. Their affinity for the IGFs is 2- to 50-fold greater than for the IGF-I receptor. The traditional view of the IGFBPs is that they function as carrier proteins for the IGFs in the circulation, regulating their turnover and transport towards the tissue. At the tissue level, IGFBPs compete with IGF receptors for IGF binding. The significant difference in the biochemical characteristics of these proteins accounts for the differences in their biological actions [6-10].

IGFBP-4, the smallest IGFBP, is expressed by a wide range of tissues and cell types. Its expression is regulated by different mechanisms in a cell-specific

manner. Several lines of evidence suggest that IGFBP-4 functions as a purely inhibitory protein *in vitro* and *in vivo*, and can exert its actions via IGF-dependent but possibly also via IGF-independent pathways (reviewed in [11]). IGFBP-5 is the most conserved IGFBP across species. Like it is well known for IGFBP-3, circulating IGFBP-5 also forms a ternary complex with one IGF molecule and a third protein termed acid labile subunit (ALS). IGFBP-5 is considered to be a stimulatory binding protein in at least two systems, bone and cultured vascular smooth muscle cells, but inhibitory actions have also been reported. The most important regulator of IGFBP-5 expression and function is IGF-I, however in many situations IGFBP-5 exerts biological activities in the absence of IGFs (reviewed in [12]).

Several studies have reported that cytokines impair the IGF-I axis and analyzed the mechanisms in various *in vitro* models [13, 14]. It has also been reported in an *in vivo* rat model, that intravenous infusion of TNF-α decreases IGF-I plasma concentrations and IGF-I mRNA expression mainly in muscle tissue including the heart [15]. The purpose of the present study is to test if TNF-α can interfere with the IGF-I axis in adult rat cardiomyocytes by regulating IGFBP-4 and IGFBP-5 expression. Consistent with earlier reports, we show that TNF-α can induce apoptotic mechanisms and decrease cell viability in particular after long-term incubation. In addition, however, we show that TNF-α has immediate early positive effects on cardiomyocyte viability, and we demonstrate that TNF-α decreases IGFBP-4 mRNA expression in a concentration- and time-dependent manner. Our data suggest a role for IGFBP-4 in mediating part of the beneficial effects of TNF-α.

Materials and methods

Cardiomyocyte isolation and culture

The protocol was performed in accordance with institutional guidelines for the care of experimental animals. Ventricular cardiomyocytes were isolated from 6-week-old Sprague-Dawley rats (IFFA Credo, L'Arbresle, France) using the method described by Belke and coworkers [16]. Cells were plated onto 100 mm dishes or 96 well plates (Sarstedt AG, Switzerland) coated with 20 μg/ml laminin (Sigma-Aldrich, Germany). The culture medium consisted of DMEM containing 1000 mg/L glucose (Invitrogen AG, Basel, Switzerland) with or without 20% heat-inactivated fetal calf serum (Amimed AG, Basel, Switzerland). Treatment with recombinant mouse TNF-α(R&D Systems, Minneapolis, MN) took place immediately after isolation.

RNase protection assay

Total RNA was isolated from treated and non-treated cardiomyocytes using Tri-Reagent (Molecular Research Centre, Cincinnati, OH) and following the manufacturer's protocol. RNA concentrations and purity were determined by spectrophotometric analyses. Solution hybridization/ribonuclease (RNase) protection assays (RPA) were performed as described previously [17]. Briefly, IGFBP-4, IGFBP-5 and 18S [^{32}P]UTP-labeled RNA probes synthesized from DNA templates using T7 polymerase were hybridized overnight at 42°C with 10 μg of total RNA. Probes and other single-stranded RNA were then digested with RNases. The remaining RNase-protected probes were proteinase K-treated, phenol-extracted and resolved on denaturing polyacrylamide gels. The autoradiograms were quantified by densitometry using ImageQuant software (Molecular Dynamics, Sunnyvale, CA). The value of each hybridized probe was normalized to that of 18S as an internal control.

Cell viability assay

Cell viability was quantified using a colorimetric assay (Cell Proliferation Reagent WST-1, Roche Diagnostics AG, Switzerland), which measured mitochondrial dehydrogenase activity. The assay was performed following the manufacturer's instructions.

Western immunoblotting

After treatment, cells were lysed in RIPA buffer, containing 50 mM Tris-HCl, pH 7.4, 1% NP-40, 150 mM NaCl, 1 μg/μl pepstatin, 1 mM PMSF plus and "Mini-

Complete" protease inhibitor cocktail (Roche Diagnostics). Protein concentrations were measured with the Micro BCA protein assay kit (PIERCE, Switzerland) and equal amounts, 20 μg, were resolved by SDS-PAGE and transferred to PVDF membranes (Millipore AG, Switzerland). The membranes were blocked with 5% nonfat dry milk in Tris-buffered saline/0.1% Tween (TBST) and probed with primary antibodies against Bax NT (Upstate, Lucerna-Chem AG, Switzerland) or Bcl-2 ΔC-21 (Santa Cruz Biotechnologies, CA) diluted 1/1000 and 1/250, respectively. After reaction with secondary antibodies (diluted 1/10000 in TBST) conjugated with horseradish peroxidase (Jackson ImmunoResearch, West Grove, PA), bands were visualized using enhanced chemiluminescence reagents (Supersignal West Pico, PIERCE, Switzerland) and exposure to autoradiographic film.

Statistical analysis

The data presented are the mean ± S.E. The statistical significance of differences between treatments was determined by one- or two-way ANOVA analysis of variance. Values of $P < 0.05$ were considered statistically significant.

Results

TNF-α decreases IGFBP-4 but not IGFBP-5 mRNA expression

Freshly isolated adult cardiomyocytes were incubated for 24 h in the presence or absence of 20 ng/ml of TNF-α. RNA was extracted and analyzed by RNase protection assays. Fig. 1 shows a representative autoradiogram, which demonstrates that the mRNA of IGFBP-4 and IGFBP-5 are both readily detectable in adult rat cardiomyocytes under baseline (control) conditions. In the presence of TNF-α, levels of IGFBP-4 were markedly decreased, while no change in IGFBP-5 mRNA expression was observed (n=3). The TNF-α effect was obtained in serum free medium as well as in medium containing 20% fetal calf serum. The rest of our experiments were carried out in serum free medium.

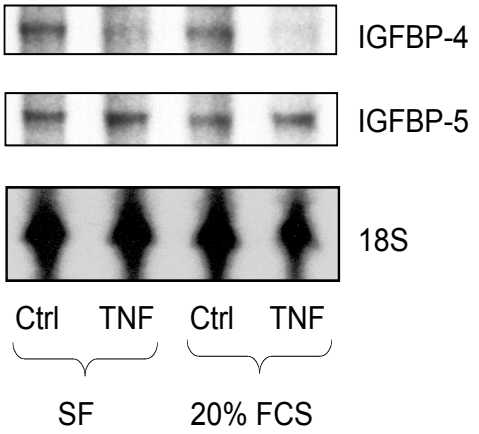

Fig. 1. TNF-α decreases IGFBP-4 but not IGFBP-5 mRNA levels.

Ventricular rat cardiomyocytes were treated for 24 h with TNF-α (20 ng/ml) in serum free (SF) medium or in medium containing 20% fetal calf serum (FCS). IGFBP-4, IGFBP-5 and 18S mRNA analysis was performed by RNase protection assay (RPA) and is shown in a representative experiment.

Concentration and time dependence of TNF-α effects

The TNF-α-induced down-regulation of IGFBP-4 mRNA expression was concentration dependent with a significant decrease of 40% already achieved at 1 ng/ml. The effect was maintained at higher concentrations of TNF-α (Fig. 2). Multiple time course experiments demonstrated that in comparison to cells taken right after their isolation from the heart (time point 0), IGFBP-4 mRNA levels decreased during the first 3 h in culture medium. This was followed by stabilization at approximately 50% expression of the levels at time point 0. Incubation with TNF-α caused a significant additional reduction in IGFBP-4 mRNA levels ($P < 0.01$), an effect already apparent at 3 h and maintained up to 18 h (Fig. 3), when lowest levels were reached.

At this time point, IGFBP-4 mRNA in TNF-α -treated cells were decreased by 58% ($P < 0.001$).

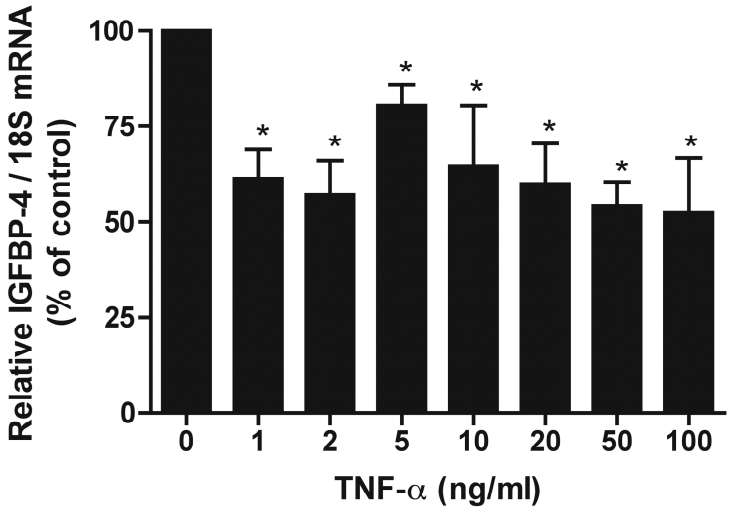

Fig. 2. Concentration dependence of TNF-α-induced downregulation of IGFBP-4 mRNA.

Ventricular rat cardiomyocytes were treated for 24 h with increasing concentrations of TNF-α (1-100 ng/ml). IGFBP-4 mRNA analysis was performed by RPA and quantification of the autoradiograms is shown as percentage of untreated control after normalization for 18S mRNA. Values represent mean ± S.E. of 3-5 independent experiments. * $P < 0.05$ vs. control.

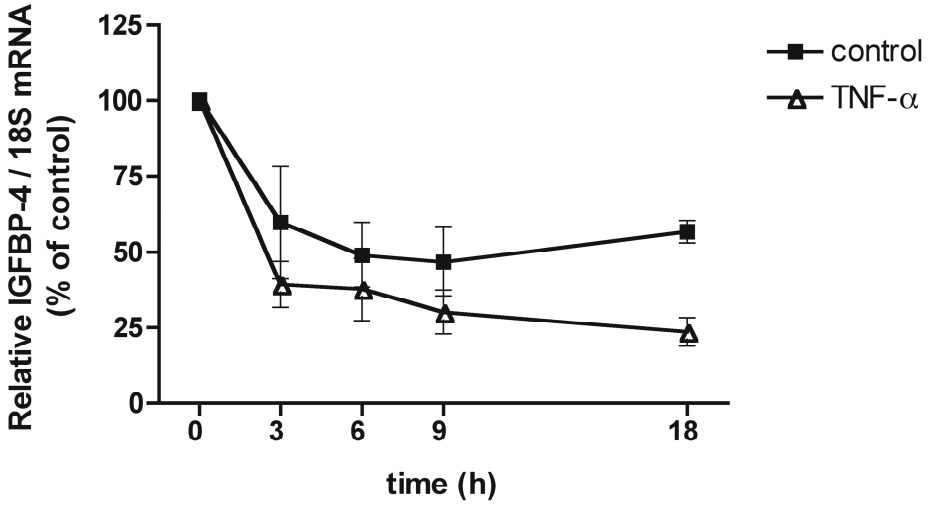

Fig. 3. Time-course of TNF-α-induced downregulation of IGFBP-4 mRNA.

Ventricular rat cardiomyocytes were treated with 20 ng/ml TNF-α, and cells were harvested at the indicated times between 0 and 18 h. IGFBP-4 mRNA analysis was performed as described for Fig. 1 and 2, and is shown as percentage of untreated control at 0 h after normalization for 18S mRNA. Values represent the mean ± S.E. of 3-4 independent experiments.

Effect of TNF-α on cardiomyocyte viability and markers of apoptosis

In parallel to the IGFBP-4 mRNA expression measurements, viability assays were performed under the same conditions. At early time points, 3 and 6 h, TNF-α markedly increased cell viability with a maximal effect at 6 h where viability was 277% higher than in control cells measured at the same time point. An opposite effect was observed after 24 h of treatment when TNF-α decreased cardiomyocyte viability by 66% (Fig. 4A). At this time point, we also analyzed the expression of two apoptotic markers. We show that in the presence of TNF-α, pro-apoptotic Bax protein levels were increased whereas anti-apoptotic Bcl-2 protein levels were decreased (Fig. 4B).

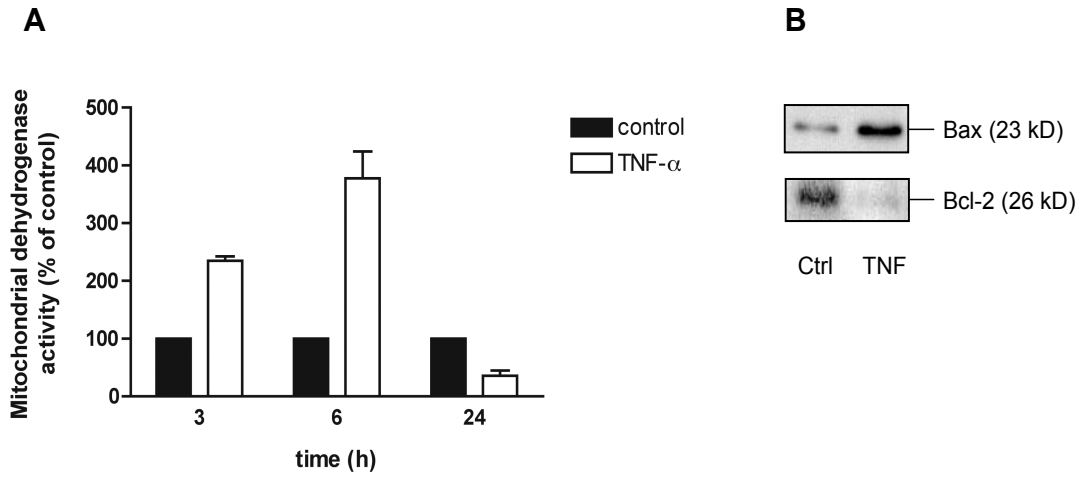

Fig. 4. Effect of TNF-α on cardiomyocyte viability and apoptotic markers.

(A) Ventricular rat cardiomyocytes were treated with 20 ng/ml TNF-α, cell viability was determined by measuring mitochondrial dehydrogenase activity at the indicated times and is shown as percentage of untreated control. Values represent mean ± S.E. of quintuplet measurements. (B) Cardiac myocytes were treated for 24 h with 20 ng/ml TNF-α. Bax and Bcl-2 protein analysis was performed by Western blot and is shown in a representative blot.

Discussion

In the present study, we showed that TNF-α lowers IGFBP-4 mRNA expression in adult rat cardiomyocytes from as early as 3 h of treatment up to 24 h. Our readout, measured as mitochondrial activity, was cell viability which was enhanced by TNF-α treatment at early time points (3 and 6 h) but was decreased after 24 h of treatment. Moreover, expression levels of two apoptotic markers, Bax and Bcl-2, indicated the activation of apoptotic cascades after 24 h of exposure to TNF-α.

Although IGF-I itself has been attributed a central role in many aspects of cardiomyocyte cell biology, our study is the first to report on the gene expression of IGFBP-4 and IGFBP-5 in cardiomyocytes, and to describe the specific down-regulation of IGFBP-4 but not IGFBP-5 by TNF-α. In contrast to TNF-α, incubation with angiotensin II did not change the expression of this binding protein in cardiomyocytes (data not shown). Since IGFBP-4 is the only binding protein described to consistently inhibit IGF-I [11], our finding suggests that IGF-I activity is enhanced in response to the cytokine TNF-α. The enhanced cell viability that we observed at early time points may therefore be related to the IGFBP-4 lowering effect of TNF-α. IGFBP-4 mRNA expression was significantly reduced also after 24 h of exposure to TNF-α; nevertheless cell viability was markedly reduced at that time point. Thus, a reduction in IGFBP-4 by itself is not sufficient to improve cell viability. At those later time points, TNF-α likely reduces viability by additional effects, which may include down-regulation of IGF-I, IGF-II and the IGF-I receptor. Indeed, the relative expression levels of the IGFs, the IGF-I receptor and IGFBPs together will ultimately determine the activation state of IGF signaling pathways that modulate cardiomyocyte viability. It remains to be established which other mechanism is activated upon long term TNF-α treatment that overrules TNF's immediate protective effects on the IGF system.

Our finding that TNF-α increases the pro-apoptotic protein Bax and decreases the anti-apoptotic Bcl-2 is consistent with reports on neonatal and adult cardiomyocytes, which describe that TNF-α induces apoptosis [18, 19]. Activation of these apoptotic mechanisms is most likely the cause of the reduced cardiomyocyte viability that we measured with sustained TNF-α treatment. These studies, together with a range of *in vivo* reports have initially led to the view that TNF-α may be harmful to the heart and contributed to the rationale behind clinical studies targeting TNF-α in heart failure patients. However, several recent studies provided evidence

for beneficial next to detrimental roles of TNF-α. Transgenic mice over-expressing TNF-α in the heart developed ventricular hypertrophy and dilation, fibrosis, and overt congestive heart failure. In this model, TNF-α induced both pro- and anti-apoptotic pathways resulting in increase of apoptosis primarily in non-myocytes. The study concluded that, *in vivo*, TNF-α by itself does not induce myocyte apoptosis [20]. Consistently, a recent study undertaken on samples from human infarcted myocardial tissue showed that cardiomyocyte loss in the acute stage of myocardial infarction was due to apoptosis but this was not directly triggered by TNF-α[21]. Several lines of evidence among which the direct activation by TNF-α of the transcription factor nuclear factor-kappa B, a mediator of anti-apoptotic pathways, support that TNF-α can activate rapid protective pathways in cardiomyocytes [2, 20]. The emerging idea from these accounts, and in line with the data of our present study, is that TNF-α expression in the acute stage in response to stress may be beneficial whereas persistent expression in chronic stages, or exposure to excessive amounts of the cytokine, may be harmful to the myocardium [22].

Indeed, whether a cell benefits or suffers following exposure to TNF-α may not solely be a function of the time of exposure, but is likely also related to the concentrations of TNF-α that it gets exposed to. Several studies have addressed this issue: murine C_2C_{12} skeletal myotubes and rat L6 myotubes both displayed a clear dual behavior upon TNF-α treatment, with both studies supporting that TNF-α can elicit anabolic as well as catabolic effects. In the first model, low TNF-α concentrations decreased both total and myofibrillar protein content, whereas higher concentrations increased protein content [23]. The second model showed that intermediate concentrations of TNF-α increased total and myofibrillar protein content, while lower and higher concentrations decreased protein content [24]. Two cell surface receptors with distinct affinities mediate the effects of TNF-α, TNFR-1 and TNFR-2. Both receptor subtypes are present in human and rat cardiac myocytes, however TNFR-1 is the main receptor subtype [25]. Dual responses to TNF-α have been attributed to activation of TNFR-1, whereas the TNFR-2 is believed to mediate cardioprotective effects only [26]. Hence, based on the fact that we detected detrimental effects, we conclude that the TNFR-1 receptor is most likely activated in our cells. The biphasic behavior of the cells with respect to their IGFBP-4 mRNA expression in response to increasing concentrations of TNF-α □□□□□□□□ that for the beneficial effect both receptors may be involved, but further studies are required to prove this.

To date, a down-regulation of IGFBPs by TNF-α has only been described in skeletal and vascular smooth muscle cells [13, 27, 28]. In 2000, Meadows and colleagues reported that in differentiating C_2C_{12} cells TNF-α blocked IGFBP-5 and IGF-II secretion, and related this to increased apoptotic cell death. The authors concluded that these TNF-α-induced mechanisms play a fundamental role in cancer-related muscle wasting [27]. Recently, Lang and colleagues confirmed the decrease in IGFBP-5 mRNA in TNF-α-treated C_2C_{12} myoblasts and established the *in vivo* relevance of the effect in the gastrocnemius from rats continuously infused with TNF-α [28]. In vascular smooth muscle cells, TNF-α reduced IGFBP-3 [13]. None of these studies, however, has reported an alteration in muscle IGFBP-4 mRNA abundance. With our study we provide the first description of IGFBP-4 down-regulation by TNF-α in a different muscle cell model, namely that of the adult cardiac myocyte. Our results provide support for the existence of a novel pathway through which TNF-α exerts its acute protective effects, and may contribute to new strategies aiming to limit or prevent cardiac damage in response to injury.

Acknowledgments

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VI. A. 2. Key findings and complementary results

Key findings

The previous study shows that TNF-α can activate apoptotic mechanisms and decreases cell viability in particular after long-term incubation. However, this cytokine also has immediate protective effects in adult rat cardiomyocytes.

Interplay between TNF-α and IGF-I signaling pathways

We have seen that TNF-α can enhance cardiomyocyte viability and that this cytokine might also increase IGF-I activity by downregulating IGFBP-4. Considering these findings, we propose to further investigate on the specific intracellular mechanisms triggered by TNFα and attempt to answer the following question: does TNF-α modulate specific signaling pathways induced by IGF-I? Additional experiments were performed to determine the effects of TNF-α on selected signaling pathways activated by IGF-I, and at the same time determine whether IGF-I also regulates pathways activated by TNF-α. These signaling pathways and their downstream cellular effects are summarized in Fig 6.

In many systems, activation of the “protein and lipid kinase” phosphoinositide 3-kinase (PI3K) and its downstream serine-threonine kinase effector, Akt (or Protein Kinase B), provide a potent stimulus for cell proliferation, growth, and survival. Akt is described as a downstream effector of the IGF-I receptor (IGF-IR). In the heart, Akt plays a key role in cardiomyocyte control of cell size, and regulation of survival and metabolism [50] (and reviewed in [24]). We analyzed phosphorylation states of Akt in cardiomyocytes.

Another signaling cascade thought to be an important regulator of cardiac hypertrophy is the mitogen-activated protein kinases (MAPK). The MAPK signaling cascade is initiated in cardiac myocytes by activation of G-protein coupled receptors, receptor tyrosine kinases (including the IGF-IR), and by stress stimuli, among which TNF-α. MAPK signaling pathways consist of a sequence of successively acting kinases that ultimately result in the dual phosphorylation and activation of terminal effector kinases [51] (and reviewed in [52]). We analyzed phosphorylation states of the following kinases: extracellular signaling-regulated kinase1/2 (ERK1/2) and p38, one of the ″stress responsive″ MAPK which is activated by TNF-α.

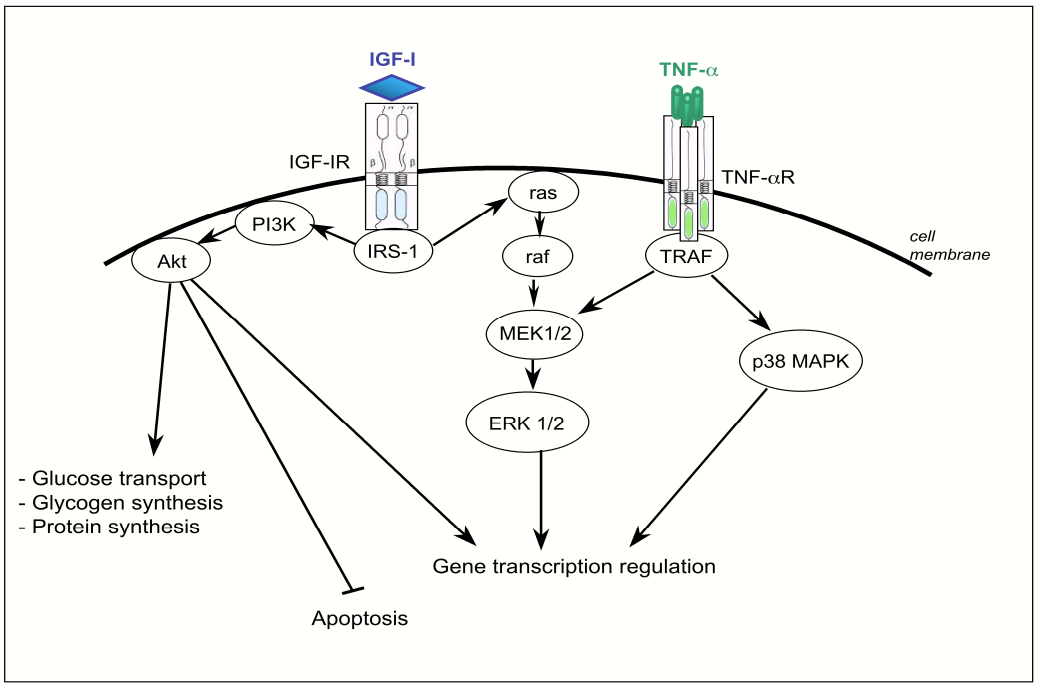

Fig 6. Intracellular signaling pathways induced by IGF-I and TNF-α

IGF-I binds to its receptor (IGF-IR) on the cellular surface and activates the receptor substrate (IRS)-1. The PI3K-Akt and ras-raf-MEK1/2-ERK1/2 signaling cascades are then activated. Akt induces glucose transport in the cell as well as protein and glycogen synthesis. Akt also inhibits apoptosis, and regulates gene transcription. TNF-α binds to its receptor (TNFα-R) and through the receptor-associated factor (TRAF) activates mitogen-activated ERK activating kinase (MEK1/2) and p38 MAPK. Both ERK1/2 and p38 MAPK are gene transcription regulators.

After isolation, the cardiomyocytes were kept for 3 h in serum free medium at 37°C in the presence of 5% CO_2 then incubated during 10 min with TNF-α (20 ng/ml), Des-IGF-I (40 ng/ml) or both together. Des-IGF-I is an analog of human IGF-I with reduced binding to the IGFBPs. Protein kinases phosphorylation was analyzed by Western blotting.

In our model we show a tremendous increase of Akt phosphorylation levels in response to Des-IGF-I treatment (Fig 7). TNF-α by itself markedly reduced Akt phosphorylation by approximately 60% (Fig 7). When the cells were treated with both TNF-α and Des-IGF-I, the IGF-I-induced increase dramatically dropped from 66-fold increase to 20-fold (Fig 7). TNF-α and Des-IGF-I alone induced phosphorylation of ERK1/2 (2- and 3-fold increase, respectively) in the adult cardiomyocytes (Fig 8). This increase was potentiated 10-fold when cells were co-incubated with TNF-α and Des-IGF-I (Fig 8). Like for the activation of Akt, opposite effects of TNF-α and Des-IGF-I were obtained with respect to the phosphorylation of p38 MAPK. TNF-α

induced a 2.5-fold increase in phosphorylation, while IGF-I decreased p38 MAPK phosphorylation by 50% (Fig 9). When cardiomyocytes were incubated with both compounds, Des-IGF-I inhibited the TNF-α-induced increase, thus lowering the phosphorylation to control levels (Fig 9).

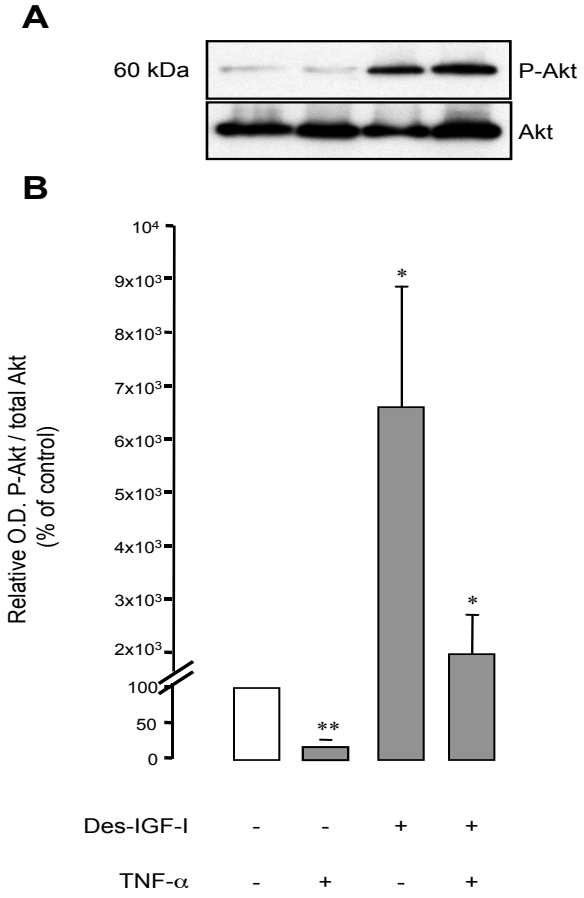

Fig 7. TNF-α interferes with IGF-I-induced Akt phosphorylation in adult cardiomyocytes

Adult rat cardiomyocytes were incubated with TNF-α (20 ng/ml), Des-IGF-I (40 ng/ml) or both together for 10 min. Cells were collected, phosphorylated as well as total Akt protein levels were analyzed by Western blot and are shown in a reprensentative experiment (**A**). 2-4 independent experiments were performed and results are expressed as percentage of untreated control (**B**). Values represent the mean ± S.E. *, significantly different from control, $P < 0.05$; **, significantly different from control, $P < 0.01$.

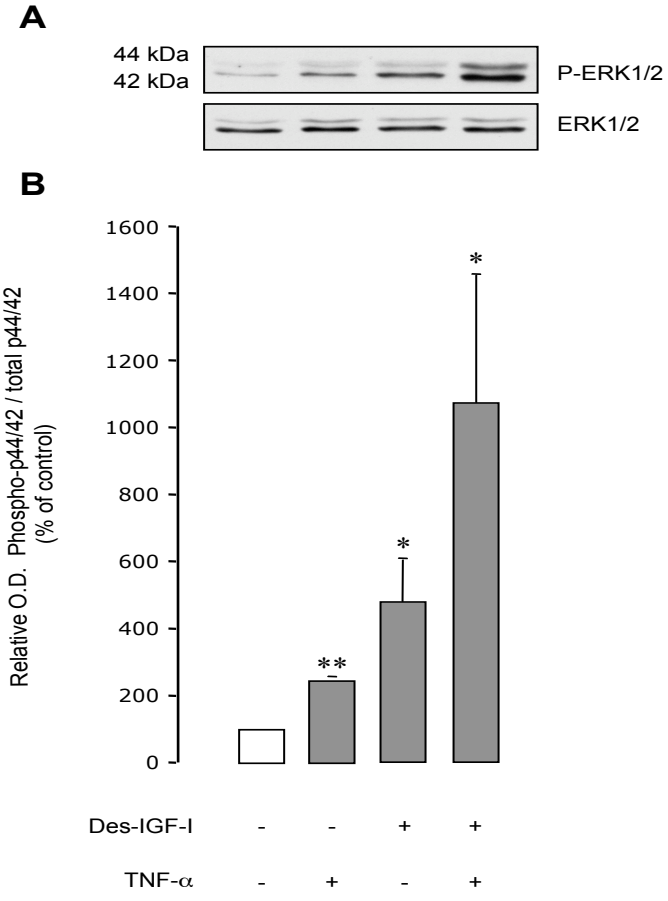

Fig 8. TNF-α and IGF-I potentiate ERK1/2 phosphorylation in adult cardiomyocytes

Adult rat cardiomyocytes were harvested after 10 min incubation with TNF-α (20 ng/ml), Des-IGF-I (40 ng/ml) or both. Protein levels of both phosphorylated and total ERK1/2 were measured as described in Fig 7 and shown in a representative experiment (**A**). Results of 3-5 independent experiments are shown (**B**) and expressed as percentage of untreated control. Values represent the mean ± S.E. *, significantly different from control, $P < 0.05$; **, significantly different from control, $P < 0.01$.

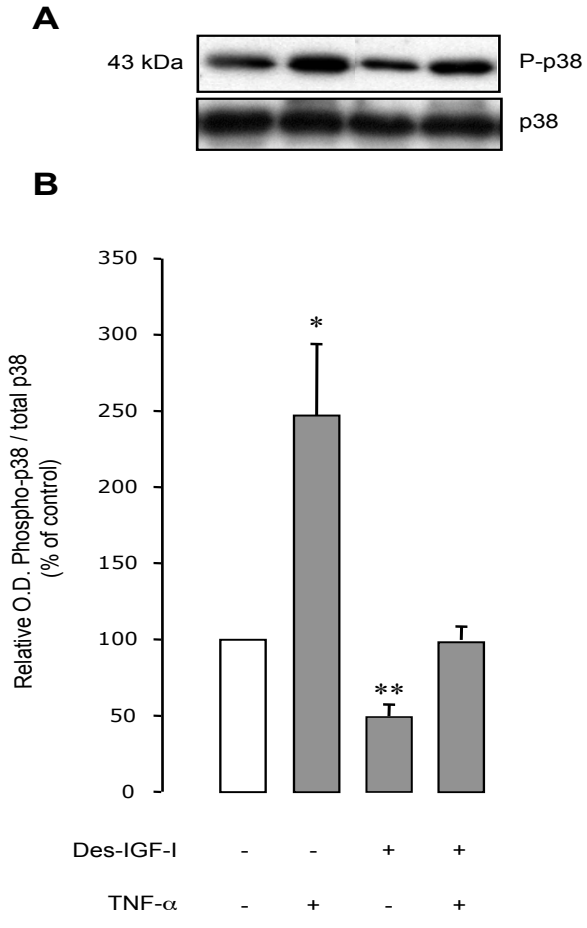

Fig 9. IGF-I inhibits TNF-α-induced phosphorylation of p38 MAPK in adult cardiomyocytes

Adult rat cardiomyocytes were treated as described in Fig 7 and 8. Protein levels of both phosphorylated and total p38 MAPK were measured by Western blot and shown in a representative experiment (**A**). 2-3 independent experiments were performed and results are expressed as percentage of untreated control (**B**). Values represent the mean ± S.E. *, significantly different from control, $P < 0.05$; **, significantly different from control, $P < 0.01$.

Altogether, these results show that:

- TNF-α interferes with the IGF-I Akt survival, metabolic and hypertrohic pathway
- IGF-I antagonises the TNF-α p38 MAPK stress activated pathway
- TNF-α and IGF-I have additional effects on ERK1/2 activation

Our data demonstrate that extensive crosstalk exists between TNF-α and IGF-I signaling in adult rat cardiomyocytes.

On the one hand, TNF-α and IGF-I antagonize each other in the Akt and p38 MAPK pathways. With Akt being a pro-survival and metabolic pathway [50], and p38 having inverse effects [52], these results may explain the opposed effects of IGF-I and TNF-α on cardiomyocyte survival: TNF-α triggers apoptosis [53, 54] whereas IGF-I inhibits it [55, 56].

On the other hand, TNF-α enhances the IGF-I-induced activation of the ERK1/2 pathway. This signaling cascade has been shown to promote cardiac hypertrophy *in vivo*, as demonstrated by transgenic mice with cardiac restricted overexpression of MEK1/2. Indeed, these animals were characterized by concentric hypertrophy, hyperdynamic function and resistance to apoptosis [51]. This effect was confirmed in a later *in vivo* study, and the same group showed that activation of the Raf/MEK/ERK pathway plays a major role in hypertrophic cardiac growth (via the initiation of protein synthesis) in adult cardiomyocytes [57]. Hence, our results suggest a potential beneficial role for IGF-I and TNF-α when expressed together in the cellular environment of cardiac myocytes. Studies in neonatal cardiomyocytes, have shown that TNF-α induces hypertrophic responses through activation of two different signaling pathways, including Akt [58, 59]. This shows differential regulation of the same cellular function by TNF-α throughout development.

VI. A. 3. Adult cardiac myocytes: KEY FINDINGS

Taken together with our previous observation that TNF-α reduces the inhibitory IGFBP-4, our data on the TNF-α-induced increase in phosphorylation of ERK1/2 MAPK (in the presence of IGF-I) support that TNF-α may have an important cardioprotective function in adult cardiomyocytes.

VI. B. In vivo cardiac IGFBPs regulation by hypertension

Our previous findings have provided evidence of specific regulation of IGFBP-4 in cardiomyocytes by a factor involved in the progression of heart failure, namely TNF-α. Our next step was to investigate on the *in vivo* relevance of IGFBP regulation during the cardiac remodeling process. In the second part of the results, we emphasize on hypertension and its effects on cardiac IGFBPs expression in two animal models.

The following article describes mRNA expression of IGFBP-3, -4 and -5 in left ventricle of two different models of hypertension, namely in rats infused with Ang II through osmotic minipumps and in salt-fed Dahl salt-sensitive (DSS) rats. Ang II infusion decreased IGFBP-3 and increased IGFBP-4 mRNA levels, whereas IGFBP-5 remained unchanged. Hypertensive DSS rats had higher IGFBP-4 and IGFBP-5 mRNA levels compared to normotensive Dahl resistant rats. Salt-induced hypertension, however did not cause any change in IGFBP-3 mRNA levels. The increase in IGFBP-4 transcripts in both models suggests a pressure-dependent response, whereas the Ang II-induced IGFBP-3 decrease is most likely mediated by pressure-independent mechanisms.

Differential Regulation of IGF Binding Protein-3, -4 and -5 Gene Expression in Angiotensin II- and Salt-Induced Cardiac Hypertrophy

Claire Murigande, Isabelle Plaisance, Christian Morandi, Bianca Mottironi, Nathalie Rosenblatt, Thomas Dieterle, Dagmar I Keller, Marijke Brink

Abstract

Insulin-like growth factor (IGF)-I is an important endocrine/autocrine mediator of cardiac hypertrophy. A family of highly specific IGF binding proteins (IGFBPs) modulates the biological activity of IGF-I, but to date the regulation and function of these binding proteins has not been assessed during the process of remodeling that follows hypertension-induced increases in workload. In this study we have measured left ventricular mRNA levels of IGFBP-3, -4 and –5 in two different models of hypertension, namely in rats infused with angiotensin II (Ang II) through osmotic minipumps and in salt-fed Dahl salt-sensitive (DSS) rats. Control groups consisted of vehicle-infused rats and Dahl salt-resistant (DSR) rats, respectively. Ang II infusion significantly decreased cardiac IGFBP-3 mRNA by 57, 52, and 36% at three, 7, and 14 days, respectively. Gene expression of IGFBP-4 was increased by 78% at day three and then decreased to levels that were still 26 and 24% above those of controls at 7 and 14 days, respectively. Cardiac IGFBP-5 mRNA was not affected in this model, but was modulated by infusion of IGF-I. In the Dahl model, hypertensive DSS rats had 42% higher IGFBP-4 and 33% higher IGFBP-5 mRNA levels compared with normotensive DSR rats after four weeks of high salt diet, but no changes in IGFBP-3 gene expression were observed. The increase in IGFBP-4 transcripts in both models suggests that this response is due to increased pressure, and additional experiments with primary cardiomyocyte cultures confirmed that the mechanism does not involve direct Ang II actions at the cellular level. As IGFBP-4 is known as an inhibitory binding protein, our findings suggest that the increase serves to limit excessive IGF action in the remodeling heart. IGFBP-3 was decreased in hypertensive Ang II-rats but not in hypertensive Dahl rats, suggesting that the effect is mediated by a pressor-independent mechanism of Ang II. Consistently, in cultured cardiac endothelial cells Ang II specifically reduced IGFBP-3 mRNA. In conclusion, the distinct regulatory patterns of cardiac IGFBPs support an important role for these proteins in modulating IGF-I action during cardiac remodeling in low and high Ang II models of pressure-overload.

Introduction

A substantial body of evidence supports that insulin-like growth factor (IGF)-I has an important function during compensatory adaptive responses to pressure overload as well as during cardiac remodeling that leads to heart failure.[1] It is induced in the heart in response to increased work load,[2-4] or in the border region of the injury caused by infarct.[5-8] Together with systemic IGF, this locally produced IGF-I increases cardiac mass in the case of hypertension,[4] or diminishes apoptosis that occurs after infarct.[9] In fact, *in vitro* and *in vivo* studies show that IGF-I increases myofibril formation, but this without re-expression of fetal cardiac proteins that usually occurs in hypertrophy.[10-12] Recent studies have furthermore shown that cardiac restricted IGF-I expression prevents senescencing of cardiac precursor cells,[13] or that IGF-I injection activates resident cardiac progenitor cells to restore cardiac tissue in rat and dog,[14,15] thus adding more mechanisms by which the peptide may potentially maintain cardiac mass and function. Finally, in a therapeutic setting, cardiac IGF-I injection together with embryonic stem cells enhanced their cellular engraftment and differentiation after myocardial infarct,[16] and IGF-I delivery with nanofibers was recently shown to improve cell therapy with transplanted cardiomyocytes.[17]

Evidence that autocrine production of IGF-I is important came from studies with mice in which IGF-I was deleted in the liver, the organ responsible for production of circulating IGF-I. The deletion had only minor consequences for organ weight or total body size.[18,19] Most of the recent data that support a beneficial function for IGF-I in cardiac pathophysiology was obtained in mice that over-express the gene under control of cardiac-specific promotors. That circulating IGF-I is important as well is supported by our previous studies in which we showed that a rise in the circulating hormone increases cardiac mass, and in several other rat models in which IGF-I or growth hormone (GH) treatment was associated with improved cardiac function.[4,20-25] GH treatment of heart failure patients has been reported to increase cardiac mass, a response most likely mediated by IGF-I, systemically and locally induced in cardiac tissue.[26-28] Despite encouraging results obtained with animal models,[24] trials with GH and IGF-I in heart failure patients gave equivocal results with respect to cardiac function.[29] This lack of clinical benefit has in part been explained by other groups as a lack of responsiveness to GH: sub-grouping of the patients analyzed showed that those patients that increased their circulating IGF-I by more than 77 ng/L in response to the GH treatment indeed improved their ejection fraction.[27] The general negative outcome of these clinical studies underscores the necessity for studies aimed at getting a better understanding of cardiac mechanisms of action of IGF-I. In addition,

several recent experimental studies suggested that long-term activation of IGF or signaling pathways downstream of IGF can even lead to maladaptive hypertrophy,[30-32] and the mechanisms that cause this transition from postive to negative consequences of IGF-action are poorly understood.

Important regulators of IGF-I activity are the highly specific IGF binding proteins (IGFBPs), a family of at least six proteins that bind IGF-I with high affinity.[33-35] IGFBPs affect IGF function by competing with IGF receptors for IGF binding. Their expression is tightly regulated in a time and tissue-specific fashion, and they can inhibit or potentiate IGF activity depending on the tissue studied and conditions used. The biologically active IGF-I is either the free IGF-I or IGFBP binary complexes, which can cross the endothelium or are formed after IGF-I has passed through the endothelium.[36] Although IGF-I itself has been attributed a central role in cardiac biology, nothing is known about the cardiac expression and regulation of the IGFBPs in the heart during the adaptative and successive maladaptive remodeling that occurs after cardiac injury. We hypothesize that changes in the IGFBPs occur during remodeling which result in inadequately regulated IGF activity. As a first step towards a better understanding of the role played by the IGFBPs during the remodeling process in cardiac tissue, we measured cardiac transcripts encoding for the IGFBPs in normotensive Dahl salt-resistant (DSR) and hypertensive salt-sensitive (DSS) rats and in angiotensin (Ang) II-infused rats. Ang II, besides its well-known effects on arterial pressure, exerts mitogenic and growth promoting effects on cardiac myocytes and non-myocyte elements. Our study shows that (1) cardiac IGFBP-4 transcripts are increased in response to Ang II and salt-induced hypertension, and (2) cardiac IGFBP-5 transcripts are increased in salt-induced hypertension, but not in Ang II-induced hypertension, however, transcript levels are regulated by IGF-I, and (3) IGFBP-3 is significantly decreased only in response to Ang II infusion.

Materials and Methods

Animals

Osmotic minipumps (Alzet model 2001, ALZA Corp., Palo Alto, CA) were implanted in male Sprague-Dawley rats (IFFA CREDO, L'Arbresle, France), to infuse diluent (controls) or Ang II at a rate of 500 ng/kg/min.[4] IGF-I was infused via minipumps at a rate of 1.4 mg/kg/d. Rats were pair-fed as described previously.[37] All procedures were performed in accordance with institutional guidelines for the care of experimental animals.

Three, 7 or 14 days after implantation of the osmotic pumps, rats were anesthetized, aortic blood was withdrawn, mixed with EDTA in prechilled glass tubes, and immediately placed in ice. Plasma samples were stored at -80°C until measured for IGF-I content by radioimmuno assay (RIA). Tissues were removed, weighed, snap frozen in liquid nitrogen and stored at -80°C until processed. Dahl salt-sensitive (DSS) and salt-resistant (DSR) rats (Harlan) were fed a high salt diet (8% NaCl) for 4 weeks, starting at 8 to 9 weeks of age.

Cardiomyocyte and Endothelial Cell Culture

Ventricular cardiomyocytes were isolated from 6-week-old Sprague-Dawley rats using methods as described.[38] After a 2 h pre-plating step, cells were distributed on 100 mm gelatin-coated dishes and treated with 1 µmol/L Ang II (Sigma in serum-free M199 medium (Invitrogen AG, Basel, Switzerland). In some experiments, additional dishes were incubated with tumor necrosis factor (TNF)-α at 10 ng/mL for comparison. Rat heart endothelial cells (RHEC) were separated from other cardiac cell types by centrifugation and differential plating, cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen AG) containing 20% fetal bovine serum (Invitrogen AG), and used for the experiments between passage 2 and 5. Cells were kept for at least 5 h in serum-free DMEM, in some experiments pre-treated with 1 µmol/L Losartan for 30 min, and then treated with Ang II in the serum-free DMEM for up to 24 h. All cultures and incubations were performed at 37°C in an atmosphere of 5% CO_2.

Plasma IGF-I Radioimmuno Assay

Plasma samples were extracted with acid-ethanol to separate IGF binding proteins (IGFBPs) from IGF-I and assayed for IGF-I immunoreactivity as described[39] using a polyclonal anti-IGF-I rabbit antiserum provided through the National Hormone and Pituitary Program of the NIH-NIDDK. Standard curves were generated using

human recombinant IGF-I, kindly provided by Dr. H.P. Guler, Ciba-Geigy Corp., Summit, NJ.

Northern Blot Analysis

Total RNA was prepared from frozen left ventricular muscle, or isolated cardiomyocytes and endothelial cells using Tri-Reagent (Molecular Research Center, Cincinnati, OH) and assessed for purity by measuring absorptions at 260 and 280 nm. Total RNA (20 μg) was separated by electrophoresis in a 1% agarose-formaldehyde gel, transferred to Hybond C membrane (Amersham Pharmacia Biotech, NJ), and cross-linked to the membrane by ultraviolet irradiation. RNA loading and transfer efficiencies were verified by methylene blue staining of membranes. RNA was hybridized with cDNA probes for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), IGFBP-3,[40] IGFBP-4, IGFBP-5 ,[41] ANF, or SM-α-actin using Quickhyb (Stratagene, La Jolla, CA). The amounts of hybridized probes for the IGFBPs were quantified by phosphorimager (Molecular Dynamics) and normalized for loading using the corresponding GAPDH values. GAPDH was not regulated by Ang II or IGF-I.

Real-Time PCR

RNA isolated from endothelial cells was analyzed by real-time PCR using routine procedures. Briefly, 1 μg RNA was reverse transcribed using the OMNISCRIPT-RT kit (Qiagen), 0.5 μg/μg RNA of random hexameres (Promega), and 20 U RNase inhibitor (Promega). Each PCR reaction contained 2.5 ng cDNA, 300 nM of each primer and ready-to-use ITaQ Syber Green mix (BioRad) in a final volume of 25 μl. The primers used were designed with assistance of the computer program Primer Express (Applied Biosystems) and synthesized by Microsynth AG (Switzerland). Primer sequences for IGFBP-3 were: Fwd: 5'- GTC TCC TGG AAA CAC CAC TGA GT -3', Rev: 5'- GGA GTG GAT GGA ACT TGG AAT C -3'. Real-time PCR was performed on a Light-Cycler from Applied Biosystems with cycles of 15 sec at 95°C and 45 sec at 60°C. After the PCR reaction, the software GeneAmp 5700 SDS (Applied Biosystems) plotted a profile of fluorescence against the cycle number. An arbitrary threshold of fluorescence was set within the exponential phase of amplification and the cycle at which the amplification of the product exceeded this threshold was determined and used as Ct value for calculation. The expression of mRNA levels of the IGFBPs was quantified relative to β-tubulin using mathematical methods published by Pfaffl.[42]

Statistical Analysis

All data represent mean ± SEM of at least six rats per experimental group in the Ang II-infusion model, and 4 rats per group in the Dahl model. Results were analyzed using Student's *t* test when results from two experimental groups were compared or analysis of variance (ANOVA) when data from three or more groups were studied. For data analyzed by ANOVA, pair-wise comparisons were made by the Tukey's test; $P < 0.05$ was considered statistically significant.

Results

Time Course of Cardiac IGFBP mRNA Levels in Response to Ang II Infusion

Sprague Dawley rats were treated with Ang II (500 ng/kg/min) for three, 7, and 14 days by infusion via osmotic minipumps. Ang II raised systolic blood pressures as reported previously[4] and caused increases in cardiac to body weight ratios of 20, 21, and 32% above controls at three, 7, and 14 days, respectively ($P < 0.001$ at all timepoints). Figure 1 shows Northern blot analysis of total RNA purified from the left ventricle of control and Ang II-treated rats at 7 days. Hybridization with probes specific for atrial natriuretic factor (ANF) and smooth muscle (SM)-α-actin showed that the expression of these two markers of hypertrophy was significantly increased (Figure 1A). After Ang II infusion for 7 days, 13- and 1.7-fold increases were measured for ANF and SM-α-actin, respectively ($P < 0.001$ for both). Figure 1B shows representative autoradiograms after hybridization of RNA from the left ventricle of control and Ang II-treated rats with probes specific for IGFBP-3, -4, and –5. A decrease in IGFBP-3 an increase in IGFBP-4, and no change in IGFBP-5 mRNAs are visible. We quantified the signals obtained for multiple rats that were sacrificed after three, 7 and 14 days of continuous Ang II infusion, using a phosphorimager (Figure 2). Figure 2A shows that at all time points, IGFBP-3 mRNA levels were decreased significantly compared to those of vehicle-infused controls (-57, -52, and –36%, $P < 0.01$, 0.01, and 0.05, for the three time-points, respectively). IGFBP-4 mRNA, on the other hand, was 78% higher in Ang II-infused rats than in controls at three days ($P < 0.01$, Figure 2B). This effect of Ang II infusion became significantly less pronounced, but was still higher than in controls at 7 (+26%) and 14 days (+24%, Figure 2B). With respect to IGFBP-5, Ang II slightly increased its mRNA at 7 days (+24%, Figure 2B), but at none of the time points did the difference between controls and Ang II-infused rats reach significance (Figure 2C).

In conclusion, these experiments show that Ang II infusion in Sprague Dawley rats increases IGFBP-4 in particular at early timepoints after start of the infusion, and that it decreases IGFBP-3 at all timepoints. To assess by which mechanisms these changes are caused, we performed the following experiments with IGF-I infusion and cultured cardiac cells.

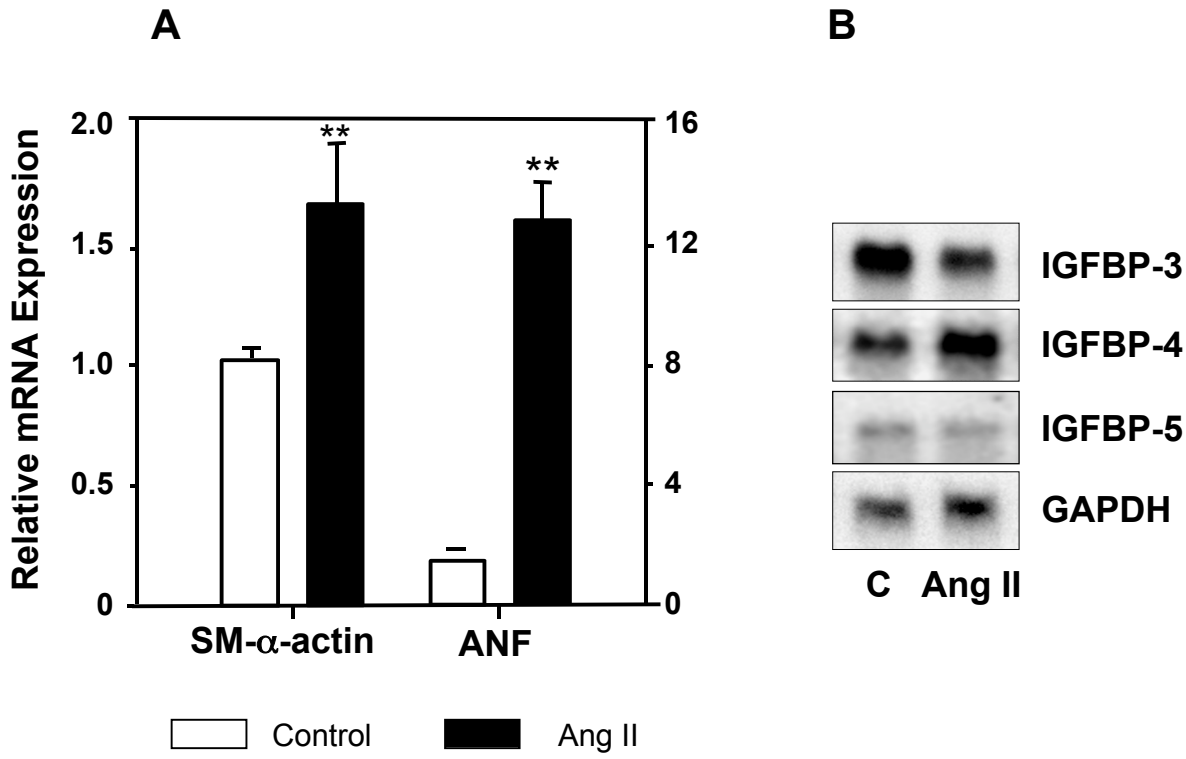

Figure 1: Northern blot analysis of total RNA isolated from the left ventricle of control and Ang II-treated rats.

(**A**) After hybridization with specific probes, signals for smooth muscle (SM)-α-actin and atrial natriuretic factor (ANF) were normalized for GAPDH, and the mean of the ratios obtained from 6 rats per experimental group are shown. (**B**) Example of a Northern blot hybridized with randomly transcribed probes specific for IGFBP-3, -4, and -5 mRNA. ** *P < 0.001* vs control

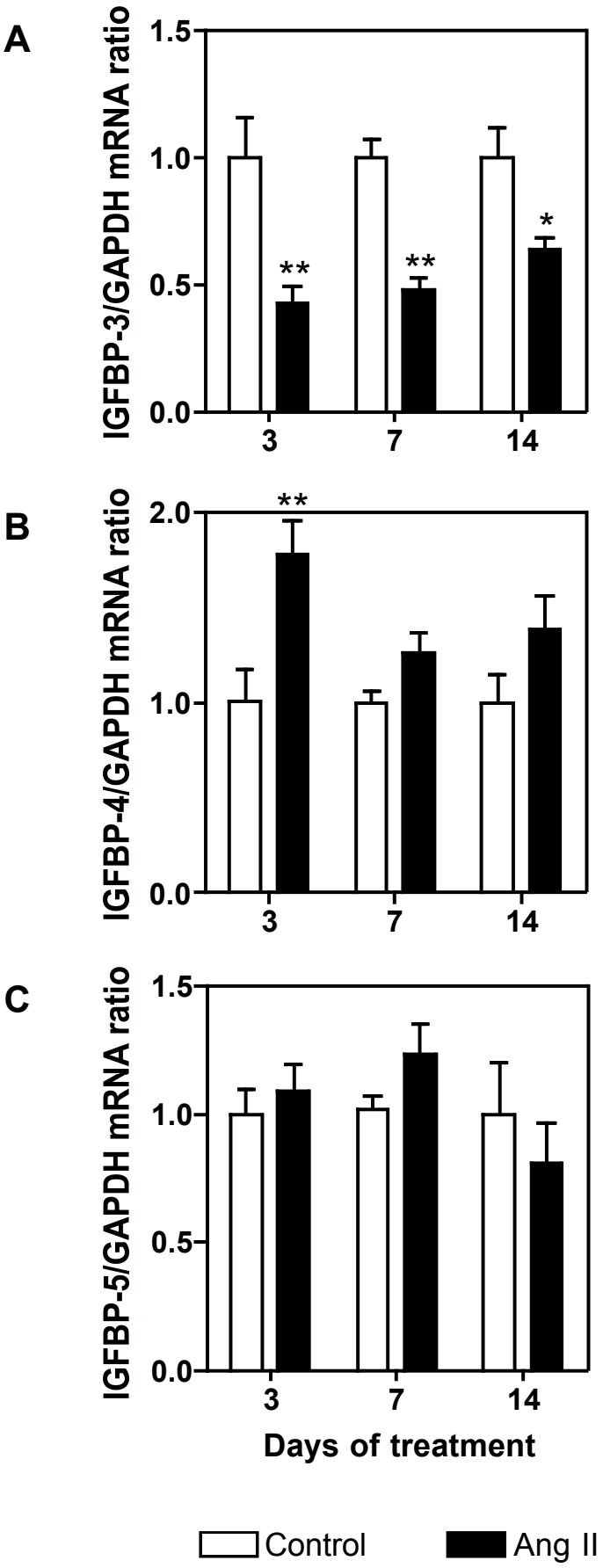

Figure 2: Time course of cardiac IGFBP mRNA levels in response to Ang II infusion

Changes in IGFBP-3 (**A**), IGFBP-4 (**B**), and IGFBP-5 (**C**) in the left ventricle of rats infused with Ang II for three, 7 and 14 days. Northern blots were prepared and hybridized as described in the materials and methods section, radioactive signals for the binding proteins were quantified using a phosphorimager, and normalized by determining the ratio with GAPDH. * $P < 0.05$ vs control, ** $P < 0.01$ vs control, $^{\#\#}$ $P < 0.01$ vs Ang II at day 3.

Effect of IGF-I Treatment on the Ang II-Induced Changes in IGFBP mRNA

In the present study, plasma IGF-I levels in Ang II-infused Sprague Dawley rats were reduced after three and 7 days of treatment compared to pair-fed controls, consistent with our previously published data (data not shown).[39] We examined whether circulating levels of IGF-I regulate cardiac expression of the IGFBPs. To this end, we re-established near-to-normal plasma levels of IGF-I or increased plasma IGF-I to above normal by infusing rats with IGF-I in the presence or absence of Ang II. The combined infusion of Ang II and IGF-I increased plasma IGF-I levels 2.2-fold at three days and 2.0-fold at 7 days compared to animals infused with Ang II alone. Compared with control rats that received no Ang II, IGF-I infusion resulted in IGF-I concentrations that were 1.4 and 1.3 times higher at three and 7 days, respectively (data not shown). Thus, in the co-infused rats, IGF-I levels were re-established to slightly above normal.

Despite these increases in circulating IGF-I, the lower IGFBP-3 and higher IGFBP-4 mRNA levels in the left ventricle of Ang II-infused rats were not reversed. This was true at three (data not shown) and 7 days of infusion (Figure 3, $P < 0.01$ for IGFBP-3; $P < 0.05$ for IGFBP-4, both vs control, P = NS vs Ang II). IGF-I infusion by itself, in the absence of Ang II, raised the IGF-I levels 2.3-fold above controls, but this still did not change cardiac IGFBP-3 or -4 mRNA expression (Figure 3). Taken together, these data support that cardiac IGFBP-3 and -4 mRNA levels are not affected by circulating IGF-I levels. In contrast, IGF-I infusion markedly increased cardiac mRNA expression of IGFBP-5 1.3-fold above controls ($P < 0.01$) in the absence of Ang II. When IGF-I was co-infused with Ang II, IGFBP-5 mRNA levels were 1.5-fold of control levels ($P < 0.05$) and 1.4-fold of the levels measured in rats infused with Ang II alone ($P = 0.09$, Figure 3). The increase induced by IGF-I alone was significantly stronger than that induced by Ang II and IGF-I co-infusion ($P < 0.05$). Taken together, we show that IGFBP-3 and -4 mRNA levels are not modulated by circulating IGF-I, but that cardiac IGFBP-5 transcript levels depend strongly on this growth factor.

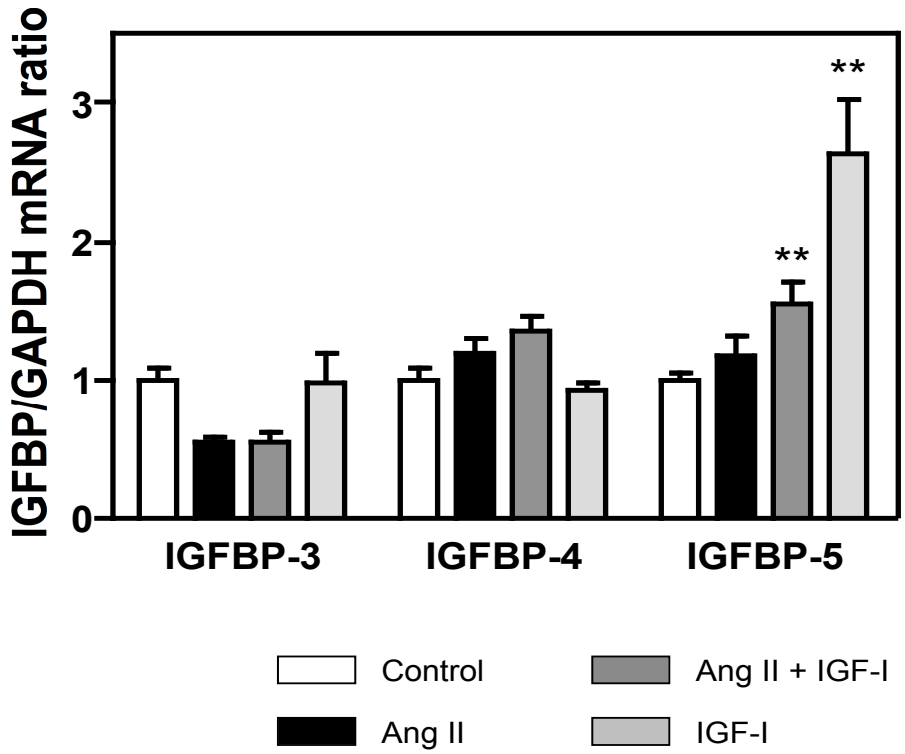

Figure 3: Effect of IGF-I infusion on the expression of IGFBP-3, -4, and -5 mRNA in Ang II-infused and control rats.

Changes in IGFBP-3, IGFBP-4 and IGFBP-5 mRNA in the left ventricle of rats infused with Ang II or IGF-I or co-infused with both during 7 days were analyzed by Northern blot as described for Figure 2. * *P < 0.05* vs control, ** *P < 0.01* vs control. N≥5 per experimental group.

Ang II Does Not Change IGFBP-4 mRNA Expression in Cultured Cardiac Cells, But it Decreases IGFBP-3 in Cardiac Endothelial Cells

Previous studies in vascular smooth muscle and endothelial cells have reported that Ang II, via its cognate cell surface receptors, can modulate IGFBP levels.[43] To assess whether such direct effects occur in cardiac cells, we treated cardiomyocytes and endothelial cells isolated from adult rat hearts with Ang II up to 24 h. No change in IGFBP-4 was observed under these conditions in multiple experiments in the cardiomyocytes (Figure 4A). For comparison, the same Northern blot revealed that tumor necrosis factor-α causes a marked decrease in IGFBP-4 (Figure 4A, and Murigande *et al*., submitted manuscript). With respect to IGFBP-3, we established in preliminary experiments that its mRNA was at least 600 times higher in cardiac endothelial cells than in the myocytes, and therefore we performed real-time PCR analysis of total RNA isolated from those cells primarily. Figure 4B demonstrates that Ang II causes a concentration-dependent decrease in IGFBP-3 mRNA. This decrease was not obtained in the presence of the angiotensin receptor AT_1 antagonist Losartan, compared with cells incubated with Losartan alone (Figure 4B). Incubation with this inhibitor by itself caused a reduction in IGFBP-3 mRNA, consistent with the partial agonistic

properties that have been reported for this compound. IGFBP-4 and –5 mRNAs were not changed after Ang II incubations (data not shown).

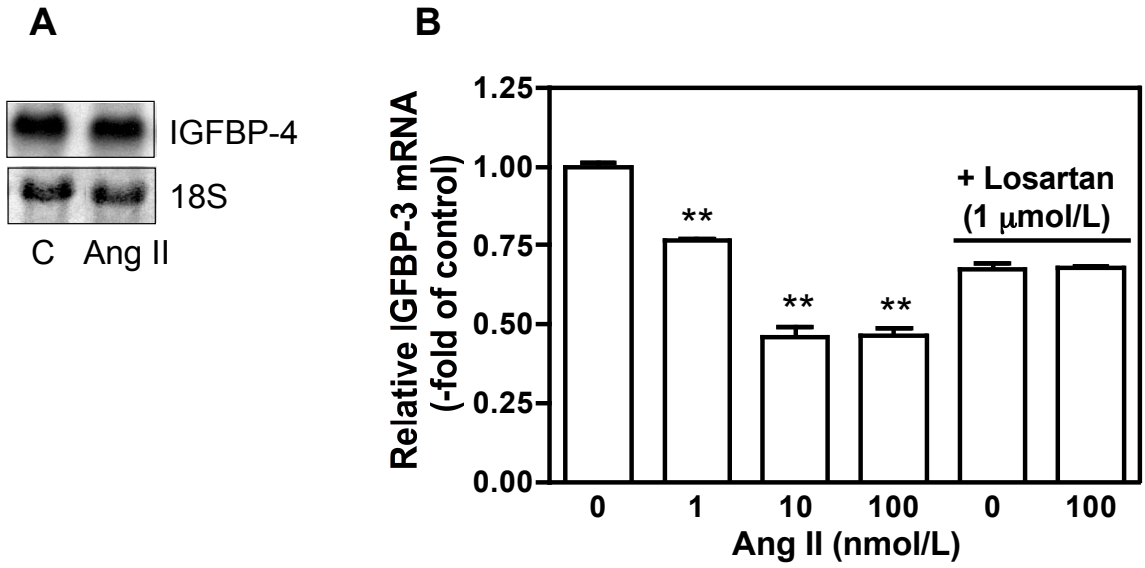

Figure 4: Effect of Ang II on mRNA expression of IGFBP-4 in cardiomyocytes and IGFBP-3 in cardiac endothelial cells.

Cells were isolated from rat hearts as described in the materials and methods section and incubated with Ang II (0-100 nmol/L) up to 24 h in serum-free medium. (**A**) IGFBP-4 mRNA levels were determined by Northern blot analysis in two different experiments and shown in a representative blot. (**B**) IGFBP-3 mRNA levels were determined by real-time PCR and values from 3 independent experiments are shown. ** $P < 0.01$ vs control

Salt-Induced Hypertension Increases Left Ventricular IGFBP-4 and –5 mRNA, and Does Not Change IGFBP-3 mRNA

To test whether the changes in expression of the cardiac IGFBPs are a more general phenomenon in pressure-induced hypertrophy we used a second model of hypertension, namely that induced by a high salt diet in Dahl salt-sensitive (DSS) rats, a model with low circulating Ang II levels. After four weeks of high salt diet, the blood pressure of these rats was increased to 200 mm Hg. Salt-resistant (DSR) control rats were fed the same diet and did not increase their blood pressure. Left ventricular weight normalized for body weight was 20% higher in DSS compared with DSR rats (Figure 5A) and associated with a significant increase in ANF expression (Figure 5B). Additional controls consisted of DSS rats fed a low salt (LS) diet. These developed moderate or no hypertension at all, and correspondingly ANF levels were not affected (Figure 5C). IGFBP-3 mRNA was not different between hypertensive DSS rats and normotensive DSR control rats. IGFBP-4 and -5 mRNA were 1.4- ($P < 0.01$) and 1.3-fold ($P < 0.05$) higher in DSS rats than in DSR controls (Figure 6). These effects were

not due to strain differences, because IGFBP-4 and IGFBP-5 mRNA levels were similar in DSR rats on a low or high salt (HS) diet and in DSS rats fed a low salt diet for 4 weeks (data not shown). Thus, levels were only increased in DSS rats on a HS diet.

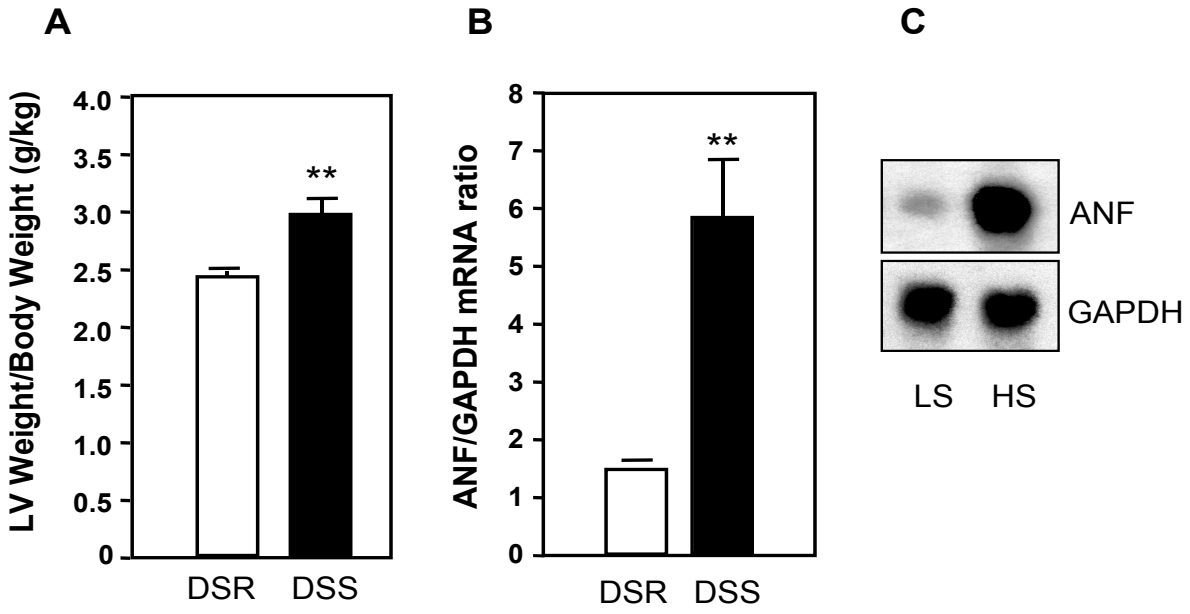

Figure 5: LV weight quantification and ANF mRNA expression in salt-induced hypertensive rats compared to salt resistant rats

After 4 weeks of high salt (HS) diet, (**A**) left ventricular (LV) weight was normalized to body weight for Dahl salt resistant (DSR) rats and Dahl salt sensitive (DSS) rats, and (**B**) ANF mRNA expression was analyzed by Northern blot as described in Figure 2. ** *P < 0.01* vs control. N=4 per experimental group. (**C**) the effect on ANF mRNA exprssion of HS diet compared to low salt diet (LS) in DSS rats is shown.

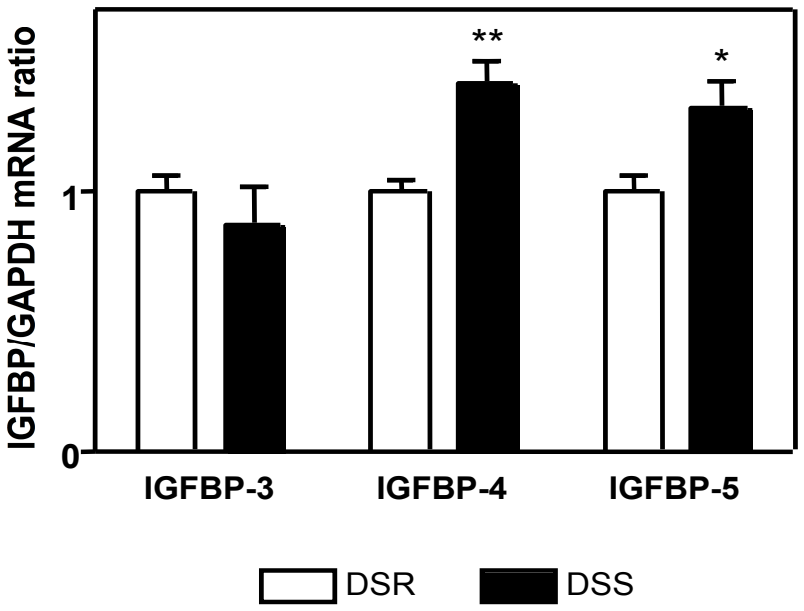

Figure 6: Cardiac IGFBP mRNA levels in salt-induced hypertension

Changes in IGFBP-3, IGFBP-4 and IGFBP-5 mRNA in the left ventricle of DSR and DSS rats were analyzed by Northern blot as described for Figure 2 and are shown from 4 rats per experimental group. * *P < 0.05* vs control, ** *P < 0.01* vs control.

Discussion

We analyzed mRNA levels of IGFBP-3, -4, and -5 in cardiac tissue of normotensive and hypertensive rats. In both models of hypertension tested in our study, cardiac IGFBP-4 mRNA was higher in the hypertensive rats than in the normotensive controls. Cardiac IGFBP-3 mRNA was decreased in Ang II-treated rats only; it remained unchanged in salt-fed salt-sensitive rats. Finally, IGFBP-5 mRNA was significantly increased under conditions of salt-induced hypertension, but not affected in the Ang II infusion model. These differential changes in IGFBP mRNA levels may importantly contribute to the distinct remodeling process that takes place in the heart in response to hypertension of different etiologies, because each can affect the activity of IGF-I in a cell specific manner. In particular, the specific down-regulation of IGFBP-3 may play an important role in the pathology of conditions characterized by high Ang II levels, such as heart failure. We further analyzed mechanisms involved in the observed changes.

Regulation of Cardiac IGFBPs by IGF-I

A range of factors has been described to modulate expression of the IGFBPs in smooth and striated muscle cells, including IGF-I itself (for review, see[33,34,44,45]). Increases in cardiac IGF-I and marked decreases in circulating IGF-I have been reported in the Ang II infusion model,[4,39] and either one of these effects could be responsible for the changes in the IGFBPs measured in the present study. Increasing circulating IGF-I levels by infusion via osmotic minipumps did not affect IGFBP-3 or -4 mRNA indicating that systemic IGF-I does not regulate cardiac IGFBP-3 and -4 gene expression. The infused IGF-I reached the cardiac tissue and was effective, as a marked induction of IGFBP-5 mRNA was achieved in the same hearts.

Blood Pressure and Cardiac IGFBP-4

Pressure-overload is known to cause the release of autocrine/paracrine factors amongst which growth factors that contribute to the compensatory hypertrophic response. We have previously published that cardiac IGF-I is increased after Ang II infusion due to the elevated blood pressure of these rats,[4] and our present study demonstrates that this is accompanied by an increase in IGFBP-4 mRNA. Hypertensive salt-sensitive Dahl rats also had higher IGFBP-4 mRNA levels than controls, providing support that indeed pressure overload causes enhanced gene expression of IGFBP-4. It is of note that, although the Dahl model of hypertension is sometimes referred to as a “low Ang II model of hypertension”, cardiac Ang II may originate from pressure-induced local cardiac production, supported by the existence of a local functioning renin-angiotensin system.[46-48] The increase in IGFBP-4 gene transcription could therefore be a secondary cellular response to autocrine/paracrine Ang II in the Dahl model, and on the other hand a direct response to high circulating Ang II in the infusion model. We tested for potential direct regulatory effects of Ang II on IGFBP-4 gene expression in cardiomyocytes in cell culture experiments, but no such effects were detected up to 24 h

after start of the treatment, supporting our conclusion that the increase in IGFBP-4 mRNA is a pressure-related effect, and that the mechanism does not involve Ang II as an intermediate autocrine/paracrine signal.

A similar up-regulation of IGFBP-4 mRNA has been reported in the past in the thoracic aorta of rats that had undergone abdominal aortic coarctation, a high renin model of hypertension, and the authors concluded that the effect was pressure-related because it was not observed in the normotensive abdominal aorta.[49] The conclusion that Ang II itself was not involved in this regulation was supported by a later study by the same investigators with cultured vascular smooth muscle cells, in which Ang II decreased IGFBP-4 transcription rather than increasing it.[43] Taken together, we conclude that pressure regulates IGFBP-4 mRNA levels via an Ang II-independent mechanism.

To our best knowledge, our report is the first to provide data on the regulation of IGFBP-4 gene expression *in vivo* in cardiac muscle. Multiple studies have reported that an increase in IGFBP-4 mRNA results consistently in an inhibition of the effects of IGF-I (for review, see[44]). In fact, IGFBP-4 is an inhibitor of IGF-mediated actions in all systems tested to date, including the arterial wall where IGFBP-4 is the most abundant IGFBP.[43,50-53] Therefore, the enhanced expression of IGFBP-4 measured in our study in response to increased afterload may limit excessive IGF-I activity in cardiac tissue. The contrary explanation, namely that the increased IGFBP-4 may enhance IGF-I action, can however not be excluded based on the data obtained with our *in vivo* models. Such an interpretation would be supported by the observation that IGFBP-4 null mice had 10-15% lower body weight at birth than their wild-type littermates. The absence of IGFBP-4 was in that model explained to diminish IGF storage capacity[54,55] and conversely, increased expression of IGFBP-4 in our models of hypertension may provide a locally stored source of IGF-I that can readily be made available when needed. Active IGF-I would get released after proteolysis of IGFBP-4 protein by pregnancy associated plasma protein A (PAPP-A). PAPP-A provides a post-translational mechanism of regulation of IGFBP-4 abundance, thereby adding an important level at which IGF-I action can be regulated.[50,56,57] PAPP-A is expressed in vascular smooth muscle cells of aortic tissue, and future determination of its expression in cardiac tissue should assess its role under conditions of hypertension and hypertrophy.

Regulation of Cardiac IGFBP-5 by Blood Pressure and IGF-I

Cardiac IGFBP-5 expression was significantly higher in hypertensive DSS rats than in DSR controls, but no changes were evident in Ang II-induced hypertension. Strain differences other than salt-sensitivity did not account for these changes because the increase was also observed when hypertensive DSS rats were compared with DSS rats that had been on a low salt diet for four weeks. Plasma IGF-I levels directly affect cardiac IGFBP-5 gene expression, because IGF-I infusion in Ang II-treated or control rats increased IGFBP-5 mRNA levels significantly. In addition, IGF-I of local cardiac origin might modulate IGFBP-5 mRNA levels in our model. A small increase in IGFBP-5 at 7 days of Ang II infusion occurs despite low

systemic IGF-I and may reflect a response to the concomitant increase in cardiac IGF-I. The absence of significant increases in IGFBP-5 in the Ang II-infusion model may be due to the fact that simultaneous low systemic IGF-I is fully blunting a potential response to local pressure-induced IGF-I production. In DSS rats, circulating IGF-I levels were similar to those of control DSR rats, and the actual trigger for the increase in cardiac IGFBP-5 could be the increase in cardiac IGF-I. Consistent with this interpretation, earlier studies showed that IGF-I enhances secretion of IGFBP-5 in various types of muscle cells.[58-60] An induction of cardiac IGFBP-5 has also been reported to follow with a short delay the increases in IGF-I and IGF-II induced by a brief coronary occlusion in the pig heart.[61] By infusing IGF-II in absence or presence of IGFBP-5, the same group demonstrated that IGFBP-5 inhibits the cardioprotection afforded by IGF-II.[61,62] Thus, the action of IGFBP-5 in the heart may be similar to that of IGFBP-4, namely to limit the effects of the IGFs. It should be mentioned, however, that a stimulatory role for IGFBP-5 on IGF actions has been reported in a range of studies, in particular in smooth[63-65] and skeletal muscle models,[66-72] where it may be involved in differentiation and regeneration. In conclusion, the distinct regulation of IGFBP-5 in two models of hypertension indicates a role for this binding protein during the remodeling in cardiac muscle, and future cell culture studies should determine its biological effects in cardiac cells. Since nutritional status affects IGF-I levels and IGF-I is reduced in cardiac cachexia,[73,74] our finding that IGFBP-5 is regulated by IGF-I levels is particularly relevant for the cardiac remodeling in those conditions.

Regulation of IGFBP-3 by Ang II

The hypertrophy induced by Ang II is in part due to the elevated pressure, but it also consists of a pressure-independent component. A significant decrease in IGFBP-3 was observed after Ang II infusion but not in hypertensive Dahl rats, and is therefore probably not directly related to the elevated blood pressure, but may contribute to the pressure-independent part of the response to Ang II at the cellular level. We have analyzed the relative mRNA levels of IGFBP-3 in cardiomyocytes and endothelial cells by real time PCR, which revealed that the latter cell type has at least 600 times higher expression of this IGFBP than cardiomyocytes (data not shown). Incubation of cardiac endothelial cells with Ang II reduced IGFBP-3 mRNA significantly in a concentration-dependent manner, indicating that the decrease measured *in vivo* is taking place in the endothelial cells of the heart. The lower IGFBP-3 may have autocrine/paracrine consequences for neighbouring endothelial cells, fibrobasts or myocytes. It has been suggested that IGFBP-3 produced by bovine aortic endothelial cells inhibits the growth of endothelial cells, in particular when the cells reach confluency, when IGFBP-3 synthesis is increased.[75] We have previously reported growth inhibitory effects of IGFBP-3 also in rat aortic vascular smooth muscle cells: TNF-α increased

transcription and secretion of IGFBP-3, and incubation with recombinant IGFBP-3 blocked proliferation of these cells.[76] Inversely, our group also published that the growth stimulatory effect of estradiol is mediated by a reduction in expression of IGFBP-3.[77] These studies, together with our novel data on cardiac endothelial cells, suggest that the Ang II-induced reduction in IGFBP-3 in the heart may be a stimulus for angiogenesis. Very little data exists with respect to the effect of IGFBP-3 on cardiomyocytes: IGFBP-3 blocked IGF-I-induced protein accumulation and hypertrophy in cultured adult rat or rabbit cardiac myocytes,[78,79] both studies supporting that low IGFBP-3 is beneficial to the cardiomyocyte. Notably, specific down-regulation of inhibitory IGFBP-3 may also contribute to the high level of cardiac fibrosis observed in Ang II-induced hypertrophy. Consistently, long term enhanced IGF-activity by over-expression of IGF-I in transgenic mice resulted in excessive fibrosis and diminished cardiac function.[30] IGFBP-3 has been detected in the human heart.[80]

In conclusion, the vast differences in abundance and tissue distribution, and the complexity of regulation of the expression of the IGFBPs would indicate that these proteins serve diverse functions in the heart. IGF-I is generally believed to be protective in the heart, but excessive IGF-I activity risks to result in fibrosis or downregulation of the molecules necessary for causing its protective effects, such as its own receptor, Akt or PI3 kinase,[32] and therefore an adequate control by the IGFBPs is crucial. Our findings could have implications for understanding the pathophysiology of hypertension-induced hypertrophy and more knowledge on underlying mechanisms and the cellular effects of the IGFBPs alone, or together with IGF-I, should help to design future approaches in which the use of IGFBPs or IGF/BP complexes may belong to the potential novel therapeutic strategies.

Acknowledgments

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Key findings

With the previous study, we show that differential regulation of cardiac IGFBPs exists in low and high Ang II models of pressure-overload. Our data suggest that up-regulation of IGFBP-4 in both models is due to pressor-dependent mechanisms. Moreover, specific down-regulation of IGFBP-3 by Ang II occurs, whereas, IGFBP-5 expression is not influenced by Ang II. The former effect may play an important role in pressor-independent cardiac effects of Ang II.

VI. C. Protein content regulation by TNF-α in skeletal myocytes

Progressive loss of muscle mass is a frequent consequence of heart failure. In this regard, we investigated the mechanisms regulating hypertrophy and atrophy in skeletal myocytes. One might wonder whether muscle atrophy is simply the converse of hypertrophy. There is indeed a distinct set of genes, which are inversely regulated by hypertrophy and atrophy [60], however, unique mechanisms are induced during skeletal muscle atrophy (reviewed in [19]). In the third and last part of the results, we focus on the effects of TNF-α on protein synthesis (PS) and protein degradation (PD) in skeletal muscle cells.

VI. C. 1. TNF-α and IGF-I increase protein synthesis through different pathways

The following article examines the signaling pathways activated by TNF-α and their effect on total protein content in comparison to IGF-I, a factor known to enhance protein synthesis. For this purpose, we used C_2C_{12} mouse myotubes. We measured an increase in PS after 4 and 24 h of treatment with TNF-α or IGF-I. This effect was concentration dependent and required *de novo* protein synthesis. In the case of TNF-α, activation of gene transcription was also required. The use of specific pathway inhibitors showed that TNF-α induces PS mainly via PI3K-Akt and to a lesser extent also via MEK-ERK1/2, whereas IGF-I acts most likely in a PI3K independent manner.

Tumor necrosis factor-α and insulin-like growth factor-I increase protein synthesis in differentiated C2C12 myotubes in a PI3-kinase-dependent and -independent manner, respectively

Isabelle Plaisance, Christian Morandi, Claire Murigande, Marijke Brink

ABSTRACT

Tumor necrosis factor (TNF)-α is generally associated with maladaptive processes in heart and skeletal muscle pathologies. However, TNF-α can also promote beneficial effects. Since its role and mechanisms of action remain poorly defined in muscle cells, we examined the signalling pathways activated by TNF-α and their effects on total protein content in comparison to insulin-like growth factor (IGF)-I, a hormone known to promote hypertrophy. A significant and concentration-dependent increase in protein synthesis (PS) was observed after 24 h treatment with TNF-α or IGF-I. DNA staining excluded a change in the number of cells, but the WST-1 assay measured a marked increase of C2C12 myotube viability induced either by TNF-α or IGF-I. After LY294002 (LY, a PI3-K inhibitor) pre-treatment, PS was still highly increased by IGF-I, whereas the action of TNF-α or insulin (used as a control) was completely abolished. Consistently, enhancement of cell viability induced by IGF-I was not modified by LY, but totally blocked when TNF-α was the stimulator. Strong increases in phosphorylation of Akt and the downstream effectors mTOR, GSK3 and p70S6K occurred after IGF-I and TNF-α stimulations. LY pre-treatment did not change the pattern of any of the IGF-I-induced phosphorylation events, while it completely inhibited TNF-α-induced phosphorylations. As confirmed by a kinase activity assay, after LY pre-treatment, Akt was still highly activated by IGF-I, whereas TNF-α-induced Akt activity was fully abrogated. In conclusion, our results provide evidence for two novel pathways, a TNF-α/PI3-K-dependent and an IGF-I/PI3-K-independent pathway, that both play an essential role in the stimulation of protein synthesis and metabolic activity in differentiated myotubes.

Key words: TNF-α, IGF-I, protein synthesis, myotubes, PI3-Kinase

INTRODUCTION

Skeletal muscle is the largest pool of protein in the body. Maintenance of its mass requires a precise balance between protein synthesis and degradation. Therefore, small changes in one of these mechanisms, if sustained, can have a significant impact on muscle mass of the whole organism (Mitch and Goldberg*,* 1996; Heszele and Price, 2004). Regulation of muscle catabolism or anabolism involves complex interactions among several mediators, including growth factors and cytokines.

Insulin-like growth factor-I (IGF-I) has long been recognized as an anabolic growth factor responsible for normal growth and development (for review, see: Florini *et al*, 1996). In skeletal muscle, IGF-I has been shown to be sufficient to induce hypertrophy either by autocrine or by paracrine mechanisms (Yakar *et al*, 1999; Musaro *et al*, 2001; Shansky *et al*, 2006). The positive actions of IGF-I on protein synthesis (Dardevet *et al*, 1996; Bark *et al*, 1998; Shen *et al*, 2005) as well as on proliferation and differentiation of myoblasts or satellite cells are very well documented (for review, see: Florini *et al*, 1996; for review, see: Butler *et al*, 1998; for review see: Glass 2003; Foulstone *et al*, 2004; Jacquemin *et al*, 2004; Zorzano *et al*, 2003) . It was also proposed that IGF-I participates in regenerative processes. In this setting, its administration in skeletal muscle of patients with muscle diseases improved recovery of muscle mass (Furling *et al*, 1999, Rabinovsky *et al*, 2003). Finally, IGF-I promotes survival by diverse mechanisms: blocking of pro-apopototic pathways (Lawlor and Rotwein, 2000; Datta *et al*, 2002;), antagonizing some specific elements of the ubiquitin proteasome system (for review see: Glass, 2003; 2005; Sacheck *et al*, 2004), and supressing proteolysis (Salvesen and Duckett, 2002; Downward *et al*, 2004; Du *et al*, 2004; Stitt *et al*, 2004).

Tumor necrosis factor-α (TNF-α) is a pro-inflammatory cytokine with pleiotropic biological effects and mediates diverse pathological processes. It is considered to play a major role in muscle catabolism for diverse reasons. Elevated circulating levels of the cytokine are generally associated with catabolic states (Argiles *et al*, 1997; Eid *et al*, 2001). TNF-α has been shown to enhance protein degradation, and muscle wasting has been observed after chronic administration of the cytokine (Tracey *et al,* 1990*;* Garcia-Martinez *et al,* 1993 A; Llovera *et al*, 1993) or in TNF-α-transgenic animals (Kubota *et al,* 1997). The cytokine was also described to inhibit differentiation by inducing apopotosis in myoblasts (Langen *et al*, 2001; Foulstone *et al*, 2001; Stewart *et al*, 2004) and in differentiated myotubes (Li *et*

al, 2000; Tolosa *et al*, 2005). Finally, *in vivo* and *in vitro* studies have demonstrated that TNF-α can up-regulate a muscle specific ubiquitin ligase (Li *et al*, 2000; 2005) or stimulate proteolysis in skeletal muscle through activation of the ubiquitin-proteasome-pathway (Garcia-Martinez *et al*, 1993 B; 1994; Llovera *et al*, 1997; Li *et al*, 2000).

However, the role of this cytokine in muscle cells remains controversial and poorly understood. It has been reported that TNF-α can increase protein synthesis (PS) (Hiraoka *et al*, 2001) or cause resistance to hypoxic stress (Yokoyama *et al*, 1997; Nakamura *et al*, 1998; Nakano *et al*, 1998) in cultured cardiomyocytes, while its involvement in chronic heart failure and cardiovascular diseases is well described (Meldrum *et al*, 1998; for review see: Mann, 2003). Similarly, in muscle cells, its action depends on cell type and conditions, but range from survival and proliferation (Mustapha et al, 2000; Tantini et al, 2002; Foulstone *et al*, 2004) to cytotoxicity, apoptosis and necrosis (Li *et al*, 2000; Tolosa *et al*, 2005).

The purpose of the present study is to provide a better understanding for the role and mechanisms of action of TNF-α in skeletal muscle cells. We have used differentiated myotubes to analyze the TNF-α-induced signalling pathways and their effects on PS , in comparison to IGF-I, a hormone known to promote hypertrophy. To further characterize interactions between these potentially hypertrophic and atrophic signalling molecules, we also examined the effect of combinations of IGF-I and TNF-α on the phosphorylation state of the relevant signalling molecules and the final outcome at the level of PS. We show that TNF-α and IGF-I both increase PS and metabolic activity (in a viability assay) of differentiated myotubes. The TNF-α-induced protein synthesis involved mainly PI3-kinase, while IGF-I-induced PS did not implicate this kinase. Our results suggest the existence of a new IGF-I-mediated pathway that plays a central role in the hypertrophic function of this growth factor in skeletal muscle cells. Furthermore, our findings also argue for a beneficial action of TNF-α on myotubes metabolism.

MATERIALS AND METHODS

Materials

Cells were treated as detailed in the results section with recombinant mouse TNF-α, (10 ng/ml, R&D Systems, Minneapolis, MN), IGF-I (20 ng/ml, IBT GmbH, Reutlingen, Germany) or both together, in the presence or absence of the specific inhibitors LY294002 (20 μM, 30 min pretreatment), PD98059 (20 μM, 1 h pretreatment) or SB203580 (10 μM, 1 h pretreatment). All inhibitors were from Calbiochem, Merck Bioscience; Darmstadt, Germany. Most of the antibodies used for the study (anti-phospho-Thr202/Tyr204-Erk1/2; anti-Erk1/2; anti-phospho-Ser473 Akt; anti-phopho-Thr308 Akt; anti-Akt; anti-phospho-Ser9/21 GSK3; anti-GSK3; anti-phospho-Ser2481 mTOR; anti-mTOR; anti-Thr389 p70S6K; anti-phospho-Thr421/Ser424 p70S6k; anti-p70S6K) were purchased from Cell Signaling Technology and diluted at 1/1000, except anti-actin from Sigma diluted at 1/500.

The C2C12 model

Myoblasts from the muscle-derived C2C12 cell line were obtained from American Type Culture Collection (Manassas, VA). The seeding density used throughout the experiments was 10^4 cells/cm^2 diameter. Undifferentiated cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated foetal calf serum at 37°C in the presence of 5% CO_2. This medium will be referred to as growth medium (GM). The myoblasts were fused into myotubes by shifting the GM to differentiation medium (DM, DMEM supplemented with 2% heat-inactivated horse serum). The DM medium was changed after 48 h (at Day 2), and the differentiation was allowed to continue for 96 more hours. All experiments were performed at Day 6 (6 days after the beginning of differentiation). To preserve the characteristics of the C2C12 cell line, the splitting of the cells was done up to a maximum of seven times.

Protein synthesis

A constant number of myoblasts (2 x 10^4 cells) were seeded into each well of 24-well polystyrene plates and grown as described in the paragraph above. In order to determine the rate of protein synthesis, cells were incubated with IGF-I, TNF-α, or both together in the presence of radio-labelled ^{3}H-phenylalanine (Amersham Biosciences) at a final activity of 1 μCi/ml for 4 h or 24 h. The reaction was stopped by washing the cell culture twice with ice cold PBS, then the cells were fixed with

10% TCA (Trichloro acetic acid) and dried with chilled ethanol (95%). The cells were solubilized by incubation with 0.2 mol/l NaOH (0.5 ml/well) for 45 min under constant agitation. The resulting lysates were mixed with liquid scintillation and counted in a β-counter.

Protein degradation

^{3}H-Phenylalanine (1 μCi/ml) was incubated with the cells for 48 h during the last two of the regular 6 days of differentiation. Then, cells were chased with excess unlabeled phenylalanine, followed by exposure to the hormones for 24 h. Protein degradation was measured by counting in a β-counter the ^{3}H-Phenylalanine release from pre-labelled cells into the culture supernatant during the 24 h of incubation with hormones.

Cell viability/metabolic assay and DNA staining

Cell viability was quantified using a colorimetric assay (Cell Proliferation Reagent WST-1, Roche Diagnostics AG, Switzerland), which measures mitochondrial dehydrogenase activity. The assay was performed following the manufacturer's instructions. After reading optical density at 450 nm, cells were rinsed with PBS and fixed with 4% formaldehyde. After washing, cells were incubated in crystal violet solution for 30 min at room temperature, then extensively rinsed and lysed in 1% SDS solution under constant agitation for 1 h. Optical density was read at 595 nm.

Western blotting

After treatment, cells were washed in PBS containing 1 mM orthovanadate, and lysed in RIPA buffer, containing 50 mM Tris-HCl, pH 7.4, 1% NP-40, 150 mM NaCl, 1 μg/μl pepstatin, and 1 mM PMSF plus and "Mini-Complete" protease inhibitor cocktail (Roche Diagnostics). For analysis of phosphorylated proteins, the lysis buffer also contained 1 mM NaF and 1 mM orthovanadate. Protein concentrations were measured with the Micro BCA protein assay kit (PIERCE, Switzerland) and equal amounts, 15 μg, were resolved by SDS-PAGE and transferred to PVDF membranes (Millipore AG, Switzerland). The membranes were blocked with 5% nonfat dry milk in Tris-buffered saline/0.1% Tween (TBST) and probed overnight with primary antibodies in TBST supplemented with 5% bovine serum albumin (BSA). After reaction with secondary antibodies (diluted 1/10000 in TBST) conjugated with horseradish peroxidase (Jackson ImmunoResearch, West Grove, PA), bands were

visualized using enhanced chemiluminescence reagents (Supersignal West Pico, PIERCE, Switzerland) and exposure to autoradiographic film. Membranes were first probed with anti-phosphopeptide antibodies. To verify the loading, membranes were stripped, then reprobed with the corresponding antibodies that recognize the same protein independently of phosphorylation state, or with actin in the case of the kinase assay. For quantification, the intensity of each phosphorylated protein band was normalized to the corresponding total protein band by image analysis using the NIH Image 1.62 software.

Measurement of Akt activity

This was achieved using the Akt kinase kit (Cell Signalling) according to the manufacturer's instructions. Briefly, cytosolic extracts were prepared, and Akt was immunoprecipitated by overnight incubation with an Akt mouse monoclonal antibody immobilized on agarose hydrazide beads. Beads were harvested by centrifugation and washed with Akt lysis buffer, before resuspending them in a kinase buffer containing 200 μM ATP and 1 μg of GSK3 fusion protein. The reaction was performed at 37°C for 30 min and stopped by addition of SDS sample buffer. Beads were pelleted by micro-centrifugation, and the supernatants boiled for 5 min before SDS/PAGE and Western Blotting with phospho-(GSK-3α/β) antibody. Detection of the phosphorylated protein was performed using routine procedures described above under "Western blotting".

Statistics

Data are expressed as means ± standard deviation (SD). All comparisons were made versus the appropriate control condition of the respective treatment and time of the experiment. Comparisons versus controls were made using the repeated measures one-way analysis of variance (ANOVA) design versus control (Dunnet's post hoc analysis; Prism v. 4.0; Graph Pad software). The significance level was set at $P < 0.05$ or $P < 0.01$ as indicated.

RESULTS

TNF-α and IGF-I increase protein synthesis in C2C12 myotubes

To investigate the effects of IGF-I and TNF-α on protein synthesis (PS) in myotubes, we induced differentiation in C2C12 cells and then incubated the myotubes for different time periods with each factor alone or the two factors together in the presence of ^{3}H-phenylalanine. Four hours of treatment with IGF-I induced a significant increase in PS of 12±0.09% above untreated controls ($P < 0.01$). TNF-α had a similar effect and increased PS by 11±0.06% over controls (Fig 1A). Increases in ^{3}H-phenylalanine incorporation were more pronounced after longer exposures (24 h) to the stimulators: IGF-I and TNF-α caused increases of 43±7.8% and 22±4.6% over controls ($P < 0.01$ for both), respectively (Fig 1B). At both time points, treatment with the two hormones together had an additive effect, suggesting that IGF-I and TNF-α use two different mechanisms to stimulate protein synthesis. Solely IGF-I affected the rate of protein degradation and decreased it by 15.7±2.2% ($P < 0.05$; Fig 1C).

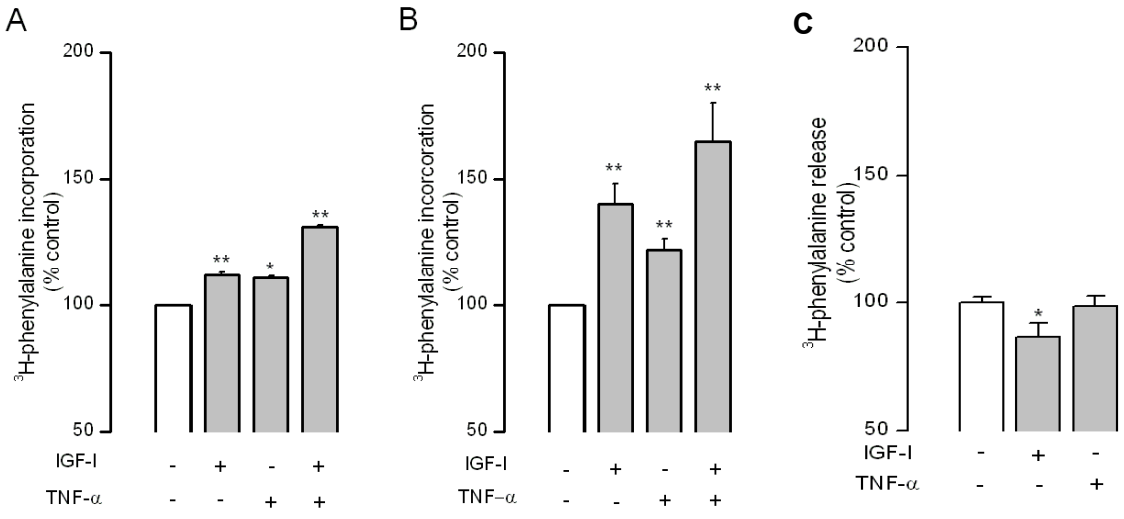

Figure 1. TNF-α and IGF-I increase protein content in differentiated myotubes

At Day 6 post-differentiation, cells were treated with IGF-I (20 ng/ml), TNF-α (10 ng/ml) or both together for 4 h (**A**), or for 24 h (**B**). Protein synthesis (PS) was measured as the incorporation of ^{3}H-phenylalanine, according to procedures described in the materials and methods. Protein degradation was measured as the release of pre-incorporated ^{3}H-phenylalanine from the cells after 24 h of treatment with the hormones (**C**). Results are expressed as the percentage change in ^{3}H-phenylalanine incorporation or release compared to the control cells (white column, set at 100%) ± standard deviation (SD). Results represent data from multiple experiments using independent preparations of C2C12 ($n \geq 3$), and duplicate measurements were obtained in each experiment. The SD is calculated from the average values obtained in the independent experiments. *$P < 0.05$ and **$P < 0.01$ vs. control.

TNF-α and IGF-I increase protein synthesis in a concentration-dependent manner

The concentration-dependence of the effects of TNF-α and IGF-I on PS was assessed at 24 h. A significant increase in PS was already observed with 6.6 ng/ml of IGF-I (25.7±1.6% compared to control values, $P < 0.01$) and reached its maximum at a concentration of 200 ng/ml (64.3±6.1% vs. control). A concentration of 20 ng/ml, which was used in all further experiments, resulted in a 43% increase in ^{3}H-phenylalanine incorporation ($P < 0.01$; Fig 2A). Very low concentrations of TNF-α (0.5 ng/ml) enhanced significantly the incorporation of radioactive label by 14.6±3.5% compared to untreated cells ($P < 0.05$), and the effect reached a plateau at 5 ng/ml (22.8±4.2%, $P < 0.01$ vs. control). Higher concentrations of TNF-α did not increase PS any further (Fig 2B).

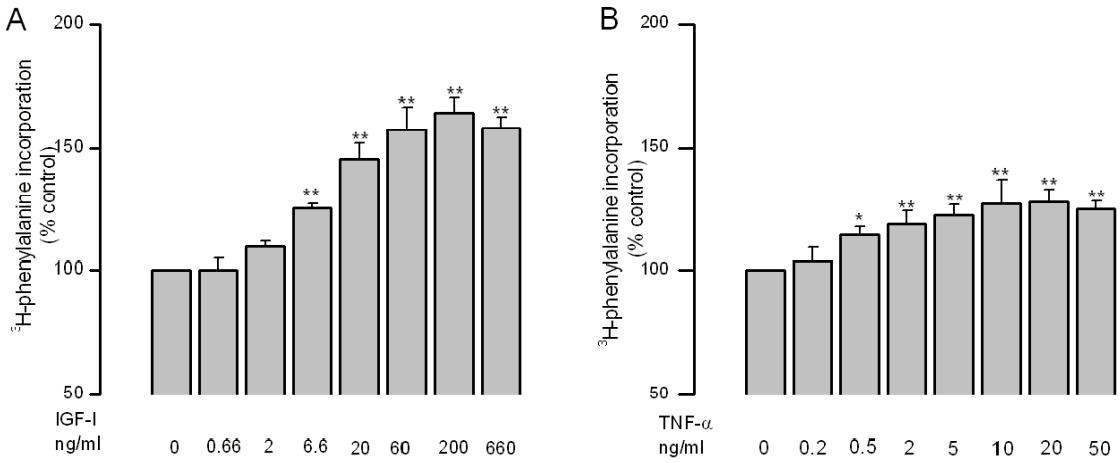

Figure 2. TNF-α and IGF-I increase protein synthesis in a concentration-dependent manner in C2C12 myotubes

C2C12 myotubes were incubated for 24 h with increasing concentrations of IGF-I (**A**) or TNF-α (**B**). At each concentration, PS was measured as the incorporation of ^{3}H-phenylalanine as described for Fig 1. Results are expressed as the percentage change (±SD) in ^{3}H-phenylalanine incorporation compared with the control cells, which were set at 100%, *$P < 0.05$ and **$P < 0.01$ vs. control.

TNF-α and IGF-I enhance metabolic activity of differentiated myotubes

It is currently accepted that TNF-α plays a major role in muscle catabolism. The cytokine appears to be associated with diverse pathological processes, including the induction of apoptosis in cardiac (Krown *et al*, 1996, Meldrum *et al*, 1998) and skeletal muscle myocytes (Tolosa *et al*, 2005). In apparent contradiction to those previous reports our results indicate that this cytokine increases the protein content

of myotubes. To test for potential effects of TNF-α on cellular viability, WST-1 assays were performed on the C2C12 myotubes. Figure 3 shows that IGF-I and TNF-α both enhance dehydrogenase activity in C2C12 myotubes by 27±1.4 and 26±1.5%, respectively (P < 0.01 for both), indicating that TNF-α increases rather than decreases viability in our cells, and on the other hand confirming the well-established protective role of IGF-I on viability. The WST-1 assay is often used to measure proliferation because in many cases enhanced dehydrogenase activity is directly proportional to increase of the cell number. To test this possibility, staining of DNA with crystal violet was performed and indicated that neither TNF-α nor IGF-I increased the number of C2C12 cells (data not shown). Interestingly, the magnitude of enhancement in metabolic activity was identical for IGF-I and TNF-α (Fig 3), this in contrast to the effects of either factor on PS, where the response to IGF-I was 2-fold stronger than that of TNF-α (Fig 1).

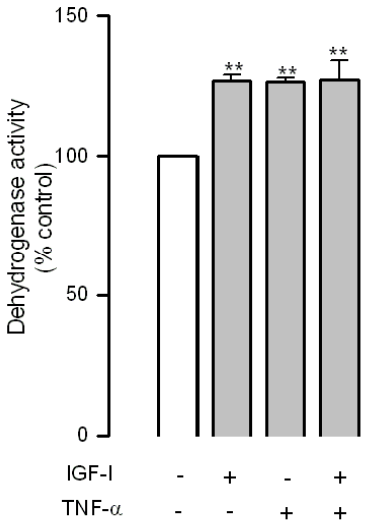

Figure 3. TNF-α and IGF-I enhance metabolic activity of C2C12 myotubes

C2C12 myotubes were cultured in 96-well plates and incubated 24 h with IGF-I (20 ng/ml), TNF-α (10 ng/ml) or both together. WST-1 reagent was added during the two last hours of treatment. Cell viability was assessed by measurement of optical density (OD) at 450 nm, resulting from mitochondrial dehyrogenase activity. Crystal violet stains were performed and the reading of OD at 595 nm allowed the calculation of OD450/OD595 ratios. Results are expressed as the percentage change (±SD) of the OD450/OD595 ratio obtained for treated cells compared to the ratio obtained in control cells, which was set at 100 %. Results represent multiple experiments using independent preparations of C2C12 ($n \geq 3$), and measurements in each experiment were performed in sextuplet. SD is calculated from the average value obtained for the independent experiments. ** P < 0.01 vs. control.

IGF-I-induced protein synthesis requires only translational mechanisms, whereas TNF-α uses both transcriptional and translational mechanisms

Cellular protein content can increase directly via activation of the translational machinery or indirectly via stimulation of gene transcription. In the C2C12 model, TNF-α has been reported to influence mRNA expression of IGF-I (Fernandez-Celemin *et al*, 2002; Frost *et al*, 2003) and of diverse cytokines or cytokine receptors (Alvarez *et al*, 2002). To test whether the effects of TNF-α on protein synthesis

depend on translational and/or transcriptional mechanisms, experiments with the protein synthesis inhibitor cycloheximide (CHX, 10 ng/ml) and the transcriptional inhibitor actinomycin D (0.5 µM) were performed (Fig 4). IGF-I, well known for its immediate effects on the translational machinery, served as a control in these experiments. Myotubes pre-treated with CHX for 30 min were incubated with IGF-I or TNF-α for 4 h. CHX abrogated completely the IGF-I- and TNF-α-induced PS, confirming that the effects observed for both hormones were mediated via translational mechanisms and not due to a none specific internalisation of the radio-labelled amino acid. Similarly, the transcription inhibitor actinomycin D was added to cultured C2C12 myotubes to assess the potential role of gene transcription in the IGF-I- and TNF-α-induced responses. IGF-I-induced PS was not modified by 30 min of actinomycin D pre-treatment, whereas the response to TNF-α was diminished (Fig 4). These findings indicate that gene transcription is not required to increase PS during IGF-I treatment, while TNF-α-induced PS involves both transcriptional and translational mechanisms.

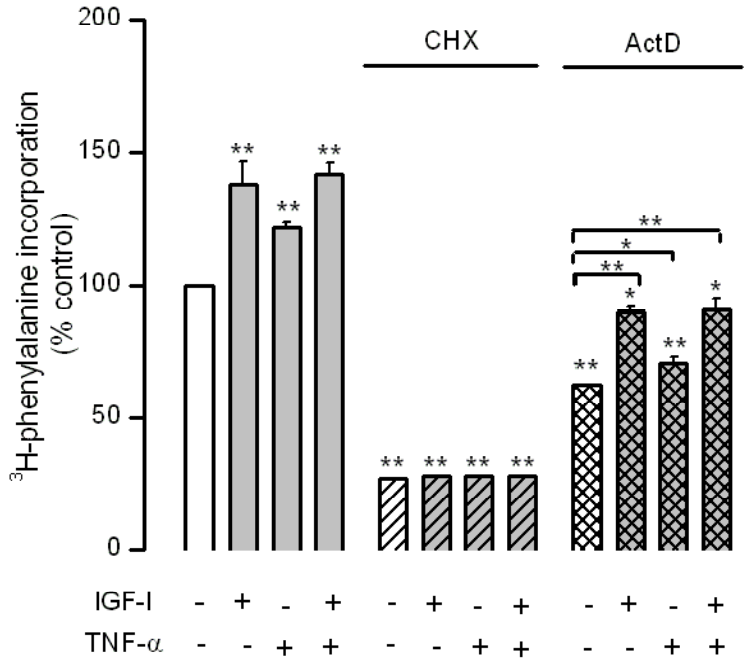

Figure 4. The TNF-α-induced increase in protein synthesis requires transcription and translation

C2C12 myotubes were pretreated with cycloheximide (CHX, 10 ng/ml) or actinomycin D (ActD, 0.5 µM) for 30 min before addition of IGF-I (20 ng/ml), TNF-α (10 ng/ml), or both together with the radiolabel for 4 h. PS was measured as the incorporation of ^{3}H-phenylalanine. An arbitrary value of 100% was assigned to untreated control cells, and results are expressed as the percentage change (± SD) in ^{3}H-phenylalanine incorporation above control. Data shown are representative of three independent experiments. Errors bars signify the SD calculated from the average values obtained for the independent experiments. *P < 0.05 and **P < 0.01 vs. control.

Analysis of signalling pathways whereby TNF-α and IGF-I change PS in myotubes

Binding of IGF-I to its receptor is known to trigger several signalling events, the most important of which are activation of the MAP kinase (MAPK) Erk1/2 via MEK, and activation of Akt/PKB via PI3-kinase (PI3-K). The latter is followed by phosphorylation of an array of substrates involved in PS, including the mammalian target of rapamycin mTOR, p70S6K, and glycogene synthase kinase 3 (GSK3), (Cantley et al, 2002). TNF-α, on the other hand, is known to trigger the stress-activated MAPK p38 and SAPK-JNK, as well as other death receptor signalling pathways that lead to apoptosis in various cells.

To analyze by which signalling pathways IGF-I and TNF-α increase PS, three inhibitors were used: PD98059 (PD, 20 μM) as an inhibitor of MEK, LY294002 (LY, 20 μM) as a specific blocker of PI3-K, and Rapamycin (Rap, 20 ng/ml) to inhibit mTOR. First, we assessed the effects of these inhibitors on basal protein synthesis. Treatment with PD, LY or Rap for 24 h decreased PS to 81±2.8%, 37±6.7% and 54±3.8% of untreated controls, respectively ($P < 0.01$ for all). These results indicate a strong impact of PI3-K and its downstream actor mTOR, as well as a more modest impact of the MEK/Erk1/2 pathway on baseline PS (Fig 5A).

We next investigated the effects of these pharmacological reagents on the IGF-I- and TNF-α-induced increases in PS. After PD pre-treatment, TNF-α-induced PS was reduced from 22±4.6% to 11±3.2%, while IGF-I-induced PS remained unchanged (43±7.8% increase vs. control compared to 45±6.1% vs. PD), implying that MEK mediates part of the TNF-α- but not the IGF-I-induced PS (Fig 5B). Moreover, the data support that other pathways must be involved in the induction of PS for both factors. LY treatment completely abolished TNF-α-induced PS, implying that this response fully depends on PI3-K, and suggesting that crosstalk exists between the MEK and PI3-K pathways induced by TNF-α in differentiated myotubes. In contrast, in the presence of LY, IGF-I was still markedly increasing ^{3}H-phenylalanine incorporation by 73.5±10.5% (vs. LY, $P < 0.01$). In fact, this increase was stronger than that induced by IGF-I in the absence of LY (Fig 5B). Similar results were obtained after pre-incubation with the alternative PI3-kinase inhibitor Wortmannin (100 nM), followed by 4 h of stimulation with IGF-I (data not shown). These results strongly support the lack of involvement of PI3-K in mediating IGF-I action in our model. Rap pre-incubation lowered the IGF-I-induced ^{3}H-phenylalanine incorporation from 43±7.8% to 25±10.8% (vs. Rap alone, $P < 0.01$), indicating that mTOR mediates part of the IGF-I-evoked response. Since LY had no effect at all on IGF-I-induced-PS, the Rap-inhibited part of the pathway must also be independent of PI3-K. TNF-α-induced ^{3}H-phenylalanine incorporation remained unchanged after Rap pre-incubation (Fig 5B).

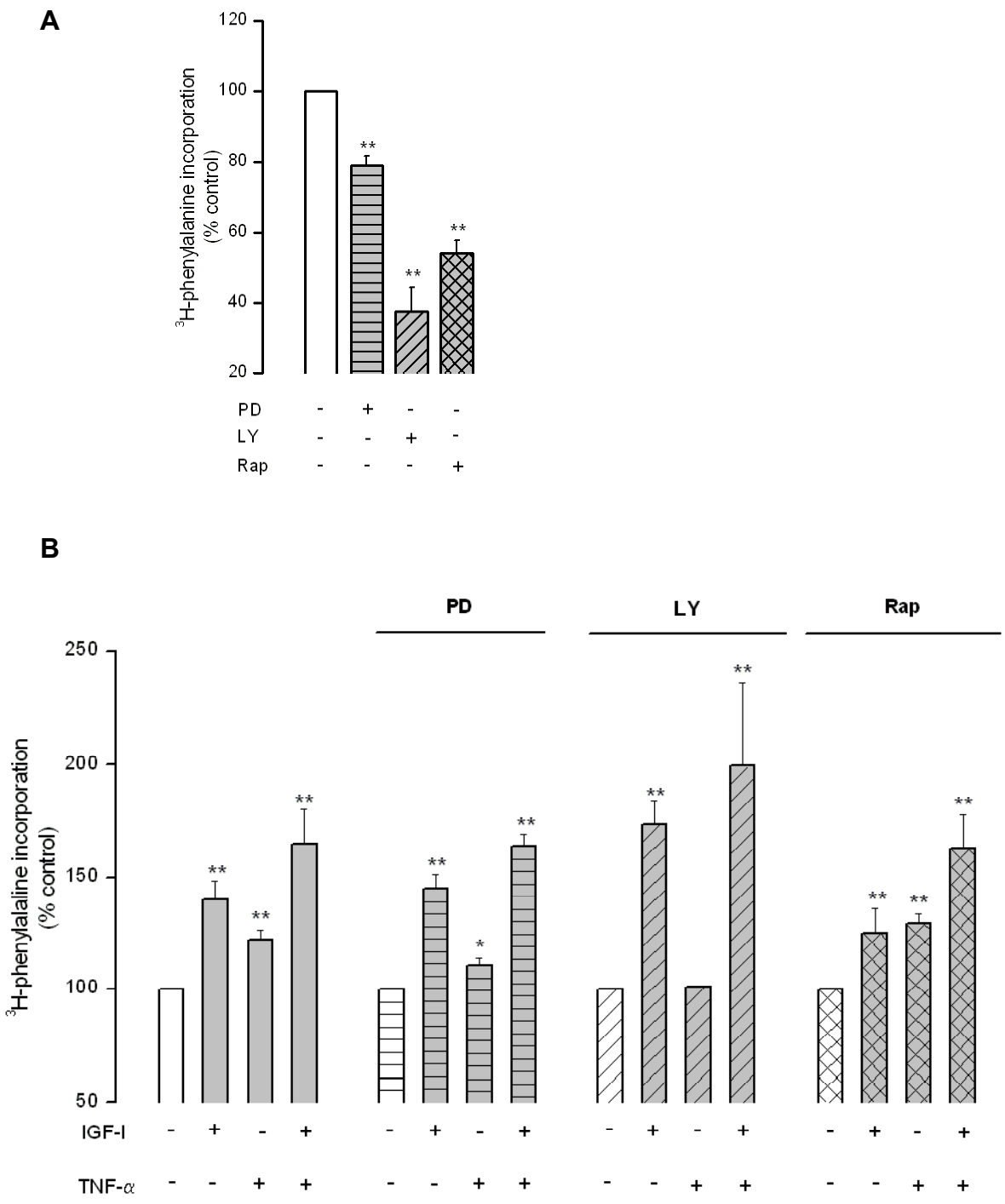

Figure 5. Effect of PD, LY and Rap on basal and on TNF-α- or IGF-I-induced protein synthesis in C2C12 myotubes

(**A**) C2C12 myotubes were incubated with vehicle (DMSO) or PD (10 µM, column with horizontal lines), LY (20 µM, hatched column) or Rap (20 ng/ml, double hatched column) for 24 h, and protein synthesis assessed by measuring ^{3}H-phenylalanine incorporation. (**B**) After pre-treatment with the inhibitors (30 min for LY and Rap, and 1h for PD), IGF-I (20 ng/ml) or/and TNF-α (10 ng/ml) were added to the culture medium for 24 h. PS was measured as described before. Results are expressed as percentage change (±SD) in ^{3}H-phenylalanine incorporation compared with the control (white columns), which was set at 100 %. Results represent multiple experiments with independent preparations of C2C12 ($n \geq 3$), and each experiment was performed in duplicate. SD is calculated from the average value obtained in the independent experiments. *$P < 0.05$, **$P < 0.001$ vs. control.

Analysis of the signalling pathways by which TNF-α and IGF-I change metabolic activity in C2C12 myotubes

To investigate which pathways are involved in the TNF-α and IGF-I-induced increases in metabolic activity, WST assays were performed after PD, LY or Rap pre-treatment. Under control conditions, all pharmacological reagents significantly reduced cell viability (data not shown). PD pre-treatment did not diminish IGF-I or TNF-α-increased cell viability. The significantly enhanced viability induced by TNF-α in myotubes was fully abrogated by LY. As a marked contrast, the effect of IGF-I on viability became even more pronounced: an increase of 75±12.3% was obtained in the presence of LY, compared to a 27±1.4% increase in the absence of LY ($P < 0.01$). Rap pre-incubations enhanced metabolic activity in C2C12 myotubes (Fig 6). Similar to our data on protein synthesis, these results indicate that TNF-α increases C2C12 viability via PI3-K, while IGF-I acts in a PI3-K-independent manner.

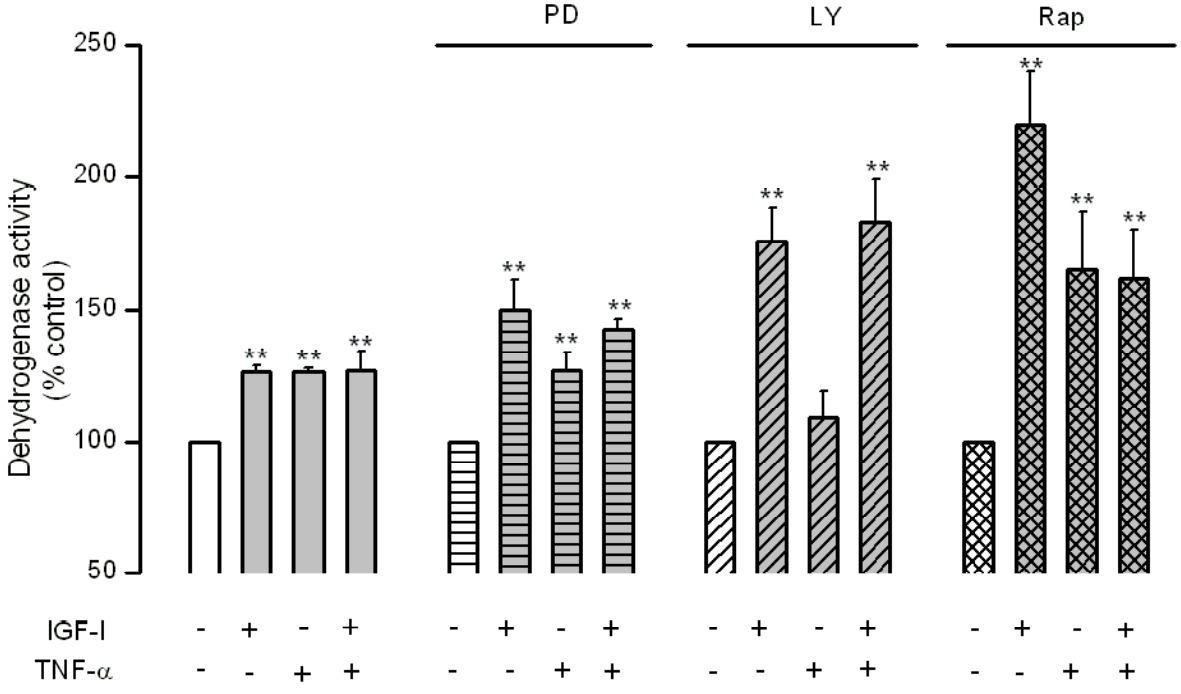

Figure 6. Effect of PD, LY and Rap on TNF-α- or IGF-I-induced increases in metabolic activity in C2C12 myotubes

C2C12 were treated with inhibitors and hormones for 24 h as described for Fig 5B. WST-1 reagent was added during the two last hours of treatment, and cell viability was assessed as indicated for Fig 3. Results represent multiple experiments with independent preparations of C2C12 ($n \geq 3$), and for each experiment, sextuplet measurements were performed. SD is calculated from the average values obtained of the independent experiments. **$P < 0.01$ vs. control.

Effect of inhibitors on IGF-I- and TNF-α-induced phosphorylation

Very few authors have described a lack of involvement of PI3-K in IGF-I-mediated effects. Similarly few papers have related the actions of TNF-α to PI3-K activity. Therefore, our next experiments were aimed at further clarifying the signalling pathways mediating these actions of TNF-α and IGF-I. We used phospho-specific antibodies on Western blots to analyze the phosphorylation state of kinases known to be involved in translational mechanisms (MEK, Akt/PKB, mTOR, GSK3, p70S6K). In time course experiments we determined that IGF-I-stimulated phosphorylation reached a maximum at 10 min and remained elevated during 1 h, while TNF-α-stimulated phosphorylation was most efficient at 30 min (data not shown). Therefore, 10 min of treatment was used for IGF-I and 30 min for TNF-α, respectively, to analyse the effects of the inhibitors PD, LY, and Rap on TNF-α and IGF-I-induced phosphorylations. IGF-I increased markedly the phosphorylation of Akt/PKB, GSK3, mTOR, p70S6K and Erk1/2. TNF-α had similar but less pronounced effects (Fig 7). PD blunted the IGF-I- and TNF-α-induced phosphorylation of Erk1/2 (Fig 7A), while the phosphorylation state of all other kinases was unchanged (data not shown). Taken together with the results on PS, this confirms that the MEK/Erk1/2 pathway contributes to the TNF-α but not to the IGF-I-induced PS. Interestingly, IGF-I was still able to induce phosphorylation of Akt and its downstream mediators in the presence of LY, whereas the same inhibitor completely abolished all TNF-α-induced phosphorylations (Fig 7B). This finding is consistent with our data on PS, and strengthened the idea that IGF-I activates Akt and PS in a PI3-K independent manner, whereas the effect of TNF-α seems to be mediated via the PI3-K/Akt pathway. Rap fully blocked the TNF-α- and nearly completely the IGF-I-induced increase in phosphorylation of p70S6K (Fig 7C). No modification of the signals for the other kinases (Erk1/2, Akt/PKB, GSK3, mTOR) was noticed (data not shown). In conclusion these observations further support a central role of PI3-K in the TNF-α-induced PS, while IGF-I acts in a PI3-K independent manner.

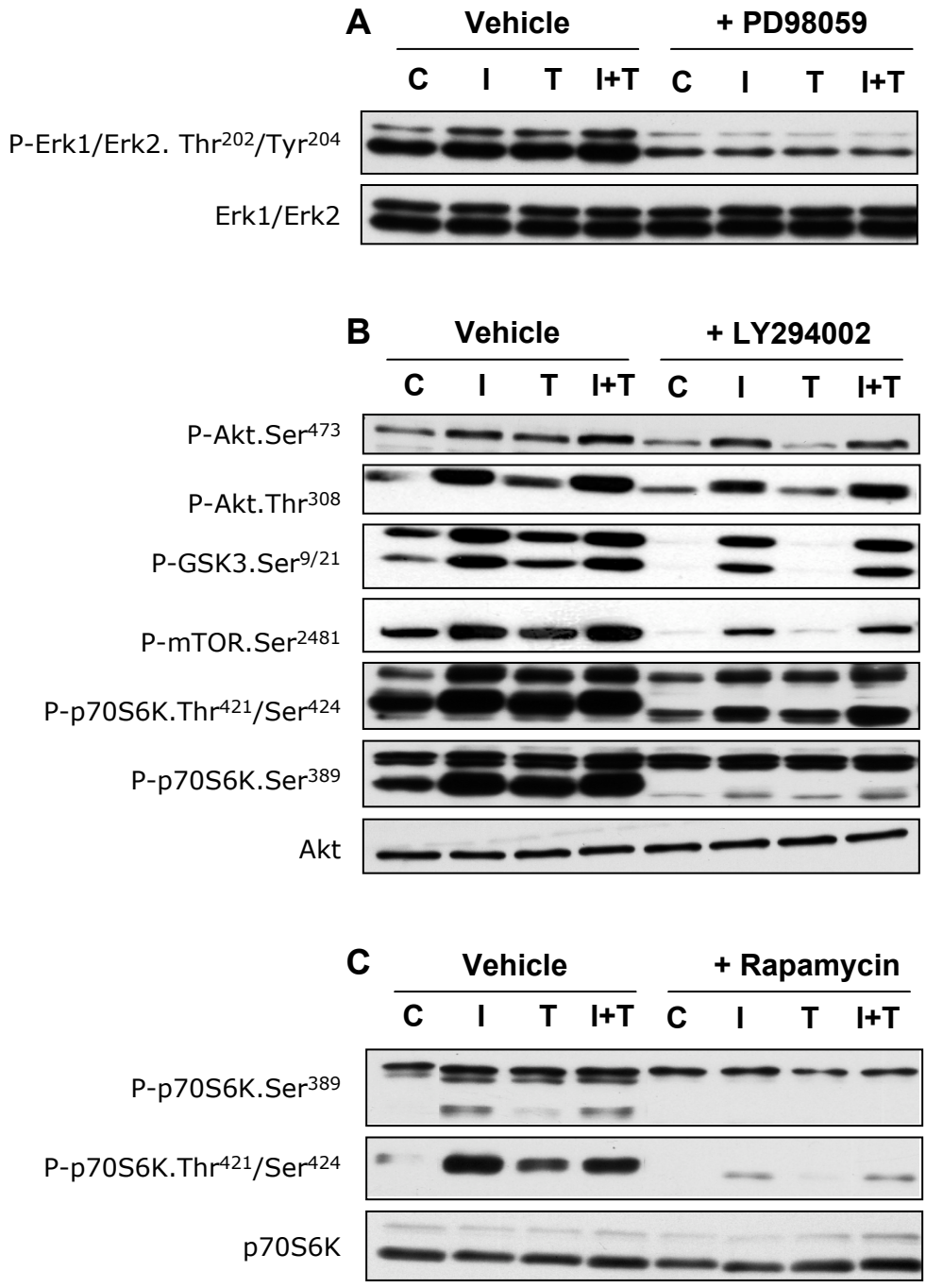

Figure 7. Effect of PD, LY and Rap on TNF-α- and IGF-I-induced phosphorylation of cellular kinases

Differentiated C2C12 were pre-treated with vehicle (DMSO) or with the pharmacological inhibitors (**A**: with PD, **B**: with LY and **C**: with Rap) as described for Fig. 5, followed by addition of IGF-I (I, 20 ng/ml) for 10 min, TNF-α (T, 10 ng/ml) for 30 min or both together (T+I) for 30 min. Cells were harvested and protein lysates were subjected to SDS/PAGE/immunoblotting. Immunoblots were analyzed for total and phosphorylated protein kinases as indicated. Results are representative of at least three independent experiments using independent preparations of C2C12.

LY inhibits insulin-induced Akt phosphorylation and protein synthesis

Insulin stimulates protein synthesis in skeletal muscle cells (Biolo et al; 1995) and cell lines (Kimball et al, 1998, Williamson et al, 2005, Shen et al, 2005), an effect reported to involve PI3-K (Scott et al, 1998). Therefore, we used insulin as a control to verify the lack of involvement of PI3-K in the IGF-induced effects. As shown in Fig 8, insulin induced strong phosphorylation of Akt at its two sites, Ser^{473} and Thr^{308} (Fig 8A), and increased PS by 35±1.2% compared to untreated cells ($P < 0.01$; Fig 8B). Consistent with previously published data (Scott et al, 1998; Shen et al, 2005), PI3-K inhibition by LY completely abolished all insulin-induced effects. In the same experiment, we reproduced the results of Figs 6 and 7: TNF-α-induced PS and Akt phosphorylation were again blocked by LY, whereas all IGF-I actions were unchanged (Fig 8).

LY blocks TNF-α- but not IGF-I-induced-Akt activation

TNF-α and IGF-I both seem to mediate their effect on PS to a large extent via Akt, even if our data indicate that the upstream signalling pathways implicated in its stimulation differ between them. In Fig 7B we showed that TNF-α-induced Akt phosphorylation completely disappeared when C2C12 myotubes were pre-incubated with LY, whereas IGF-I-induced Akt phosphorylation was still strong. To ensure that Akt phosphorylation at Ser^{473} and Thr^{308} is associated with enhanced Akt activity and to verify the effect of LY pre-treatment on TNF-α or IGF-I-induced Akt activation, the kinase activity was analyzed by the means of a kinase assay. As seen in Figure 9, TNF-α and IGF-I strongly increased Akt activity, with a more pronounced effect for IGF-I. The enhancement of Akt activity, measured by assessing the phosphorylation state of a down-stream fusion protein, was fully abrogated by LY pre-treatment for TNF-α. In contrast, in the presence of LY, the phosphorylation activity of Akt was still high after IGF-I stimulation.

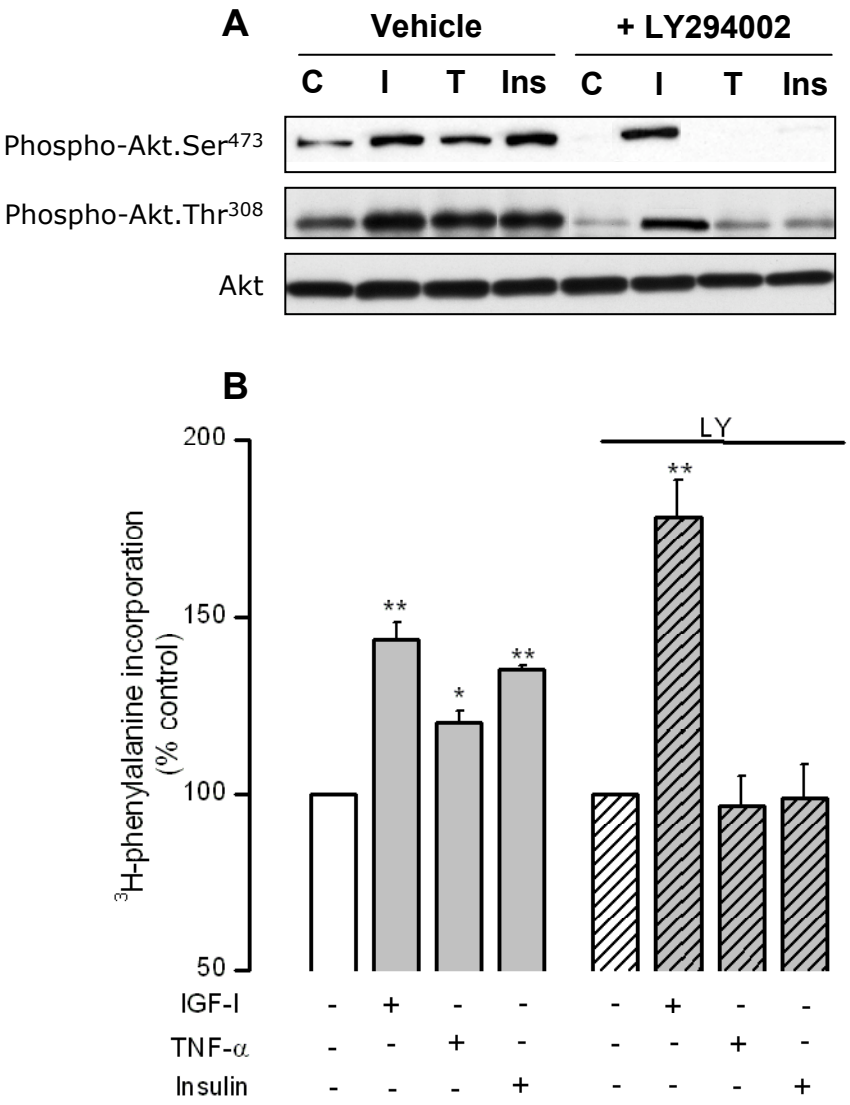

Figure 8. Effect of LY on insulin-induced protein synthesis and Akt phosphorylation

(**A**): Cells were pre-treated 30 min with LY (20 μM), before addition of IGF-I (I, 20 ng/ml) for 10 min, TNF-α (T, 10 ng/ml) for 30 min, or insulin (Ins, 15 ng/ml) for 10 min. Cell extracts were prepared and analyzed for total and phosphorylated Akt as for Fig 7. Result is representative of three experiments with independent preparations of C2C12. (**B**): C2C12 were pre-treated with LY for 30 min (hatched columns) or vehicle (DMSO, gray columns) before addition of IGF-I (20 ng/ml), TNF-α (10 ng/ml), or insulin (15 ng/ml) for 24 h. Protein synthesis was measured as for Fig 1. Results represent multiple experiments with independent preparations of C2C12 ($n \geq 3$), and each experiment was performed in triplicate. SD is calculated from the average values of the independent experiments. *$P < 0.05$, **$P < 0.001$ vs. control.

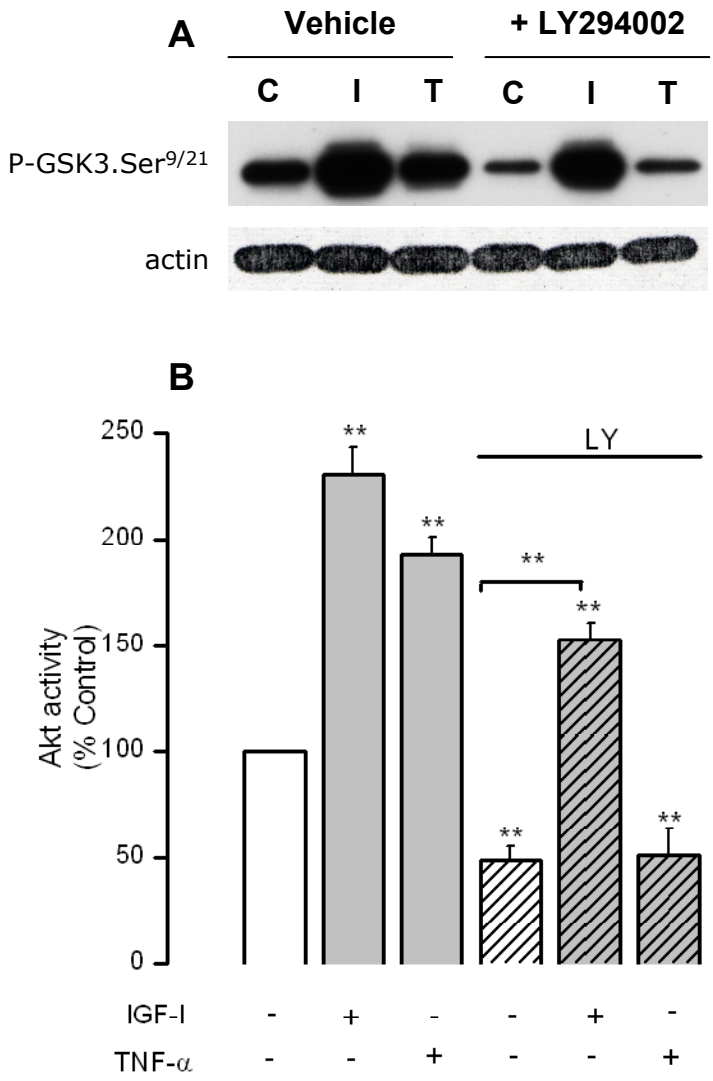

Figure 9. Effect of LY on IGF-I- and TNF-α-induced Akt activity

(**A**): C2C12 myotubes were pre-treated with LY (20 µM) for 30 min (hatched columns) or vehicle (DMSO, gray columns) before the addition of IGF-I (I, 20 ng/ml) or TNF-α (T, 10 ng/ml). Cytosolic extracts were prepared and PKB activation was determined by measuring phosphorylation of a GSK3 fusion protein as described in the experimental section. The membranes were reprobed with anti-actin to control loading. (**B**): The signals for phosphorylated GSK3 fusion protein were normalized with the corresponding signals for actin. Results are expressed in percentage of change of the phosphorylation of GSK3 fusion protein (± SD) compared to the control (set at 100%). Results are representative of three independent experiments using independent preparations of C2C12. SD is calculated from the average value obtained for the independent experiments.**$P < 0.01$ vs. control.

DISCUSSION

Our results provide evidence for two novel pathways, a TNF-α/PI3-K-dependent and an IGF-I/PI3-K-independent pathway, that both play an essential role in the stimulation of protein synthesis and metabolic activity in differentiated myotubes. Our findings are interesting for different reasons that will be discussed hereafter.

The role of TNF-α in muscle protein metabolism is controversial

A large body of evidence associates elevated circulating levels of TNF-α with skeletal muscle pathology (for review see Reid and Li, 2001; Argiles *et al* 1997). *In vivo* studies have described a loss of muscle mass and enhancement of protein breakdown when animals were treated with the cytokine (Garcia-Martinez *et al*, 1993 A) or expressed the TNF-α transgene (Cheng *et al*, 1992). However, beside these negative effects, other authors have indicated that the cytokine is involved in regeneration of skeletal myocytes after stress injury (Zador *et al*, 2001), or is expressed in regenerating muscle fibers during myopathy (De Bleecker *et al*, 1999; Kuru *et al*, 2003). In line with the emerging idea that TNF-α can be beneficial in skeletal muscle cells, here we show that TNF-α dose-dependently increases protein content in differentiated myotubes. This conclusion is supported by multiple experiments showing that TNF-α treatment significantly increases ^{3}H-phenylalanine incorporation and that it does not affect protein breakdown. These observations corroborate those that related stimulatory effects of TNF-α on protein synthesis (Langen *et al*., 2001; El Naggar *et al*, 2004; Foulstone *et al*, 2004), but are opposed to those where increases in protein degradation (Li *et al*, 1998) or decreases in PS (Frost *et al*, 1997) by the cytokine were observed. Similar to *in vivo* studies, results obtained with TNF-α on *in vitro* preparations are controversial. Several lines of evidence support that effects of TNF-α apparently depend on concentration, time of exposure as well as the type and state of the cell that is examined, including influences of culture conditions and the differentiation state of the cells (for review see: Argilés *et al*, 2000). In this context, recent studies have reported, even in the same cell line, opposite effects of the cytokine on protein content as a function of its concentration (Alvarez *et al*, 2001; El Naggar *et al*, 2004), or of culture conditions (Li *et al*, 1998; Langen *et al*, 2001). TNF-α mediates its actions through two cell surface receptors with distinct affinities: TNFR-1 and TNFR-2. Both receptors are expressed in skeletal muscle cells and in particular in C2C12 cells (Alvarez *et al*, 2002;

Fernandez-Celemin *et al*, 2002). TNFR-1 contains a death domain in its cytoplasmic tail that is important to induce apoptosis. Its stimulation also results in activation of the nuclear factor κB (NFκB) transcription factor that is involved in the regulation of pro- and anti-apoptotic genes (Liu *et al*, 1996; for review see: Liu, 2005). TNFR-2 does not contain a death domain in its cytoplasmic tail (for review see: MacEwan, 2002). Because most of the research efforts from many laboratories is devoted to TNFR-1, the function of the TNFR-2 is poorly understood. However, this receptor described to mediate in majority protective pathways (Rothe *et al*, 1995; for review see: Mann, 2002). Extensive crosstalk between pro- and anti-apoptotic pathways mediated by TNF-α could explain the large variety of cellular responses to this cytokine.

TNF-α uses both transcriptional and translational mechanisms to increase PS

TNF-α-induced PS was partially blocked by actinomycin D, supporting that TNF-α uses in part transcriptional mechanisms to exert its effect. One of the most important transcription factors rapidly stimulated by TNF-α is NFκB, which leads to transcription of a range of genes. Since NFκB was shown to promote survival in muscle cells (Mustapha *et al*, 2000. Catani *et al*, 2004; Ma *et al*, 2006), the actinomycin D-inhibited part of TNF-α-induced PS could be related to this pathway. In addition, up-regulation of mRNA of anti-inflammatory cytokines like IL-15 after TNF-α treatment was recently mentioned by Alvarez *et al* (2002). The decrease of muscle wasting associated with cachexia in tumor-bearing animals was partially reverted by this cytokine (Carbo *et al*, 2000). Therefore, regulation of expression of diverse genes by TNF-α can lead to beneficial effects.

CHX fully abrogated the effect of TNF-α on PS, proving that the increased of radioactivity is a consequence of the incorporation of the radio-labelled amino acid into peptides. We also provided evidence that the mechanism involves the PI3-K/Akt pathway. This conclusion is supported by data showing that 1) TNF-α-induced increases in PS were completely abolished by LY or Wortmannin, 2) TNF-α-increased phosphorylation of Akt and Akt-downstream effector molecules was fully blocked by LY pre-incubation, and 3) TNF-α-induced Akt activation was abrogated in the presence of this PI3-K inhibitor. These findings are in line with previous studies indicating that TNF-α increases PS or protein content in neonatal cardiac myocytes (Hiraoka *et al*, 2001) or in differentiated myotubes (El Naggar *et al*, 2004). However, while the effect of TNF-α was mediated via the PI3-K/Akt pathway in cardiac myocytes (Hiraoka *et al*, 2001), its effect was described to involve Erk1/2 and not

PI3-K in L6 myotubes (El Naggar *et al*, 2004). The PI3-K/Akt pathway was also reported to be activated after TNF-α stimulation in fibroblasts (Kim *et al*, 1999), retinal cells (Fontaine *et al*, 2002) or in a murine sarcoma cell line, the WEHI-164 cells (O'Toole *et al*, 2001). In addition, we provided evidence for the involvement of MEK in TNF-α-induced PS, since its inhibition with PD reduced partially TNF-α effects. Full inhibition with LY, however, supports that PI3-K is dominant over MEK.

Effect of TNF-α on cell viability

Interestingly, we also demonstrated that TNF-α does not reduce viability in C2C12 myotubes. Two results supported this: 1) Optical density reading for crystal violet staining did not decrease indicating that the number of cells was not reduced after TNF-α-treatment, and 2) the cytokine increased mitochondrial dehydrogenase activity. To date, most of the published data indicated an induction of apoptosis after TNF-α treatment in myoblasts (Langen *et al*, 2001; Foulstone *et al*, 2001; Stewart *et al*, 2004) or in differentiated myotubes (Li *et al*, 2000; Tolosa *et al*, 2005). A few papers have on the other hand mentioned that the cytokine does not induce apoptosis in primary human skeletal cell culture (Fousltone *et al*, 2004) or in neonatal cardiomyocytes (Mustapha *et al*, 2000; Hiraoka *et al*, 2001). In our work, we provide the first evidence that TNF-α enhances metabolic activity in differentiated myotubes. Interestingly, the degree of this enhancement was comparable to that induced by IGF-I. Similarly to the effect of TNF-α on PS, this action was mediated through the PI3-K pathway.

IGF-I can induce protein synthesis and metabolic activity in a PI3-K-independent manner

The stimulatory action of IGF-I on protein synthesis in muscle cells has been well documented and an important role for PI3-K in this effect has been demonstrated in cardiac (Seimi *et al*, 2004) as well as skeletal muscle preparations (Dardevet *et al*1996; Shen *et al*, 2005). Our findings for IGF-I are therefore surprising, because we showed that PI3-K is not involved in its effects on PS and metabolic activity. Very few other papers, and generally they were on myoblast cultures, have described IGF-I-actions that are not mediated through PI3-K. Only Rousse *et al* (1998) and Czifra *et al* (2006) have reported that IGF-I could evoke responses that are PI3-K independent in muscle cells. The first group described that in a C2 myoblast cell line IGF-I induced IGFBP-5 expression in a manner that is not

sensitive to LY or Wortmannin. The second article demonstrated the lack of involvement of the MAPK and PI3-K systems, but reported a central role of PKCδ in mediating mitogenic actions of IGF-I in a primary human skeletal muscle and in C2C12 myoblasts. IGF-I mediated protection from apoptosis independent of PI3-K/Akt pathway was already described in a rhabdomiosarcoma cell line (Thiammaiah *et al*, 2003).

To our best knowledge, our study demonstrates for the first time that IGF-I is able to enhance PS and metabolic activity in skeletal myotubes without the involvement of PI3-K, and we support this conclusion with the following results. PI3-K inhibition by LY did not affect any of the IGF-I-evoked cellular responses: Akt and its downstream effectors mTOR, GSK3 and p70S6K were all still phosphorylated after IGF stimulation in the presence of LY, and consistently, protein synthesis and metabolic activity were not affected either. The lack of blockade of LY on all actions of IGF-I is not due to a defect of the pharmacological reagent because Wortmannin, the alternative inhibitor of PI3-K, also failed to inhibit IGF-I effects (data not shown). Furthermore, insulin, known to share common signalling pathways and effects with IGF-I including IRS and PI3-K activation (Kimball *et al*, 1998), increased Akt phosphorylation and PS in C2C12 myotubes and this response was fully abolished by LY.

Using an activity assay, strong phosphorylation of a GSK3 fusion protein by Akt was observed after IGF-I stimulation in the presence of LY, confirming that the lack of effect of LY on phosphorylation is indeed associated with a lack of effect on the kinase activity. Since Akt is still highly activated by IGF-I in the presence of LY, we assume that Akt is the kinase that mediates the IGF-I-evoked increase in PS and viability that we measured, although strictly spoken this remains to be proven by using Akt inhibitors. Although most previously published data have implied PI3-K in the growth factor-dependent activation of Akt (Franke *et al*, 1997; Coffer *et al*, 1998; Downward *et al*, 1998), other pathways, independent of PI3-K have been mentioned (Konishi *et al*, 1996; Sable *et al*, 1997; Filippa *et al*, 1999), some of them implicating PKA (Sable *et al*, 1997; Filippa *et al*, 1999).

TNF-α and IGF-I act synergistically

Treatment with combinations of TNF-α and IGF-I always consistently induced higher increases in PS compared to the responses obtained with each factor alone (43 and 22% increases over control for IGF-I and TNF-α, respectively, compared to a 65% increase when they were co-incubated). This observation is in contradiction with

other studies that demonstrated an inhibition of the effects of IGF-I by TNF-α. TNF-α has been shown to diminish IGF-I expression in myoblasts (Fernandez-Celemin *et al*, 2002, Frost 2003) or to inhibit IGF-I-induced differentiation in primary skeletal human myoblasts (Foulstone *et al*, 2004). Even in the same cell line that we used, TNF-α was reported to impair signals and biological activity downstream of the IGF-IR (Broussard *et al*, 2003) and to inhibit IGF-I (Grzelkowska-Kowalczyk *et al*, 2006) or insulin-induced PS (Williamson *et al*, 2005). However, all these studies were performed in myoblasts, and in some of them, TNF-α was added during the differentiation process, whereas all our treatments were performed on differentiated myotubes. These different conditions (treatment, culture conditions and state of the cells) may explain in part the difference between our results and the previous studies.

The fact that TNF-α and IGF-I have additive effects on protein synthesis suggests that IGF-I and TNF-α use two different mechanisms to stimulate protein synthesis, and our results support this. The hypothetic signalling pathways and interactions involved in TNF-α or IGF-I-induced protein synthesis in C2C12 myotubes are presented in Figure 10. Akt causes in the phosphorylation of diverse substrates mainly involved in PS: mTOR, which consequently activates p70S6K and promotes liberation of the eukaryotic initiation factor 4 (eIF)4E (Kimball *et al*, 1998), and GSK3, which phosphorylation activates the eukaryotic initiation factor 2B (eIF)2B (Cross *et al*, 1995). Rap blocked around 50% of the response to IGF-I, which implies that mTOR/p70S6K mediates partially the IGF-I-induced PS. Since Rap did not change the GSK3 phosphorylation state (data not shown), Akt/GSK3 is the other pathway that could mediate a part of the IGF-I-induced protein synthesis (Fig 10A). Rap did not modify the TNF-α-induced effect, indicating that mTOR and p70S6K are not implicated in TNF-α-induced PS. Therefore, we conclude that TNF-α enhances PS in differentiated myotubes mainly via GSK3. PD blocked partially TNF-α-induced PS. The molecular mechanisms by which MAPK regulate PS remain poorly understood, but MAPK can phosphorylate eIF4 through Mnk1 (Ishida *et al*, 2003) and regulate protein synthesis (Fig 10B).

In summary, our results provide evidence for two novel pathways: a TNF-α/PI3K-dependent and an IGF/PI3K-independent pathway. Both play an essential role in the stimulation of protein synthesis in muscle cells. This study underlines the complexity of TNF-α's mechanism of action. This cytokine activates many signaling cascades, and cross talk between these cascades induces a large variety of cellular responses. In our study we have shown that TNF-α can, either by itself or together

with other factors, enhance beneficial survival pathways in skeletal myotubes: it increases PS and metabolism and improves the IGF-I-evoked responses. A better understanding of these mechanisms could help to design new strategies against muscle wasting that occurs during pathologies such as heart failure, where changes in hormonal and cytokine levels are generally observed.

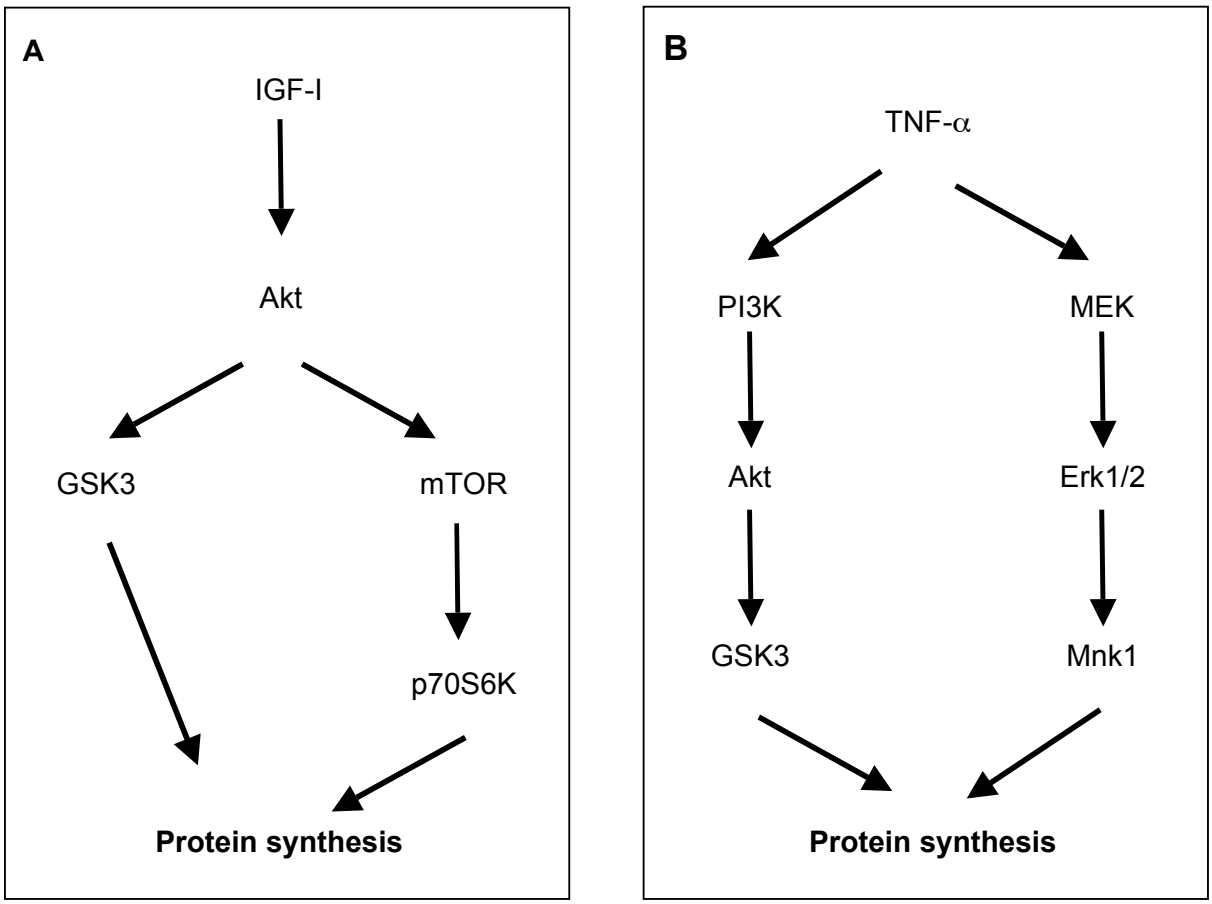

Figure 10. Diagram showing the hypothetic signalling pathways and connections involved in TNF-α or IGF-I-induced protein synthesis in C2C12 myotubes

Our results suggest that two pathways are implicated in the IGF-I-induced PS: the Akt/mTOR and the Akt/GSK3 pathways (**A**). The response to TNF-α may also involve two signalling pathways: the Akt/GSK3 pathway and the MEK/Erk1/Mnk1 system (**B**).

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VI. C. 2. Key findings and complementary results

Key findings

The previous study shows that both TNF-α and IGF-I increase the protein content of cultured skeletal muscle myotubes, and we demonstrate that TNF-α acts via PI3K, whereas IGF-I acts via a different kinase that remains to be identified.

IGF-I inhibits the transient up-regulation of Atrogin-1 mRNA expression induced by TNF-α

The positive effect of TNF-α on PS was surprising because this cytokine is generally thought to cause atrophy. However, one should keep in mind that the final cellular response is due to the balance between PS and PD. Therefore additional experiments were performed to determine the effects of TNF-α on protein degradation mediated by the UPP, in particular on Atrogin-1 gene expression, and the signaling pathways involved. IGF-I was used as a reference because it is known to downregulate Atrogin-1 expression.

Atrogin-1, also called MAFbx (for muscle atrophy F-box protein), is a ubiquitin ligase, an enzyme that binds and mediates ubiquitination of specific substrates. Atrogin-1 is considered a marker of atrophy because the corresponding gene has been shown to be upregulated in multiples models of skeletal muscle atrophy [61-63] (and reviewed in [19]). Fig 10 depicts the regulation of Atrogin-1 gene expression by IGF-I and by insulin [64], and shows its role in muscle protein degradation. This enzyme presents a major interest for three principal reasons. Firstly, its enzymatic activity seems to be required for muscle atrophy; secondly, it is expressed specifically in muscle skeletal and cardiac muscle cells; thirdly, it does not seem to be required for normal muscle growth and function [65].

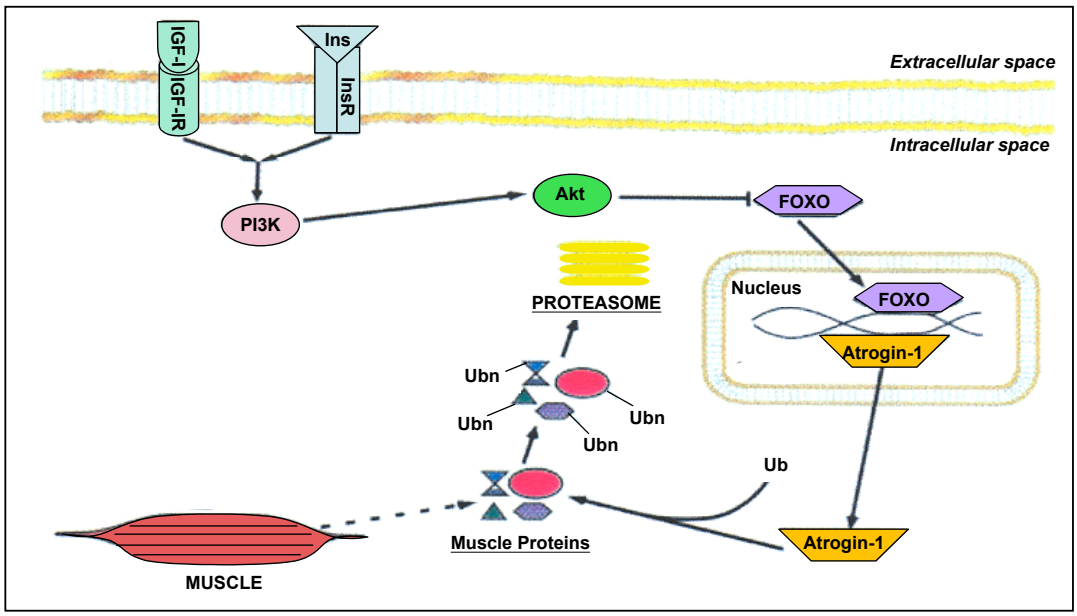

Fig 10. Atrogin-1 gene expression regulation by IGF-I and insulin in muscle cells

The ubiquitin ligase Atrogin-1 binds and mediates specific ubiquitination of muscle proteins. The polyubiquitinated (Ubn) proteins are then degraded by the proteasome. IGF-I and insulin activate the PI3K-Akt signaling pathway with subsequent inhibition of the FOXO transcription factor. This leads to inhibition of Atrogin-1 gene expression. Modified from [61].

We hypothesized that TNF-α increases Atrogin-1 expression and that IGF-I can reverse this effect, hence providing protection to muscle cells against protein degradation observed in pathologic conditions. To test this hypothesis we compared the effects of TNF-α alone or in combination with IGF-I on Atrogin-1 mRNA expression. C_2C_{12} myotubes were depleted of serum during 3 h, then incubated with TNF-α (10 ng/ml) or IGF-I (20 ng/ml) for various periods of time in the presence or absence of LY, PD or SB. Atrogin-1/MAFbx mRNA levels were measured by real-time PCR.

After 2 h of incubation, TNF-α induced a transient increase in Atrogin-1 gene expression of 1.7-fold, and subsequently decreased Atrogin-1 mRNA levels around 50% of the control value at 4 and 24 hours (Fig 11). When IGF-I was added together with TNF-α to the cells, the Atrogin-1 mRNA up-regulation was significantly reduced compared to TNF-α alone, indicating that IGF-I prevents TNF-α-induced Atrogin-1 mRNA increase. IGF-I by itself decreased Atrogin-1 mRNA levels by approximately 50% at all time points (Fig 11).

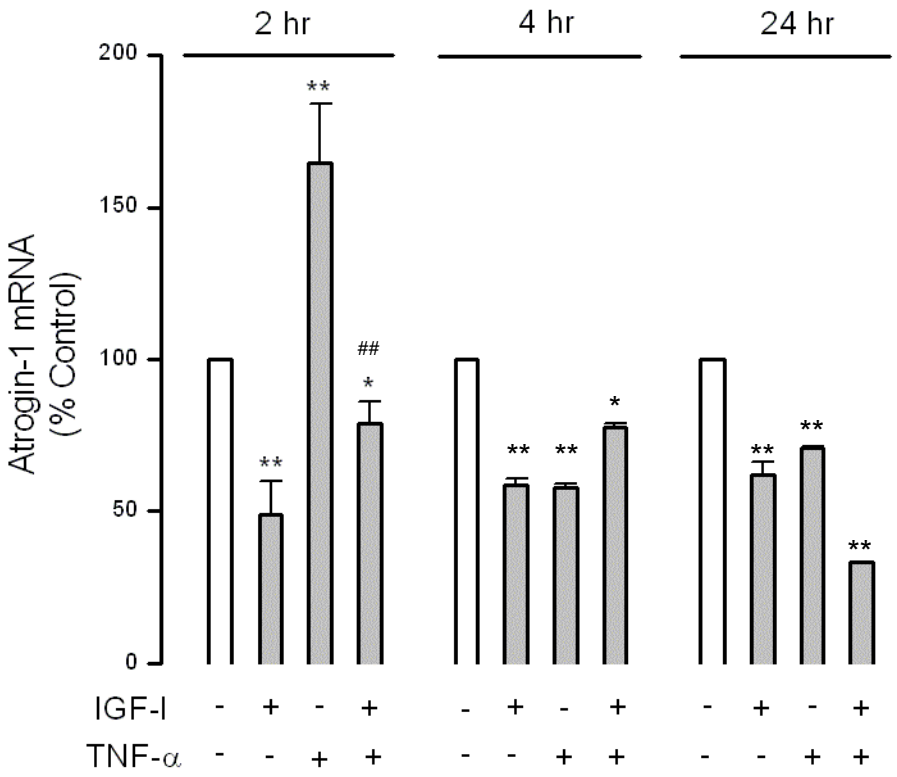

Fig 11. IGF-I prevents TNF-α-induced Atrogin-1 expression in C2C12 myotubes

C2C12 myotubes were treated with IGF-I (20 ng/ml) or TNF-α (10 ng/ml) or both for 2 h, 4 h or 24 h. At each point, cells were collected, total RNA was extracted and RT-PCR was performed. Atrogin-1 mRNA regulation was analysed by quantitative real-time PCR. Results are expressed as percentage of change in Atrogin-1 mRNA expression compared to the control at each time point (white columns). Values represent the mean ± S.E. of 3 independent experiments. *, significantly different from control, $P < 0.05$; **, significantly different from control, $P < 0.01$; ##, significantly different from TNF-α, $P < 0.01$.

We then examined the pathways involved in the responses by using three specific inhibitors: LY (blocker of PI3K), PD (blocker of MEK1/2) and SB (blocker of p38 MAPK). First, as a control we assessed the effect of these inhibitors on basal Atrogin-1 gene expression. All the pharmacological agents increased Atrogin-1 mRNA level, in particular LY and PD (3.5-fold and 2.7-fold increase respectively) (Fig 12). This result indicates an inhibitory role for PI3K and MEK1/2 on Atrogin-1 gene expression. SB pretreatment blunted the TNF-α-induced increase of Atrogin-1 mRNA, while LY and PD had no effect (Fig 13). Fig 13 shows again that IGF-I inhibits the increase of Atrogin-1 mRNA induced by TNF-α, but none of the inhibitors used was able to block this effect. In all cases, when IGF-I was added to TNF-α, IGF-I significantly reduced atrogin-1 mRNA level (Fig 13).

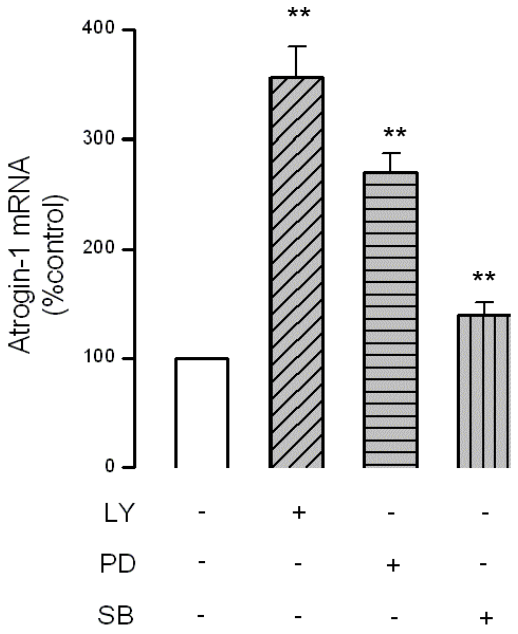

Fig 12. LY, PD and SB increased basal Atrogin-1 expression in C2C12 myotubes

C2C12 myotubes were incubated with DMSO (vehicle control) LY (20 µM for 1h30 min, hatched column) PD (10 µM for 3h, column with horizontal lines), or SB (10 µM, for 3h, column with vertical lines) and real time PCR was assessed after total RNA extraction and RT-PCR. Results are expressed as percentage of change in Atrogin-1 mRNA expression compared to the control (white column). Values represent the mean ± S.E. of 3 independent experiments.**, significantly different from control, $P < 0.01$.

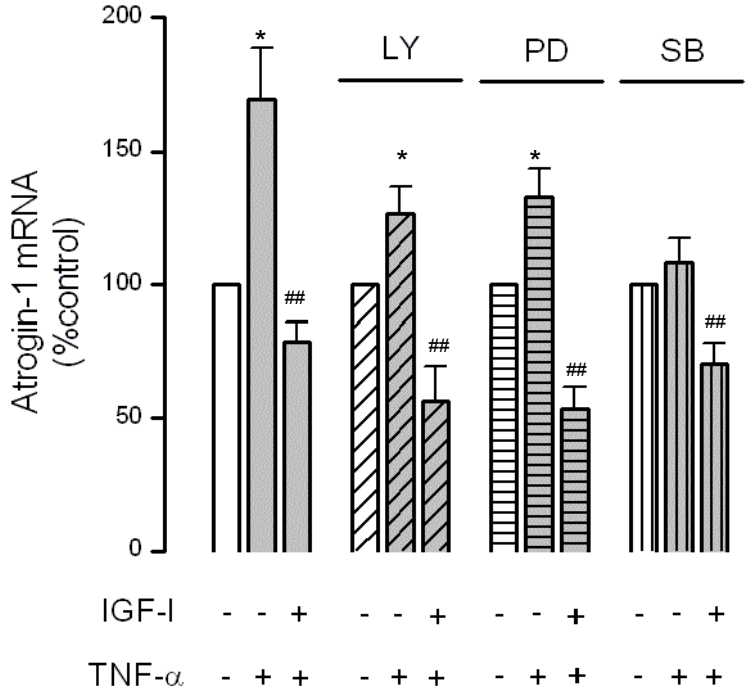

Fig 13. TNF-α and IGF-I regulate Atrogin-1 gene expression

C2C12 myotubes were pretreated with the inhibitors (LY, 20 µM, 30 min, hatched columns or PD, 10 µM, 1 h, columns with horizontal bars or SB, 10 µM, 1 h, columns with vertical lines) then the hormones were added for 2h (IGF-I, 20 ng/ml or TNF-α, 10 ng/ml). Cells were collected, total RNA was extracted and RT-PCR was performed. Atrogin-1 mRNA expression was analysed by quantitative real-time PCR. Results are expressed as percentage of change in Atrogin-1 mRNA expression compared to the control (white columns). Values represent the mean ± S.E. of 3 independent experiments. *, significantly different from control, $P < 0.05$; ##, significantly different from TNF-α, $P < 0.01$.

Altogether these results show that:

- TNF-α upregulates Atrogin-1 mRNA levels, via the p38 MAPK signaling pathway
- This effect is blocked by IGF-I in a PI3K, MEK-ERK1/2, p38 MAPK independent manner

Our data indicate that in cultured skeletal myotubes, TNF-α transiently (2 h) activates protein degradation mechanisms in a p38 MAPK dependent manner and that this effect can be inhibited by IGF-I. Surprisingly, the marked effect of IGF-I is not mediated by PI3K, which is well described as the IGF-I-induced mechanism downregulating Atrogin-1 gene expression in cardiac [19, 66] and skeletal muscle cells [60, 62] (and reviewed in [19]). The IGF-I-induced activation of the UPP in the presence of TNF-α involves a signaling pathway that still remains to be identified.
At later time points (4 and 24 h) however, TNF-α decreases Atrogin-1 mRNA levels suggesting inhibition of protein breakdown. This was again an astonishing result, given the fact that TNF-α levels are increased in catabolic conditions leading to muscle mass loss [13-15]. Involvement of the UPP in skeletal muscle atrophy has been well established, nonetheless control of apoptosis is also involved in the regulation of musle protein degradation [67]. The latter mechanism may also be induced by TNF-α in our model.

VI. C. 3. C_2C_{12} skeletal myocytes: KEY FINDINGS

The TNF-α-induced inhibition of protein breakdown mechanisms complements in a consistent way our previous observation that TNF-α increases protein content at 4 and 24 h. Our *in vitro* results therefore suggest that TNF-α may have a beneficial role in preventing muscle wasting.
Furthermore, the “classical” PI3K-Akt pathway is not involved in both responses to IGF-I that we measured in our model, namely the increase in PS and the inhibition of TNF-α-induced Atrogin-1 expression.

VII. FINAL CONCLUSIONS

In the present thesis, we have shown different effects mediated by factors involved in the cardiac remodeling process, namely TNF-α and Ang II. These factors were shown to (1) modulate the IGF-I system, at the level of the binding proteins and specific signaling cascades, and (2) to have combined effects with IGF-I or opposite effects to IGF-I.

- Our study provides the first description of regulation of cardiac IGFBPs by Ang II in an *in vivo* model of hypertrophy. At this point, further studies are required to determine the physiological significance of the increase in IGFBP-4 and reduction of IGFBP-3 by Ang II in cardiac tissue.
- Our study in cardiac cells showed dual effects of TNF-α: cell viability was decreased and expression of apoptotic markers was enhanced by TNF-α in the long-term. However, our findings also suggest a beneficial role for TNF-α in cardiomyocytes through its down-regulation of IGFBP-4 and enhancement of IGF-I-induced ERK signaling.
- We also demonstrate that TNF-α is beneficial in skeletal muscle cells through its induced increase in metabolic activity and protein synthesis. Furthermore TNF-α acts synergistically with IGF-I on protein synthesis. In the same model, TNF-α showed a transient increase at 2 h in activation of protein degradation mechanisms, but after 4 h this effect was reversed as shown by reduced Atrogin-1 expression. The latter findings suggest a harmful effect of TNF-α only at short time, which can be counteracted by IGF-I, while in the long-term TNF-α may play a beneficial role in the prevention of muscle wasting.

In conclusion, at the cellular level fine-tuning is required to integrate all these signals and determine the cell fate: hypertrophy and survival or death and atrophy.
These mechanisms may play a fundamental role in regulating the cardiac remodeling process as well as muscle atrophy, in heart failure.

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