List of abbreviations

4EBP1	eIF4E-binding protein 1
5'-TOP mRNA	5' terminal oligopyrimidine messenger RNA
ALS	acid labile subunit
ATP	adenosine triphosphate
ca	constitutively active
dn	dominant negative
DNA	deoxyribonucleic acid
cDNA	complementary DNA
CMV	cytomegalovirus
CSA	cross sectional area
Ctrl	control
DAB	diaminobenzidine
eIF2	eukaryotic initiation factor 2
eIF4E	eukaryotic initiation factor 4A
ERK	extracellular signal-regulated kinase
Foxo3a	forhead box O 3a
Gal	galactosidase
GC	glucocorticoid
GH	growth hormone
GSK-3β	glycogen synthase kinase-3β
HA	hemaglutinin A
IGF-I	insulin like growth factor I
IGF-II	insulin like growth factor II
IGFBP	IGF binding protein
IGF-IR	IGF-I receptor type I
IGF-IIR	IGF-I receptor type II
IR	insulin receptor
IRS	insulin receptor substrate
kDa	kilodalton
KO	knock out
LID	liver gene-deletion

MAPK	mitogen-activated protein kinase
MGF	mechano growth factor
MHC	myosin heavy chain
mRNA	messenger Ribonucleic Acid
mTOR	mammalian Target of Rapamycin
MuRF1	muscle ring finger 1
NFATc	nuclear factor of activated T-cells
ns	not significant
PIP2	phosphatidylinositol biphosphate
PIP3	phosphatidylinositol triphosphate
PTEN	phosphatase and tensin homolog
p70S6K	protein 70 kDa ribosomal S6 kinase
PI3kinase	phosphoinositide kinase-3
S6	ribosomal protein S6
SHIP2	SH2 domain containing phosphatidylinositol 5-phosphate
TA	tibialis anterior
VSV-G	vesicular stomatitis virus-glycoprotein
UT	untransfected

Chapter 1: Introduction

1. Regulation of muscle mass by IGF-I

1.1. Generalities

1.1.1. The IGF family

The Insulin Like Growth factor (IGF)s family is constituted by IGF-I and IGF-II, two molecules playing a critical role for normal growth and development. IGF-I and IGF-II have a structure close to that of pro-insulin (1). IGF-I, also called Somatomedin C, is composed by 70 amino acid residues (7.6kDa). This growth factor is required for normal development and post-natal growth (2;3). In contrast, IGF-II, which presents 70% of homology with the IGF-I amino acid sequence, plays a critical role in embryonic growth (4;5).

1.1.2. IGF-I gene and protein structure

The human IGF-I gene locus is composed of 6 exons (6) and its transcription gives rise to multiple mRNAs. The different transcripts result from different promoter usage, alternative splicing and various polyadenylation sites. Nevertheless, the coding sequence for the mature peptide is the same throughout all the different transcripts. The transcription of IGF-I can be initiated either at the first or at the second exon (black arrow, Figure 1A) giving respectively class 1 and class 2 transcripts. mRNAs initiated at exon 1 are expressed in many tissues and especially in muscle whereas those initiated at exon 2 are mainly expressed in the liver. Mature peptide (B, C, A, D, light grey box, Figure 1A) and E domain (dotted box, figure 1A) are encoded by exons 3, 4, 5 and 6. Isoform precursors IGF-I EA and IGF-I EB result from an alternative splicing of exons 5 and 6. Exon 4 is followed either by exon 5 (IGF-I $1E_B$) or by exon 6 (IGF-I E_A) (Figure 1B) (7). Exon 6, encoding the 3' untranslated region, presents multiple polyadenylation sites. Finally, another alternative splicing site has been described in exon 5 giving rise to IGF-I E_C isoform. The two isoforms which appear most relevant in muscle development are: IGF-I E_A (termed muscle IGF-I or mIGF-I) and IGF-I E_C (termed mechano-growth factor or MGF). Based on the work of Rosenthal and co-workers, muscle

overexpression of IGF-I E_A causes muscle hypertrophy. Interestingly, in the transgenic model described by Musaro and coworkers, overexpressed IGF-I remains in the muscle bed and does not enter into the circulation (8). It is not clear which of the isoform or of the promoter is responsible for restricting the IGF-I protein to muscle. The IGF-I E_C mRNA, largely described by Goldspink *et al*, seems to be produced only by damaged or loaded skeletal muscle (9-11). In a recent review, Goldspink and Yang have reported that administration of IGF-I E_C peptide or overexpression of IGF-I E_C cDNA in normal and in dystrophic muscles more effectively increases the muscle strength than administration of IGF-I E_A isoform (12). The reason why IGF-I E_C might be more effective in producing muscle hypertrophy compared to IGF-I E_A is potentially linked to its stimulatory effect on the proliferation of satellite cells (13). Based on these observations, the IGF-I E_C isoform would seem therefore more suitable to counteract muscle atrophy.

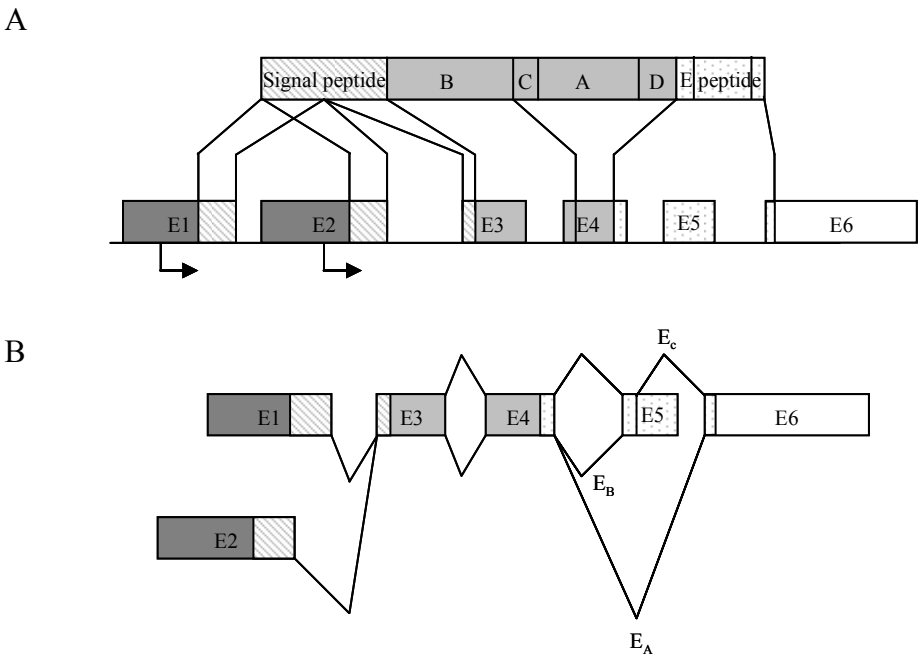

Figure 1. *Human IGF-I gene organization (A) and Human IGF-I mRNA transcripts (B)*. Mature peptide (B, C, A, D, light grey box, top) and E domain (dotted box, top) are encoded by exons 3, 4, 5 and 6. Isoform precursors IGF-I E_A, IGF-I E_B, and IGF-I E_C result from an alternative splicing of exon 5 and 6. Exon 4 is followed either by exon 5 (IGF-I $1E_B$) or by exon 6 (IGF-I E_A) or by exon 5 and 6 (IGF-I E_C) (Schakman *et al.* 2006)

1.1.3. Tissular expression and regulation

The main source of circulating IGF-I is the liver (75%) (14). Hepatic IGF-I is produced in response to Growth Hormone (GH) and acts mainly in an endocrine manner. IGF-I is also produced by most of the other tissues and especially by skeletal muscle where it acts in a more paracrine/autocrine manner (15). Production of muscle IGF-I can be influenced by many different factors (see table 1).

Conditions	Muscle IGF-I expression
GH administration	↗
Hypophysectomy	↘
IGF-I infusion	↘
Exercice	↗
Hindlimb suspension	↘
Testosterone administration	↗
Hypogonadism	↘
Glucocorticoids	↘
TNF-alpha	↘
IL-1beta	↘
Endotoxin	↘
Caloric restriction	↘

Table.1 *Rodent muscle IGF-I expression*. Muscle IGF-I expression is influenced by different factors. Interestingly, in the conditions represented in table 1, muscle mass gain or loss follow the same trend of muscle IGF-I expression.

1.1.4. General biological actions

IGF-I is essential for normal growth and development. It is the major mediator of the post-natal growth promoting actions of GH. Indeed, growth deficiency observed in hypophysectomised rats can be corrected with IGF-I administration (16) and administration of GH does not stimulate growth in IGF-I null mice (3). The necessity of IGF-I for normal growth has also been demonstrated in knockout mice models. Mice carrying null mutations in the IGF-I gene are born small and grow very poorly postnatally (2;17). Moreover, animals which do not express the IGF-I receptor do not survive after birth (18). Interestingly, mice carrying a liver IGF-I gene-deletion (LID) (19), (20) show no major impairment of growth, despite a large decline of circulating IGF-I concentrations. In the same manner, in the absence of peripheral tissue IGF-I expression, endocrine IGF-I derived from liver is sufficient to

maintain normal growth and development (21). Thus, its appears that local production of IGF-I is as also important as circulating IGF-I in promoting post-natal growth. It is to be noted that administration of GH has been shown to be more effective in promoting longitudinal bone growth, whereas IGF-I is more effective to simulate kidney and spleen growth (22). IGF-I is also able to mimic most of the metabolic effects of insulin. Indeed, administration of IGF-I induces a hypoglycemia mainly due to an increase of glucose uptake by peripheral tissues. Like insulin, IGF-I also inhibits lipolysis and proteolysis (23).

1.1.5. IGF-I binding proteins and IGF-I receptor

IGF-I binding proteins (IGFBPs)

In the blood, IGF-I is bound by a number of well characterized high affinity binding proteins (IGFBPs) (24). These act as carrier proteins, transporting IGF-I of the circulation to the target tissues and prolonging its half-life. Seven IGFBPs have been cloned and sequenced. In the blood, most IGF-I (>90%) is found in a ternary complex of 150 kDa composed of IGFBP-3 or 5 and the acid labile subunit (ALS) (25;26), which stabilizes the complex and prevents rapid degradation of IGF-I (27;28). Adult skeletal muscle expresses IGFBP-4, IGFBP-5 and IGFBP-6 (29). These binding proteins modulate the biological action of IGFs and especially muscle IGF-I action. Indeed, it has been demonstrated that IGFBP-4 inhibits the proliferation and differentiation of myoblast induced by IGF-I. IGFBP5 has dual effects on skeletal myogenesis: IGFBP5 inhibits the IGF-I induced proliferation but stimulates the IGF-I induced differentiation of L6 myoblasts (30). IGFBP-6 inhibits myoblast differentiation induced by IGF-I. (31). Finally, i*n vivo*, deletion of IGFBP-3, -4 or -5 has a minimal impact on skeletal muscle development (32) whereas their generalized overexpression impairs muscle growth (33).

IGFs receptors

IGF-I can interact with the three following receptors but with different affinity: the type I IGF-I receptor (IGF-IR, high affinity), the type II IGF-I receptor (IGF-IIR, low affinity) and the insulin receptor (IR, low affinity). In contrast, IGF-II has the ability to bind to the three receptors with comparably high affinities (34). IGF-I does not bind to the IR, except at high concentrations, since IGF-I has a relative affinity almost two orders of magnitude higher for the IGF-IR, as compared to the IR (35). As represented on figure 2, IGF-IR is a trans-membrane tyrosine kinase receptor structurally identical to the IR. IGF-IIR is identical to the

previously characterized cation-independent mannose-6-phosphate (M6P) receptor, which is thought to function as a clearance receptor for IGF-II (36). Skeletal muscle expresses the three kinds of receptor and most of IGF-I and IGF-II biological effects are thought to be mediated by IGF-IR. Surprisingly, although IGF-I is critical for load-induced skeletal muscle hypertrophy, a functional IGF-IR does not seem mandatory (37).

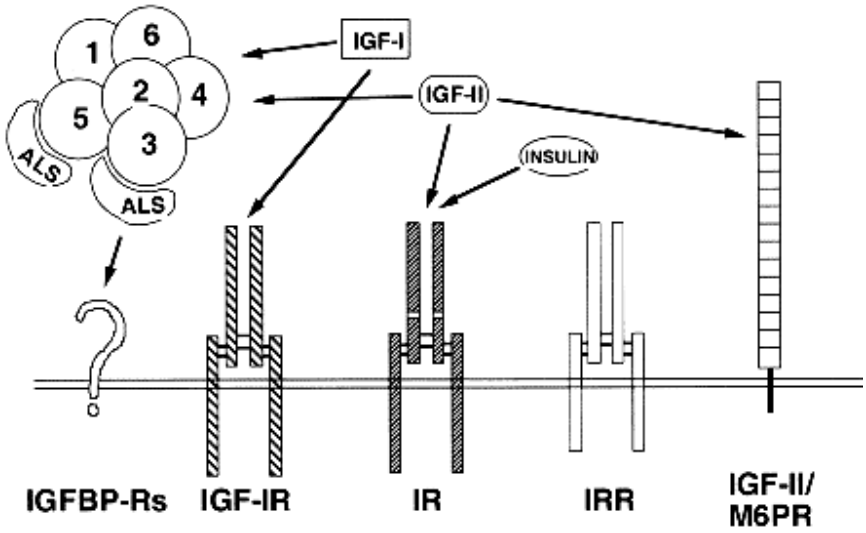

Figure 2. *The IGF-I system*. As indicated, IGF-I interacts with the IGF-IR and with IGF-binding proteins (see text), IGF-II interacts with IGF-IR, IGF-IIR and IGF-binding proteins while insulin interacts with IR. Interestingly, some of the IGFBPs effects could be exerted independently of their modulation of IGF-I signaling (35).

1.2. Mechanisms of IGF-I anabolic and anticatabolic action in muscle

1.2.1. Muscle anabolic action

1.2.1.1. Induction of muscle hypertrophy

Skeletal muscle hypertrophy is defined by an increase in muscle fiber cross sectional area. Muscle hypertrophy induced by IGF-I results from autocrine/paracrine effects on preexisting myofibers and on satellite cells (8;38). Locally, IGF-I induces myofibers hypertrophy through anabolic (stimulation of satellite cells proliferation and differentiation, increase of protein and DNA synthesis) and anticatabolic effects (inhibition of proteolysis and apoptosis). The muscle hypertrophic effect of IGF-I has been clearly demonstrated in muscle-specific transgenic mice in which local muscle IGF-I overexpression induces muscle hypertrophy (8;39).

1.2.1.2. Stimulation of muscle cell proliferation and differentiation

IGF-I stimulates myogenesis by inducing the activation of muscle stem cells, also called satellite cells to become myoblasts which then proliferate and differentiate into myotubes/myocytes and finally in mature myofibers (41). IGF-I stimulates proliferation by inducing expression of intracellular mediators such as cycline D and c-fos. This mitogenic response is mainly mediated by the MAPK/Erk (Mitogen-Activated Protein Kinase/ Extracellular signal-regulated kinase) pathway (40). In the first step of the differentiation, the myoblasts express many myogenic transcription factors such as MyoD and myogenin leading to their fusion with preexisting mature myofibers (41) (Figure 3). During the late phase of differentiation, muscle cells produce myofibrillar proteins, actin and myosin, which play a crucial role in the muscle contractile machinery. The myogenic response is mediated by the PI3kinase (phosphoinositide kinase-3) signaling pathway (42). Since IGF-I induced muscle hypertrophy is dependent of muscle satellite cells proliferation and differentiation (43), it is not surprising that inhibition of the MAPkinase pathway (44) or destruction of the proliferative capacity of satellite cells by gamma irradiation (45) blunt IGF-I induced muscle hypertrophy *in vivo*.

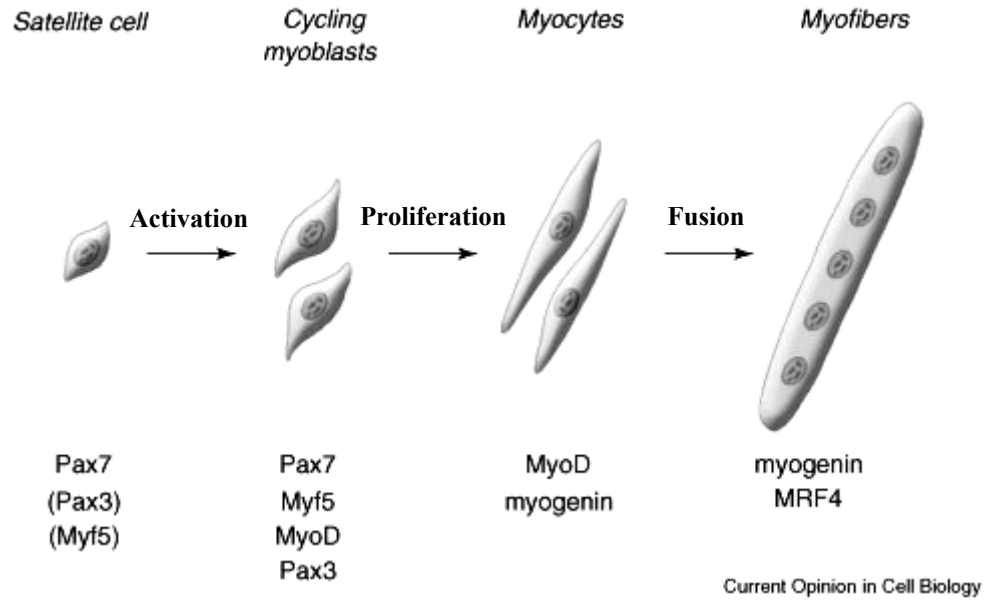

Figure 3**.** Schematic representation of adult myogenesis. Quiescent skeletal muscle satellite cells can become activated and express the paired-box transcriptions factors Pax7 and Pax3, as well as the myogenic regulatory factors Myf5 and MyoD. Once committed to differentiation, myoblasts lose expression of Pax7, Pax3, and Myf5. During the differentiation, myocytes will then align and fuse to form multinucleated myofibers (41).

1.2.1.3. Stimulation of protein synthesis

The muscle anabolic action of IGF-I is also mediated by an increase in protein synthesis (46). *In vitro*, IGF-I increases amino acid uptake and protein accretion (47). *In vivo*, administration of IGF-I directly increases protein synthesis in skeletal muscle (48). IGF-I stimulates protein synthesis by increasing gene transcription, ribosomal biogenesis and translation of mRNAs (40). The latter effect results from activation of different factor mandatory for translation such as eIF2 and eIF4E (49). The effect of IGF-I on mRNA translation is mediated by activation of the PI3Kinase/Akt/mTOR pathway. mTOR enhances protein synthesis by inhibiting 4E-BP1 and by stimulating the p70S6kinase (50).

1.2.2. Muscle anticatabolic action

1.2.2.1. Inhibition of proteolysis

The muscle anticatabolic action of IGF-I results from an inhibition of proteolysis. This is observed both *in vitro* on myotubes (51), *ex vivo* on isolated muscle (52) and *in vivo* in animals and humans (53;54). The intracellular mechanism of the anti-proteolytic action of IGF-I results from the inhibition of protein degradation by the lysosomal system, the calcium dependent calpain system and the ubiquitin proteasome system (55) (Figure 4). By activating the PI3K/Akt pathway, IGF-I down-regulates the expression of Atrogenes such as Atrogin-1, MuRF-1, Cathepsin-L involved in these different proteolytic systems (56-58). Other proteolytic systems responsible for myofibrillar degradation are also inhibited by IGF-I. Indeed, actin proteolysis by caspase-3 is inhibited by IGF-I (59). Inhibitory action of IGF-I on muscle proteolysis is mediated by the stimulation of PI3kinase pathway leading to the inhibition of GSK-3β (60;61) and Foxo3a (62).

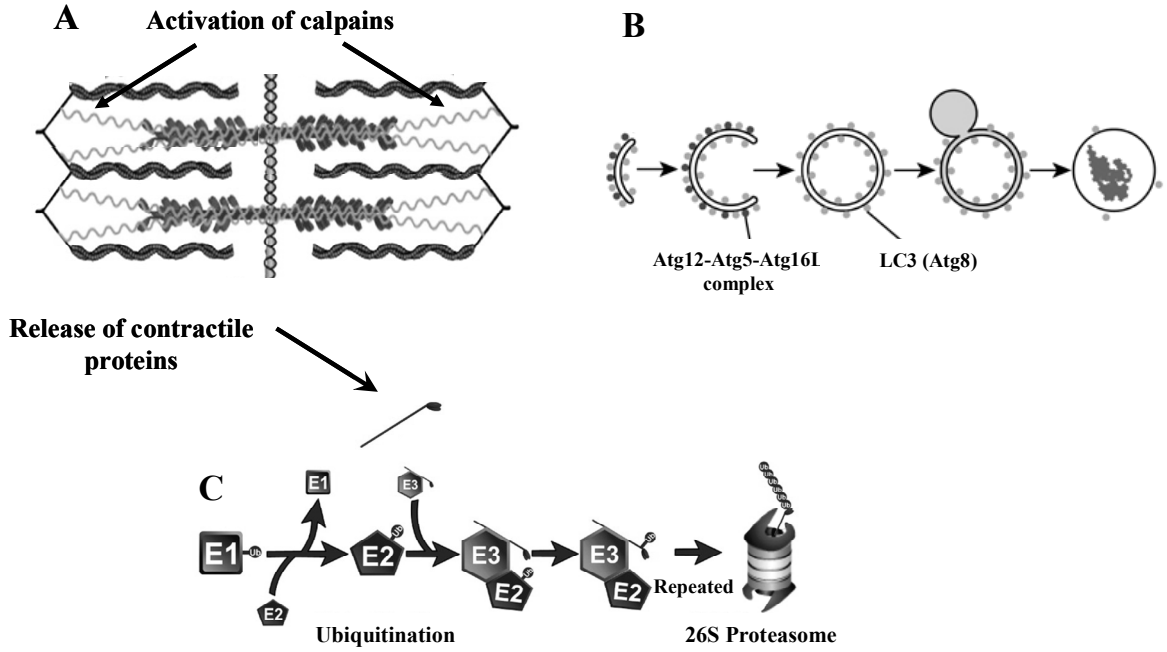

Figure 4. *The three major proteolytic systems implicated in muscle atrophy*. The intracellular mechanism of anti-proteolytic action of IGF-I results from the inhibition of the calcium-dependent calpain system (A), the lysosomal protease system (cathepsins; B), and the ubiquitin (Ub; C)-proteasome system. Figure adapted from Jackman *et al*. (63).

1.2.2.2. Inhibition of apoptosis

IGF-I is also implicated in cell survival. In serum free medium, IGF-I promotes myoblast survival via the PI3kinase/Akt pathway (64). This anti-apoptotic effect requires the phosphorylation of p21 protein, an inhibitor of cycline-dependent kinase (CDK). It has also been demonstrated that IGF-I decreases apoptosis by inhibition of BAD and BAX, which are implicated in Bcl-2 activation, a pro-apoptotic factor, and by inhibition of caspase-3, an enzyme involved in apoptosis (65).

1.3. Muscle IGF-I transduction pathways

1.3.1. Main muscle IGF-I transduction pathways

IGF-I exerts its biological action on skeletal muscle through multiple signaling mechanisms, including the PI3-kinase, MAP kinase and calcineurin pathways. The first pathway leads to the activation of Akt via activation of the insulin receptor substrate-1 (IRS-1). Indeed, the

binding of IGF-I induces a conformational change in the IGF-IR, activating its tyrosine kinase activity. This results in its trans-phosphorylation and the subsequent phosphorylation of IRS-1 on tyrosine residues. In turn this induces the activation of the PI3-Kinase. Once phosphorylated, IRS-1 binds the SH2 domains of p85 subunit of PI3-Kinase a the critical step in its activation (66). The activation of this kinase results in production of phosphatidylinositol-3, 4, 5-triphosphate, which provides a membrane docking site for the serine/threonine kinase Akt (also known as PKB) (67). Once activated, Akt phosphorylates an array of substrates involved in muscle anabolic and anticatabolic action of IGF-I (protein synthesis, cell survival…) (68;69). The second pathway activated by IGF-I, the MAP-kinase pathway, is more involved in the mitogenic action of IGF-I (40). Finally, IGF-I can also activate the calcineurin/NFAT pathway. Calcineurin is a Ca^{++} Calmodulin-activated serine/threonine phosphatase. Downstream targets of calcineurin include members of the nuclear factor activated T-cells (NFAT) and myocyte enhancer factor 2 (MEF2) family of transcription factors (70;71). Calcineurin is able to bind directly and to dephosphorylate the transcription factor NFAT within the cytoplasm, allowing its translocation to the nucleus where it participates in activating gene expression (70). Calcineurin-mediated NFAT and MEF2 activation regulates expression of muscle specific genes including MYF5, myoglobin, type I myosin heavy chain and slow troponin I (72-74). While the calcineurin pathway seems to be essential for mediating fiber-type switching towards the slow/oxidative phenotype, it appears not mandatory for skeletal muscle hypertrophy (Figure 5) (69;75-77).

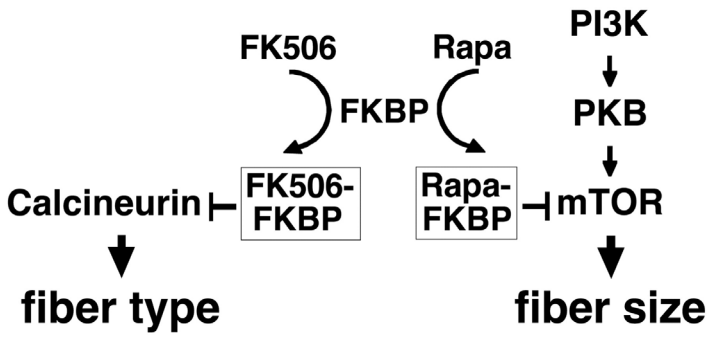

Figure 5. *Main muscle signaling pathways induced by IGF-I.* PI3kinase/Akt pathway is essentially involved in fiber size regulation whereas calcineurin is involved in the determination of muscle fiber phenotype (76).

1.3.2. The PI3K-Akt pathway

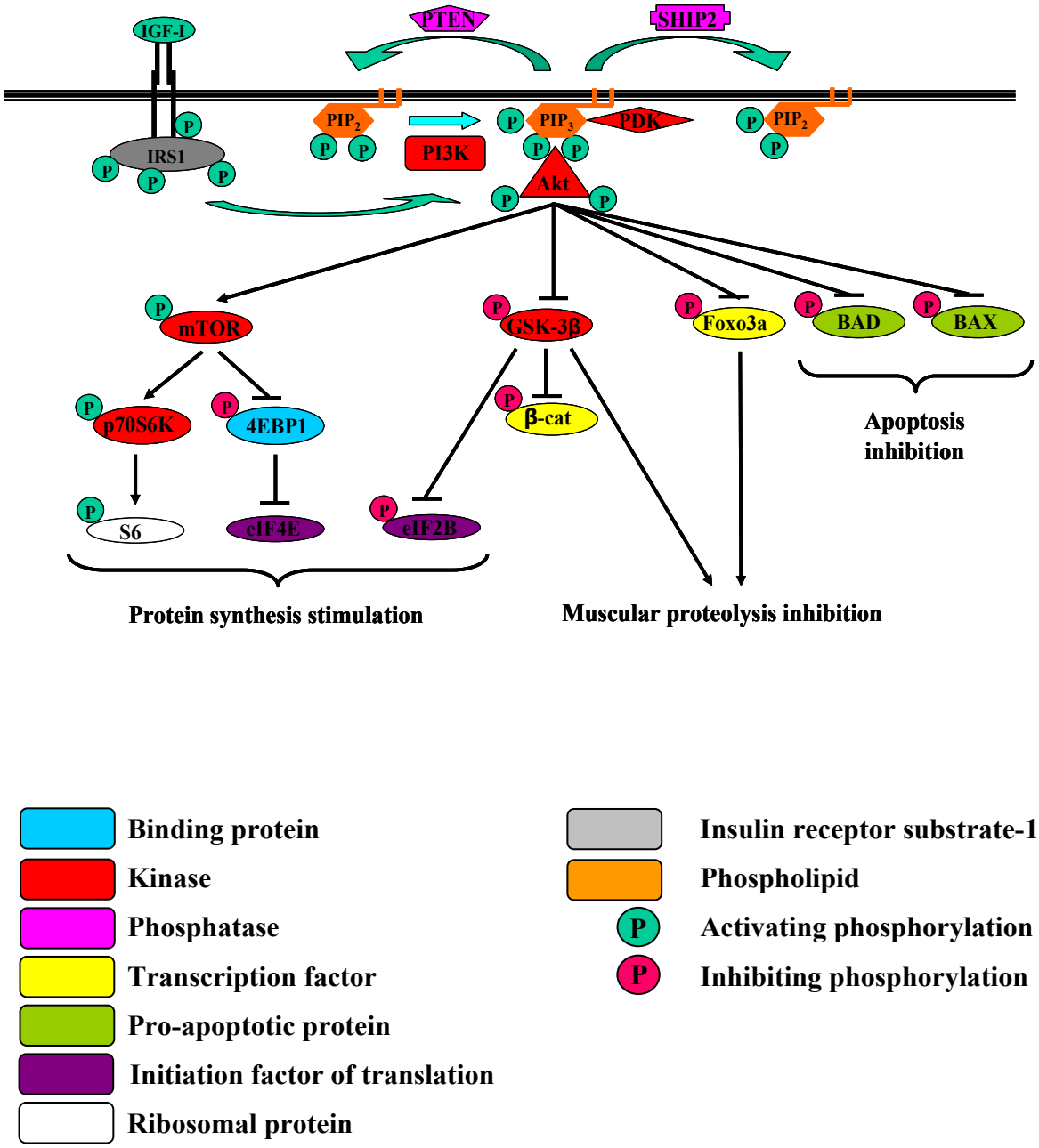

Figure 6. *The PI3kinase/Akt signaling pathways downstream of IGF-I.* By stimulating protein synthesis and inhibiting muscular proteolysis and apoptosis, the PI3kinase/Akt signaling pathways mediates most of the anabolic and the anticatabolic effects of IGF-I leading to muscle hypertrophy.

1.3.2.1. Akt

↳ Definition

Akt, also known as protein kinase B (PKB), is a serine/threonine kinase. In mammals there are three known Akt isoforms: Akt1/PKBα, Akt2/PKBβ and Akt3/PKBγ encoded by separate genes. These proteins have a similar domain structure (Figure 7) with about 80% amino acid identity (78). Akt1 and Akt2 are the most ubiquitously expressed isoforms in skeletal muscle, thymus, heart, liver and fat while Akt3 expression is predominant in brain (79). Although Akt isoforms are assumed to have identical or similar substrate specificity (80), Akt1 and Akt2 have distinct role in skeletal muscle. Indeed, it is well established that Akt 2 mediates IGF-I muscle glucose metabolism effects whereas Akt1 is directly responsible for muscle anabolic and anticatabolic effect of IGF-I (81). Recently, Akt 2 has also been implicated in skeletal muscle differentiation (82)

↳ Mechanism of regulation

Akt is activated via its pleckstrin homology (PH) domain, which binds the phosphatidylinositol-3, 4, 5-triphosphate (PIP_3) generated by the catalytic subunit of the PI3kinase. Once translocated to the plasma membrane, Akt is phosphorylated by PDK1 on two amino acids residues (Thr 308 and Ser 473) for full kinase activation (83;84). Then, phosphorylated Akt is released from plasma membrane and is translocated to both cytosol and nucleus (85), where it phosphorylates its downstream targets such as mTOR, GSK-3β and Foxo3a (62;81;86;87). Akt activity can be controlled by regulating the levels of PIP_3, which can be dephosphorylated by two different type of phosphatases: PTEN (Phosphatase and TENsin homolog) and SHIP2 (SH2-domain-containing inositol 5'phosphate) and thus limiting the recruitment of Akt to the plasma membrane mandatory for its activation (88).

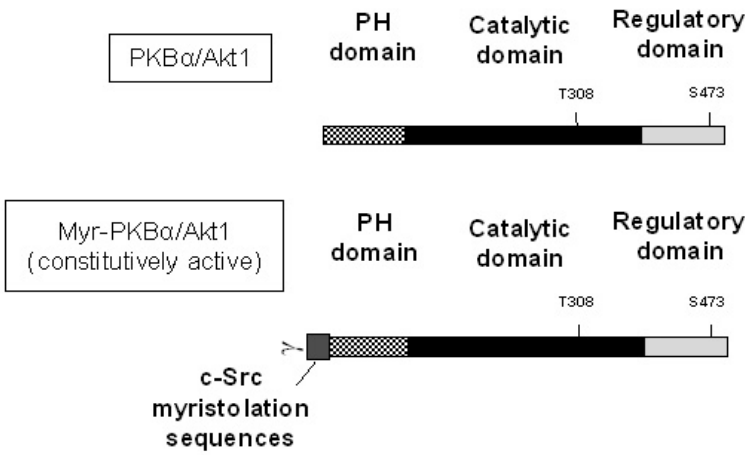

Figure 7. *Schematic representation of the structure of wild-type and constitutively active Akt/PKBα.* The three main isoforms of PKB/Akt identified in mammalian cells derived from three distinct genes, contain a C-terminal regulatory domain, a catalytic domain and an N-terminal pleckstrin homology (PH) domain that is highly conserved in all three members (top). Post post-translational modification (N-terminal myristoilation) is able to mimic the activation of Akt by IGF-I by maintaining Akt attached in permanence at the plasma membrane (89).

↳ Role of Akt in the muscle anabolic and anticatabolic action of IGF-I

Akt plays a central role in the muscle anabolic and anticatabolic action of IGF-I.

Muscle Hypertrophy

IGF-I induced myotube hypertrophy is totally blocked by inhibition of Akt activation with LY294002, a PI3Kinase inhibitor (69). Moreover, muscle hypertrophy induced by IGF-I can be reproduced by overexpression of a constitutively active (ca) form of Akt: myristoylated-Akt (90). This post-translational modification mimics the activation of Akt by IGF-I by maintaining Akt permanently attached to the plasma membrane (Figure 7). Indeed, overexpression of caAkt induced a marked hypertrophy both *in vitro* (69) and *in vivo* (68) (81;91). As observed for hypertrophy induced by IGF-I, muscle hypertrophy induced by caAkt overexpression is associated with an increased of phosphorylation of p70S6kinase (69), (76) and GSK-3β (91;81).

Stimulation of protein synthesis

Akt activation is implicated in stimulation of protein synthesis by IGF-I. Indeed, inhibition of Akt phosphorylation by Wortmannin or LY294002, two inhibitors of PI3kinase, abolishes the stimulation of protein synthesis by IGF-I (50).

Stimulation of myogenesis

Akt plays an important role in myogenic differentiation. First, expression of constitutively active forms of Akt dramatically enhances myotube formation and expression of muscle-specific proteins whereas a dominant negative form of Akt inhibits myogenesis (42). Second, the inhibition of myotube formation and the reduced expression of muscle-specific proteins caused by LY294002 are completely reversed by constitutively active forms of Akt (92). MyoD activation could be one of the mechanisms underlying Akt-mediated muscle differentiation (93). Indeed, by binding prohibitin2 (PHB2), a repressor of MyoD-dependent gene transcription, Akt reduces PHB2 binding to MyoD and relieves its repressive effect (94). Moreover, Akt-induced myogenesis could also be mediated by Foxo transcription factor (95). However, even if Akt activation is mandatory for induction of myogenic differentiation, it must be accompanied by a decrease in ERK1/2 phosphorylation (96).

Inhibition of proteolysis

Akt activation is also required for the anticatabolic effects of IGF-I. Indeed, inhibition of protein degradation by IGF-I in dexamethasone-treated myotubes is totally blunted by LY294002 which inhibits activation of Akt by IGF-I (61). The anti-proteolytic effect of Akt is probably mediated by inhibition of GSK-3β (61) and Foxo3a (62;97). Moreover, overexpression of constitutively active form of Akt blocks muscle atrophy induced by denervation (69;76).

Inhibition of apoptosis

Akt inhibits apoptosis by inhibiting BAD and BAX, two pro-apoptotic proteins (98).

1.3.2.2. GSK-3β

↳ Definition

Glycogen synthase kinase 3 (GSK-3), a ubiquitously expressed serine/threonine kinase, was originally identified as an enzyme that regulates glycogen synthesis in response to insulin (99). The GSK-3 kinase family is highly conserved throughout evolution. In humans, two genes encode two distinct but closely related GSK-3 forms, GSK-3α (51 kDa) and GSK-3β (47 kDa). They display 84% overall identity (98% within their catalytic domains). GSK-3β

plays a role in many cellular functions and especially in energy metabolism and transcriptional regulation (100).

↳ Mechanism of regulation

GSK-3β activity is regulated at multiple levels. GSK-3β is regulated by phosphorylation of serine 9 (inhibitory) and tyrosine 216 (activating) (Serine 21 and tyrosine 279 respectively in GSK-3α). In this way, Akt inactivates GSK-3β by phosphorylation on serine 9 (101) (figure 8). GSK-3β phosphorylation can also be stimulated in an Akt independent manner via stimulation of the Wnt pathway (102).

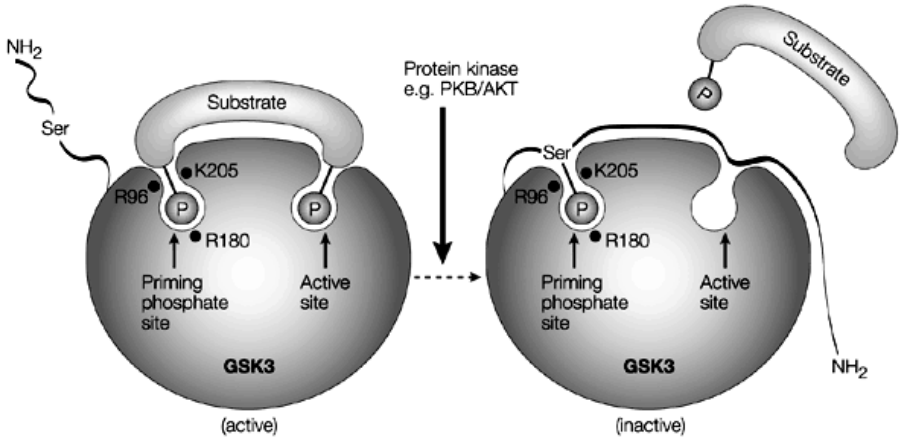

Figure 8. *Post-translational inhibition of GSK-3β induced by Akt*. In the absence of insulin or IGF-I, GSK3 is fully active. In this state, substrates that already have a 'priming phosphate' bind to a specific pocket, aligning them in such a way that GSK3 can phosphorylate a serine or threonine. As a result of phosphorylation by PKB/AKT, GSK-3β becomes phosphorylated at a serine residue near its amino terminus. This transforms the amino terminus into a 'pseudosubstrate' inhibitor, the phosphoserine occupying the same binding site as the priming phosphate of the substrate, and blocking access to the active site and thus GSK3 activity is inhibited (100).

GSK-3β activity can also be regulated by its intracellular localization and interaction with other proteins (103). Finally, GSK-3β activity can be inhibited by pharmacological inhibitors such as CHIR (104) or SB (105) acting by competition with the ATP in the ATP-binding site or by lithium acting by competition with magnesium (106) both mandatory for GSK-3β activity.

↳ Role of GSK-3β in the muscle anabolic and anticatabolic action of IGF-I

Several evidences indicate that inhibition of GSK-3β plays a role in anabolic and anti atrophic action of IGF-I:

Muscle hypertrophy and protein synthesis

Inhibition of GSK-3β by LiCl (107) or by overexpression of a dominant negative form of GSK-3β (69) is sufficient to induce a hypertrophy of myotubes. This hypertrophy could result from an increase of protein translation. Indeed, GSK-3β inhibits protein synthesis by inhibiting translation via inhibition of eIF2B (108). Moreover, it has been shown that GSK-3β inhibition contributes to the myotube hypertrophic effects of IGF-I (107).

Inhibition of proteolysis

Several observations indicate that GSK-3β plays a crucial role in muscle proteolysis. First, inhibition of GSK-3β attenuates muscular proteolysis induced by dexamethasone *in vitro* (109;110) and *in vivo* in muscle of septic (109) or burned (60) rats. Interestingly, as IGF-I, inhibition of GSK-3β prevents the induction of MuRF1 and Atrogin-1 in dexamethasone-treated myotubes (109). This is one of the potential anti-proteolytic mechanism resulting from GKS-3β inactivation by IGF-I.

Stimulation of myogenesis

Pharmacological inhibition of GSK-3β is sufficient to stimulate myogenic differentiation (111). This is one of the potential mechanism by which IGF-I stimulates mytogenesis.

1.3.2.3. β-catenin

↳ Definition

β-catenin is a small intracellular multifunctional protein. β-catenin can serve a dual function in cells dependent of its location. First, in the cytoplasm, the majority of β-catenin is associated with adherens junctions, where it interacts with the cytoplasmic region of cadherin, which is important in cell adhesion and cell structure (112). Second, in the nucleus, β-catenin can serve as a transcriptional activator by interacting with transcription factors from the T-cell

factor/lymphocyte enhancer factor (Tcf/Lef) family (113). In this context, it stimulates the expression of many genes relevant for the regulation of cellular cycle, including the proto-oncogenes *c-myc*, *c-fos* and *c-jun* and *cyclin D1* (114). These proteins play critical regulatory roles in many biological processes, including embryonic development and stem cell maintenance (115).

↳ Mechanism of activation

β-catenin levels are regulated by GSK-3β. Indeed, phosphorylation by GSK-3β of serine 33 and 37 and threonine 41 in the N-terminal region of β-catenin induces the degradation of β-catenin by the ubiquitin proteasome system (116). Thus, inhibition of GSK-3β, particularly via Akt phosphorylation (Ser9), is essential for stabilization and accumulation of β-catenin and its translocation to the nucleus (Figure 9). GSK-3β activation and thus that β-catenin degradation can also be inhibited by the Wnt signaling pathways stimulation (102).

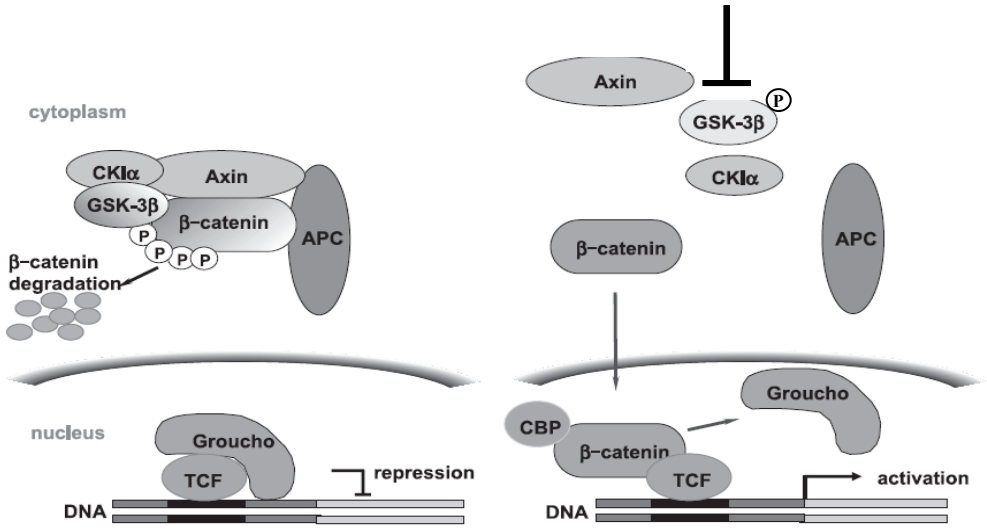

Figure 9. *Regulation of β-catenin levels by GSK-3β.* When GSK-3β is activated (non-phosphorylated), β-catenin forms a complex with axin, APC (adenomatous polyposis), GSK-3β and CK (casein kinase) 1α, resulting in its phosphorylation and degradation by the 26 S proteasome (left panel). In contrast, when GSK-3 is inhibited (phosphorylated), β-catenin escapes from phosphorylation. Unphosphorylated β-catenin accumulates in the cytoplasm and translocates to the nucleus. In the nucleus, β-catenin activates the transcription of target gene with TCF and other transcription cofactors. (Adapted from Takahashi-Yanaga, (117)).

↳ Role of β-catenin in the muscle anabolic and anticatabolic action of IGF-I

Hypertrophy

Recent studies indicate that β-catenin plays a role in muscle mass regulation. Indeed, overexpression of β-catenin is sufficient to induce hypertrophy of myotubes (118) and cardiomyocytes *in vitro* and *in vivo* (119). Moreover, β-catenin is mandatory for physiological growth of adult skeletal muscle in response to muscle loading (120;121).

Stimulation of myogenesis

β-catenin is implicated in muscle development by interacting with MyoD, a transcription factor essential in muscle differentiation. Indeed, this interaction appears to be critical for MyoD function (122). Moreover, β-catenin promotes self-renewal of skeletal-muscle satellite cells and thus contributes to the maintenance of the stem cell pool in adult skeletal muscle (123).

1.3.2.4. mTOR

↳ Definition

Mammalian target of rapamycin (mTOR) is an evolutionarily conserved serine/threonine kinase that integrates signals from nutrients (amino acids and energy) and growth factors to regulate cell growth and cell cycle progression coordinately. (124-126). In mammal cells, mTOR is implicated in two functionally distinct complexes: mTOR complex (C) 1 and 2. The best characterized of the two complexes is mTORC1, which comprises mTOR, Raptor (regulatory associated protein of mTOR) and LST8 (lethal with SEC13 protein 8). mTORC2 consists of mTOR, Rictor (rapamycin-insentive companion of mTOR), SIN1 (stress-activared-kinase-interacting protein 1) and LST8 (127). In skeletal muscle, it has been well established that mTORC1 plays a central role in muscle mass regulation (128).

↳ Mechanism of regulation

mTORC1 activity can be regulated by multiple signaling pathways. mTORC1 activation in response to growth factors has been linked to activation of the PI3k/Akt1 pathway. Indeed, activated Akt1 is able to inhibit by phosphorylation the TSC (tuberous sclerosis complex) 1-TSC2 complex, a critical negative regulator of mTORC1. mTORC1 can also be activated by amino acid availability independently of TSC1-TSC2. mTORC1 activity is also sensitive to rapamycin inhibition. Indeed, Rapamycin binds to FKBP12 (FK506-binding protein 12) and the resulting complex binds Raptor and inhibits mTORC1 (124).

↳ Role of mTORC1 in the muscle anabolic action of IGF-I

Muscle hypertrophy

Several observations indicate that mTORC1 plays a role in muscle hypertrophy induced by IGF-I. First, muscle hypertrophy induced by IGF-I is associated with the phosphorylation of p70S6K1 and 4E-BP1, two intracellular mediators activated by mTORC1 (69). Second, *in vitro* (69) as well as *in vivo* (68), muscle hypertrophy induced by IGF-I is totally blocked by Rapamycin, an inhibitor of mTORC1.

1.3.2.5. p70S6K

↳ Definition

In mammals, there are two isoforms of the protein 70 kDa ribosomal S6 kinase (p70S6K): p70S6K 1 and 2. Both are cytoplasmic serine/threonine kinases which phosphorylate the S6 protein, an integral component of the 40 S ribosomal subunit. The phosphorylation of S6 by the p70S6K enables the efficient translation of a subset of mRNAs containing a terminal oligopolypyrimide (TOP) track at the 5' end. Many of these mRNAs encode proteins of the translational machinery such as ribosomal proteins and elongation factors (129). Thus p70S6K plays a crucial role in protein synthesis but also in the control of cell cycle progression (130) and in the regulation of mammalian cell size (131). In skeletal muscle, it seems that p70S6K1, rather than p70S6K2, plays a crucial role in muscle hypertrophy (134).

↳ Mechanism of regulation

Activation of the p70S6K1 by Akt is mTOR (mammalian target of rapamycin) dependent. Studies using rapamycin, an inhibitor of mTOR, have shown that mTOR phosphorylates several sites of p70S6K: threonine 389, 229 and serine 404 (132;133).

↳ Role of p70S6K1 in the muscle anabolic and anticatabolic action of IGF-I

Muscle hypertrophy
Several evidences indicate that p70S6K1 plays a role in muscle hypertrophy induced by IGF-I. First, *in vitro*, overexpression of constitutively active form of p70S6K1 induces a hypertrophy of myotubes (69). Second, *in vivo*, deletion of the p70S6K1 gene in KO mice decreases muscle weight and muscle fiber cross sectional area. Furthermore, *in vitro*, IGF-I-induced muscle hypertrophy is blunted in p70S6K1 -/- myotubes (134).

1.3.2.6. Foxo3a

↳ Definition

Forkhead box O (Foxo) transcription factors are one of the numerous classes of the forkhead transcription factor superfamily. In mammals, this class of protein includes Foxo1 (FKHR), Foxo3a (FKHRL1), Foxo4 (AFX) and Foxo6. These transcription factors are an important family of proteins that modulate the expression of genes involved in apoptosis, the cell cycle, DNA damage, oxidative stress, cell differentiation, glucose metabolism and other cellular functions (135). In skeletal muscle, overexpression of Foxo1 and Foxo3a causes muscle atrophy (136;62).

↳ Mechanism of regulation

Foxo protein action is regulated by multiple post-translational modifications. The transcriptional activity of Foxo factors can be regulated by phosphorylation on multiple threonine and serine residues (T1, T2, S1, S2 and S3) (Figure 10). Indeed, in response to the activation of the PI3K/Akt pathway by IGF-I, Akt-induced phosphorylation (T1, S1 and S2 sites) inhibits transcriptional activity of Foxo members (137).

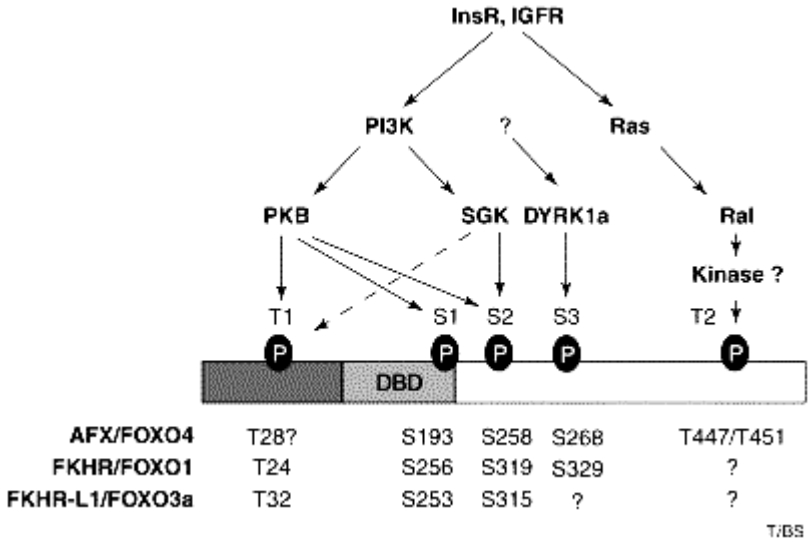

Figure 10. *Signaling pathways and kinases that affect Foxo-protein activity*. The Foxo members become phosphorylated on several residues during growth factor signaling. The general position of phosphorylation sites is indicated by S1, S2, S3, T1 and T2; the specific amino acid residue number for each Foxo factor is also indicated ('?' indicates that the residue number is either unknown or not reported). IGF-I can activate the PI3K/Akt , PI3K–serum- and glucocorticoid-inducible kinase (PI3K–SGK) and Ras–Ral signaling pathways. The serine (S1) located at the end of the DNA binding domain (DBD) is probably exclusively phosphorylated by PKB. Both PKB and SGK can phosphorylate the Foxo members on T1 as well as on S2. Activation of the Ras–Ral pathway results in the phosphorylation of Foxo4 on T2, which encompasses both Thr447 and Thr451 (137).

As indicated in figure 11, once phosphorylated by Akt, Foxo transcription factors are transported out the nucleus. Foxo members can shuttle continuously between the nucleus and the cytosol. Accessory proteins for import (α- and β-importins) and export (exportins such as Crm1) facilitate this shuttling. Under conditions of low Akt activity, Foxo proteins are predominantly unphosphorylated and nuclear (active form). Following activation of Akt by IGF-I, Akt translocates to the nucleus. A 14-3-3 protein devoid of ligand enters the nucleus passively. Akt phosphorylates Foxo, probably on multiple sites (T1, S1 and S2), and Akt-mediated phosphorylation induces the binding of 14-3-3 protein to the Foxo proteins in the nucleus, which results in the release of the Foxo protein from the DNA. Following translocation to the cytosol, the bound 14-3-3 protein prevents re-entry into the nucleus. This results in an increase of Foxo in the cytosol (inactive form). Foxo proteins are also regulated in an Akt independent manner by ubiquitination and acetylation (135).

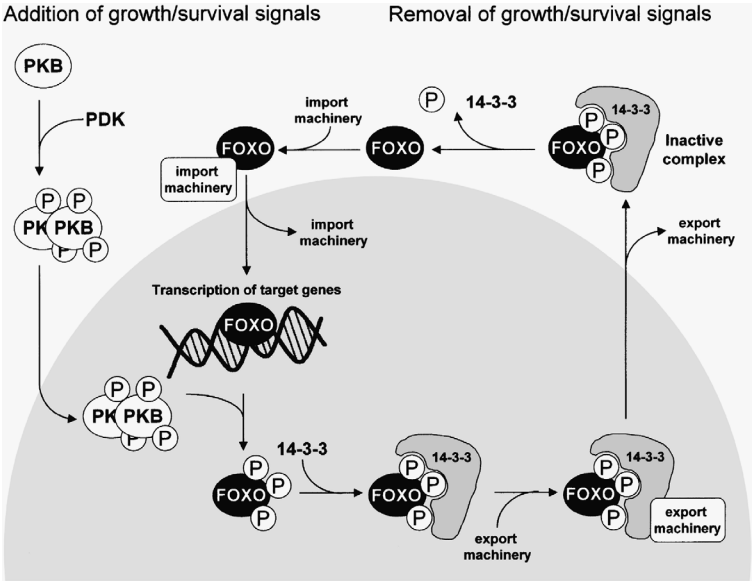

Figure 11. *Regulation of nucleocytoplasmic shuttling of Foxo by Akt/PKB.* Phosphorylation of Foxo by Akt/PKB results in release from DNA, and binding to 14-3-3 proteins. This complex is then transported out of the nucleus, where it remains inactive in the cytoplasm. Without growth/survival signals, Foxo is dephosphorylated, 14-3-3 is released, and Foxo is transported back into the nucleus where it is transcriptionally active (Figure adapted from Birkenkamp, 2003).

↳ Role of Foxo3a in the anticatabolic action of IGF-I

Muscle Atrophy

Several observations indicate that inhibition of Foxo3a plays a role in the anticatabolic action of IGF-I. First, Foxo3a is sufficient to induce muscle atrophy. Indeed, muscle overexpression of Foxo3a *in vitro* as well *in vivo* induces skeletal muscle fiber atrophy (62). This atrophy could result from an induction of several genes (Atrogin-1, MuRF-1) involved in the SUP (62;97) and in autophagy (LC3b, Bnip3) (138;139). Moreover, overexpression of a dominant negative form of Foxo3a, mimicking the IGF-I inhibition, blunts myotubes atrophy induced by glucocorticoids (62).

1.4. Role of IGF-I in muscle atrophy

IGF-I plays a crucial role in the loss of muscle mass observed in catabolic states. First, as described above, autocrine IGF-I production plays a critical role in muscle growth by exerting anabolic and anticatabolic actions towards skeletal muscle. Second, circulating and muscle IGF-I levels are frequently reduced in catabolic states. Finally, systemic administration of IGF-I or overexpression of IGF-I gene prevents loss of muscle in several animal models of muscle atrophy such as aging and muscle dystrophy (140;141). These evidences therefore suggest that IGF-I might prevent loss of muscle mass in hypercatabolic states such the one induced by glucocorticoids.

2. Mechanisms of glucocorticoid-induced myopathy

Schakman O, Gilson H, Thissen JP. Journal of Endocrinology. 2008 April; 197:1-10

2.1. Generalities

The catabolic effects of glucocorticoids have been well known for many years. Either as drugs used to treat several medical conditions or as endocrine hormones released in response to many stress situations, glucocorticoids may cause skeletal muscle atrophy. Administration of high doses of glucocorticoids to animals causes not only decreased muscle mass but also muscle dysfunction characterized by reduced force and weakness (142). In humans also, a significant relationship between steroid usage and both peripheral and respiratory muscle strength has been reported in chronic pulmonary disease (143) and cystic fibrosis (144). Peripheral muscle weakness has also been observed in patients with Cushing's syndrome who exhibit high levels of endogenous glucocorticoids (145), (146). The resulting weakness of peripheral and respiratory muscles may have major clinical implications such as loss of quality of life, fatigue, impaired wound healing, compromised lung function and poor immune response. Therefore, glucocorticoid-induced atrophy may have significant clinical implications.

2.2. Characterization of the glucocorticoid-induced muscle atrophy

Skeletal muscle atrophy is characterized by a decrease in the size of muscle fibers. Glucocorticoids have been shown to cause atrophy of fast-twitch or type II muscle fibers (particularly IIx and IIb) with less or no impact observed on type I fibers (147;148). Therefore, fast-twitch, glycolytic muscles (i.e. tibialis anterior) are more susceptible than oxidative muscles (i.e. soleus) to glucocorticoid-induced muscle atrophy. The mechanism of such fiber specificity is not known.

2.3. Mechanisms of the glucocorticoid-induced muscle atrophy

In skeletal muscle, glucocorticoids decrease the rate of protein synthesis and increase the rate of protein breakdown (149-151), both contributing to atrophy. The severity and the

mechanism for the catabolic effect of glucocorticoids may differ with age. For example, glucocorticoids cause more severe atrophy in older rats compared with younger rats. Furthermore, glucocorticoid-induced muscle atrophy results mainly from increased protein breakdown in adult rats but mostly from depressed protein synthesis in the aged animals (152).

2.3.1. Catabolic action of glucocorticoids

2.3.1.1. Inhibition of protein synthesis

The inhibitory effect on protein synthesis results from different mechanisms. First, glucocorticoids inhibit the transport of amino acids into the muscle (153), which could limit the protein synthesis. Second, glucocorticoids inhibit the stimulatory action of insulin, IGF-I and amino acids (in particular leucine), on the phosphorylation of eIF4E-binding protein 1 (4E-BP1) and the p70 ribosomal protein S6 kinase 1 (p70S6K), two factors which play a key role in the protein synthesis machinery by controlling the initiation step of mRNA translation (154-157).

2.3.1.2. Inhibition of cell differentiation

There is also evidence that glucocorticoids cause muscle atrophy by inhibiting myogenesis through down-regulation of myogenin (158) or through degradation of MyoD (159), two myogenic regulatory factor's (MRFs) mandatory for muscle cells differentiation and development.

2.3.2. Anti-anabolic action of glucocorticoids

2.3.2.1. Stimulation of proteolysis

The stimulatory effect of glucocorticoids on muscle proteolysis results from the activation of the major cellular proteolytic systems (160), namely the ubiquitin proteasome system (UPS), the lysosomal system (cathepsins) and the calcium-dependent system (calpains). The protein degradation caused by glucocorticoids affects mainly the myofibrillar proteins as illustrated by the increased excretion of 3-methyl histidine (161;162). To activate the protein

degradation, glucocorticoids stimulate the expression of several components of the UPS either involved in the conjugation to ubiquitin of the protein to be degraded (ubiquitin; 14kDa E2, a conjugating enzyme; Atrogin-1 and MuRF-1, two muscle specific E3 ubiquitin-ligases (163)) or directly responsible for the protein degradation by the proteasome (several subunits of the 20S proteasome) (164). This gene transcription activation is associated with an increased rate of protein ubiquitination and increased proteolytic activities of the proteasome (165). For example, MuRF1 is responsible for the ubiquitination of the myosin heavy chain proteins and their degradation in response to glucocorticoids (166). Because the proteasome does not degrade intact myofibrils, it is thought that actin and myosin need to be dissociated, probably by calpains, from the myofibrils before they can be degraded by the UPS (167). Finally, some *in vivo* data also suggest that caspase-3 can be implicated in myofibrillar proteins breakdown. Indeed, in glucocorticoid-dependent muscle wasting models, such as diabetes mellitus and chronic renal failure, caspase-3 activity in muscle is increased and inhibition of caspase-3 by Ac-DEVD-CHO suppresses the accelerated muscle proteolysis (59). However, the role of glucocorticoids in the induction of caspase-3 activity in these models does not seem to have been explored.

2.3.2.2. Stimulation of apoptosis

Glucocorticoids can promote apoptotic cell death of differentiated skeletal muscle by increasing caspase 8 (168) or by inhibiting the muscle anti-apoptotic action of IGF-I (169).

2.4. Role of glucocorticoids in muscle atrophy in catabolic states

Many pathological conditions characterized by muscle atrophy (sepsis, cachexia, starvation, metabolic acidosis, severe insulinopenia…) are associated with an increase in circulating glucocorticoids levels (170), suggesting that these hormones could trigger the muscle atrophy observed in these situations. In the case of sepsis, cachexia, starvation and severe insulinopenia, adrenalectomy or treatment with a glucocorticoid receptor antagonist (RU-486) attenuates muscle atrophy, indicating that glucocorticoids are in part responsible for this muscle loss. In addition to glucocorticoid excess, several other factors such as poor nutrition, cytokines and bed resting may contribute to muscle atrophy observed in these wasting conditions (160;170). In contrast, glucocorticoids do not appear to be required for disuse

atrophy (171), but may clearly exacerbate the deleterious effects of disuse on skeletal muscle mass (172).

2.5. Signaling pathways involved in glucocorticoid-induced muscle atrophy

2.5.1 Foxo

The muscle cell catabolism caused by glucocorticoids is thought to be mediated by the transcription factors Foxo. The role of these transcription factors in the glucocorticoid-induced muscle cell atrophy has been established by different observations. First, exposure of myotubes to glucocorticoids increases the Foxo gene expression, particularly –1 and –3 (173). Second, *in vitro* as well *in vivo*, Foxo overexpression causes muscle cell atrophy (62;136) together with induction of several genes characteristic of muscle cell atrophy or Atrogenes such as Atrogin-1, MuRF-1 and Cathepsin-L (62;174). Finally, overexpression of a dominant negative form of Foxo3a prevents muscle cell atrophy together with Atrogin-1 induction caused by glucocorticoids *in vitro* (62). Because Foxo, but not Atrogin-1, overexpression is sufficient to cause muscle atrophy, it is thought that Foxo transcription factors activate a variety of genes, in addition to Atrogin-1, that leads to atrophy. Taken together, these data indicate that increased expression of Foxo by glucocorticoids activates a gene transcriptional program responsible for triggering muscle atrophy. Among the genes most strongly induced in muscle atrophy due to a variety of wasting diseases are several genes (Atrogin-1, MuRF-1, Cathepsin-L, PDK4, p21, Gadd45, 4E-BP1) controlled by the Foxo transcription factors (175-178). The establishment of an active transcriptional program necessary for the induction of muscle atrophy has thus challenged the view that atrophy is a passive adaptation of the muscle to a lack of anabolic stimuli. All these observations support the role of Foxo in muscle atrophy induced by glucocorticoids but *in vivo* evidence for the requirement of Foxo in this muscle atrophy model is still lacking (Figure 12).

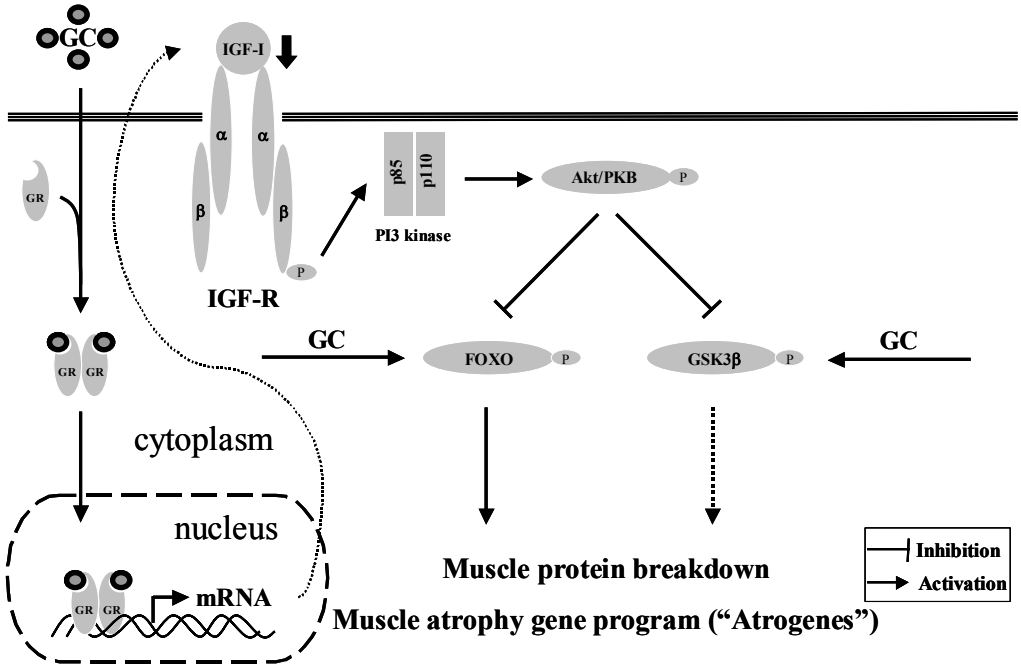

Figure 12. *Alterations in protein breakdown signaling induced by glucocorticoids.* Stimulatory effects on protein breakdown results from different mechanisms. First, glucocorticoids (GC) stimulate several proteolytic systems by activating transcription factor Foxo. Second, stimulation of GSK3-β may also be involved in the stimulatory effects of glucocorticoids on protein breakdown.

2.5.2 mTOR

The inhibition of protein synthesis by glucocorticoids mainly results from the inhibition of mTORC1, the kinase responsible for the phosphorylation of 4E-BP1 and p70S6K. Repression of mTORC1 signaling results in a reduction in the initiation phase of mRNA translation with down-regulation of protein synthesis. Recent studies indicate that the repression of mTORC1 signaling in response to glucocorticoids is the result of enhanced transcription of REDD1, a repressor of mTORC1 signaling (179). Through an as yet unidentified mechanism, REDD1 represses mTORC1 function, leading to decreased phosphorylation of both 4E-BP1 and p70S6K. Recent evidence suggests that mTORC1 signaling could also be inhibited directly by Foxo (180). Whereas the effects of glucocorticoids on protein synthesis have been explained at the molecular level, comparatively little is known about how these hormones alter anabolic gene expression. Recent studies identified ATF-4 as an anabolic transcription factor that is repressed by glucocorticoids (181). ATF-4 has been shown to be required for the activation of a genetic program for cellular uptake of essential amino acids and the synthesis of non-

essential amino acids and aminoacyl-t-RNAs. This observation suggests that glucocorticoids inhibit protein synthesis at least partially by down-regulating ATF-4, which could limit intracellular amino acid availability. It is interesting to note that insulin, an anabolic hormone, has been shown to induce ATF-4 transcription, even in the presence of glucocorticoids (Figure 13).

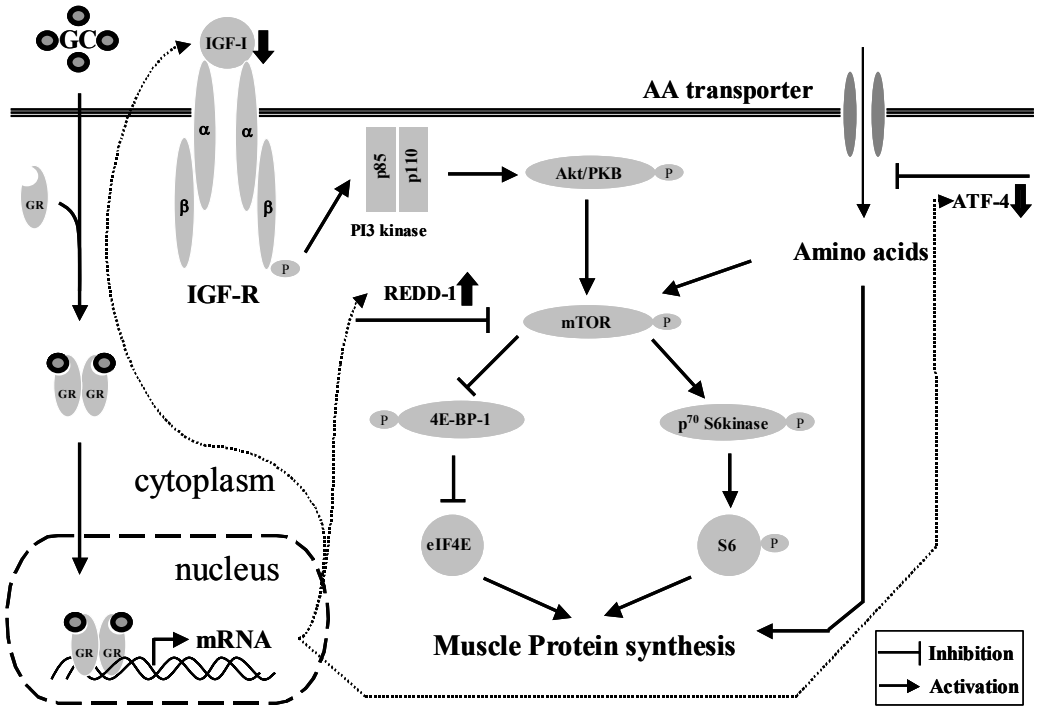

Figure 13. *Alterations in protein synthesis signaling pathway induced by glucocorticoids.* Inhibitory effects on protein synthesis results from different mechanisms. First, glucocorticoids (GC) impair protein synthesis by inhibiting the transport of amino acids into the muscle. Second, glucocorticoids inhibit the stimulatory action of insulin, IGF-I and amino acids on eIF4E-binding protein 1 (4E-BP1) and the P70 ribosomal protein S6 kinase (p70S6K1) through mTOR activity repression.

2.5.3 GSK-3β

Glycogen synthase kinase 3β (*GSK-3β*), a downstream target of IGF-I signaling which is phosphorylated and inhibited by Akt, could also be involved in the atrophic effect of glucocorticoids. Indeed, GSK-3β is known to suppress protein synthesis by inhibiting eukaryotic transcription factor 2B (eIF2B) dependent translation (108). Furthermore, not only inhibition of GSK-3β is sufficient to cause myogenic differentiation (111) and muscle cell

hypertrophy (107), but also contributes to the hypertrophic effect of IGF-I on skeletal muscle cells (107). More interestingly, inhibition of GSK-3β by overexpression of a dominant negative GSK3β or siRNA targeting GSK-3β or by pharmacologic inhibitors prevents the proteolysis and cell atrophy caused by glucocorticoids *in vitro* (60;69;109;110). The mechanism by which GSK-3β inactivation inhibits muscle protein degradation caused by glucocorticoids is not known. However, the observation that inhibition of protein degradation by GSK-3β inhibitors is associated with the blockade of the up-regulation of Atrogin-1 and MuRF-1 gene expression suggests that this reduction in muscle proteolysis is mediated at least in part by inhibiting the UPS (109). Although the role of GSK-3β in muscle atrophy induced by glucocorticoids has not yet been demonstrated *in vivo*, the anticatabolic effect of GSK-3β inhibitors suggests that GSK-3β may become an important target to inhibit muscle wasting in the future (Figure 11).

2.5.4 p300-C/EBPβ

Finally, recent *in vitro* data suggest that glucocorticoid-induced muscle proteolysis is at least in part regulated by p300-histone acetyl transferase (HAT) activity. Indeed, p300 protein levels and activity are increased, in a time- and dose- dependent manner, in dexamethasone-treated myotubes (182). Additionally, dexamethasone increases protein-protein interaction between p300 and C/EBPβ, which increases the transcription activity of C/EBPβ by acetylation (182). This interaction is particularly important since C/EBPβ may regulate multiple genes in the UPS pathway (183). Finally, treatment of myotubes with p300 small interfering RNA prevents the dexamethasone-induced increase in protein degradation, whereas overexpression of wild type p300 potentiates the effect of dexamethasone on protein degradation (184). Taken together, these results point the main role of p300 in muscle proteolysis induced by glucocorticoids. Given that several transcription factors involved in muscle wasting are regulated in part by acetylation (185-188), it appears crucial for the future to determine which proteins are acetylated in muscle atrophy and whether their acetylation controls glucocorticoid-induced muscle proteolysis.

2.6. Role of local growth factors in glucocorticoid-induced muscle atrophy

2.6.1 IGF-I

Glucocorticoids can also cause muscle atrophy by altering the production of growth factors which control muscle mass development locally. Glucocorticoids inhibit production of IGF-I by muscle (189). For these reasons, decreased muscle IGF-I has been thought to play a key role in glucocorticoid-induced muscle atrophy. This hypothesis has been recently confirmed both *in vitro* and *in vivo*. First, by activating the PI3K/Akt/mTOR pathway and blocking nuclear translocation of the transcription factor Foxo, IGF-I down-regulates the different proteolytic systems (lysosomal, proteasomal and calpain-dependent) and the expression of Atrogenes such as Atrogin-1, MuRF-1, Cathepsin-L induced by glucocorticoids (56-58). Second, IGF-I suppress the muscle cell atrophy caused by glucocorticoids *in vitro* (55;190). Third, systemic administration of IGF-I prevents glucocorticoid-induced muscle atrophy (148;191-193). Taken together, these results indicate that IGF-I has a dominant effect, overriding glucocorticoids to turn off catabolism. Therefore, restoration of IGF-I may provide a strategy to reverse the catabolic effects of glucocorticoid excess (Figure 14).

2.6.2 Myostatin

In addition to decreased muscle IGF-I production, GC also stimulate the production by the muscle of myostatin (Mstn) (194-196), a growth factor which inhibits muscle mass development and causes muscle cell atrophy by down-regulating proliferation and differentiation of satellite cells (197;198) as well as protein synthesis (199;200). Recent evidence indicates that Mstn decreases muscle mass by inhibiting the Akt/mTOR pathway leading to inhibition of ribosomal protein S6 and activation of 4E-BP1, two intracellular factors involved in protein synthesis (201). For these reasons, increased muscle Mstn has been thought to play a key role in GC-induced muscle atrophy. This hypothesis has been recently demonstrated *in vivo* using a model of Mstn knock out mice. Indeed, in contrast to wild type mice, Mstn KO mice did develop neither a reduction of muscle mass nor fiber cross-sectional area in response to GC treatment (202). This observation indicates that Mstn is mandatory for the atrophic effects of GC on skeletal muscle. The mechanism by which Mstn deletion prevents muscle atrophy caused by GC is not known. However, the observation that prevention of muscle atrophy by Mstn deletion is associated with the blockade of the up-

regulation of Atrogenes expression and proteosomal activity caused by GC suggests this protection of muscle mass results at least in part from the inhibition of the muscle proteolysis (202). Taken together, these results suggest that increased Mstn contributes to the atrophic effects of GC on skeletal muscle. Therefore, besides stimulating IGF-I, inhibition of Mstn may provide another strategy to reverse the catabolic effects of GC excess (Figure 14).

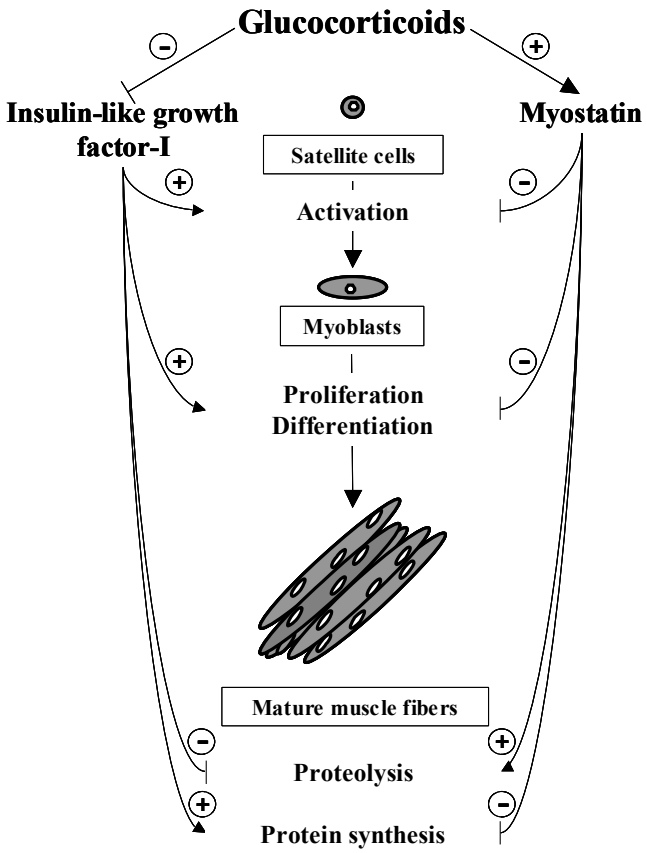

Figure 14. *Local growth factors production plays a crucial role in glucocorticoid-induced muscle atrophy.* Glucocorticoids can cause muscle atrophy by altering the muscle production of IGF-I and myostatin, two growth factors exhibiting opposite effects on muscle mass development. Decrease of IGF-I together with increase of myostatin both induced by glucocorticoids inhibit satellite cells activation as well as myoblast proliferation and differentiation. In mature muscle fibers, these growth factors changes cause down-regulation of protein synthesis and stimulation of protein degradation.

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Chapter 2: Aim of the thesis

General goal

Catabolic situations such as cancer, sepsis, AIDS, or chronic inflammatory diseases are characterized by an accelerated loss of lean body mass, mainly skeletal muscle. This change in body composition is associated with an increase in morbidity and mortality.

As illustrated in the introduction, increased glucocorticoid production plays a major role in the induction of muscle atrophy in many catabolic conditions. Several observations indicate that decreased IGF-I might mediate the loss of muscle mass caused by glucocorticoids. Therefore, IGF-I administration seems to constitute an attractive and promising avenue to limit loss of muscle mass observed in catabolic states. However, IGF-I administration must mimic as closely as possible the autocrine/local production of IGF-I. Indeed, local production and thus autocrine action of IGF-I seem to play a more important role in the regulation of muscle mass than its endocrine action. Furthermore, use of systemic IGF-I administration is limited by its hypoglycemic and cardiac hypertrophic actions and by its association with cancer risk.

Based on these observations, we hypothesized that the local decrease in muscle IGF-I content might be responsible for the muscular atrophy induced by glucocorticoids. The aim of this thesis was to determine if the restoration of IGF-I muscle content could reverse muscle atrophy induced by glucocorticoids.

Objective 1:

To demonstrate the anabolic effect of local IGF-I gene overexpression in skeletal muscle of hypophysectomized rats.

Objective 2:

To demonstrate the anticatabolic effect of local IGF-I overexpression in skeletal muscle of rats made catabolic by glucocorticoid treatment.

Objective 3:

To characterize the intracellular signaling pathways responsible for the IGF-I anti-atrophic action in glucocorticoid-induced muscle atrophy.

List of publications of Olivier Schakman:

1. **Schakman O, Gilson H, de C, V, Lause P, Verniers J, Havaux X, Ketelslegers JM, Thissen JP** 2005 Insulin-like growth factor-I gene transfer by electroporation prevents skeletal muscle atrophy in glucocorticoid-treated rats. Endocrinology 146:1789-1797 (see chapter 3, point 2)

2. **Schakman O, Thissen JP** 2006 Gene therapy with anabolic growth factors to prevent muscle atrophy. Curr Opin Clin Nutr Metab Care 9:207-213 (see annexe)

3. **Gilson H, Schakman O, Combaret L, Lause P, Grobet L, Attaix D, Ketelslegers JM, Thissen JP** 2007 Myostatin gene deletion prevents glucocorticoid-induced muscle atrophy. Endocrinology 148:452-460

4. **Schakman O, Kalista S, Bertrand L, Lause P, Verniers J, Ketelslegers JM, Thissen JP** 2008 Role of Akt/GSK-3beta/beta-catenin transduction pathway in the muscle anti-atrophy action of insulin-like growth factor-I in glucocorticoid-treated rats. Endocrinology 149:3900-3908 (see chapter 3, point 3)

5. **Schakman O, Gilson H, Thissen JP** 2008 Mechanisms of glucocorticoid-induced myopathy. Journal of Endocrinology 197:1-10 (see chapter 1, point 2)

Chapter 3: Results

1. Insulin-like growth factor-I gene transfer by electroporation promotes skeletal muscle hypertrophy in hypophysectomized rats.

Abstract (presented at the 86th Annual Meeting of The Endocrine Society, New Orleans, LA, p 3-187)

Hypercatabolic states caused by injury are characterized by a loss of skeletal muscle which is associated with an increased morbidity and mortality. The anabolic action of Insulin-like Growth Factor (IGF)-I on muscle and the reduction of its muscle content in response to injury suggest that IGF-I overexpression might prevent skeletal muscle loss caused by injury. We therefore investigated whether local overexpression of IGF-I gene by gene transfer could promote skeletal muscle hypertrophy. We developed a model of localized overexpression of IGF-I in tibialis anterior (TA) muscle by injection of DNA followed by electroporation. A total of 100 μg of IGF-I DNA inserted in a plasmid under control of the CMV promoter was injected in 10 injections of 10 μg each, followed by 10 pulses of 200 V/cm applied in 20 ms square wave at 1Hz. Both treated and contralateral TA muscles were electroporated. To validate the efficacy of our gene transfer method, we assessed the IGF-I muscle content in hypophysectomized rats, which have a low basal IGF-I muscle content. Three days after gene transfer, the IGF-I content was 2.9-fold higher in the treated TA than in contralateral TA (12.3±2.4 vs 4.2±1.0 ng/g of muscle, $P<0.01$, n=4). Muscle IGF-I content decreased with time (5.0±0.6 vs 1.7±0.4 ng/g at 14 days, $P<0.01$, n=4), but remained still higher 28 days after electroporation (2.7±0.2 vs 1.7±0.1 ng/g, $P<0.001$, n=8) in the IGF-I DNA-injected TA compared to the contralateral muscle. This difference was not anymore significant at 56 days (1.9±0.2 vs 1.7±0.2 ng/g, NS, n=7). To investigate the anabolic action of IGF-I overexpression, we then assessed the effect of IGF-I gene transfer on muscle parameters. Fifty-six days after electroporation, IGF-I gene transfer increased the muscle mass (330±8 vs 313±5 mg, $P<0.05$, n=13), the muscle area (27.8±0.8 vs 26.6±0.7 mm2, $P<0.05$, n=6) and the fiber cross-sectional area (1816±77 vs 1504±56 μm2, $P<0.001$, n=6) of the IGF-I DNA-injected TA compared to the contralateral muscle. These anabolic effects were already observed at 28 days. In conclusion, our results show that IGF-I gene transfer by electroporation stimulates, up to 56 days, muscle hypertrophy in hypophysectomized rats. In further investigations, we plan to test whether IGF-I overexpression might prevent skeletal muscle loss in animal models of catabolic states

Introduction

Hypercatabolic states such as cancer, sepsis, AIDS, or chronic inflammatory diseases are characterized by an accelerate loss of lean body mass. This change in body composition is associated with an augmentation of morbidity and mortality. The loss of lean body mass is mainly due to a loss of skeletal muscle (1).

Insulin-like growth factor I (IGF)-I is an important endocrine and autocrine anabolic growth factor responsible for normal growth and development (2). Autocrine IGF-I production has been reported to play a crucial role in muscle growth (3,4). Several evidences suggest that IGF-I plays an important role in the loss of lean body mass observed in hypercatabolic states. In skeletal muscle, IGF-I has an anabolic and anticatabolic action in vivo and in vitro. Anabolics effects of IGF-I result from the stimulation of protein synthesis (5) as well as proliferation and differentiation of muscle stem cells (6,7). The anticatabolic effects result from the inhibition of muscle proteolysis (8) and apoptosis (9). Moreover, circulating and muscle IGF-I levels are reduced in hypercatabolic states (10). Finally, high levels of IGF-I also cause significant muscle hypertrophy, which as been shown in numerous studies where IGF-I has been infused, introduced by virus or overexpressed in transgenic animals (11-16). These evidences suggest that IGF-I could be used to prevent loss of lean body mass in hypercatabolics states. However, systemic IGF-I administration is limited by hypoglycemic and cardiac hypertrophic action. Moreover, autocrine action and then local production of IGF-I seems play a more important role in the regulation of muscular mass. Thus to limit loss of muscle mass, IGF-I administration must mimic as close as possible the autocrine production of IGF-I.

There are two principal ways to transfer a therapeutical gene : viral and non viral methods. Methods using virus vectors show a high rate of transfected cells but the risk of mutagenesis (17) or inflammatory reaction is important and actually it cannot completely avoided (18). In addition, virus can cause an immunologic response given rise to neutralizing antibodies who reduced the efficiency of the transfection method (19). In vivo DNA electrotransfer seems to be the best non viral gene delivery method (20-22) largely tested in several tissues (23-26).

The aim of this study is to demonstrate that long term muscle IGF-I overexpression via electroporation could promote muscle hypertrophy in intact rats.

Materials and methods

Expression plasmids and DNA preparation

Plasmid pM1-hIGF-I was constructed by inserting a 770 bp human IGF-I cDNA (a generous gift from Dr Paul Steenbergh, University of Utrecht, The Netherlands) into the SalI-NotI sites, between the cytomegalovirus (CMV) promoter and a 3' flanking sequence of the bovine growth hormone polyadenylation signal, in the pM1-MT Expression Vector (Roche Molecular Biochemicals, Indianapolis, USA). Plasmids were amplified in E.Coli top 10 F' (Invitrogen, Carlsbad, USA) and purified with an EndoFree Plasmid Giga Kit (QIAGEN, Hilden, Germany). Plasmids were stocked at -80°C. The day before the injection, 100µg of plasmid was lyophilized and resuspended in 100 µl of NaCl 0.9% solution.

Animals

Six-week-old male Wistar hypophysectomized (hypox) rats (Charles River laboratories, Lyon, France) (140-150 g) were used to assess the anabolic action of transfected IGF-I on muscle. The animals were housed under controlled conditions of lighting (12-h light, 12-h dark cycle) and temperature (22 ± 2°C.). Access to animals chow and water containing 0.9% NaCl was unrestricted. Animals were weighed every other day to confirm the absence of spontaneous growth. One week after their reception, animals were treated with daily injections of thyroxine (1 µg/100 g BW, Sigma Aldrich, St. Louis, USA) and cortisol (50 µg/100 g BW, Solu-Cortef®, Pharmacia Upjohn, NY, USA) until sacrifice.

DNA injection and electroporation

Eight days after the beginning of thyroxine and cortisol injections, rats were anaesthetized with a mixture of 75 mg/kg ketamine (Ketalar®, Pfizer, NY, USA) and 15 mg/kg xylazine hydrochloride (Rompun®, Bayer, Leverkusen, Germany) administered by intraperitoneal injection. Ten µL of pM1-hIGF-I plasmid solution (1 µg/µL) were injected in 10 different sites in one TA muscle (total volume = 100 µl). The contralateral TA muscle was injected, in a same manner, with 100 µL of pM1-MT plasmid solution (NaCl 0.9%). Both muscles were electroporated. Transcutaneous pulses were applied by two stainless steel plate electrodes with distance between the two plates of 1 cm. Electrical contact with the leg skin was ensured by shaving each leg and applying conductive gel. Electric pulses with a standard square wave were delivered by an electroporator (# PA-4000, Four parameter Pulsagile Electroporation

System, Cytopulse Science, Inc, Columbia, Maryland, USA). Ten pulses of 200 V/cm were administered to the muscle with a delivery rate of 1 pulse/s with each pulse of 20 ms in duration. Animals were sacrificed 3 and 14 days (experiment A) or 28 and 56 days (experiment B) after DNA injection and electroporation. For biochemical analysis, TA muscles were removed, weighed, deep frozen in liquid nitrogen and stored at -80° until further analysis. For histological analysis, TA muscles were dissected and a transversal slice of 0.5 cm thickness was made in the middle belly of the muscle. This slice was embedded in Tissue Tek (Sakura, Zoeterwoude, The Netherlands), frozen with isopentane supercooled by liquid nitrogen and stored at -80°C until further analyzed.

Muscle protein extraction

Briefly, 100 mg of TA muscle, previously pestled in liquid nitrogen, were homogenized with Ultraturrax (IKA-Labortechnik, Staufen, Germany) in 1 ml ice-cold lysis buffer (10 mM Tris/HCl [pH7.5], 5 mM EDTA, 0.5% NP40, 1 mM phenylmethylsulfonylfluoride, 5 µg/mL aprotinin, 2 µg/ml leupeptin). Homogenates were centrifuged 10 min. at 10.000 rpm. (Sorvall SS-34 rotor) to pellet myofibrillar proteins. Myofibrillar proteins were resuspended in 8 M urea/50 mM Tris/HCl, pH 7.5. The supernatant contained soluble proteins. Myofibrillar and soluble muscle protein content was determined by using Bradford's protein assay (Bio-Rad, Munich, Germany).

Muscle and circulating IGF-I extraction

Five hundred µl of supernatant, containing soluble proteins, were mixed and incubated 2 h on ice with 500 µL of acetic acid (2 M). The resulting homogenate was lyophilized and resuspended in the RIA buffer (0.03 M NaH_2PO_4, 0.02% protamine, 0.1 M EDTA, 0.05% tween, 0.02% azide, pH 7.5). Serum IGF-I was extracted as previously described (27,28).

IGF-I Radioimmunoassay

The muscle and circulating IGF-I concentrations were measured by specific radioimmunoassay (RIA). Briefly, two hundred microliters of muscle extract or 50 µl of serum extract (or standard), IGF-I antiserum, and ^{125}I-IGF-I (10 000 cpm) were pipetted into duplicate RIA tubes. The rabbit anti-IGF-I antiserum (UB-487, a kind gift from Dr. L.E. Underwood, University of North Carolina, Chapel Hill, NC, USA) was used at a dilution of 1:40.000 for muscle IGF-I and at a dilution of 1:15.000 for serum IGF-I. Tubes were incubated at 4°C for 16 to 24 h. At the end of the incubation, 1 ml of a precipitate solution

(6% polyethyleneglycol, 0.5% goat anti-rabbit, 0.25% cellulose) and 100 µL of normal rabbit serum were added to each tube. The tubes were then centrifuged at 14 000 rpm at 4°C for 30 min to separate bound ^{125}I-IGF-I from unbounded. Immediately after centrifugation the supernatant was removed by aspiration. All RIA tubes were then placed in a Gamma counter (LKB-Wallac CliniGamma Gamma counter) and the bound IGF-I fraction radioactivity was counted 2 min. per tube. The sample IGF-I concentration was determined using an IGF-I standard curve. The recovery of recombinant IGF-I added to supernatant-acetic acid mixture was 48.3±1.8% (mean ± SEM, n=3). The results are expressed as nanograms of IGF-I per gram of muscle and are not corrected for recovery.

RNA extraction and analysis by real time quantitative RTQ-PCR

Total RNA was isolated from the TA muscle using TRIzol reagent as described by manufacturer. Recovery was 1 µg/mg TA. Reverse transcription using 1µg of total RNA was done as previously described (29). Specifics primers (Table 1) were determined using the Primer Express software (Applied Biosystems, Foster City, CA, USA). Accession number for the sequences used were AH002176 (Rat IGF-I), S85346 (Human IGF-I) and AF106860 (glyceraldehyde-3-phosphate dehydrogenase (GAPDH) used as reporter gene. Primers were tested in order to avoid primers dimers, self-priming formation or unspecific amplification. Human and rat IGF-I primers were tested to demonstrate the absence of cross reaction with the heterologous IGF-I cDNA sequence. Primers were designed to have standardized optimal PCR condition. RTQ-PCR was carried out using following cycle parameters: 10 min at 95°C, followed by 40 cycles of 1 min at 60°C and 15 sec at 95°C. For each gene, RTQ-PCR was conducted in duplicate with 25 µl reaction volume of 1 ng of cDNA. To ensure the quality of the measurements, each plate included for each gene a negative control and a positive control. The threshold cycle (Ct) from a positive sample was used to calculate the intra and inter-assay coefficient of variation (CV). For each gene, the intra-assay CVs were calculated as standard deviation/mean of five Ct determined for a same sample on the same plate with the same mix. The inter-assay CVs was calculated as standard deviation/mean of the Ct determined on five different plates and with different mixes. The intra-assay and the inter-assay CVs obtained for the different gene studied were comprised between 1% and 3%. Results were expressed using the comparative cycle threshold (Ct) method as described in the User Bulletin #2 (Applied Biosystem). Briefly, the ΔCt values were calculated in every sample for each gene of interest as followed: $Ct_{\text{gene of interest}} - Ct_{\text{reporter gene}}$ with GAPDH as the reporter gene. The calculation of the relative changes in the expression level of one specific gene (ΔΔCt) was performed by

subtraction of the ΔCt from the control group to the corresponding ΔCt from the treated groups (29, 31). The values and ranges given in different figures were determined as followed: $2^{-\Delta\Delta Ct}$ with ΔΔCt+SEM and ΔΔCt –SEM, where SEM is the standard error of mean of the ΔΔCt value (User Bulletin #2, Applied Biosystem).

Histological analysis of muscle

Cryostat serial sections (10 μm thick) were cut and mounted onto glass slides. The sections were fixed 2 min. with acetone for Hematoxylin-Eosin staining. Muscles and fibers cross sectional area (CSA) were measured with a microscope (Leitz Wetzlar) coupled to an image analyzer system (Kontron image analysis division MOP-Videoplan, Eching, Germany). To evaluated muscle fiber CSA, 5 or 6 fields were randomly chosen and 200 fibers were counted per sections and 2 sections were analyzed per muscle.

Statistical analysis

Results are presented as means ± standard error of the mean (S.E.M.). Statistical analyses were performed using Student t-test. Cross sectional fibers area distribution statistical analysis was performed using χ^2 Pearson test. Statistical significance was set at $P < 0.05$.

Results

ASSESSMENT OF IGF-I OVEREXPRESSION

IGF-I muscle content.

To demonstrate IGF-I overexpression after IGF-I DNA transfection, we determined the IGF-I muscle content in hypophysectomized rats, which have a low basal IGF-I muscle content. Three and fourteen days after IGF-I DNA injection and electroporation, the IGF-I content was 2.9-fold higher in the treated TA muscle than in the untreated contralateral muscle (12.3±2.4 vs. 4.2±1.0 ng/g of muscle, P <0.01, n=4 at 3 days and 5.0±0.6 vs. 1.7±0.4 ng/g of muscle at 14 days, P <0.01, n=4) (Fig. 1). With time, the IGF-I muscle content decreased, but remained still higher 28 days after electroporation in the IGF-I DNA injected TA muscle compared to the contralateral muscle (2.7±0.2 vs. 1.7±0.1 ng/g of muscle, P <0.001, n=8). The difference in IGF-I content between the two sides was not anymore significant 56 days after electroporation (1.9±0.2 vs. 1.7±0.2 ng/g of muscle, NS, n=7) (Fig.1).

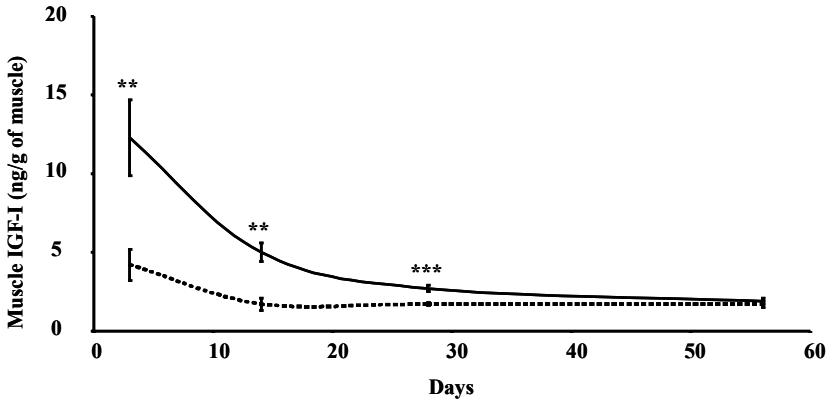

Figure1. *IGF-I DNA transfer increases IGF-I muscle content*. Tibialis anterior muscles were injected and electroporated with a total of 100 µg of pM1-hIGF-I plasmid at a concentration of 1 µg/µl (——). Contralateral tibialis anterior muscles were injected and electroporated with an insert-less plasmid 1 µg/µl (-----) (pM1). Muscle IGF-I content was determined by RIA 3 (n=4), 14 (n=4), 28 (n=8) and 56 (n=7) days after IGF-I DNA injection and electroporation. Results are expressed as mean ± SEM. ** P <0.01, *** P <0.001, vs. contralateral muscle.

mRNA IGF-I analysis.

To distinguish the contribution of endogenous rat and ectopic human IGF-I mRNAs in the increased muscle IGF-I content, we designed two specific pairs of primers. Using human and rat tissues, we confirmed that rat IGF-I mRNA was not detectable by human primers and the opposite (data not shown). As expected, human IGF-I mRNA was not detected in the control

TA muscle. In contrast, in the IGF-I DNA injected and electroporated TA muscle, ectopic human IGF-I mRNA was detected. At all time points examined, the ectopic human IGF-I mRNA was significantly more expressed than the endogenous rat IGF-I mRNA (5-fold at 14 days, P <0.05; 3-fold at 28 days, P <0.001 and 2-fold induction at 56 days, P <0.05) (Fig.2). Furthermore, IGF-I DNA transfection did not impair the endogenous rat IGF-I mRNA, as indicated by the similar rat IGF-I mRNA levels in both muscles.

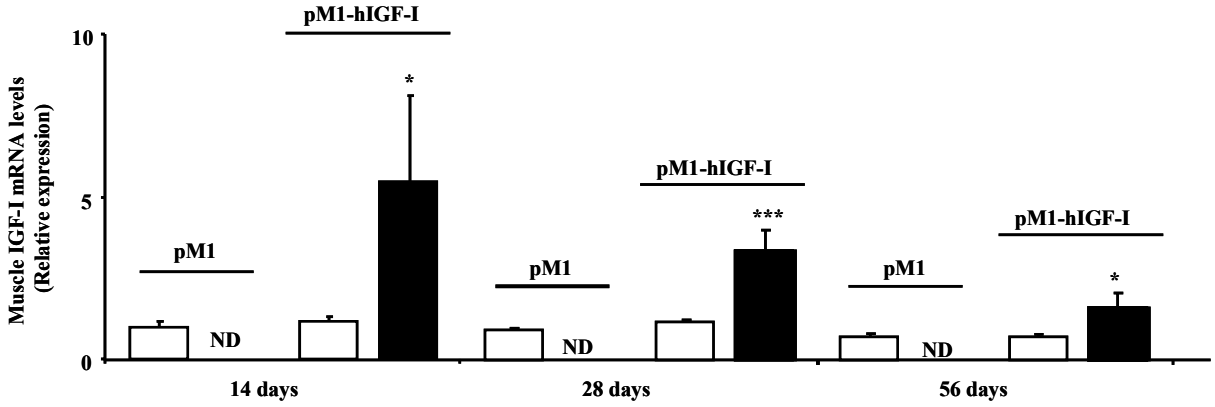

Figure 2. *IGF-I DNA transfer increases human IGF-I mRNA (black box) muscle content without down-regulating rat IGF-I mRNA (white box).* Muscle IGF-I mRNA content was determined 14 (n=3) days, 28 (n=8) and 56 (n=7) days after DNA injection and electroporation. Results are expressed as mean ± SEM. * P <0.05 and *** P <0.001, vs. rat IGF-I mRNA in the same muscle.

Circulating IGF-I concentrations.

Serum IGF-I concentrations did not change significantly as the result of treatment or elapsed time out to 56 days (Table 1).

	Day 0	Days 28	Days 56
Serum IGF-I (ng/ml)	**62 ± 5**	**61 ± 5**	**64 ± 5**

Table 1. *Muscle IGF-I overexpression did not change circulating IGF-I concentrations.* Plasma IGF-I concentrations were determined by RIA at 0 (n=6), 28 (n=15) and 56 (n=13) days after DNA injection and electroporation. Data are expressed as mean ± SEM.

Muscle mass, muscle area and fiber cross sectional area.

Despite the IGF-I mRNA and peptide overexpression observed 3 and 14 days after DNA injection and electroporation in the treated TA muscle, the weight of the two opposite TA muscles did not differ at those time points (data not shown). The anabolic effect of IGF-I appeared with time. Indeed, the TA muscle weight was significantly increased after 28 (treated: 295±5 vs. control: 285±5 mg, P <0.01, n=15) and 56 days (treated: 330±8 vs. control: 313±5 mg, P <0.05, n=13) of IGF-I overexpression (Fig.3A). The muscle weight gain induced by IGF-I overexpression was associated with significant increase in the average TA muscle cross-sectional area at 28 days (treated: 23.3±0.6 vs. control: 21.6±0.7 mm^2, P <0.05, n=7) and 56 days (treated: 27.8±0.8 vs. control: 26.6±0.7 mm^2, P <0.05, n=6) (Fig.3B). This increase in muscle area was explained by an increase in the average cross sectional area of muscle fibers in the treated TA muscle compared to the control one, as observed at 56 days (1816±77 vs. 1504±56 μm^2, P <0.001, n=6) (Fig.3C).

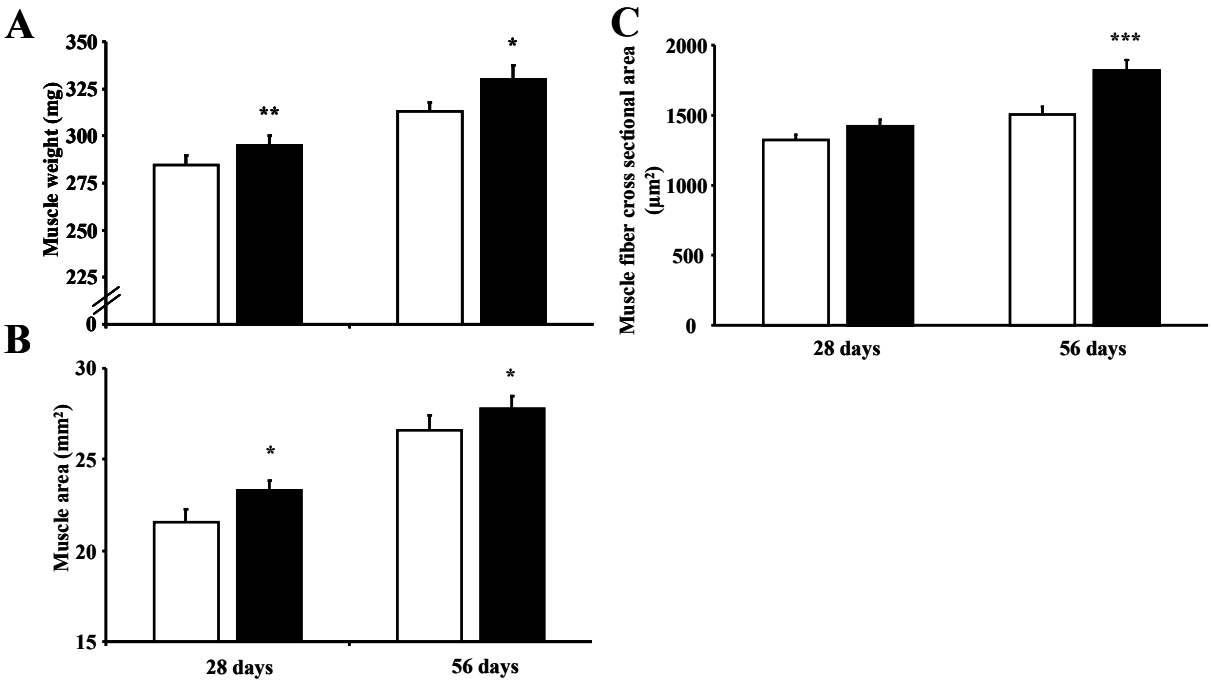

Figure 3. *IGF-I DNA transfer stimulates muscle hypertrophy*. Tibial anterior muscles were injected and electroporated with a total of 100 µg of pM1-hIGF-I plasmid at a concentration of 1 µg/µl (black box). Contralateral tibial anterior muscles were injected and electroporated with pM1 plasmid at a concentration of 1µg/µl (white box). Muscle mass (A), total muscle CSA (B) and mean fibers CSA

(C) were determined 28 and 56 days after DNA injection and electroporation. Results are expressed as mean ± SEM. * P <0.05, ** P <0.01,*** P <0.001, vs. contralateral muscle.

The fiber size increase was confirmed by the analysis of fiber size distribution showing that the proportion of larger fibers (over 1500-1750 μm^2) was significantly greater in the treated muscle than the control one at 28 days (P <0.001) and even more markedly at 56 days (P <0.001) (Fig.4).

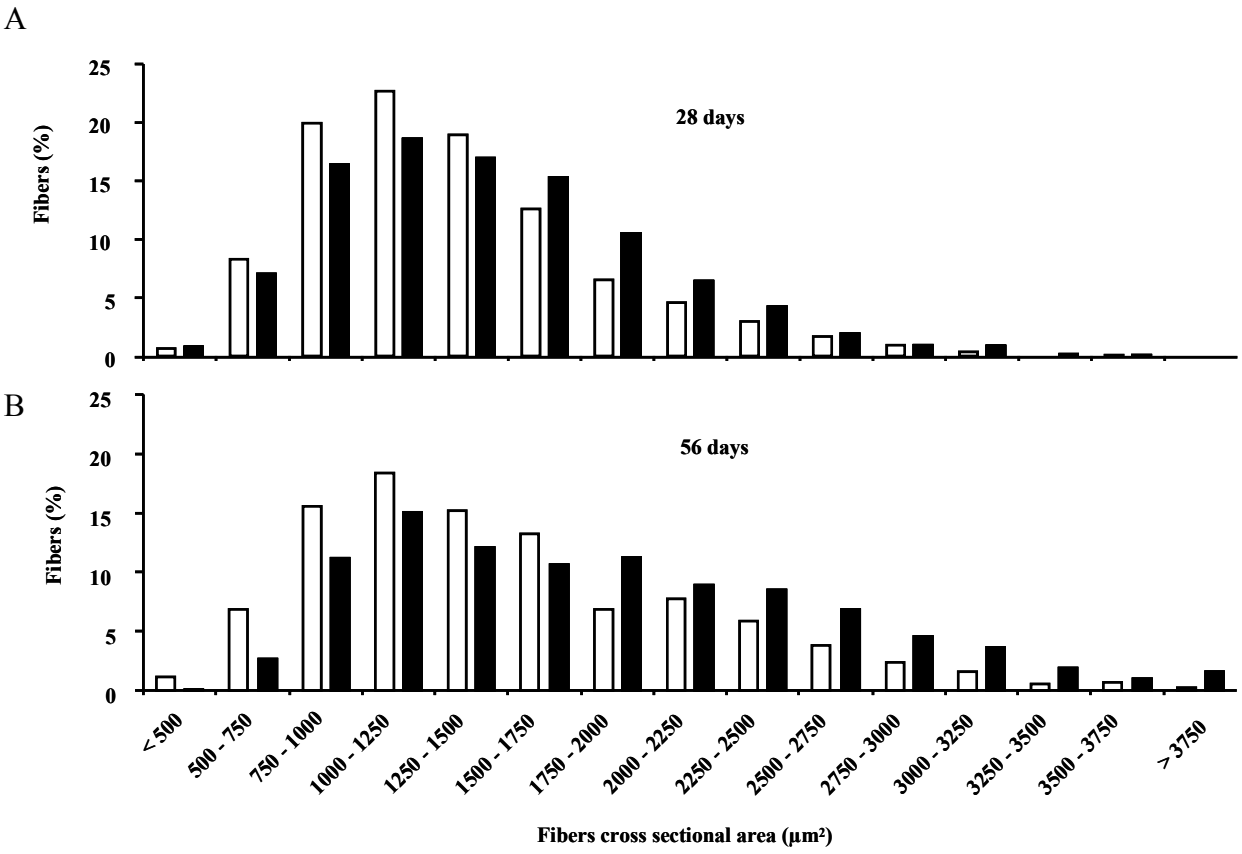

Figure 4. *IGF-I DNA transfer increases the proportion of large fibres*. The proportion of large fibers was greater in IGF-I DNA injected TA muscle (black column) than in contralateral TA muscle (white column) at 28 days (A) and at 56 days (B). Statistical analysis was performed using χ^2 Pearson test. *** P <0.001.

Myofibrillar and soluble muscle protein content.

At 28 and 56 days, the myofibrillar and the soluble muscle protein contents were not significantly different between the treated and contralateral muscles (Table 2).

	Day 28		Day 56	
	Control muscle (n=8)	**Treated muscle (n=8)**	**Control muscle (n=7)**	**Treated muscle (n=7)**
Soluble fraction (mg/g of muscle)	**60±1**	**63±3**	**73±1**	**72±2**
Myofibrillar fraction (mg/g of muscle)	**56±4**	**54±4**	**82±.2**	**83±3**

Table 2. *Muscle IGF-I overexpression did not change myofibrillar and soluble muscle protein contents.* Myofibrillar and soluble muscle protein contents were determined 28 and 56 days after DNA injection and electroporation as described in Materials and Methods. TA muscles from control were injected with 10 µl of pM1 plasmid solution (1µg/µl) in 10 injection sites (total volume=100µl) and electroporated. TA muscles from treated were injected with 10 µl of pM1-hIGF-I plasmid solution (1µg/µl) in 10 injection sites (total volume=100 µl) and electroporated. Data are expressed as mean ± SEM.

Discussion

Our observations show that localized overexpression of IGF-I by gene electrotransfer results in a long term skeletal muscle hypertrophy in a non muscle-injury model. Human IGF-I gene electrotransfer resulted in increased muscle IGF-I mRNA and protein levels associated with increased muscle mass mainly caused by increased size of muscle fibres.

In this work, we show that delivery of human IGF-I gene to the tibialis anterior muscle of hypophysectomized rats increases muscle IGF-I peptide as early as three days and for approximatively four weeks. Despite the doubling of muscle IGF-I content, electrotransfer of IGF-I DNA into muscle of hypophysectomized rats did not restore IGF-I concentrations to the values observed in intact animals (10 ng/g of muscle) (42). Because a similar relative induction of IGF-I content is observed in intact animals after IGF-I DNA electrotransfer (data not shown), we hypothesized that muscle of hypophysectomised rats lacks some factors mandatory for full expression of the transgene. The doubling of IGF-I muscle content obtained with our electrotransfer protocol is close to the increase in IGF-I observed in mIGF-I transgenic mice (15), but lower than 5-fold increase reached by local infusion of the peptide (11).

The sudden and transient increase of IGF-I peptide in control muscle three days after electroporation was probably caused by some degree of injury. It is known that some muscle injury can occur as a result of DNA electroporation (43). This damage may have induced a regenerative response accompanied with increased endogenous IGF-I expression (44).

Despite increased muscle IGF-I peptide, local IGF-I overexpression was not accompanied by increase in serum concentrations of IGF-I. Two explanations may be proposed to explain the absence of increased circulating IGF-I concentrations in our model. First, the expression level of our transgene was to low to support increased systemic levels of IGF-I. Indeed, IGF-I muscle content reached in our work is far lower than that obtained in other IGF-I overexpression models (15) in which elevation of circulating IGF-I was noted. Second, the IGF-I isoform (Ea) overexpressed in our work was not associated with production of endocrine IGF-I. Indeed, three proIGF-I isoforms (Ea, Eb and Ec) have been described in skeletal muscle. While isoform Ea has been considered as the most abundant in most tissues, isoform Eb seems almost inexistant in muscle in contrast to liver, and isoform Ec has been reported to be exquisitely expressed in response to strech. Overexpression of IGF-I Ea isoform by transgenesis (45) or virus (12-16) has not been so far associated with increased

circulating IGF-I concentrations. The mechanism by which the IGF-I Ea isoform is locally restricted remains unclear. Changes in muscle IGF-I concentrations were reflected by parallel changes in human IGF-I mRNA levels. So the progressive decrease with time of muscle IGF-I peptide was directly related to the decrease of human IGF-I mRNA in transfected muscle, suggesting the extinction of our transgene expression. Several hypothesises may be proposed to explain the diminution of the transgene expression. First, the transgene introduced in rat muscle cells is not from murine origin and thus the expression of the human IGF-I peptide may have cause an immune response. Indeed, previous works have shown that long-term overexpression of a foreign protein such as β-galactosidase required immunosuppression (36). But, in our case, the probability that human IGF-I produces an immune response is very small, because there is a great homology (96%) between the two animo-acid sequences entering in the composition of the different IGF-I proteins. Second, the extinction of the pCMV promoter (47) or the progressive degradation of extrachromosomal DNA might result in premature decline in muscle IGF-I expression. Finally, it is also possible that increased muscle IGF-I content by electrotransfer might have contribute to downregulate the expression of rat endogenous IGF-I expression. Previous observations showed down-regulation of IGF-I expression by exogenous IGF-I added to muscle cells in culture (48). However, this hypothesis is refuted by our observation showing that local increased IGF-I was not associated with a dow-regulation of rat IGF-I in the muscle overexpressing human IGF-I.

Overexpression of IGF-I in tibialis anterior muscle of hypophysectomized animals lead to muscle hypertrophy as demonstrated by increase in muscle mass. The absence of any decrease in muscle protein concentrations supports a protein gain and not a water gain. As showed in other models of postnatal muscle IGF-I overexpression (15,45), muscle hypertrophy induced by IGF-I electrotransfer is essentially due to increased fibre size. Compared with the IGF-I transgenic mice model (45), the fibre size increase is smaller in our IGF-I electroporated rat model (+25% vs +17%). There are two major explanations for this difference. First, in the transgenic animal model, all fibres expressed IGF-I, assuming a perfect distribution of the peptide into the muscle mass. The second principal reason is the time duration of overexpression. Muscle fibres of transgenic mice have been exposed for 6 months to IGF-I. If our results are compared with those observed after 4 months of overexpression using a viral vector (12), increases in muscle size fibre are similar (+14% vs +17%). This fibre hypertrophy is directly correlated with an increased in muscle force in normal and pathological animals models (12, 15, 45).

Although the muscle anabolic effects of electrotransfered IGF-I have already been reported (49, 35), the models previously used assessed its anabolic effects after a strong catabolic stimulus (hindlimb suspension and muscle injury). Furthermore, when muscle from intact animals was electroporated with IGF-I DNA, only the transfected fibers and the fibers immediately adjacent to the positive fibres were considered to assess the hypertrophic effect due to overexpressed IGF-I (Alzghoul, 2003). These fibres represent however a small part of the muscle because only 10% of the muscular fibres overexpressed IGF-I (35). In contrast, in our work, we have considered the whole muscle. Finally, we reported that muscle anabolic effects of electrotransfered IGF-I persist until two months after DNA injection and electroporation, despite progressive decline of IGF-I peptide and mRNA.

In conclusion, our results demonstrated that muscle IGF-I gene electrotransfer causes muscle IGF-I overexpression for a month and hypertrophic effects lasting at least 2 months in hypophysectomized animals. In further experiments, we will test if this long term local IGF-I overexpression can prevent loss of muscle mass encountered in catabolic states.

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2. Insulin-like growth factor-I gene transfer by electroporation prevents skeletal muscle atrophy in glucocorticoid-treated rats.

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Abstract

Catabolic states caused by injury are characterized by a loss of skeletal muscle. The anabolic action of Insulin-like Growth Factor (IGF)-I on muscle and the reduction of its muscle content in response to injury suggest that restoration of muscle IGF-I content might prevent skeletal muscle loss caused by injury. We investigated whether local overexpression of IGF-I protein by gene transfer could prevent skeletal muscle atrophy induced by glucocorticoids, a crucial mediator of muscle atrophy in catabolic states. Localized overexpression of IGF-I in tibialis anterior (TA) muscle was performed by injection of IGF-I cDNA followed by electroporation 3 days before starting dexamethasone injections (0.1 mg/kg/day SC). A control plasmid was electroporated in the contralateral TA muscle. Dexamethasone induced atrophy of the TA muscle as illustrated by reduction in muscle mass (403±11 vs. 461±19 mg, $P<0.05$) and fiber cross-sectional area (1759±131 vs. 2517±93 μm^2, $P<0.05$). This muscle atrophy was paralleled by a decrease in the IGF-I muscle content (7.2±0.9 vs. 15.7±1.4 ng/g of muscle, $P<0.001$). As the result of IGF-I gene transfer, the IGF-I muscle content increased 2-fold (15.8±1.2 vs. 7.2±0.9 ng/g of muscle, $P<0.001$). In addition, the muscle mass (437±8 vs. 403±11 mg, $P<0.01$) and the fiber cross-sectional area (2269±129 vs. 1759±131 μm^2, $P<0.05$) were increased in the TA muscle electroporated with IGF-I DNA compared to the contralateral muscle electroporated with a control plasmid. Our results show therefore that IGF-I gene transfer by electroporation prevents muscle atrophy in glucocorticoid-treated rats. Our observation supports the important role of decreased muscle IGF-I in the muscle atrophy caused by glucocorticoids.

Key words: IGF-I, skeletal muscle, gene therapy, glucocorticoids.

Introduction

Catabolic states such as cancer, sepsis, AIDS, or chronic inflammatory diseases are characterized by an accelerate loss of lean body mass mainly skeletal muscle. This change in body composition is associated with an increase in morbidity and mortality (1). Despite recent better understanding of the metabolic and molecular derangements leading to muscle wasting, therapy of muscle atrophy has still a poor success rate.

Glucocorticoids play a major role in the induction of muscle atrophy in catabolic conditions (2, 3). Indeed, administration of glucocorticoids induces muscle atrophy mainly due to stimulation of muscle proteolysis (4, 5) and inhibition of protein synthesis (4-6). Furthermore, circulating glucocorticoids levels are frequently increased in catabolic states (2). Finally, the inhibition of glucocorticoids action by RU 486, an antagonist of the glucocorticoid receptor, markedly reduces the loss of muscle mass encountered in such catabolic states (3, 7, 9). Recent evidence indicates that the loss of muscle mass induced by glucocorticoids might be due to decreased Insulin-like Growth Factor (IGF)-I levels, an important endocrine and autocrine anabolic growth factor (9). Glucocorticoids treatment indeed decreases skeletal muscle IGF-I expression (10) and systemic administration of IGF-I has been reported to prevent corticosteroid-induced muscle atrophy (6, 11).

Several evidences indicate that IGF-I plays a crucial role in the loss of muscle mass observed in catabolic states. First, autocrine IGF-I production plays a critical role in muscle growth (9,12). IGF-I plays an anabolic and anticatabolic action in skeletal muscle *in vivo* and *in vitro*. Anabolics effects of IGF-I result from the stimulation of protein synthesis (13) as well as proliferation and differentiation of muscle satellite cells (14, 15), while its anticatabolic effects result from the inhibition of muscle proteolysis (16) and apoptosis (17). Moreover, circulating and muscle IGF-I levels are frequently reduced in catabolic states (18). Finally, IGF-I gene overexpression prevents loss of muscle in some muscle atrophy models such as aging and muscle dystrophy (19, 20). These evidences suggest therefore that IGF-I might prevent loss of muscle mass in hypercatabolics states

However, use of systemic IGF-I administration is limited by its hypoglycemic (21) and cardiac hypertrophic actions (22, 23). Moreover, local production and thus autocrine action of IGF-I seem to play a more important role in the regulation of muscle mass than systemic and endocrine IGF-I (9). Therefore, to limit loss of muscle mass observed in catabolic states, IGF-I administration must mimic as close as possible the autocrine production of IGF-I.

Taken together these observations, we hypothesized that the local decrease in muscle IGF-I content might be responsible for the muscular atrophy induced by glucocorticoids. The aim of this study was therefore to demonstrate that local IGF-I overexpression by electroporation of IGF-I DNA could prevent muscle atrophy caused by glucocorticoid treatment. In order to demonstrate our hypothesis, we have studied the anticatabolic effect of local IGF-I overexpression on skeletal muscle of rats made catabolic by glucocorticoid treatment. The study was performed on the tibialis anterior muscle, a skeletal muscle quite homogenous (fibers IIb/d 96%) (24) and highly sensitive to the catabolic effects of glucocorticoids (11, 25, 26). To avoid problems encountered with viral vectors (27-29), *in vivo* DNA electrotransfer was used to deliver the IGF-I gene into muscle. This method has been already successfully used in several tissues (30-32) and especially in skeletal muscle (33-36).

Materials and methods

Expression plasmids and DNA preparation

Plasmid pM1-hIGF-I was constructed by inserting a 770 bp human IGF-I cDNA (a generous gift from Dr Paul Steenbergh, University of Utrecht, The Netherlands) into the SalI-NotI sites, between the cytomegalovirus (CMV) promoter and a 3' flanking sequence of the bovine growth hormone polyadenylation signal, in the pM1 Expression Vector (Roche Molecular Biochemicals, Indianapolis, USA). A pM1-β-Gal Expression Vector (Roche Molecular Biochemicals) was used to determine the optimal transfection's conditions. Plasmids were amplified in E.Coli top 10 F' (Invitrogen, Carlsbad, USA) and purified with an EndoFree Plasmid Giga Kit (QIAGEN, Hilden, Germany). Plasmids were stocked at -80°C. The day of injection, 100 µg of plasmid was lyophilized and resuspended in 100 µl of NaCl 0.9% solution.

DETERMINATION OF OPTIMAL TRANSFECTION'S CONDITIONS BY ELECTROPORATION IN MUSCLE

Animals

Six week-old male Wistar rats (150-160 g) provided by Janvier Breeding (Le Genest St-Isle, France) were housed under controlled conditions of lighting (12-h light, 12-h dark cycle) and temperature (22 ± 2°C.). Access to animals chow and water was unrestricted. Animals were weighed daily.

DNA injection and electroporation

Rats were anaesthetized with a mixture of 75 mg/kg ketamine (Ketalar®, Pfizer, NY, USA) and 15 mg/kg xylazine hydrochloride (Rompun®, Bayer, Leverkusen, Germany) administered by intraperitoneal injection. In a first step, we determined the effects of electroporation on muscle transfer of naked DNA. In this experiment, the two tibial anterior (TA) muscles were injected transcutaneously with 100 µl of pM1-β-Gal plasmid solution using an insulin syringe with 30-gauge needle. One TA muscle was electroporated and the contralateral muscle was not. Transcutaneous pulses were applied by two stainless steel plate electrodes with distance between the two plates of 1 cm. Electrical contact with the leg skin was ensured by shaving each leg and applying conductive gel. Electric pulses with a standard square wave were

delivered by an electroporator (# PA-4000, Four parameter Pulsagile Electroporation System, Cytopulse Science, Inc, Columbia, Maryland, USA). Ten pulses of 200 V/cm were administered to the muscle with a delivery rate of 1 pulse/s with each pulse of 20 ms in duration. In a second step, we tested the influence of multiple injection sites on the efficiency of transfection by electroporation. In the TA of one side, 100 µl of pM1-β-Gal plasmid solution were injected in one single injection and muscle was electroporated as previously described. In the contralateral TA muscle, the 100 µl of pM1-β-Gal plasmid solution was divided into 10 injection sites. Electroporation conditions were as previously described, except that the ten pulses were replaced by two series of five pulses applied on each half of the muscle to cover most of the muscle volume.

Assessment of β-galactosidase gene expression

Rats were killed by decapitation 3 days after DNA injection and electroporation. TA muscles were dissected, weighed, snap frozen in liquid nitrogen, and stored at -80°C. According to manufacturer recommendation, 100 mg of TA muscle, previously pestled in liquid nitrogen, were homogenized with Ultraturrax (IKA-Labortechnik, Staufen, Germany) in 1 ml Reporter Lysis Buffer (Promega, Madison, WI) and centrifuged 10 min at 10 000 rpm (Sorvall SS-34 rotor). Supernatant (150 µL) was then mixed and incubated 30 min with 150 µl of Reaction buffer (Promega, Madison, WI). The reaction was stopped with 500 µL of carbonate de lithium (1 M) and the absorbance was measured with a spectrophotometer at 420 nm. Muscle protein content was determined by using Bradford's protein assay (Bio-Rad, Munich, Germany). Results were expressed in mU of β-galactosidase per gram of muscle protein. The variation coefficient was calculated as standard deviation/mean of five measurements of β-galactosidase activity determined in the same electroporation conditions.

Histochemical staining for β-galactosidase activity

Rats were killed by decapitation 3 days after DNA injection and electroporation. TA muscles were dissected and 0.5 cm thick transverse slices were made in the middle belly of the muscle. Five micron thick sections were obtained with a cryotome (Micron HM500, Micron international, Walldorf, Germany, T = -26°C) and directly applied onto Superfrost Plus slides (Menzel Gläser, Germany). Sections were dried (15 min, RT) and then stored at -80°C until use. In order to detect β-galactosidase activity, cryosections were successively air dried (10 min, 37°C), fixed in formaldehyde (10min, RT), washed under tap water, rinsed in phosphate

buffered saline (PBS) (10 min,RT) and finally incubated (30min, 37°C) with a commercial solution of X-Gal (β-gal staining set, ref 1828673, Roche Diagnostic, Mannheim, Germany).

EVALUATION OF THE ANTICATABOLIC EFFECT OF IGF-I DNA TRANSFECTION IN GLUCOCORTICOID-TREATED RATS

Animals

Six-week-old male Wistar rats (190-215 g) provided by Janvier Breeding were used to assess the muscle anticatabolic action of transfected IGF-I in rats treated with dexamethasone. The animals were housed under controlled conditions of lighting (12-h light, 12-h dark cycle) and temperature (22 ± 2°C.).

Experimental design

Thirty rats were divided in three groups: *ad libitum* (n=6), pair-fed (n=12) and dexamethasone (n=12). The *ad libitum* and pair-fed groups received daily subcutaneous injections of saline solution (vehicle, 0.9% NaCl). The glucocorticoid-treated group received daily subcutaneous injections of saline solution of dexamethasone (10 µg/100 g of BW, Aacidexam®, Organon, Oss, The Netherlands). Because dexamethasome treatment is known to decreased food intake, the pair-fed group received the same amount of food as that consumed by the dexamethasone-treated group during the previous day. The *ad libitum* and dexamethasone-treated animals were allowed to free access to chows and water. Animals were weighed and food intake measured every day. Animals were sacrificed after 7 days of dexamethasone treatment. For biochemical analysis, TA muscles were removed, weighed, deep frozen in liquid nitrogen and stored at -80° until further analysis. For histological analysis, TA muscles were dissected and a transversal slice of 0.5 cm thickness was made in the middle belly of the muscle. This slice was embedded in Tissue Tek (Sakura, Zoeterwoude, The Netherlands), frozen in precooled isopentane and stored at -80°C until further analyzed.

DNA injection and electroporation

Three days before dexamethasone treatment, each rat was anaesthetized with a ketamine/xylazine hydrochloride mixture, as previously described. In the *ad libitum* group, the two TA muscles were injected with 10 µL of pM1 plasmid solution (1 µg/µl) in 10 different sites (total volume per muscle = 100 µl). In the two other groups, 10 µL of pM1-

hIGF-I plasmid solution (1 µg/µl) were injected in 10 different sites in one TA muscle (total volume = 100 µl). The contralateral TA muscle was injected, in a same manner, with 100 µl of pM1 plasmid solution. Both muscles were electroporated using the electroporation conditions previously validated.

Muscle protein extraction

Briefly, 100 mg of whole TA muscle, previously pestled in liquid nitrogen, were homogenized with Ultraturrax (IKA-Labortechnik, Staufen, Germany) in 1 ml ice-cold lysis buffer (10 mM Tris/HCl [pH7.5], 5 mM EDTA, 0.5% NP40, 1 mM phenylmethylsulfonylfluoride, 5 µg/mL aprotinin, 2 µg/ml leupeptin). Homogenates were centrifuged 10 min at 10 000 rpm (Sorvall SS-34 rotor) to pellet myofibrillar proteins. Myofibrillar proteins were resuspended in 8 M urea/50 mM Tris/HCl, pH 7.5. The supernatant contained soluble proteins. Myofibrillar and soluble muscle protein contents were determined by using Bradford's protein assay (Bio-Rad, Munich, Germany).

Muscle and circulating IGF-I extraction

Five hundred µl of supernatant, containing soluble proteins, were mixed and incubated 2 h on ice with 500 µl of acetic acid (2 M). The resulting homogenate was lyophilized and resuspended in the RIA buffer (0.03 M NaH_2PO_4, 0.02% protamine, 0.1 M EDTA, 0.05% tween, 0.02% azide, pH 7.5). Serum IGF-I was extracted as previously described (37, 38).

IGF-I Radioimmunoassay

The muscle and circulating IGF-I concentrations were measured by specific radioimmunoassay (RIA) (37, 38). Briefly, two hundred microliters of muscle extract or 50 µl of serum extract (or standard), IGF-I antiserum, and ^{125}I-IGF-I (10 000 cpm) were pipetted into duplicate RIA tubes. The rabbit anti-IGF-I antiserum (UB-487, a kind gift from Dr. L.E. Underwood, University of North Carolina, Chapel Hill, NC, USA) was used at a dilution of 1:40 000 for muscle IGF-I and at a dilution of 1:15 000 for serum IGF-I. The recovery of recombinant IGF-I added to the crude homogenate was 20.7±0.6% (mean ± SEM, n=3). The results are expressed as nanograms of IGF-I per gram of muscle and are not corrected for recovery.

RNA extraction and analysis by real time quantitative (RTQ)-PCR

Total RNA was isolated from the TA muscle using TRIzol reagent as described by manufacturer. Recovery was 1 µg/mg TA. Reverse transcription using 1µg of total RNA was done as previously described (39). Specific primers (Table 1) were determined using the Primer Express software (Applied Biosystems, Foster City, CA, USA). Accession number for the sequences used were AH002176 (Rat IGF-I), S85346 (Human IGF-I) and AF106860 (glyceraldehyde-3-phosphate dehydrogenase (GAPDH) used as reporter gene. Primers were tested in order to avoid primers dimers, self-priming formation or unspecific amplification. Human and rat IGF-I primers were tested to demonstrate the absence of cross reaction with the heterologous IGF-I cDNA sequence. Primers were designed to have standardized optimal PCR conditions.

RTQ-PCR was carried out using following cycle parameters: 10 min at 95°C, followed by 40 cycles of 1 min at 60°C and 15 sec at 95°C. For each gene, RTQ-PCR was conducted in duplicate with 25 µl reaction volume of 1 ng of cDNA. To ensure the quality of the measurements, each plate included for each gene a negative control and a positive control. The threshold cycle (Ct) from a positive sample was used to calculate the intra and inter-assay coefficient of variation (CV). For each gene, the intra-assay CVs were calculated as standard deviation/mean of five Ct determined for a same sample on the same plate with the same mix. The inter-assay CVs were calculated as standard deviation/mean of the Ct determined on five different plates and with different mixes. The intra-assay and the inter-assay CVs obtained for the different genes studied were comprised between 1% and 3% (Table 1). Results were expressed using the comparative cycle threshold (Ct) method as described in the User Bulletin #2 (Applied Biosystem). Briefly, the ΔCt values were calculated in every sample for each gene of interest as followed: $Ct_{\text{gene of interest}} - Ct_{\text{reporter gene}}$ with GAPDH as the reporter gene. The calculation of the relative changes in the expression level of one specific gene (ΔΔCt) was performed by subtraction of the ΔCt from the control group to the corresponding ΔCt from the treated groups (39, 40) The values and ranges given in different figures were determined as followed: $2^{-\Delta\Delta Ct}$ with ΔΔCt+SEM and ΔΔCt –SEM, where SEM is the standard error of mean of the ΔΔCt value (User Bulletin #2, Applied Biosystem).

Gene	5' - 3' Sequences	Amplicon (bp)	Intra-assay variation coefficient	Inter-assay variation coefficient
Rat IGF-I	Forward primer CAG GCT ATG GCT CCA GCA T Reverse primer GGA AGC AAC ACT CAT CCA CA	63	1%	1%
Human IGF-I	Forward primer CTT CAA CAA GCC CAC AGG GT Reverse primer CAT CCA CGA TGC CTG TCT GA	65	1%	1%
GAPDH	Forward primer TGC ACC ACC AAC TGC TTA Reverse primer GGA TGC AGG GAT GAT GTT C	72	1%	3%

Table 1. *Oligonucleotide sequences and variation coefficient of RTQ-PCR.* Human and rat IGF-I primers were tested to demonstrate the absence of cross reaction with the heterologous IGF-I cDNA sequences.

Histological analysis of muscle

Cryostat serial sections (10 μm thick) were obtained and mounted onto glass slides. The sections were fixed 2 min with acetone for Hematoxylin-Eosin staining. Muscle and fiber cross-sectional area (CSA) were measured with a microscope (Leitz Wetzlar) coupled to an image analyzer system (Kontron image analysis division MOP-Videoplan, Eching, Germany). To evaluate muscle fiber CSA, 5 or 6 fields were randomly chosen and 200 fibers were counted per muscle.

Statistical analysis

Results are presented as means ± standard error of the mean (SEM). Statistical analyses were performed using a one-way ANOVA followed by a Newman-Keuls Multiple Comparison Test to compare muscles from different animals undergoing different experimental conditions (*ad libitum*-pair fed-dexa) or by a paired *t*-Test to compare muscles undergoing different experimental conditions within the same animal (pM1-pM1hIGF-I). Fiber cross-sectional area distribution statistical analysis was performed using χ^2 Pearson test. Statistical significance was set at $P < 0.05$.

Results

OPTIMAL TRANSFECTION CONDITIONS

With electroporation (10 pulses of 200 V/cm administered at 1 pulse/s with each pulse of 20 ms in duration), the β-galactosidase expression in the β-gal DNA-injected TA muscle was increased about 30-fold in comparison with non-electroporated β-gal reporter plasmid injected contralateral TA muscle (66±17 vs. 2±1 mU/mg of protein, P <0.001, n=5). When the number of injection's sites was increased (10 µg of pM1-βgal plasmid injected in 10 injection sites) and two series of five pulses were applied on each half of the muscle, the β-galactosidase expression in the TA muscle was increased 6.3-fold compared to contralateral TA muscle injected with a single injection of β-gal reporter plasmid and electroporated with one serie of ten pulses (360±46 vs. 58±24 mU/mg of protein, P <0.01, n=5) (Table 2).

Experimental groups	β-galactosidase expression (mU/mg of protein)	β-galactosidase expression (Fold induction)	Variation coefficient
1X pM1-β-gal without electroporation	**2 ± 1**	**1**	
1X pM1β-gal <u>with</u> electroporation	**58 ± 24 *****	**30**	**53%**
<u>10X</u> pM1-β-gal <u>with</u> electroporation	**360 ± 46 °°**	**189**	**29%**

Table 2. *Summary of optimal transfection's conditions using the pM1-β-GAL reporter plasmid.* Lane 1, TA muscles were injected transcutaneously with a single injection of 100 µl of pM1-β-Gal plasmid solution (1 µg/µl) and TA muscles were not electroporated (n=5). Lane 2, TA muscles were injected with a single injection of 100 µl of pM1-β-Gal plasmid solution (1µg/µl) and TA muscles were electroporated (n=5). Lane 3, 10 µl of pM1-β-Gal plasmid solution were injected in 10 injection sites (total volume=100µl, n=5) and TA muscles were electroporated. β-galactosidase expression was determined 3 days after DNA injection and electroporation. Induction were calculated by comparison with lane 1. Data are expressed as mean ± SEM. *** P<0.001 vs group without electroporation. °° P<0.01 vs one injection site with electroporation.

Under these optimal conditions of DNA injection (10 µg of plasmid injected in 10 injection sites) and electroporation (two series of 5 pulses on each half of the muscle), histological analysis of TA muscles injected with β-galactosidase reporter plasmid showed an average of 37±8% (mean ± SEM, n=8) of the fibers expressing β-gal DNA 3 days after DNA injection and electroporation (Fig. 1). These transfection's conditions were therefore used to overexpress the IGF-I gene in the muscle of rats.

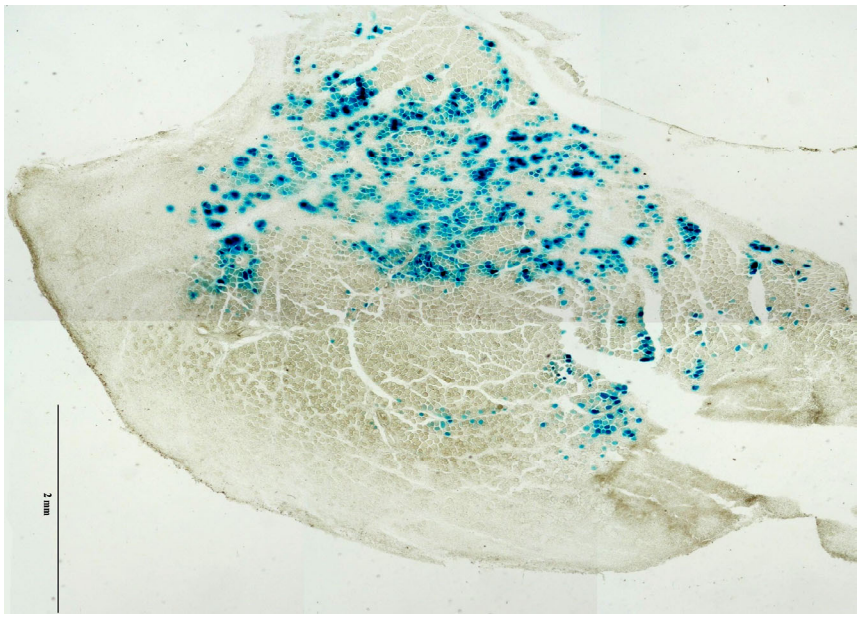

Figure 1. *Histochemical staining of β-Gal gene electrotransfered tibialis anterior muscle*. Tibialis anterior muscles were analyzed 3 days after DNA injection and electroporation as described in Materials and Methods.

ANTICATABOLIC EFFECTS OF TRANSFECTED IGF-I IN MUSCLE IN DEXAMETHASONE-TREATED RATS.

Animal weight and food intake

During the 7-day treatment, the body weight gain was lower in animals treated with dexamethasone (14±2 g) than in the *ad libitum* rats (64±2 g, P <0.001) and even in the pair-fed rats (28±2 g, P <0.001) (Fig. 2a). Compared to the *ad libitum* group, the mean daily food intake was markedly decreased in animals treated with dexamethasone (28.2±0.7 g vs. 22.7±0.6 g, P <0.001). According to our experimental design, the mean daily food intake of the pair-fed group was also reduced at 22.7 g (Fig.2b).

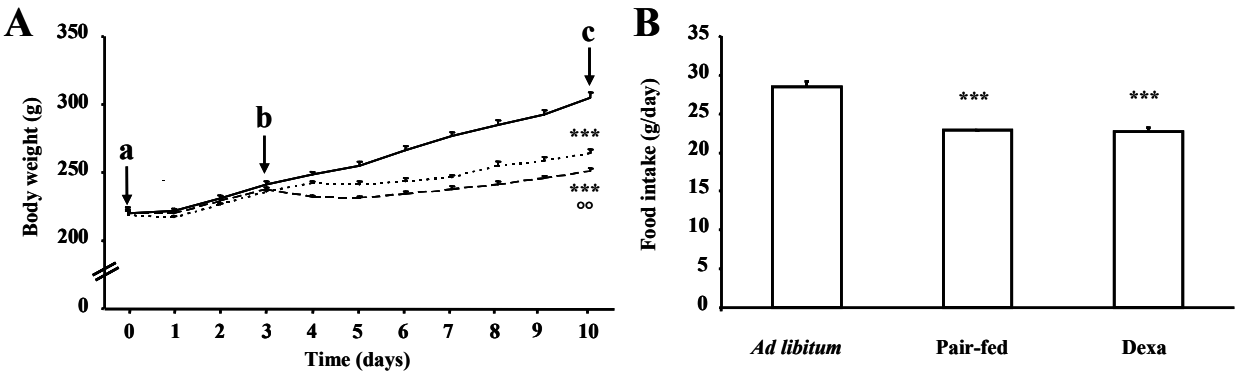

Figure 2. *Dexamethasone treatment reduced body weight gain and food intake*. Panel A represents the evolution of body weight of the *ad libitum* (—), pair-fed (·····) and dexamethasone-treated rats (----). Three days after DNA injection and electroporation (a), rats were treated with dexamethasone (0.1 mg/kg/d) (b) during seven days and sacrificed (c). Panel B represents the daily mean food intake (g/day) of the three experimental groups. Results are expressed as mean ± SEM. *** P <0.001 vs. *ad libitum*. °° P <0.01 vs. pair-fed animals.

IGF-I muscle content

After seven days of dexamethasone injections, the IGF-I muscle content was decreased by about 50 % in the TA muscle electroporated with the pM1 plasmid compared to muscle of *ad libitum* animals (7.2±0.9 vs. 15.7±1.4 ng/g of muscle, P <0.001, n=6). Food intake restriction imposed in the pair-fed group was also associated with a significant, albeit smaller (30%), fall in muscle IGF-I content (11.1±0.8 vs 15.7±1.4 ng/g of muscle, P <0.05, n=6). However, in the muscle electroporated with the IGF-I plasmid, the IGF-I muscle content was 2-fold increased by comparison with the contralateral muscle transfected with the pM1 both in the pair-fed (20.7±1.9 vs. 11.1±0.8 ng/g of muscle, P <0.001, n=6) and in the dexamethasone animals (15.8±1.2 vs. 7.2±0.9 ng/g of muscle, P <0.001, n=6). Transfection of IGF-I gene was therefore able to completely prevent the decline of the muscle IGF-I content caused by dexamethasone (Fig 3a).

IGF-I mRNA muscle levels

Muscle endogenous IGF-I mRNA levels were significantly reduced in the animals treated with dexamethasone (44%, P <0.05, n=6) in comparison with *ad libitum* rats. In contrast, food restriction did not affect rat IGF-I mRNA expression. Using species specific primers, hIGF-I mRNA was not detected in the TA muscle electroporated with pM1 plasmid. However, in the IGF-I plasmid electroporated TA muscle, ectopic hIGF-I mRNA was abundantly present. In these muscles, the level of hIGF-I mRNA was even higher than that of endogenous rat IGF-I mRNA (5-fold in the pair-fed and in the dexamethasone treated groups, P <0.001; n=6) (Fig 3b).

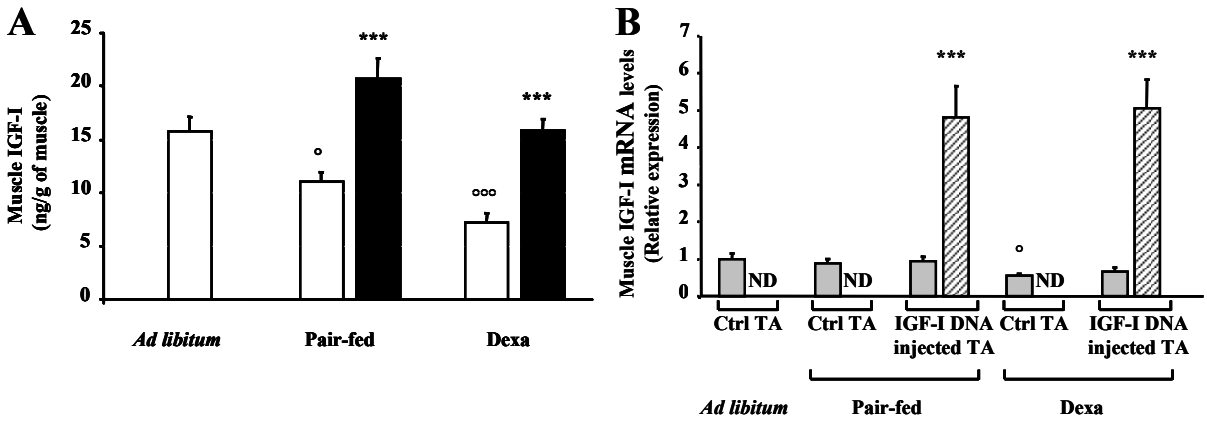

Figure 3. *IGF-I gene electrotransfer restores IGF-I muscle content and increases human IGF-I mRNA in dexamethasone-treated rats*. In panel A, tibial anterior muscles were injected and electroporated with a total of 100 µg of pM1 plasmid at a concentration of 1 µg/µl (white box). In pair-fed and dexamethasone treated-rats, contralateral tibial anterior muscles were injected and electroporated with 100 µg of pM1-hIGF-I plasmid at a concentration of 1 µg/µl (black box). In panel B, dexamethasone injection reduced expression of rat IGF-I mRNA (grey box), while IGF-I gene electrotransfer increased human IGF-I mRNA (lined box) by comparison with *ad libitum* control rats. Both muscle IGF-I protein and muscle IGF-I mRNA content were determined 10 days after IGF-I DNA injection and electroporation according to the experimental design described in material and methods. Results are expressed as mean ± SEM. Panel A, *** P <0.001, vs contralateral muscle and ° P <0.05, °°° P <0.001 vs. *ad libitum* animals. Panel B, *** P <0.001, vs. rat IGF-I mRNA in the same muscle and ° P <0.05 vs. rat IGF-I mRNA in the muscle of *ad libitum* animals.

Circulating IGF-I concentrations

Serum IGF-I concentrations did not change significantly as the result of dexamethasone treatment (992±39 ng/ml of serum, P > 0.05, n=11) or food restriction (898±41 ng/ml of serum, P > 0.05, n=12) compared with the levels observed in *ad libitum* animals (960±53 ng/ml of serum, n=6).

Muscle weight

The muscle weight of the TA electroporated with pM1 plasmid was significantly 13% lower in dexamethasone-treated rats than in *ad libitum* rats (dexamethasone: 403±11 mg, n=5 vs. *ad libitum*: 461±19 mg, n=6; P <0.05). IGF-I transfection increased by about 8% the TA muscle weight in dexamethasone-treated animals (TA electroporated w/ pM1-hIGF-I: 437±8 vs. TA electroporated w/ pM1: 403±11 mg, P <0.05, n=5). Therefore, by comparison with *ad libitum* rats, the loss of muscle mass due to the dexamethasone injections was partially prevented in the TA muscle transfected with IGF-I gene (*ad libitum* w/ pM1: 461±19 mg vs. dexamethasone w/ pM1-hIGF-I: 437±8 mg, ns). There was no significant effect of IGF-I overexpression on the muscle weight in the pair-fed animals (Fig.4a).

Muscle area and fiber cross-sectional area

Dexamethasone decreased by about 11% the TA muscle cross-sectional area (*ad libitum*: 35.8±0.8, n=6 vs. dexamethasone: 31.7±1.7 mm^2, n=5, ns). This decrease in muscle area was caused by a reduction in the muscle fiber cross-sectional area. Indeed, the muscle fiber size was significantly 30% lower in the dexamethasone-treated than in the *ad libitum* animals (dexamethasone: 1759±131 n=5 vs. *ad libitum*: 2517±93 μm^2, P <0.001, n=6). Food restriction caused also a significant reduction by about 19% in muscle fiber size (pair-fed: 2049±111 vs. *ad libitum*: 2517±93 μm^2, P <0.01, n=6). IGF-I gene transfection increased average muscle fiber cross-sectional area by about 22% in dexamethasone-injected rats (pM1-hIGF-I: 2269±129 vs. pM1: 1759±131 μm^2, P <0.05, n=5) and by about 9% in pair-fed rats (pM1-hIGF-I: 2250±183 vs. pM1: 2049±111 μm^2, P <0.05, n=6). In parallel, the average TA muscle cross-sectional area tended to increase when IGF-I was overexpressed in the muscle of dexamethasone-injected rats (pM1-hIGF-I: 35.5±1.2 vs. pM1: 31.7±1.7 mm^2, n=5, ns) (Fig.4b and 4c).

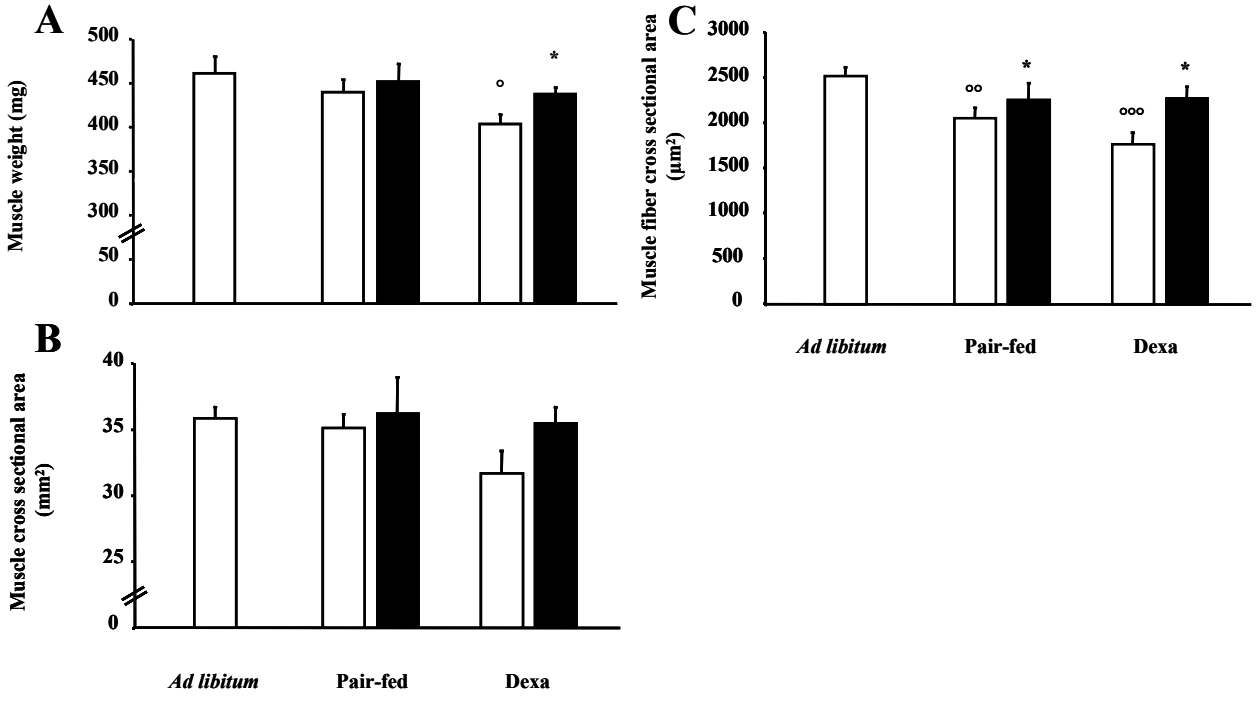

Figure 4. *IGF-I gene electrotransfer prevents muscle atrophy caused by dexamethasone treatment*. Tibial anterior muscles were injected and electroporated with a total of 100 µg of pM1 plasmid at a concentration of 1 µg/µl (white box). In pair-fed and dexamethasone treated-rats, contralateral tibial anterior muscles were injected and electroporated with pM1-hIGF-I plasmid at a concentration of 1µg/µl (black box). Muscle mass (A), total muscle cross-sectional area (B) and mean fiber cross-sectional area (C) were determined 10 days after DNA injection and electroporation. Results are expressed as mean ± SEM. * P <0.05 vs. contralateral muscle. ° P <0.05, °° P <0.01, °°° P <0.001 vs. muscle of *ad libitum* animals.

The increase in the average muscle fiber cross-sectional area induced by IGF-I transfection was confirmed by the analysis of fiber size distribution showing that the proportion of large fibers was significantly greater in the IGF-I transfected muscle than the pM1 control one in the pair-fed group (P <0.001) (Fig.5b) and even more markedly in the dexamethasone group (P <0.001) (Fig.5c). In agreement with the results obtained for the muscle weight, IGF-I overexpression prevented the catabolic effects of the dexamethasone on muscle fiber cross-sectional area.

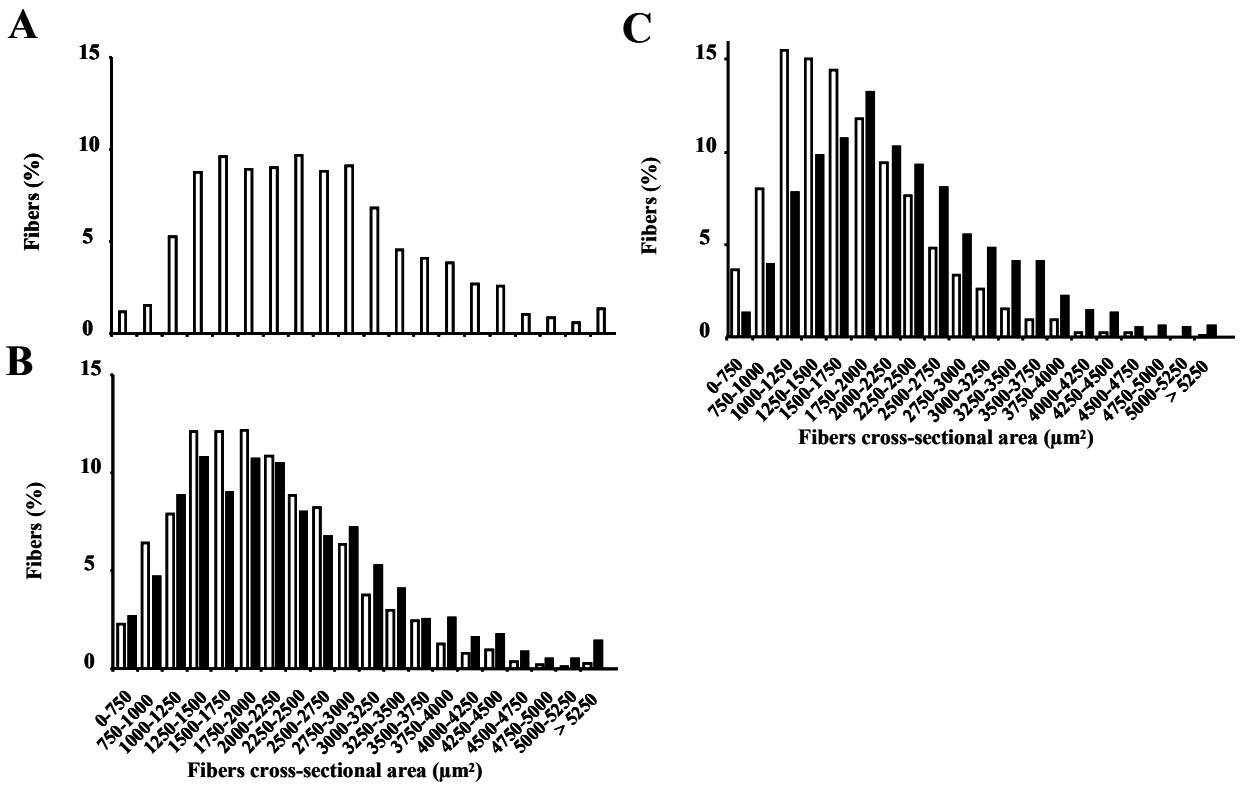

Figure 5. *IGF-I gene electrotransfer prevents decrease of fiber cross-sectional area*. The proportion of large fibers in the pair-fed (B) and dexamethasone groups (C) was larger in the IGF-I DNA injected TA muscle (black box) than in the controlateral pM1 DNA injected TA muscle (white box). Statistical analysis was performed using χ^2 Pearson Test. *** P <0.001 for both B and C.

Myofibrillar and soluble muscle protein concentration

The myofibrillar and the soluble muscle protein concentrations were not significantly different between all the groups considered in this work. As observed in other muscle atrophy models (41), the muscle atrophy caused by glucocorticoids is characterized by a reduction in muscle mass without any reduction in myofibrillar protein concentration (mg/g muscle). Furthermore, the absence of any decrease in muscle protein concentrations in the pM1-hIGF-I groups supports a protein gain and not a water gain (Table 3).

	Ad libitum (n=6)	**Pair-fed (n=6)**		**Dexamethasone (n=6)**	
	pM1	**pM1**	**pM1-hIGF-I**	**pM1**	**pM1-hIGF-I**
Soluble fraction (mg/g of muscle)	**62±3**	**62±1**	**59±2**	**60±1**	**60±3**
Myofibrillar fraction (mg/g of muscle)	**75±3**	**80±4**	**78±2**	**83±3**	**80±4**

Table 3. *Myofibrillar and soluble muscle protein concentrations in the TA muscle of ad libitum, pair-fed and dexamethasone-treated rats whose one TA muscle was injected and electroporated with pM1-hIGF-I and the other with pM1*. Myofibrillar and soluble muscle protein concentrations were determined 10 days after DNA injection and electroporation as described in Materials and Methods. TA muscles were injected with 10 µl of pM1 plasmid solution (1µg/µl) or with 10 µl of pM1-hIGF-I plasmid solution (1µg/µl) in 10 injection sites (total volume=100 µl) and electroporated. Data are expressed as mean ± SEM.

Discussion

Our observations show that localized overexpression of IGF-I by gene electrotransfer prevents muscle atrophy in glucocorticoid-treated rats. In this work, high rate of fibers transfection and gene expression was reached by combining multiple injection sites of DNA with electroporation. Human IGF-I gene electrotransfer using this optimised protocol resulted in increased muscle IGF-I mRNA and protein levels associated with the prevention of loss of skeletal muscle mass induced by dexamethasone.

Electrotransfer of naked DNA is a powerful tool for *in vivo* muscle transfection and a promising avenue for treatment of muscle disorders (42). However, the migration of injected DNA into muscle is very limited (43) and, therefore, distribution over the whole muscle often poor and non-homogenous. The number of transduced muscle fibers after DNA electroporation has been reported to be around 10% (44). Our observations show that injection of the β-galactosidase reporter plasmid at multiple sites in the tibialis anterior muscle followed by electroporation with square wave pulses delivered by plate electrodes increased by several orders of magnitude the reporter gene expression and the number of transfected fibers together with reduction of the inter-individual variability (35, 36, 45. In our case, the number of transfected fibers is an important issue given the fact that IGF-I exerts its anabolic actions toward muscle essentially in an autocrine and paracrine fashion (46). So a marked but restricted expression of IGF-I might not be sufficient to reach a large number of cells and stimulate whole muscle development. With our injection procedure and electric parameters, the average number of β-galactosidase positive cells (37%) obtained was higher than with a single injection of naked DNA (10-20%) (34, 36, 47) and equivalent with multiple injection sites (45). Recently, Dona *et al.* obtained by gene electroporation a percentage of transfected muscle cells as high as 80% (48). However, this high percentage was obtained with an invasive method susceptible to heighten the inflammatory response due to the electroporation. According to our results, the number of transfected cells achieved with our protocol seems largely sufficient to induce autocrine and paracrine effects of IGF-I. The number of transfected fibers is probably less critical in the case of secreted factors with an endocrine action such as erythropoietin (49). Because the tibialis anterior muscle is almost exclusively composed with type IIb/d fibers (96%) (24), β-galactosidase positive cells were for most of them type IIb/d fibers. While electroporation decreased inter-individual variability

(35), our data indicate that combination of electroporation with multiples injections was able to further reduce this variability.

Glucocorticoid treatment caused a 13% decrease in tibialis anterior muscle weight. Although this decrease was smaller than that reported by previous studies (15-20%) (10, 11, 50), the difference can be easily explained by the low dose used (0.1 vs. 80 even 5 mg/kg/d) , (10; 50) and also by the short period of treatment (7 days vs. 28 days) (11) in our study. This decrease in muscle mass resulted from a decrease in mean muscle fiber cross-sectional area by 30%, a value comparable with the results of Kanda *et al.* (-26%) (5) obtained on the EDL and soleus muscle, and of Fournier *et al.* (-26%) (11) obtained on diaphragm muscle. We did not investigate the effects of glucocorticoids on muscle fiber phenotype, because the tibialis anterior muscle is almost exclusively composed of type II fibers, a fiber type considered to be more sensitive to the catabolic effects of glucocorticoids than the type I (25, 26). As the modest food restriction associated with glucocorticoids treatment was sufficient to induce muscle atrophy, the catabolic effects of glucocorticoids in our model partially result from reduced food intake (20%).

Muscle atrophy caused by dexamethasone was associated with a decrease in the IGF-I mRNA and IGF-I peptide muscle content, without any decrease in serum IGF-I concentrations. Nevertheless, some studies have reported a decrease in circulating IGF-I concentrations after glucocorticoid administration (10, 51). This decrease was however only observed with large dose of glucocorticoids. Indeed, while a high dose of glucocorticoids (80 mg/kg) markedly decreases serum IGF-I (68%) (10), a lower dose (0.3 mg/kg) decreases serum IGF-I only slightly (10%) (51). Compared to these results, the even lower dose of glucocorticoids used in our study (0.1 mg/kg/d) may explain the absence of significant changes in serum IGF-I concentrations. Furthermore, the reduction in serum IGF-I levels observed in the previous studies might be also related to the major reduction in food intake observed in animals treated with high doses of glucocorticoids (52, 53). Indeed, a dose of 80 mg/kg/d of glucocorticoids decreased food intake by about 90% (10), whereas this reduction was only 20% in our study. Finally, it is also possible that high doses of glucocorticoids may inhibit liver IGF-I production independently of the decrease in food intake (51). Altogether these results suggest that the fall in circulating IGF-I levels is not mandatory for glucocorticoids to cause muscle atrophy and indicate therefore that the reduction of the IGF-I muscle content plays a crucial role in the loss of skeletal muscle weight induced by glucocorticoid administration.

The main observation of our study is that overexpression of IGF-I in tibialis anterior muscle partially prevents muscle atrophy caused by glucocorticoid injections. The increase in muscle

mass induced by IGF-I overexpression resulted mainly from increase in the muscle fiber cross-sectional area. This increase in tibialis anterior muscle fiber cross-sectional area (+22%) was similar to that found in glucocorticoid-treated animals receiving systemic IGF-I (+15% for the EDL and soleus muscle cross-sectional area) (5). In this study, as in our work, IGF-I did not completely prevent muscle atrophy induced by glucocorticoids. Only Fournier *et al*. totally prevented muscle atrophy caused by glucocorticoids by systemic administration of IGF-I in hamsters (11).

Interestingly, the muscle anticatabolic effects of electrotransfered IGF-I gene have been recently reported in a hind limb suspension muscle atrophy model (44). In this model, Alzghoul *et al.* showed that IGF-I overexpression increases gastrocnemius/soleus muscle fiber cross-sectional area by about 36% after 7 days of hind limb suspension compared to the gain of 29% observed after 7 days of glucocorticoid treatment in our work. However, in contrast to our study which assessed the whole muscle, this study has only considered the transfected fibers and the fibers immediately adjacent to the transfected ones to assess the hypertrophic effect of overexpressed IGF-I (44).

Our results strongly suggest that the anticatabolic action of IGF-I gene transfer results from a complete restoration of IGF-I muscle content in the tibialis anterior muscle of dexamethasone-treated rats. The doubling of IGF-I muscle content obtained with our electrotransfer protocol is close to the increase in IGF-I observed in mIGF-I transgenic mice (20), but lower than the 5-fold increase reached by local infusion of the peptide (54). Only one group have measured the muscle IGF-I content reached after muscle IGF-I gene transfer in a regeneration animal model and showed a modest increase of 30-40% only after two weeks (55). The results obtained with our local administration of IGF-I contrast with those of Fournier *et al.* (2003) showing that the systemic administration of IGF-I to glucocorticoid-treated animals completely prevents the muscle atrophy without any restoration of the IGF-I levels nor in the muscle neither in the circulation (11).

Changes in muscle IGF-I concentrations were reflected by parallel changes in human IGF-I mRNA levels. Previous observations showed *in vitro* a down-regulation of IGF-I expression by exogenous IGF-I added to muscle cells in culture (56). However, our observation shows that local increased IGF-I was not associated with a dow-regulation of rat IGF-I gene expression. The absence of a negative feedback of IGF-I on its own expression have been already reported *in vivo* in mIGF-I transgenic mice (23).

Some hypotheses can be raised to explain the muscle anticatabolic effect of IGF-I in glucocorticoid-treated rats. First, IGF-I could have inhibited proteolysis induced by

glucocorticoids. Indeed, in *vivo* as *in vitro* IGF-I inhibits total (57-59) and myofibrillar protein (5, 6) breakdown induced by glucocorticoids. This anticatabolic action of IGF-I could be mediated by the inhibition of the ubitiquin-proteasome-dependent pathway induced by glucocorticoids. Indeed, IGF-I is known to down-regulate glucocorticoid-induced mRNA for several components of the ubitiquin-proteasome-dependent pathway such as Ubiquitin, Ubiquitin-conjugated enzymes (E2) (50, 60) and especially two ubiquitin ligases, Mafbx/Atrogin-1 and Murf1 (59, 61) crucial in muscle atrophy. IGF-I can also block the lysosomal proteolytic pathway by inhibiting the glucocorticoid-induced cathepsin L (62) and B (58). A second hypothesis is that IGF-I could have increased the protein synthesis inhibited by glucocorticoids. However, the IGF-I anticatabolic action seems unlikely to be due to an increase in protein synthesis because stimulation of protein synthesis by IGF-I seems blunted in presence of glucocorticoids (4)

In conclusion, we have demonstrated that muscle IGF-I gene electrotransfer prevents muscle atrophy caused by glucocorticoids. Our results suggest that the fall in IGF-I muscle content induced by glucocorticoids plays a critical role in the loss of muscle mass observed in this model. Since circulating IGF-I levels are not reduced by glucocorticoids, these observations also suggest that local IGF-I levels play a more critical role in the regulation of muscle mass than endocrine IGF-I. Given that glucocorticoids play a major role in muscle atrophy, our data may be transposable in most catabolic models including sepsis and immobilization where glucocorticoids are induced. The preservation of muscle mass by IGF-I does not allow to assert that muscle function was improved in parallel. Nevertheless, results obtained in IGF-I transgenic mice indicate that the muscle function was preserved in parallel to the muscle mass (19). Preventing decrease of IGF-I muscle by local administration of IGF-I genes may provide a clinically relevant avenue for the preservation, attenuation or reversal of disease-related muscle loss.

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3. Role of Akt/GSK-3β/β-catenin transduction pathway in the muscle anti-atrophy action of Insulin-like growth factor (IGF)-I in glucocorticoid-treated rats

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Abstract

Decrease of muscle Insulin-like Growth Factor (IGF)-I plays a critical role in muscle atrophy caused by glucocorticoids (GC), as IGF-I gene electrotransfer prevents muscle atrophy caused by GC. The goal of the present study was to identify the intracellular mediators responsible for the IGF-I anti-atrophic action in GC-induced muscle atrophy. We first assessed the IGF-I transduction pathway alterations caused by GC administration and their reversibility by local IGF-I overexpression performed by electrotransfer. Muscle atrophy induced by dexamethasone (dexa) administration occurred with a decrease in Akt (-53%, P <0.01) phosphorylation together with a decrease in β-catenin protein levels (-40%, P <0.001). Prevention of atrophy by IGF-I was associated with restoration of Akt phosphorylation and β-catenin levels. We then investigated whether muscle overexpression of these intracellular mediators could mimic the IGF-I anti-atrophic effects. Overexpression of a constitutively active form of Akt induced a marked fiber hypertrophy in dexa-treated animals (+175% of cross sectional area [CSA]; P <0.001), and prevented dexa-induced atrophy. This hypertrophy was associated with an increase in phosphorylated GSK-3β (+17%, P <0.05) and in β-catenin content (+35%, P <0.05). Furthermore, overexpression of a dominant negative GSK-3β or a stable form of β-catenin increased fiber CSA by respectively 23% (P <0.001) and 29% (P <0.001) in dexa-treated rats, preventing completely the atrophic effect of GC. In conclusion, this work indicates that Akt, GSK-3β and β-catenin probably contribute together to the IGF-I anti-atrophic effect in GC-induced muscle atrophy.

Key words: Insulin-like growth factor-I, glucocorticoids, skeletal muscle atrophy

Introduction

Many pathological states characterized by muscle atrophy (sepsis, cachexia, disuse atrophy, fasting, metabolic acidosis, severe insulinopenia…) are associated with an increase in circulating glucocorticoids (GC) levels (1), suggesting that they could trigger the muscle atrophy observed in these situations. Indeed, adrenalectomy or treatment with an antagonist of GC receptor (RU-486) attenuate, and in some cases, abolish muscle atrophy (1, 2). GC-induced muscle atrophy results from decreased protein synthesis and increased protein degradation rates (3-6). The inhibitory effect of GC on protein synthesis is thought to result from the inhibition of the p70 ribosomal S6 protein kinase (p70S6K) involved in the regulation of protein synthesis (7-10). On the other hand, the stimulatory effect of GC on muscle proteolysis results from the activation of two major cellular proteolytic systems (2), namely the ubiquitin proteasome system (UPS) (11-13) and the lysosomal system (cathepsins) (14-16).

Several observations indicate that a decrease in the production of IGF-I (17), a growth factor which stimulates the development of muscle mass by increasing protein synthesis and myogenesis while decreasing proteolysis and apoptosis (18, 19), could contribute to GC-induced muscle atrophy. Although the down-regulation of IGF-I gene expression by GC has been previously reported (20), the key role of decreased muscle IGF-I in GC-induced muscle atrophy has been only recently demonstrated. First, by activating the phosphatidylinositol-3-kinase (PI3K)/Akt pathway, IGF-I down-regulates the different proteolytic systems (lysosomal and proteasomal) stimulated by GC (16, 21-23). Second, IGF-I inhibits GC-induced proteolysis in myotubes through PI3K/Akt /GSK-3β and PI3K/Akt/mTOR-dependent mechanisms (24). Third, systemic administration (25-28) and local overexpression of IGF-I into skeletal muscle, as reported by our group (29), prevent GC-induced muscle atrophy and cause muscle fiber hypertrophy (30). Taken together, these results indicate that IGF-I has a dominant effect, overriding GC to turn off catabolism. However, the mechanisms by which IGF-I inhibits *in vivo* muscle atrophy induced by GC are still unclear.

As a direct extension of our previous work, the present study was undertaken to characterize the intracellular signaling pathways responsible for the IGF-I anti-atrophic action in GC-induced muscle atrophy. Although *in vitro* observations had already assessed the role of Akt, GSK3β, p70S6k in GC-myotube atrophy, we decided to determine whether such factors were also involved *in vivo* and to evaluate the potential role of β-catenin. Then we determined whether local IGF-I overexpression could prevent these alterations. Finally, we tested whether

muscle overexpression of these intracellular mediators themselves could mimic the IGF-I anti-atrophic effects in GC-induced muscle atrophy.

Materials and methods

Expression plasmids and DNA preparation

pM1-hIGF-I and pM1-ΔNβ-catenin plasmids were constructed by inserting respectively the IGF-I cDNA (gift from Dr P Steenbergh, University of Utrecht, The Netherlands) and the ΔNβ-catenin cDNA (gift from Dr T Force, Thomas Jefferson University of Philadelphia, USA) into the pM1 Expression Vector (Roche Molecular Biochemicals). ΔNβ-catenin cDNA codes for a vesicular stomatitis virus-glycoprotein (VSV-G) tagged β-catenin where the N-terminal 134 amino acids, a region that contains the GSK-3β phosphorylation sites, were deleted (31). pCMV5-(m/p)Akt plasmid (gift from Dr D Alessi, University of Dundee, Scotland) codes for hemagglutinin (HA) tagged constitutively active (ca) Akt form where a Lck myristoylation/palmitylation signal was added to the NH2-terminal (32). pcDNA3-dnGSK-3β plasmid (gift from Dr J Woodgett, Samuel Lunenfeld Research Institute, Toronto, Canada) codes for a HA tagged catalytic inactive GSK-3β where lysine residue at position 85 of the ATP binding site was mutated to alanine (33). Empty pCMV5 (gift from Dr D Alessi, University of Dundee, Scotland) and pM1 (Roche Molecular Biochemicals) were used as control plasmids. Plasmids were amplified in E.Coli top 10 F' (Invitrogen) and purified with an EndoFree Plasmid Giga Kit (QIAGEN). Plasmids were stocked at -80°C. The day before injection, 100 µg of plasmid was lyophilized and resuspended in 100 µl of NaCl 0.9% solution.

General *in vivo* experimental design

Animals

Six-week-old male Wistar rats (180-220 g) provided by Janvier Breeding (Le Genest St-Isle, France) were used. The animals were housed under controlled conditions of lighting (12-h light, 12-h dark cycle) and temperature (22 ± 2°C). The experiments were conducted and the animals were cared for in accordance with the directives of the institutional animal care and use committee of University of Louvain.

Dexamethasone administration

Rats were divided in three groups: ad libitum, pair-fed and dexamethasone. The ad libitum and pair-fed groups received daily subcutaneous injections of saline solution (vehicle, 0.9% NaCl). The glucocorticoid-treated group received daily subcutaneous injections of saline

solution of dexamethasone (10 µg/100 g of BW; Aacidexam®, Organon). Since this dosage is able in rats to mimic the muscle atrophy observed in catabolic conditions characterized by an increase of endogenous GC, this dose must be considered at the upper limit of the physiological range. Because dexamethasone treatment is known to decrease energy intake, the pair-fed group received the same amount of energy as that consumed by the dexamethasone-treated group during the previous day (-20%; energy intake (kcal/day): 82±2 kcal in ad libitum rats vs. 66±2 kcal in dexamethasone-treated rats, P <0.001). The *ad libitum* and dexamethasone-treated animals were allowed to free access to chow and water. Animals were weighed and food intake was measured every day. After seven days of treatment, animals were sacrificed and tibialis anterior (TA) muscles were removed.

DNA electrotransfer

Three days before starting dexamethasone treatment, each rat was anaesthetized with a mixture of 75 mg/kg ketamine (Ketalar®, Pfizer) and 15 mg/kg xylazine hydrochloride (Rompun®, Bayer) administered by intraperitoneal injection. Plasmid solutions (1 µg/µl) were injected in 10 different sites (total volume per muscle = 100 µl) in each TA muscle and then muscles were electroporated using the electroporation conditions previously described (29). To assess the IGF-I transduction pathway alterations caused by dexamethasone and their reversibility by local IGF-I overexpression, the two TA muscles of the *ad libitum* rats were injected with the pM1 plasmid. In pair-fed and dexamethasone-treated animals, one TA muscle was injected with the pM1-hIGF-I plasmid and the contralateral TA muscle with the pM1 plasmid. Previous experiments have shown the absence of exogenous IGF-I overexpression in contralateral muscle together with the absence of increased circulating IGF-I indicating that IGF-I overexpression occurs only in the TA muscle electroporated with the pM1-hIGF-I plasmid (34). To assess the anti-catabolic effect of caAkt transfection in dexamethasone-treated rats, all animals were injected with the pCMV5-(m/p)Akt plasmid in one TA muscle and with the pCMV5 plasmid in the contralateral TA muscle. To assess the anti-catabolic effect of dnGSK-3β and ΔNβ-catenin DNA transfection in dexamethasone-treated rats, the two TA muscles of all animals were injected either with the pcDNA3-dnGSK-3β plasmid or with the pM1-ΔNβ-catenin plasmid. As shown in a previous work (29), dexamethasone treatment did not alter the CMV promoter activity driven exogenous expression of IGF-I. Indeed, the levels of exogenous hIGF-I mRNA achieved by pM1-hIGF-I overexpression were similar in GC-treated animals and in pair-fed animals. Since signaling

molecules expressed in this work are under control of the same promoter (CMV), expression of all these overexpressed molecules is unlikely to be altered by dexamethasone treatment.

Western blots and antibodies

Briefly, 100 mg of whole TA muscle, previously pestled in liquid nitrogen, were homogenized with Ultraturrax (IKA-Labortechnik) in 1 ml ice-cold lysis buffer I (50 mM Tries/Hl [pH7.5], 1 mM EDTA, 1mM EGTA, 0.5% NP40, 1 mM phenylmethylsulfonylfluoride, 10 µg/mL aprotinin, 10 µg/ml leupeptin, 10 mM β-glycerophosphate, 1 mM K_2PO_4, 1 mM vanadate, 50 mM NaF, 10 mM Nappi) or in 1 ml ice-cold lysis buffer II (lysis buffer I with 1% NP40 and 150 mM NaCl). Lysis buffer I was used for the assessment of IGF-I transduction pathway alterations caused by dexamethasone administration and their reversibility by local IGF-I overexpression. Lysis buffer II was used in all other experiments. Homogenates were then centrifuged 10 min. at 10 000 rpm (Sorvall SS-34 rotor) and the supernatant muscle protein content was determined by using Bradford's protein assay (Bio-Rad). Equal amounts of proteins were resolved by a SDS-polyacrylamide gel 10% electrophoresis and transferred to polyvinylidene difluoride membranes (Immobilon P/PVDF, Millipore). Membranes were probed with anti-phospho-Akt (Ser 473, 1:1000; Cell Signaling), anti-Akt (1:1000, Upstate), anti-phospho-GSK-3β (Ser 9, 1:3000; Cell Signaling), anti-GSK-3β (1:10 000, Cell Signaling), anti-β-catenin (1:30 000, Sigma), anti-phospho-p70S6K (Thr 389, 1:1000; Santa Cruz Biotechnology), anti-p70S6K (1:1000, Santa Cruz Biotechnology), anti-HA (1:1000, Bethyl Laboratories), and anti-VSV-G (1:5000, Sigma) followed by horseradish peroxidase-coupled secondary antibody, anti-mouse or anti-rabbit (1:40 000, Amersham Biosciences), and developed by a chemiluminescence-based detection system (ECL Plus, Amersham Biosciences). Developed film was scanned (Image Scanner, Amersham Pharmacia Biotech) and analyzed using ImageMaster TotalLab software v2.0 (Amersham Pharmacia Biotech). Phospho data blots were normalized for the signal with the cognate pan antibodies.

Immunohistochemistry

For the evaluation of the anti-catabolic effect of caAkt transfection, transversal slices of 0.5 cm thickness made in the middle belly of the muscle were embedded in O.C.T. compound (Tissue-Tek) and frozen in precooled isopentane. Cryostat serial sections (10 µm thick) were cut and mounted onto glass slides (Superfrost®, Menzel-Glaser). The sections were fixed 10

min. with buffered formol. For immunohistochemistry, sections were incubated at room temperature (RT) with a solution of bovine serum albumin (BSA) 5% for 30 min and incubated with a rabbit polyclonal anti-HA antibody (1:400, Bethyl Laboratories) in PBS containing 1% BSA overnight (ON). Primary antibody was detected by applying for 1 hour at RT a second antibody, which was a goat anti-rabbit conjugated to peroxidase-labeled polymer (En Vision, Dako). Peroxidase activity was revealed with diaminobenzidine (DAB) substrate (Liquid DAB+ Substrate chromogen System, Dako), which produces a brown stain. Sections were mounted in Depex (VWR). For the evaluation of the anti-catabolic effect of dnGSK-3β and ΔNβ-catenin DNA transfection, transversal slices of 0.5 cm thickness made in the middle belly of the muscle were fixed with buffered formol for 48 to 72 hours and embedded in paraffin. For immunohistochemistry, sections were deparaffinized and pretreated in a microwave oven in Tris–citrate buffer (pH 6.5) for one cycle of 3 min at 750 W and three cycles of 3.5 min at 350 W. Thereafter, sections were blocked in PBS-BSA (5%) containing normal goat serum (4%) for 30 min at RT and incubated with a rabbit polyclonal anti-HA (1:400, Bethyl Laboratories) for 1h or with a rabbit polyclonal anti-VSV-G (1:800, Sigma) for 2h. Primary antibodies were detected by applying for 30 min. at RT a biotinylated second antibody (Vector Laboratories) followed by applying for 30 min. at RT an avidin/biotinylated peroxidase complex (Vectastain®ABC kit Peroxydase Standard, Vector Laboratories) . Peroxidase activity was revealed with diaminobenzidine (DAB) substrate (Chemicon), which produces a brown stain. Sections were counterstained with Mayer's hematoxylin, rinsed, and mounted in Faramount (Dako). Fiber cross-sectional area (CSA) were measured with a microscope (Leitz Wetzlar) coupled to an image analyzer system (Kontron image analysis division MOP-Videoplan, Eching, Germany). To evaluate muscle fiber CSA, all the positive muscle fibers were counted. Untransfected (UT) muscle fibers surrounding positive muscles fibers were considered as control (Ctrl). The use of insert-less plasmid transfected fibers as control would not affect the results, as there is no difference between CSA of surrounding UT fibers and CSA of muscle fibers transfected with an insert-less plasmid.

Statistical analysis

Results are presented as means ± standard error of the mean (SEM). Statistical analyses were performed using a Student *t*-test or a one-way ANOVA followed by a Newman-Keuls Multiple Comparison Test to compare muscles from different animals undergoing different experimental conditions. A paired *t*-Test was used to compare muscles undergoing different

experimental conditions within the same animal. Fiber cross-sectional area distribution statistical analysis was performed using χ^2 Pearson test. Statistical significance was set at $P < 0.05$.

Results

Inhibition of Akt/p70S6K and GSK-3β/β-catenin signaling pathways by dexamethasone administration is reversed by local IGF-I overexpression.

To determine whether local muscle IGF-I decrease observed in dexamethasone-treated rats (29) is associated with the inhibition of IGF-I signaling pathways, we studied the phosphorylation state of several key intracellular mediators of IGF-I action. Dexamethasone injection decreased Akt phosphorylation by about 53 % (dexa pM1 [n=6]: 47±8% vs. *ad libitum* pM1 [n=5]: 100±15%, P <0.01) (Fig.1A). Interestingly, transfection of pM1-IGF-I plasmid in the muscle of dexamethasone-treated rats restored Akt phosphorylation to the levels observed in *ad libitum* rats (dexa pM1-hIGF-I [n=6]: 86±15% vs. *ad libitum* pM1 [n=5]: 100±15%, ns). Changes in Akt phosphorylation occurred without significant modification in Akt protein content (insert Fig.1A). Furthermore, the Akt inactivation caused by dexamethasone was associated with decreased phosphorylation of p70S6K (dexa pM1 [n=6]: 33±7% vs. *ad libitum* pM1 [n=5]: 100±20%, P <0.01) (Fig.1.B) without any changes of GSK-3β phosphorylation (dexa pM1 [n=6]: 101±16% vs. *ad libitum* pM1 [n=5]: 100±13%, ns) (Fig.1C), two downstream targets of Akt. As observed for Akt1, p70S6K (dexa pM1-hIGF-I [n=6]: 79±20% vs. *ad libitum* pM1 [n=5]: 100±20%, ns) phosphorylation was restored by IGF-I overexpression in dexamethasone-treated rats. Changes in p70S6K phosphorylation could not be explained by changes in p70S6K protein content (insert Fig 1.B). In contrast, we observed in the muscle of dexamethasone-treated rats a significant decrease in GSK-3β protein content (dexa pM1 [n=6]: 72±5% vs. *ad libitum* pM1 [n=5]: 100±12%, P <0.05), which might explain the absence of changes of the phospho-GSK-3β/total-GSK-3β ratio (insert Fig 1.C). We also investigated whether β-catenin protein, a downstream target of GSK-3β is down regulated by dexamethasone. Dexamethasone decreased by about 40% β-catenin protein levels in the TA muscle (dexa pM1 [n=6]: 60±6% vs. *ad libitum* pM1 [n=5]: 100±4%, P <0.001). IGF-I overexpression in the muscle of dexamethasone-treated rats increased the β-catenin protein levels which remained however significantly lower than in the muscle of *ad libitum* animals (dexa pM1 hIGF-I [n=6]: 74±7% vs. *ad libitum* pM1 [n=5]: 100±4%, P <0.05) (Fig.1D). Taken together, the results indicate that Akt, p70S6K, GSK-3β and β-catenin are potentially involved in the IGF-I anti-catabolic actions in dexamethasone-induced muscle atrophy.

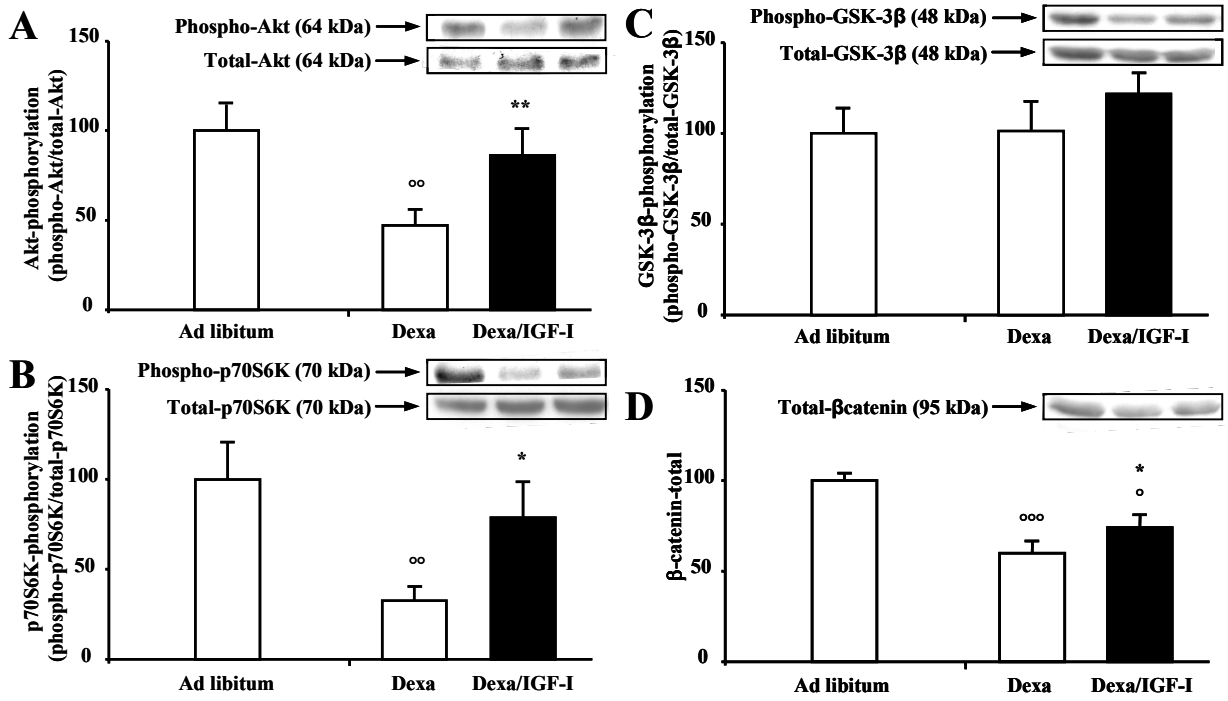

Figure 1. *Decrease of Akt and p70S6K phosphorylation and β-catenin levels by dexamethasone administration is reversed by local IGF-I overexpression.* Tibial anterior muscles were injected and electroporated with a total of 100 µg of pM1 plasmid at a concentration of 1 µg/µl (white column) while contralateral tibial anterior muscles were injected and electroporated with 100 µg of pM1-hIGF-I plasmid at a concentration of 1 µg/µl (black column). Insert:: representative Western blot of phosphorylation forms (top) and total forms (bottom). Seven days after dexamethasone administration, Akt phosphorylation (A), p70S6K phosphorylation (B) and β-catenin (D) were decreased without any changes in GSK3-β phosphorylation. IGF-I overexpression prevented these transduction pathway alterations observed in dexamethasone-treated rats. Results are expressed as mean ± SEM. ° P <0.05, °° P <0.01,°°° P <0.001 vs. *ad libitum*. * P <0.05, ** P <0.01 vs. contralateral muscle.

Constitutively active (ca)Akt muscle overexpression induces a marked fiber hypertrophy and prevents dexamethasone-induced muscle atrophy.

To explore the role of Akt in the anti-catabolic effects of IGF-I, we overexpressed a constitutively active (ca) form of Akt in the muscle of dexamethasone-treated rats. Expression of the caAkt transgene was detected by western blot and immunohistochemistry in TA muscle electroporated with the pCMV5-(m/p)Akt using an antibody raised against HA tag (Fig.2A and Fig.2B). As expected, dexamethasone treatment for seven days decreased by about 20% the cross-sectional area (CSA) of the untransfected (UT) muscle fibers (dexa UT: 1 394±48 [n=6] vs. *ad libitum* UT: 1 738±69 [n=6] μm^2, P <0.001). Muscle fiber size was not affected by food restriction (Fig.2B). As showed by immunohistochemical analysis, caAkt gene transfection induced marked muscle fiber hypertrophy in the three groups of animals (Fig.2B). Indeed, we observed an increase in the muscle fiber CSA by about 176% in dexamethasone-injected rats (pCMV5-(m/p)Akt: 3 846±210 vs. UT: 1 394±48 μm^2, P <0.001,

[n=6]), by about 169% in pair-fed rats (pCMV5-(m/p)Akt: 4 733±160 vs. UT: 1 760±22 µm^2, *P* <0.001, [n=5]) and by about 177% in *ad libitum* rats (pCMV5-(m/p)Akt: 4 813±224 vs. UT: 1 738±69 µm^2, *P* <0.001, [n=6]) (Fig.2B). The increase in the muscle fiber CSA induced by caAkt gene transfection was confirmed by the analysis of fiber size distribution showing a greater proportion of large fibers among the caAkt transfected fibers than among the UT control fibers in the three groups of rats (*P* <0.001) (Fig.2C).

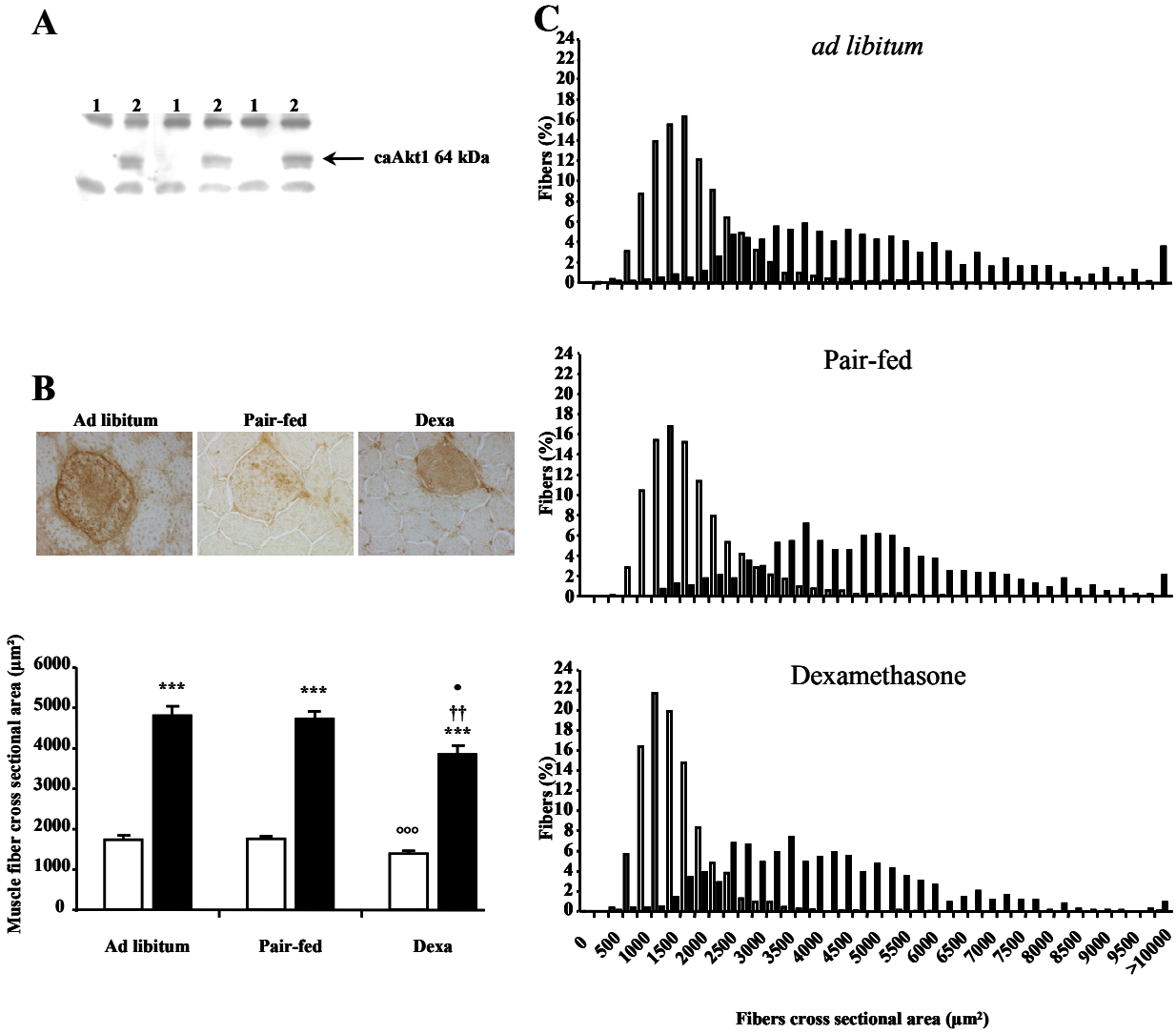

Figure 2. *Constitutively active (ca)Akt muscle overexpression induces a marked fiber hypertrophy and prevents dexamethasone-induced muscle atrophy***.** Overexpression of caAkt was detected by western blot (A) and by immunohistochemistry (B, top) in TA muscles electroporated with pCMV5-(m/p)Akt plasmid. caAkt overexpression induced a marked muscle fiber hypertrophy (black column) compared to surrounding untransfected (UT) muscle fibers (white column)(B, bottom). Results are expressed as mean ± SEM. *** *P* <0.001 vs. UT muscles fibers of the same muscle. °°° P <0.001 vs. *ad libitum* UT muscles fibers. †† *P* <0.01 vs. pair fed caAkt transfected muscles fibers. • *P* <0.05 vs. *ad libitum* caAkt transfected muscles fibers. The proportion of large fibers in the three groups was larger in the caAkt DNA transfected muscle fibers (black column) compared to surrounding untransfected (UT) muscle fibers (white column) (C). Statistical analysis was performed using χ^2 Pearson Test. *** *P* <0.001 for the three groups. A total of 1 991 positive and 8 280 untransfected muscle fibers were analyzed for measurements of fiber size.

To identify the intracellular mediators involved in the prevention of muscle atrophy by caAkt overexpression in dexamethasone-treated rats, we analyzed the phosphorylation state of several key targets of Akt. As observed for IGF-I overexpression, caAkt transfection increased p70S6K phosphorylation by about 130% in the TA muscle of dexamethasone-treated rats (pCMV5-(m/p)Akt: 230±29% vs pCMV5: 100±7%, *P* <0.01 [n=6]) (Fig.3A) without affecting total p70S6K protein levels (insert Fig 3.A). Similarly, caAkt overexpression increased significantly both GSK-3β phosphorylation (pCMV5-(m/p)Akt: 117±30% vs. pCMV5: 100±29%, *P* <0.05 [n=6]) and β-catenin protein levels (pCMV5-(m/p)Akt: 135±13% vs. pCMV5: 100±8%, *P* <0.05 [n=6]) in the TA muscle of dexamethasone-treated rats (Fig.3B and Fig.3C). Total GSK-3β protein levels did not changed (insert Fig 3.B). These results indicate that p70S6K, GSK-3β and β-catenin are potentially implicated in the anti-catabolic action of caAkt overexpression in this model.

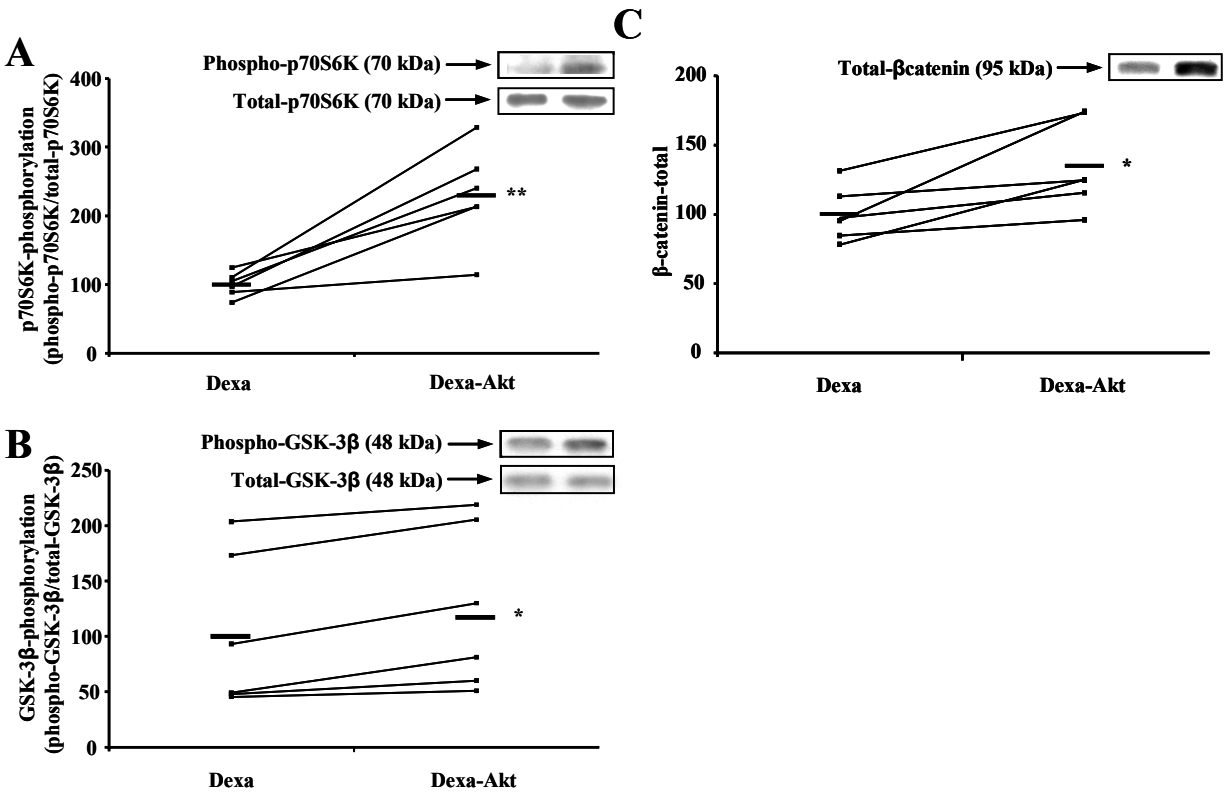

Figure 3. *caAkt overexpression stimulates p70S6K and GSK-3β phosphorylation and β-catenin levels in the muscle of dexamethasone-treated rats.* Prevention of dexamethasone-induced muscle atrophy by caAkt overexpression was associated with a significant increase of phosphorylated p70S6K (A), GSK-3β (B) and total β-catenin levels (C), indicating that these molecules potentially contribute to the hypertrophic effects of Akt overexpression. Insert:: representative Western blot of phosphorylation forms (top) and total forms (bottom).* *P* <0.05, ** *P* <0.01 vs. contralateral muscle.

Dominant negative GSK-3β overexpression induces a modest muscle fiber hypertrophy and prevents dexamethasone-induced muscle atrophy.

To explore the role of GSK-3β in the anti-catabolic effects of Akt, we overexpressed a dominant negative form of GSK-3β, dnGSK-3β, in the muscle of dexamethasone-treated rats. Expression of the dnGSK-3β transgene was detected by western blot and immunohistochemistry in TA muscle electroporated with the pcDNA3-dnGSK-3β using an antibody raised against the tag HA (Fig.4A and Fig.4B). Compared to caAkt overexpression, dnGSK-3β gene transfection induced only a modest fiber muscle hypertrophy in the three groups of animals (Fig.4B). Indeed, we observed an increase of the muscle fiber CSA by about 23% (P <0.001, n=10) in dexamethasone-treated rats, by about 9% in pair-fed rats and in *ad libitum* rats (P <0.01, n=8) (Fig.4B). The increase in the muscle fiber CSA induced by dnGSK-3β gene transfection was confirmed by the analysis of fiber size distribution showing a greater proportion of large fibers among the dnGSK-3β transfected fibers than among the UT control fibers in the three groups (P <0.001) (Fig.4C).

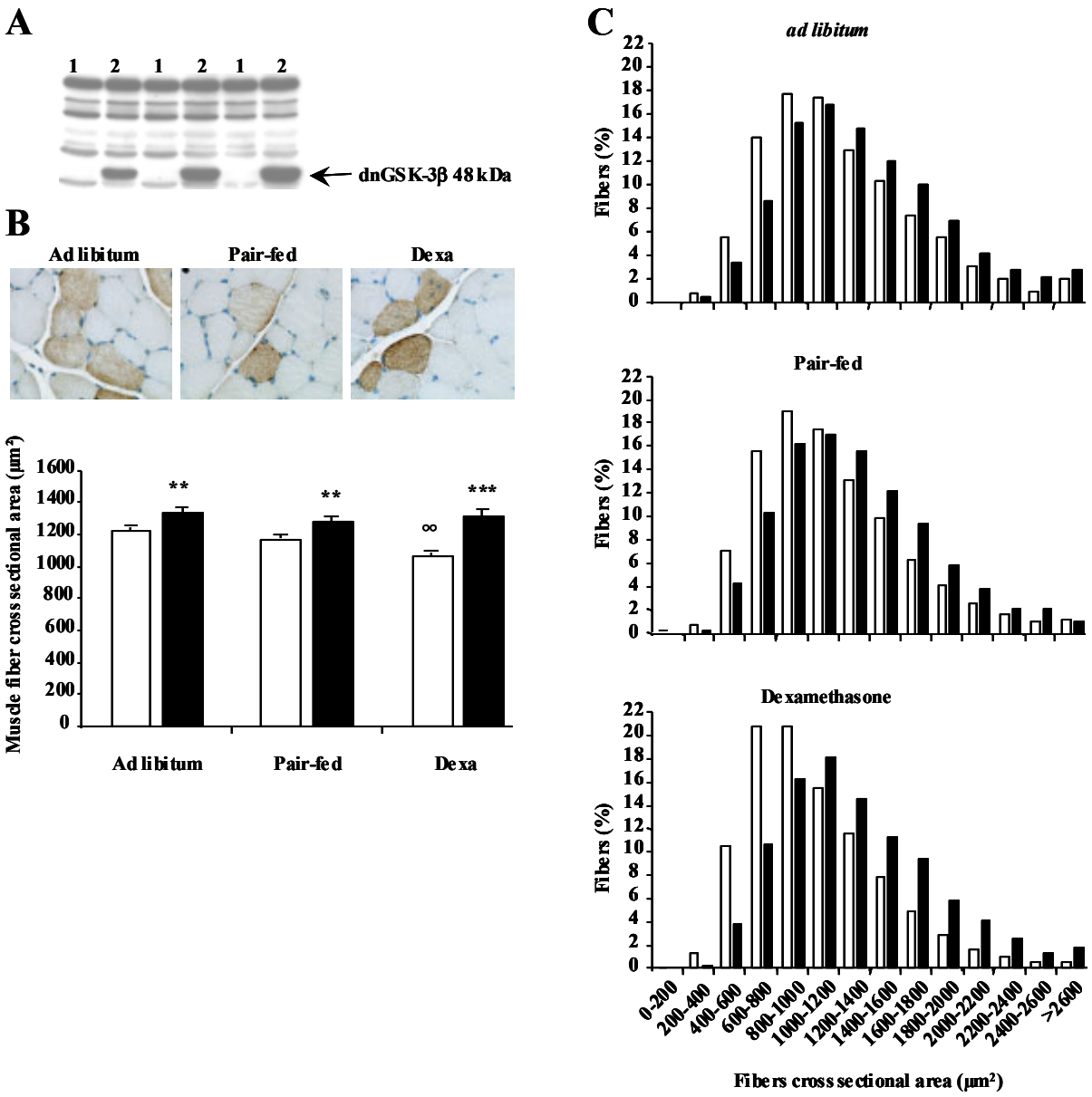

Figure 4. *Dominant negative (dn)GSK-3β overexpression induces a modest muscle fiber hypertrophy and prevents dexamethasone-induced muscle atrophy.* Overexpression of dnGSK-3β was detected by werstern blot (A) and by immunohistochemistry (B, top) in TA muscles electroporated with pcDNA3-dnGSK-3β plasmid. dnGSK-3β overxepression induced a modest muscle fiber hypertrophy (black column) compared to surrounding untransfected (UT) muscle fibers (white column)(B, bottom). Results are expressed as mean ± SEM. ** P <0.01,*** P <0.001 vs. UT muscles fibers of the same muscle. °° P <0.01 vs. *ad libitum* UT muscles fibers. The proportion of large fibers in the three was greater in the dnGSK-3β DNA transfected muscle fibers (black column) compared to surrounding untransfected (UT) muscle fibers (white column)(C). Statistical analysis was performed using χ^2 Pearson Test. *** P <0.001 for the three groups. A total of 6 354 positive and 16 431 untransfected muscle fibers were analyzed for measurements of fiber size.

ΔNβ-catenin overexpression prevents muscle atrophy in dexamethasone-treated rats.

To explore the role of β-catenin in the anti-catabolic effects of Akt, we overexpressed a N-terminal deleted form of β-catenin, the ΔNβ-catenin, in the muscle of dexamethasone-treated rats. Expression of the ΔNβ-catenin transgene was detected by western blot and immunohistochemistry in TA muscle electroporated with the pM1-ΔNβ-catenin using an antibody raised against the VSV-G (Fig.5A and Fig.5B). As dnGSK-3β gene transfection, ΔNβ-catenin overexpression induced a modest increase of muscle fiber CSA by about 29% in the dexamethasone-treated rats (P <0.001, n=11), by about 16% (P <0.001, n=10) in the pair-fed rats and by about 17% (P <0.001, n=10) in *ad libitum* rats (Fig.5B). The increase in the average muscle fiber CSA induced by ΔNβ-catenin gene transfection was confirmed by the analysis of fiber size distribution showing that the proportion of large fibers was significantly greater among the ΔNβ-catenin transfected fibers than among the UT control fibers in the three groups (P <0.001) (Fig.5C).

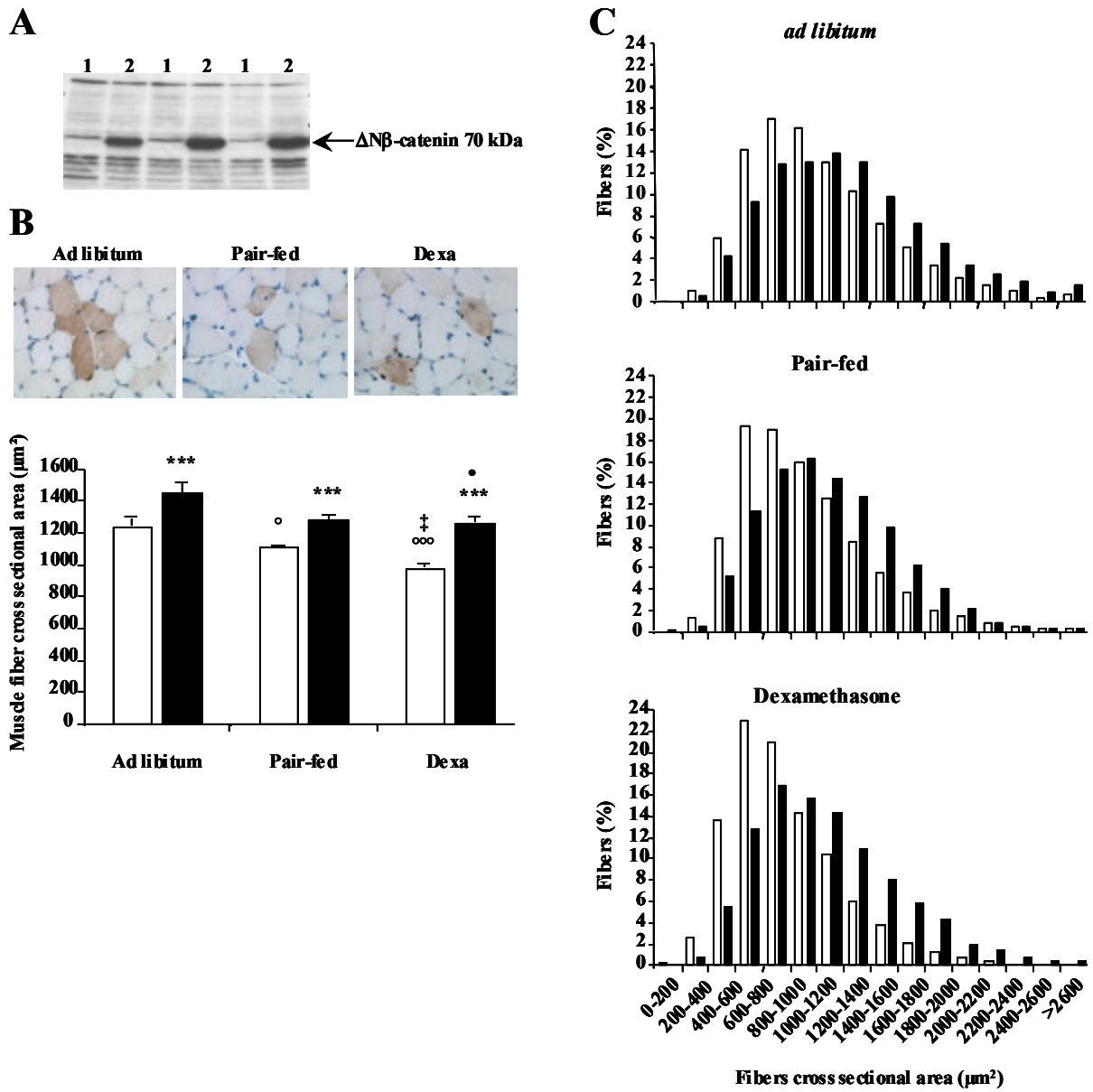

Figure 5. *ΔNβ-catenin overexpression prevents muscle atrophy in dexamethasone-treated rats.* Overexpression of ΔNβ-catenin was detected by werstern blot (A) and by immunohistochemistry (B, top) in TA muscles electroporated with pM1-ΔNβ-catenin plasmid. ΔNβ-catenin overxepression increase muscle fiber CSA (black column) compared to surrounding untransfected (UT) (white column)(B, bottom). Results are expressed as mean ± SEM. *** P <0.001 vs. UT muscles fibers of the same muscle . ° P <0.05, °°° P <0.001 vs. *ad libitum* UT muscles fibers. ‡ P <0.05 vs pair-fed UT muscles fibers. • P <0.05 vs. *ad libitum* ΔNβ-catenin transfected muscles fibers. The proportion of large fibers in the three groups was greater in the ΔNβ-catenin DNA transfected muscle fibers (black column) compared to surrounding untransfected (UT) muscle fibers (white column)(C). Statistical analysis was performed using χ^2 Pearson Test. *** P <0.001 for the three groups. A total of 15 312 positive and 31 759 untransfected muscle fibers were analyzed for measurements of fiber size.

Discussion

The present study demonstrates that muscle atrophy caused by glucocorticoids (GC) is associated with alterations in the Akt/GSK-3β/β-catenin signaling, a transduction pathway crucial for muscle growth. More interestingly, we show that prevention of the GC-induced muscle atrophy by local IGF-I gene overexpression (29) is associated with the correction of these transduction alterations. Furthermore, muscle overexpression of caAkt, dnGSK-3β and ΔNβ-catenin themselves is sufficient to alleviate the catabolic effects of GC and thus mimic the anti-atrophic action of IGF-I in this model. Taken together, our results show, for the first time *in vivo*, the role of the Akt/GSK-3β/β-catenin pathway in the skeletal muscle atrophy caused by GC and its prevention by IGF-I.

It is well established *in vitro* (33) and *in vivo* (35,37-39) that Akt exerts an anabolic action on skeletal muscle. However, the role of Akt in muscle atrophy caused by GC had not yet been investigated. In the present study, we demonstrate *in vivo* that muscle atrophy induced by GC is associated with a decrease in Akt phosphorylation levels. Such decrease in Akt phosphorylation state has been directly correlated to a large decrease in Akt activity (36), suggesting the role of decreased Akt activity in the atrophic effect of GC. Moreover, our data indicate that prevention of muscle atrophy by IGF-I overexpression is associated with the restoration of Akt phosphorylation levels, a result which extends previous *in vitro* observations. Indeed, in L6 myotubes, inhibition by IGF-I of GC-induced protein degradation was associated with increased Akt phosphorylation (24). Here we show for the first time *in vivo* that Akt overexpression overcomes muscle atrophy caused by GC, supporting the role of Akt in the prevention GC-induced muscle atrophy by IGF-I. These results are in line with a recent report in which evidence was found that the PI3K/Akt pathway mediates the anti-atrophic effect of IGF-I in myotubes. The myristoylation of the Akt form overexpressed in our experiment might explain the dramatic degree of hypertrophy observed. Indeed, the myristoylation maintains Akt attached at the cellular membrane in permanence and so suppresses the negative feed back exerted by PTEN and SHIP2, two important phosphatases regulating Akt activity (40). In contrast with its anabolic effect, the anti-atrophic effect of Akt has only been demonstrated in a denervation muscle atrophy model (37). The concept that caAkt overexpression is able to prevent muscle atrophy in another condition such as that induced by glucocorticoids is supported by our data. Indeed, caAkt overexpression in glucocorticoid-treated animals totally overcomed muscle atrophy. The degree of increase of

fiber CSA induced by caAkt overexpression was similar (170%) in GC-treated and ad libitum animals, although the absolute values of CSA achieved were slightly larger in ad libitum animals than in GC-treated animals.

Inhibition of the phosphorylation of p70S6K, a molecule which plays a key role in the protein synthesis machinery, is thought to be one of the mechanisms by which GC cause muscle atrophy (41;42). Our data indicate that the inhibition of p70S6K by GC is not transient but persists (7) with time in the skeletal muscles of animals exposed to GC. Moreover, we show for the first time *in vivo* that prevention of muscle atrophy by IGF-I overexpression in the muscle of GC-treated rats is associated with a restoration of p70S6K phosphorylation to normal levels. These results are in line with a report by Li et al showing that rapamycin, which inhibits the IGF-I induced phosphorylation of p70S6K, blunts partially the antiproteolytic effects of IGF-I observed in GC-treated L6 myotubes (24). Together, these observations support a role for p70S6K phosphorylation in the anti-atrophic action of IGF-I in muscle exposed to GC. Decrease of p70S6K phosphorylation may result from decreased Akt activity caused by GC administration. Indeed, as shown by our data, Akt overexpression in skeletal muscle was sufficient to increase p70S6K phosphorylation in GC-treated animals. Alternatively, decreased phosphorylation of p70S6K in response to GC might result from enhanced transcription of REDD1, a repressor of mTOR, the kinase responsible for p70S6K phosphorylation (43). Although muscle REDD1 expression was dramatically increased after GC, its induction was not prevented by IGF-I overexpression (data not shown). This observation indicates that IGF-I can overcome the inhibitory effect of REDD1. Although p70S6K is mandatory for muscle hypertrophy induced by IGF-I and amino acids (44), overexpression of p70S6K alone into muscle seems however unable to induce muscle hypertrophy (data not shown).

Several reports, especially *in vitro*, have demonstrated the role of GSK-3β in muscle atrophy induced by GC. Indeed, stimulation of protein degradation by GC in myotubes was blocked by pharmacological inhibitors (24;45) and siRNA (36) targeting GSK-3β. These observations suggest therefore that increased GSK-3β activity may contribute also to the catabolic effects of GC *in vivo*. The role of GSK-3β in the anti-atrophic action of IGF-I in myotubes exposed to GC has been also suggested. Indeed, inhibition by IGF-I of GC-induced protein degradation was associated with an increase in GSK-3β phosphorylation (24). Our results are the first to demonstrate *in vivo* the role of GSK-3β in dexamethasone catabolic and IGF-I anti-catabolic effects. Indeed, overexpression of a dominant negative form of GSK-3β prevented

totally muscle atrophy induced by GCs. However, the mechanisms by which GSK-3β inactivation inhibits muscle atrophy caused by GC had not yet been investigated. Evidence has been provided *in vitro* suggesting that GSK-3β inhibition blocks GC-induced up-regulation of Atrogin-1 and MuRF-1 gene expression, two important molecular markers of muscle atrophy in different wasting conditions (45). However, in our conditions, muscle Atrogin-1 mRNA was not increased after GC treatment (data not shown), suggesting that other mechanisms are operational. Considering the targets of GSK-3β, it is possible that GSK-3β inactivation inhibits muscle atrophy by blocking the degradation of β-catenin, a transcription factor crucial for physiological muscle growth (46). Indeed, muscle atrophy induced by GC is associated with a large decrease in total β-catenin levels, consistent with the fact that, once phosphorylated by GSK-3β, β-catenin is targeted and degraded by the proteasome (47;48). Furthermore, IGF-I overexpression was associated with a partial restoration of β-catenin levels. Interestingly, besides regulating the β-catenin stability, IGF-I is also known to regulate its localization and transcriptional activity (49). Finally, when we overexpressed a stabilized form of β-catenin, resistant to the proteasomal degradation, we blocked totally muscle fiber atrophy induced by GC. To our knowledge, this work provides the first data regarding the role of β-catenin in GC-induced muscle atrophy and its reversibility by IGF-I. The mechanisms by which β-catenin may inhibits muscle atrophy are still unclear. β-catenin, in tandem with T cell factor (TCF)/lymphocyte enhancer factor (Lef), is known to activate transcription of several genes (50) including some genes directly implicated in muscle growth. Besides early activation of myogenesis (51;52), β-catenin pathway regulates several genes such as IGFs (53), PTEN (54), and Follistatin (55) involved in the regulation of muscle cell size.

In conclusion, our results show that GC-induced muscle atrophy is associated with several alterations in the IGF-I transduction pathway. Given that these mediators play a key role in the anabolic and anti-catabolic action of IGF-I, their alterations may contribute to the muscle atrophy induced by GC. The fact that muscle overexpression of caAkt, dnGSK-3β and ΔNβ-catenin is sufficient to alleviate the catabolic effects of GC and thus mimic the anti-atrophic action of IGF-I in this model suggests that Akt, GSK-3β, β-catenin and presumably others mediators (i.e., p70S6K) play a critical role in the anti-atrophic action of IGF-I in skeletal muscle atrophy caused by GC.

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Chapter 4: General discussion, perspectives and conclusion

1 General discussion and perspectives

Increased circulating levels of glucocorticoids observed in many catabolic conditions play a major role in the induction of muscle atrophy. Indeed, inhibition of glucocorticoid action by glucocorticoid receptor antagonist attenuates and, in some cases, abolishes muscle atrophy.
In response to glucocorticoids, circulating and tissue levels of IGF-I, a growth factor that stimulates the development of muscle mass, are frequently reduced. This decline could therefore trigger muscle atrophy in catabolic conditions. Indeed, systemic administration of IGF-I prevents glucocorticoid-induced muscle atrophy. However, use of systemic IGF-I administration is limited by its hypoglycemic and cardiac hypertrophic actions and by its association with cancer risk. Moreover, local IGF-I seems to play a more important role in the regulation of muscle mass than systemic IGF-I. Therefore, to limit loss of muscle mass observed in catabolic states, IGF-I administration must mimic as close as possible the autocrine production of IGF-I.

The aim of this thesis was to investigate whether the restoration of IGF-I muscle content could reverse muscle atrophy induced by glucocorticoids. In this work we have tested the hypothesis that the local decrease in muscle IGF-I content might be responsible for the muscular atrophy induced by glucocorticoids.

In our work, we have demonstrated that localized overexpression of IGF-I by gene electrotransfer prevents muscle atrophy in glucocorticoid-treated rats. High rate of fiber transfection and long term gene expression were obtained by combining multiple injection sites of DNA with electroporation. Human IGF-I gene electrotransfer using this optimised protocol resulted in increased muscle IGF-I mRNA and protein levels together with prevention of loss of skeletal muscle mass. Furthermore, alterations in the Akt/GSK-3β/β-catenin signaling pathway caused by glucocorticoids were prevented by local IGF-I gene overexpression. Finally, muscle overexpression of caAkt, dnGSK-3β and ΔNβ-catenin was

sufficient to mimic the anti-atrophic effect of IGF-I supporting the role of this signaling pathway in muscle atrophy caused by glucocorticoids. Taken together, our results show, for the first time *in vivo*, the role of the IGF-I/Akt/GSK-3β/β-catenin pathway in the skeletal muscle atrophy caused by glucocorticoids.

Our work has identified Akt-1, GSK-3β and β-catenin as crucial intracellular mediators involved in the anti-atrophic effect of IGF-I. *In vitro* data, however, indicate that muscle cell catabolism caused by glucocorticoids is thought to be mediated at least in part by the transcription factor Foxo3a. Indeed, exposure of myotubes to glucocorticoids increases Foxo3a gene expression (1). Furthermore, Foxo3a overexpression causes muscle cell atrophy (1) together with activation of several genes involved in the ubiquitin proteasome system (i.e., Atrogin-1, MuRF-1) (1;2) and in autophagy (Lc3b) (3;4). Finally, inhibition of Foxo3a by IGF-I addition as well as overexpression of a dominant negative form of Foxo3a prevents muscle cell atrophy caused by glucocorticoids (1). All these observations support the role of Foxo3a in muscle atrophy induced by glucocorticoids and in its inhibition by IGF-I. To explore the role of Foxo3a in the catabolic effects of glucocorticoids *in vivo*, we have quantified Foxo3a expression by RTQ-PCR and its transcriptional activity by electroporating a Foxo1-3a-dependent reporter plasmid (6XDBE plasmid). No increase in Foxo3a expression or activity was observed three and ten days after glucocorticoid administration. In line with this observation, no increase in MuRF-1 was observed in these conditions. These preliminary results suggest that neither Foxo3a activation nor MuRF-1 induction are required for glucocorticoid-induced muscle atrophy at least in our experimental conditions. Our observation, as the one recently made by Clarke *et al.* (5), force to reevaluate the role of Foxo and MuRF-1 in glucocorticoid-induced muscle atrophy. To totally rule out a role of Foxo3a in glucocorticoid-induced atrophy, we tested a 10 fold higher dose of glucocorticoids (1mg/kg/day vs. 0.1mg/kg/day). Given the fact that 0.1 mg/kg/day of dexamethasone is able in rats to mimic the muscle atrophy observed in catabolic conditions characterized by an increase of endogenous glucocorticoids, this dose must be considered already at the upper limit of the physiological range. In our hands, a higher dose (1mg/kg/d) caused a significant increase in Foxo3a expression (+100%) and activity (+50%) at three days, in agreement with previous *in vitro* observations (1). In this experiment, increased Foxo3a activity was associated with increased expression of Foxo3-dependent genes such as Atrogin-1, MuRF1 and Lc3b, confirming the observations of others with similar (1 mg/kg) (5) or event higher dose (10 mg/kg) (6) of glucocorticoids. Thus, it is possible that the Foxo3a activation

contributes to muscle atrophy only in response to very high dose of glucocorticoids. Furthermore, even with high doses of glucocorticoids, the role of Foxo in the glucocorticoid-induced muscle atrophy has been recently questioned by Waddell *et al.* (6). Indeed, despite the marked attenuation of the induction of Foxo and Murf1 expression, very high doses of glucocorticoids retain the ability to cause muscle atrophy in GR homodimerization mutant mice. Before to conclude regarding the role of Foxo3a in glucocorticoid-induced muscle atrophy *in vivo,* it would be interesting to determine whether the blockade of Foxo either by overexpression of a dominant negative form or by expression of siRNA targeting Foxo prevents muscle atrophy induced by glucocorticoids *in vivo*.

In our study, we have shown that IGF-I overexpression by gene electrotransfer prevents muscle atrophy in glucocorticoid-treated rats. Seen that it has been reported that glucocorticoid administration alters muscle strength (7;8), it would be interesting to explore the possibility for IGF-I to restore muscle function in parallel to muscle mass. Evaluation of functional parameters is in general performed on the Extensor Digitorum Longus (EDL) muscle in mice. Indeed, the small size of this skeletal muscle in mice is more appropriate to perform this kind of study. Thus, to answer this question, we administrated glucocorticoids in muscle-specific IGF-I transgenic mice. Compared to our animals overexpressing IGF-I after muscle electroporation, these transgenic mice, received from the group of Nadia Rosenthal (9), are characterized by a long lasting stable IGF-I overexpression exclusively in skeletal muscle. Moreover, muscle function has been already investigated in this transgenic mice model (9). However, after administration of glucocorticoids, we observed a down-regulation of the IGF-I transgene expression in these mice, precluding any study of the muscle function in these animals. In contrast to the cytomegalovirus (CMV) promoter used in our electrotransfer protocol, it seems that the activity of the MLC (myosin light chain) promoter driven the expression of IGF-I in this transgenic model is inhibited by glucocorticoids. To avoid this problem, it would be interesting to transfect the IGF-I gene under the control of a CMV promoter in EDL mice muscle either by electroporation (as already done in this work on rat TA muscle) or by using viral transfection. The in situ stretch protocol of TA muscle may provide a valuable assay for investigation of the contractile properties of the transfected muscle (10). Interestingly, viral mediated expression of IGF-I has been already demonstrated to prevent loss of muscle function caused by aging (11).

Among all the intracellular mediators considered in this work, GSK-3β may provide one of the most potent clinical avenues for the preservation, attenuation or reversal of disease-related muscle loss. Indeed, even if the muscle hypertrophic action of caAkt is more dramatic, there is no pharmacological molecule available able to stimulate Akt. In contrast, several research groups have developed specific pharmacological inhibitors of GSK-3β such as SB, TDZD8, CHIR and the L803-mts peptide (12-14). Most of these inhibitors have been tested essentially *in vitro* or *ex vivo*. Recently, *in vivo* administration of a GSK-3β peptide inhibitor has been shown to mimic some of the insulin actions towards muscle (14). Thus, administration of these specific inhibitors should be tested in an attempt to prevent muscle atrophy induced by glucocorticoids.

In muscle IGF-I overexpression, it is essential to know whether the overexpressed IGF-I protein remains in the muscle that produces it (autocrine/paracrine effect) or enters in the circulation. Indeed, restricting the effects of overexpressed IGF-I to the targeted muscle seems preferable to avoid adverse side effects, such as heart hypertrophy (15), and increased cancer risk (16), ... Based on the two different muscle IGF-I transgenic models (17;9), it seems that the levels of IGF-I overexpression reached into the muscle, rather than the IGF-I isoforms overexpressed, influence the circulating IGF-I concentrations. Indeed, in the transgenic model described by Coleman, muscle levels of IGF-I were increased by about 45 up to 300-fold versus wild type animals. This huge increase of muscle IGF-I leads, with time, to an increase of circulating IGF-I by about 10 up to 17 % (15). In contrast, the transgenic model described by Musaro exhibits only a mild increase of muscle IGF-I (2-fold) which was not associated with increased circulating IGF-I levels. However, both models were developed in animals with the same genetic FVB background and the same class 1 IGF-I Ea isoform transgene was overexpressed. More probably, the difference in the level of IGF-I overexpression in these two models might be explained by some features of the IGF-I cDNA. Indeed, in the model of Coleman, to increase the stability of the transgenic transcripts, 1.8 kb of the alpha skeletal actin 3'UTR was substituted for the native 3'-UTR of the human IGF-I gene. This modification may be responsible for the dramatic increase of muscle IGF-I observed in this model. Alternatively, the promoter (skeletal α-actin or myosin light chain) used to drive the transgene expression may also influence the rate of overexpression. Whether specific IGF-I isoforms expressed in the skeletal muscle are normally released into the circulation is therefore unclear.

Although, local restoration of muscle IGF-I content could preserve muscle mass in catabolic conditions characterized by high levels of circulating glucocorticoids, we speculate that this therapeutical approach will be ineffective in muscle atrophy models associated with NFκB activation such as sepsis (18) and immobilization (19). In sepsis, proinflammatory cytokines play a crucial role in skeletal muscle proteolysis (20). Despite the fact that in this model a decrease in muscle IGF-I production is observed (21), several observations suggest that restoration of IGF-I muscle content will fail to prevent muscle atrophy. Indeed, data from our Lab have shown that myotube atrophy induced by proinflammatory cytokines is not prevented by IGF-I (22). Furthermore, we have also shown that restoration of circulating IGI-I levels in animals injected with LPS does not prevent atrogenes induction. Finally, in septic rats, IGF-I administration does not inhibit muscle proteolysis (23). Muscle atrophy induced by immobilization, is also associated with an induction (24) of NFκB and with a decrease of muscle IGF-I expression. But, as observed for sepsis, IGF-I fails to prevents muscle atrophy (25). These observations suggest that the mechanisms involved in these two skeletal muscle atrophy models are clearly different from those involved in glucocorticoid-induced muscle atrophy.

The IGF-I decline associated with muscle atrophy caused by glucocorticoids, together with the atrophy prevention by muscle IGF-I restoration, strongly supports the role of muscle IGF-I in this model of atrophy. As for the role of IGF-I decrease in muscle atrophy, it is often suggested that increase IGF-I is responsible and critical for the induction of muscle hypertrophy. Surprisingly, it has been demonstrated by the group of Spangenburg *et al.* that a functional IGF-I receptor is not necessary for load-induced skeletal muscle hypertrophy (26). Indeed, transgenic mice (MKR mice) overexpressing a mutated type 1 IGF-I receptor especially in skeletal muscle that prevents its activation by IGF-I, retains the capacity to undergo muscle hypertrophy in responds to mechanical loading. Some of the hypothesizes raised by the authors could be the fact that IGF-I acts via another receptor or that other factors are responsible for muscle hypertrophy. Indeed, it has been shown that one of the IGF-I isoform dramatically increased in the overload muscle is the IGF-IEb or mechano-growth factor (MGF) (27;28). MGF, largely described by Goldspink *et al.*, seems to be more effective than the other IGF-I isoforms to stimulate the proliferation of satellite cells independently of the type 1 IGF receptor. This effect might be mediated by the release from the MGF of an Eb peptide which may function independently from the rest of the IGF-I molecule (29). The

possibility exists therefore for this E peptide to contribute to the loading-induce hypertrophy by a different receptor than the one activated by mature IGF-I. In further investigation, it should be interesting to decipher the role of this small peptide in load-induced muscle atrophy. These observations do not exclude therefore the role of IGF-I in this form of hypertrophy.

Data obtained from different models of muscle specific overexpression of IGF-I suggested the use of IGF-I for different pathological conditions in humans such as cachexia, Duchenne muscular dystrophy, amyotrophic lateral sclerosis, … Before considering translating to clinical situations these various observations on beneficial effects of IGF-I obtained using rodent models, different crucial points should be considered. First, one of the major limitations of human gene therapy for halting muscle wasting is the difficulty to deliver genes to muscles throughout the body. At the present time, gene electroporation must be considered more as an experimental tool than a real therapeutic strategy to limit muscle atrophy encountered in human diseases. Indeed, this gene transfer method induces some damage to the muscle electroporated and transfection is limited to the few muscles. In contrast, systemic administration of specific rAAV serotypes (S6) offer a more promising therapeutic way to prevent muscle atrophy. Indeed, a single intravenous injection of rAAV enables muscle-specific expression of the transgene in widely dispersed skeletal muscles (30). This method might be therefore applicable for systemic administration of a wide variety of genes including IGF-I to the skeletal muscles of adult mammals including humans. Second, as already discussed, we must control transgene expression rate to limiting the increased systemic IGF-I and the expected deleterious effects such as heart hypertrophy (15) and higher cancer risk (16) should also be fully recognized. Finally, differences between human and rodent muscles should also be considered. Thus, even if overexpression models are clearly a very powerful experimental tool, it is important to understand the mechanisms of action of the artificially expressed gene.

2. Conclusion

In conclusion, this work brings up relevant new information about the mechanism by which glucocorticoids induce muscle atrophy. Given that glucocorticoids play a major role in muscle atrophy, our data may be transposable in several catabolic models including sepsis, immobilization and burn injury model where glucocorticoids play a critical role. Indeed, all these muscle atrophy models are characterized by an increase of circulating glucocorticoids together with a decrease of muscle IGF-I. Finally, as illustrated on figure 1, our work highlights the crucial role of decreased muscle IGF-I in glucocorticoid-induced muscle atrophy. Indeed, the data presented in this thesis support the fact that the atrophic action of glucocorticoids is in part due to the down-regulation of IGF-I, leading to the inhibition of its signaling pathways while the restoration of normal muscle IGF-I levels is able to counteract totally muscle atrophy.

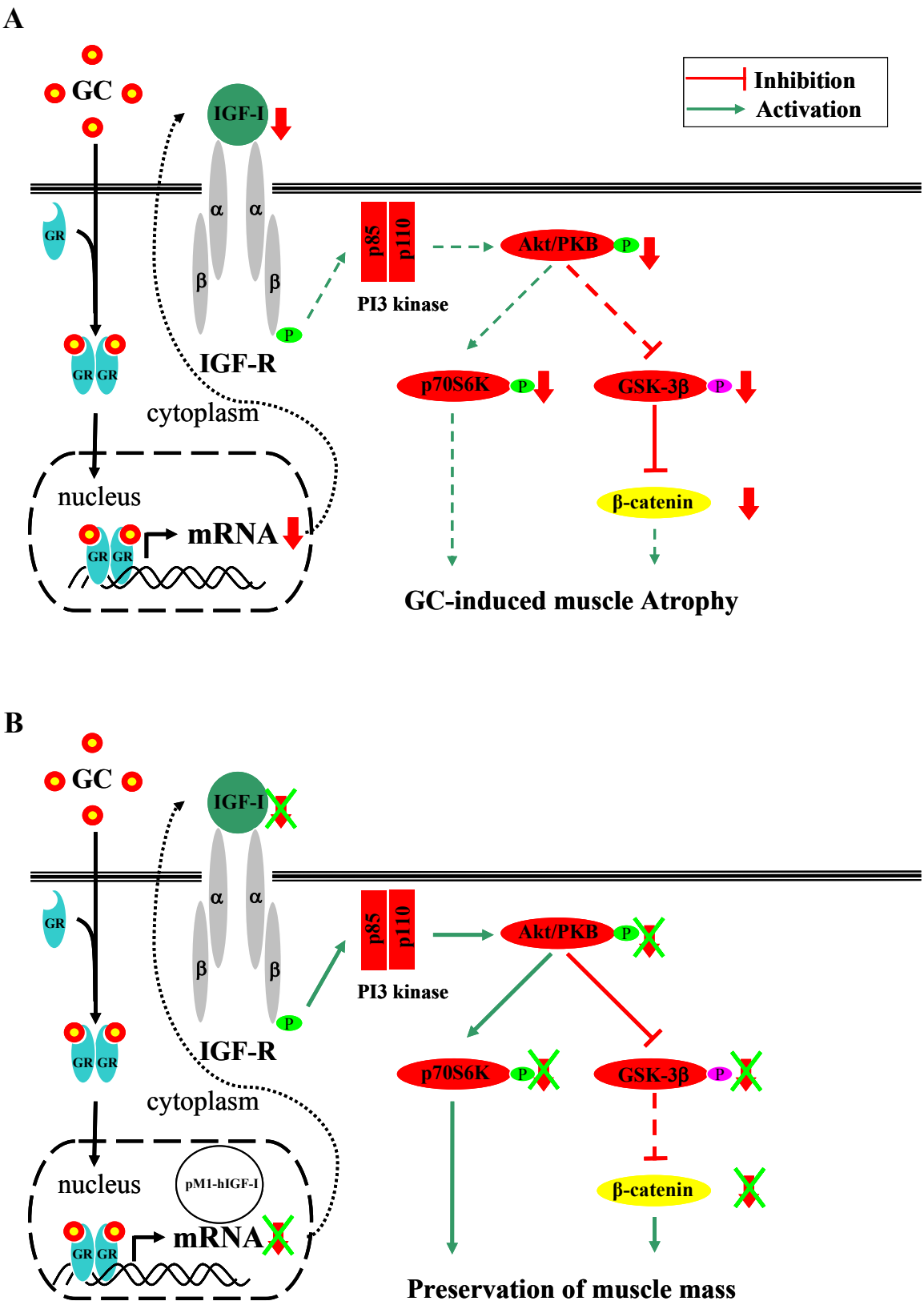

Figure 1. ***Schematic representation of the role of muscle IGF-I in glucocorticoid-induced muscle atrophy.*** Muscle atrophic action of glucocorticoids is in part due to the down-regulation of IGF-I, leading to the inhibition of its signaling pathways (A). Restoration of muscle IGF-I levels is able to counteract totally muscle atrophy (B).

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Annexes

Gene therapy with anabolic growth factors to prevent muscle atrophy

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Current Opinion in Clinical Nutrition and Metabolic Care. 2006 May; 9(3):207-13.

Abstract

Purpose of review: Many situations cause muscle atrophy. When severe, muscle atrophy is associated with an increase in morbidity and mortality. This loss of muscle mass is thought to be due to an imbalance between catabolic and anabolic pathways resulting in an increase of muscle protein proteolysis and in a decrease in protein synthesis. Changes in muscle levels of muscle growth factors are thought to play a major role in this imbalance. Despite recent better understanding of the metabolic and molecular derangements leading to muscle wasting, therapy of muscle atrophy has still a poor success rate.

Recent findings: The recent demonstration that changes in local growth factors, such as Insulin-like Growth Factor (IGF)-I and Myostatin (Mstn), occur during muscle atrophy has stimulated research interest to prevent muscle mass loss by delivering these growth factors or their inhibitors into the muscle. During the last years, several progresses made in the field of muscle gene transfer by electroporation or by rAAV vectors have opened novel therapeutic ways to deliver growth factors able to counteract loss of muscle mass.

Summary: Preventing decrease of IGF-I muscle or inhibiting Mstn action by local genes overexpression may provide a clinically relevant avenue for the attenuation or reversal of disease-related muscle loss.

Key words: Gene therapy, Growth factors, Skeletal muscle, Atrophy

INTRODUCTION

Many clinical situations may cause muscle atrophy. When severe, muscle atrophy is associated with an increase in morbidity and mortality [1]. Indeed, loss of skeletal muscle is associated with infectious (septicemia, pneumonia) and non infectious (cardio-respiratory insufficiency) complications. Moreover, a loss of more than 40% of muscle mass leads in general to patient death [2].

Despite recent better understanding of the metabolic and molecular derangements leading to muscle wasting, therapy of muscle atrophy has still a poor success rate. Loss of muscle mass is thought to be due to an imbalance between catabolic and anabolic pathways. In many situations, muscle protein synthesis is decreased. But, the muscle mass loss is considered to result mainly from increased proteolysis. These pathways are regulated by a set of signaling molecules belonging to family of hormones, growth factors, cytokines, nutrients, and neurotransmitters.

The recent demonstration that changes in local growth factors occur during muscle atrophy has stimulated research interest to prevent muscle mass loss by delivering these growth factors or their inhibitors into the muscle.

During the last years, several progresses made in the field of muscle gene transfer have opened novel therapeutic ways to deliver growth factors able to counteract loss of muscle mass. This review will focus on the therapeutic potential of a local gene delivery-based approach for the attenuation of muscle loss in catabolic situations. We will, in particular, consider the appropriate gene candidates, the methodology, the feasibility and the limits of growth factor gene therapy.

GROWTH FACTORS INVOLVED IN MUSCLE MASS REGULATION

In the last ten years, two key skeletal muscle mass regulators have been identified: Insulin-like Growth Factor (IGF)-I and Myostatin (Mstn). These two autocrine/paracrine factors exert an antagonistic action on muscle development. Autocrine IGF-I plays a positive role in muscle growth [3,4]. Indeed, IGF-I or IGF-I receptor knock out mice [5,6] display a severe muscular hypoplasia. Furthermore, a local muscle IGF-I infusion or overexpression induces muscle hypertrophy [7,8]. Anabolic effects of IGF-I result from the stimulation of protein synthesis [9] as well as proliferation and differentiation of muscle satellite cells [10,11]. On the other hand, its anticatabolic effects result from the inhibition of muscle proteolysis [12] and apoptosis [13]. In contrast, Mstn acts as a negative regulator of muscle mass. Indeed, Mstn knock out mice display a huge increase in muscle mass resulting from fiber hyperplasia

and hypertrophy [14]. Moreover, transgenic mice overexpressing the Mstn propeptide or the follistatin, two binding proteins inhibiting the Mstn activity, also exhibit a marked increase in muscle mass [15]. Indeed, Mstn propeptide, produced by cleavage of the precursor form of Mstn [15] and follistatin, inhibit the interaction of Mstn with his receptor [16,17]. Finally, Mstn blockage by administration of specific Mstn antibodies induces an increase in muscle mass in adult mice [18]. Antianabolic effects of Mstn result mainly from the inhibition of proliferation [19] and differentiation [20] of muscle cells and from the inhibition of protein synthesis [21].

ROLE OF MSTN AND IGF-I IN MUSCLE ATROPHY

Several evidences suggest that muscle overexpression of IGF-I and inhibitory binding proteins of Mstn are potentially interesting approaches to prevent muscle atrophy. Indeed, the decrease of IGF-I and the increase of Mstn in the muscle of several muscle atrophy models (immobilization, sepsis, burn, glucocorticoids excess) suggest that these two growth factors play a crucial role in muscle atrophy [22-24,25**]. Moreover, IGF-I gene overexpression have been already used to prevent loss of muscle mass in some muscle atrophy models such as aging, muscle dystrophy, immobilization, heart failure and glucocorticoids excess [8,25**,26,27,28**,31**]. In addition, inhibition of Mstn by administration of specific antibodies or by gene deletion has improved muscle mass, muscle organization and functional muscle parameters in a mouse model of muscular dystrophy [32].

A GENE-BASED THERAPEUTIC STRATEGY TO PREVENT MUSCLE ATROPHY

Considering their action on muscle mass development and their regulation in muscle atrophy models, overexpression of IGF-I and Mstn inhibition by binding proteins overexpression appear to be a promising therapeutic way to prevent muscle atrophy. From a technical point of view, IGF-I, Mstn propeptide and follistatin genes can be easily cloned in an expressing vector such as plasmid or recombinant adeno-associated virus (rAAV). Indeed, these three proteins are encoded by well characterized short cDNA sequences (< 2000 bp). Furthermore, the overexpression of these proteins within the muscle may exert a therapeutic effect for the entire muscle. Indeed, as these proteins are secreted extracellularly, the muscle cells, even the non-transfected ones, may also benefit from the protective effects of the gene overexpression.

ADVANTAGES OF A LOCAL SKELETAL MUSCLE OVEREXPRESSION

There are two major reasons to administrate local growth factors by gene therapy. First, local production and thus autocrine action of growth factors seem to play a more important role than systemic delivery in the regulation of muscle mass [4,25**]. Indeed, muscle compensatory hypertrophy induced by muscle overloading is associated with an increase in muscle IGF-I levels without any elevation of circulating IGF-I levels [33]. Moreover, specific invalidation of liver IGF-I gene does not affect muscle development, despite a severe reduction in circulating IGF-I levels [4]. Second, to avoid side effects on other organs or systemic effects, the overexpression must be restricted to the skeletal muscle. Indeed, in IGF-I transgenic animal model, the overexpression of the transgene in the heart induced first a physiological cardiac hypertrophy that rapidly progressed to a maladaptive hypertrophy [34]. Furthermore, high circulating IGF-I levels carry an increased risk of hypoglycemia and may positively influence tumour development [35]. In contrast to IGF-I, role of circulating Mstn and of its binding proteins is still unclear. Despite cardiac Mstn expression, heart hypertrophy has not been observed in mice lacking Mstn [14]. Taken together, these observations suggest that growth factors administration must mimic as close as possible the autocrine production of growth factors to limit loss of muscle mass.

DESIGN OF THE GROWTH FACTORS TRANSGENE

There are two parameters to consider for designing the transgene to express: the nature of the protein isoform and the nature of the promoter driving the transcription. The isoform used in muscle gene therapy is an important issue for IGF-I as for Mstn binding proteins.

The human IGF-I gene locus is composed of 6 exons [36] and its transcription gives rise to multiple mRNAs. The different transcripts result from different promoter usage, alternative splicing and various polyadenylation sites. Nevertheless, the coding sequence of the mature peptide is the same throughout all the different transcripts. The transcription of IGF-I can be initiated either at the first or at the second exon (black arrow, figure 1A) giving respectively class 1 and class 2 transcripts. mRNAs initiated at exon 1 are expressed in many tissues and especially in muscle whereas those initiated at exon 2 are mainly expressed in the liver. Mature peptide (B, C, A, D, light grey box, figure 1A) and E domain (dotted box, figure 1A) are encoded by exons 3, 4, 5 and 6. Isoform precursors IGF-I EA and IGF-I EB result from an alternative splicing of exon 5 and 6. Exon 4 is followed either by exon 5 (IGF-I $1E_B$) or by exon 6 (IGF-I E_A) (figure 1B) [37]. Exon 6, encoding the 3' untranslated region, presents multiple polyadenylation sites. Finally, another alternative splicing site has been described in

exon 5 giving rise to IGF-I E_C isoform. The two isoforms which appear most relevant in muscle development are: IGF-I E_A (termed muscle IGF-I or mIGF-I) and IGF-I E_C (termed mechano-growth factor or MGF). Based on the work of Rosenthal and co-workers, muscle overexpression of IGF-I E_A is associated with muscle hypertrophy. Interestingly, in this model, IGF-I overexpressed remains in the muscle bed and does not enter into the circulation [8]. It is not clear which of the isoform or of the promoter is responsible for restricting the IGF-I protein to muscle. The IGF-I E_C mRNA, largely described by Goldspink *et al*, seems to be produced only by damaged or loaded skeletal muscle [38-40]. In a recent review, Goldspink and Yang have reported that administration of IGF-I E_C peptide or overexpression of IGF-I E_C cDNA in normal and in dystrophic muscles increases more effectively the muscle strength than administration of IGF-I E_A isoform [41*]. The reason why IGF-I E_C might be more effective in producing muscle hypertrophy compared to IGF-I E_A is potentially linked to its stimulatory effect on the proliferation of satellite cells [42]. Based on these observations, the IGF-I E_C isoform would seem therefore more suitable to counteract muscle atrophy.

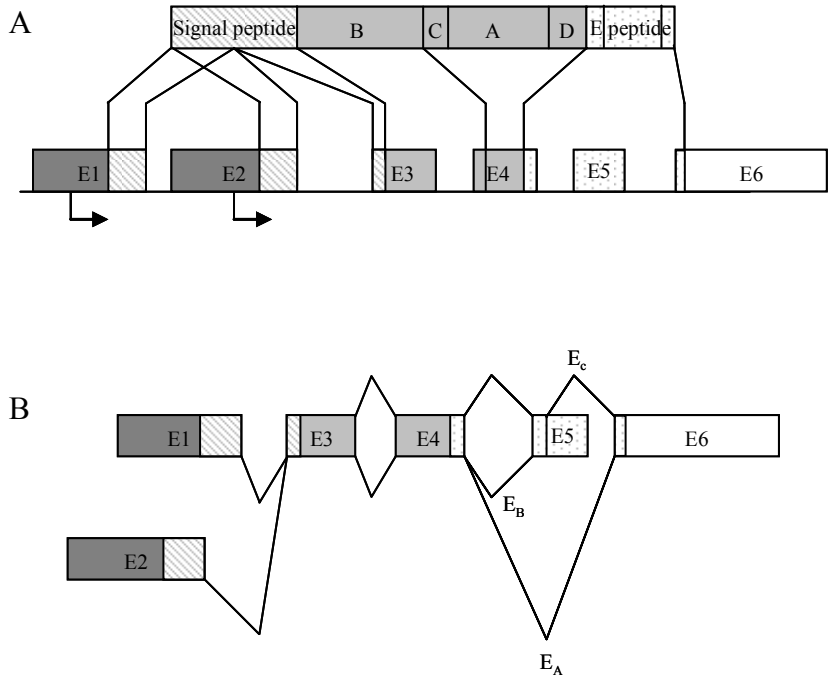

Figure 1. *Human IGF-I gene organisation (A) and Human IGF-I mRNA transcripts(B).*

As for IGF-I, the human follistatin gene encodes different protein isoforms, FS315 and FS288. The follistatin gene locus is composed of 6 exons each encoding distinct functional regions of the proteins: the signal peptide, the N-domain, the three FST domains and the C

terminal region (figure 2A) [43,44]. The N-domain is responsible for the Mstn binding activity of follistatin [44] and the C-terminal acidic tail is only present in the full-length mRNA coding for a protein of 315 amino acids (FS315). Through an alternative splicing of intron 5, a second mRNA is produced that codes for a protein of 288 amino acids (FS288) lacking the C-terminal region (figure 2B) [43]. This FS288 form of follistatin binds the extracellular matrix thanks to the FST domain [45], a property that is inhibited by the acidic C-terminal tail present in the FS315 [46]. As the short form is trapped by the extracellular matrix of the cell transfected (or neighbouring the transfected cells), it is therefore less diluted in the circulation. Thus, the FS288 isoform seems more suitable to use to inhibit local Mstn and counteract muscle atrophy.

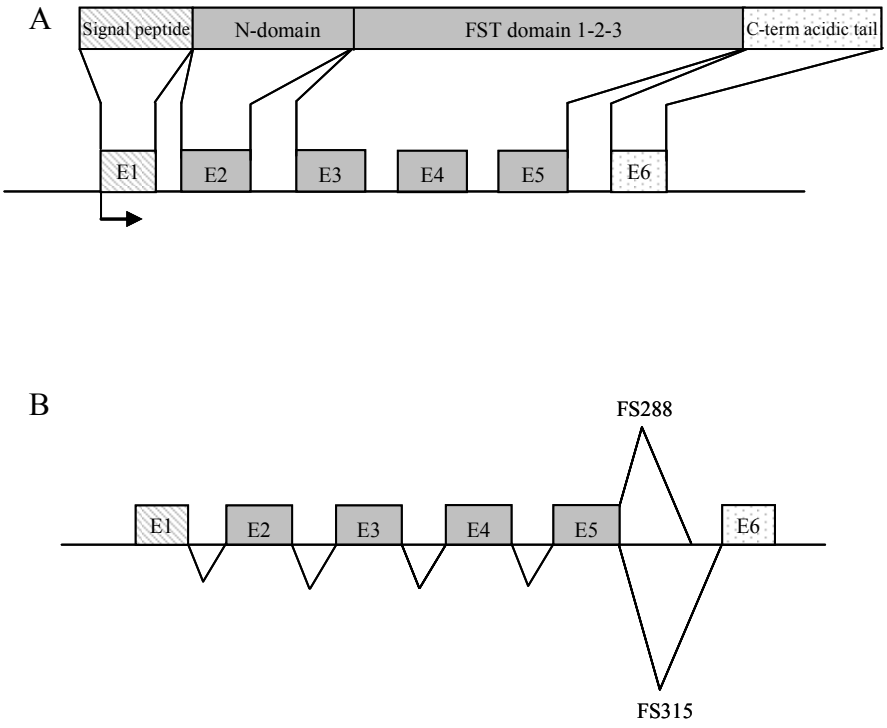

Figure 2. *Human follistatin gene organisation (A) and Human follistatin mRNA transcripts(B).*

The most important point to restrict the transgene expression to the muscle is the choice of the promoter driven the transcription. For this reason, a specific promoter only active in skeletal muscle and not in other tissues must be selected. The myosin light chain 1/3 promoter/enhancer [8,26] and the α-skeletal actin regulatory element [47] are two skeletal muscle specific promoters largely used. The utilization of such promoters is crucial in the elaboration of a muscle restricted transgenic mice lineage. The inconvenient with a muscle specific promoter is the weak levels of transgene expression obtained [48,49,50**]. As an

alternative to these specific muscle promoters, ubiquitously strong viral promoters, such as the cytomegalovirus (CMV), the Rous sarcoma virus (RSV) and the simian virus 40 (SV40) promoters are often used to drive gene expression. The inconvenient with this kind of promoter is that they drive expression in most tissues, which may cause expression of the transgene in all cell types of the transfected organs (endothelial cells, …).

SKELETAL MUSCLE GENE TRANSFER METHODS

The utilization of the skeletal muscle as a bioreactor has became real with the work of Wolf and co-workers in 1990 [51]. These authors were indeed the first to show that a simple injection of nude DNA plasmid encoding a reporter gene is able to cause expression of the gene product in muscle fibres. The inconvenient with this simple transfection method is the poor efficiency. The application of an external electric field surrounding the injected muscle has greatly increased transfection efficiency. Indeed, when square wave electric pulses are applied around the plasmid injected muscle, the levels of gene expression are increased by 2 of 3 log of magnitude [52-54]. The electric pulses ameliorate gene transfer by creating transitory pores allowing the entry of the injected DNA into the muscle cells. This method named electroporation allows to obtain up to 80% of positive transfected cells [55]. These electric pulses are delivered thanks to plate or needle electrodes connected to an electroporator. Different electric parameters influence electrotransfer efficiency, especially the field strength, the duration, the frequency of delivery and the number of electric stimuli. In the muscle electrotransfer literature, two combinations of voltage strength and pulse duration have been successfully tested. Indeed, short length pulses (100 µs) with high voltage (900 to 1000 V/cm) or long length pulses (20 ms) with low voltage (100 to 400 V/cm) give the same electrotransfer efficiency [54,56]. There are two main limitations to this transfection method. Firstly, muscles only directly placed under the skin such as gastrocnemius or tibialis anterior muscle can be transfected. Second, electroporation creates some damage and necrosis restricted to the part of the muscle near the electrodes. Another way to increase transfection efficiency is to associate cationic liposomes to nude plasmid DNA. This transfection method has been broadly used to transfer plasmid in cultured muscle cells, but seems less suitable for *in vivo* transfection [50*].

Viral gene transfer methods can be utilized as an alternative to electroporation. As electroporation, viral gene transfer has also largely used for *in vivo* gene transfer [26,57,58]. Compared to plasmid expression vectors, rAAV vectors give the possibility to express longer cDNA sequences and provide a higher level of expression. However, a major constraint in the

use of viral vectors is that efficient gene transfer occurs only in immature muscle or in regenerating muscle [59-60]. These viral vectors are relatively easy to manipulate in laboratory and have a high safety profile in clinical trials. Some rAAV, such 1, 6 and 8 are particularly expressed in skeletal muscle [61]. With these rAAVs, a persistent expression in the differentiated muscle may be achieved without an immune response. Furthermore, there is no evidence for integration of rAAV into muscle DNA. The serotype 6 seems very attractive, as it has a greater muscle tropism and some vascular permeabilizing activity, enhancing transfer to muscle from capillaries [62**]. With this new generation of rAAV, a transgene directly administrated in the blood stream may be transferred to all muscle cells into the body. Combined with a growth factor/vascular permeability factor such as vascular endothelial growth factor (VEGF) to achieve acute permeabilization of the peripheral vasculature, this rAAV6 vector displays a highly efficient targeting of skeletal and cardiac muscle throughout the body [62**]. The transfection of microdystrophin with this kind of viral vectors has been successfully used to prevent muscle degeneration in a muscle dystrophy animal model [63*].

FUTURE OF HUMAN GENE THERAPY

The major limitation of gene therapy for halting muscle wasting is the difficulty to deliver genes to muscles throughout the body. At the present time, gene electroporation must be considered more as an experimental tool than a real therapeutic strategy to limit muscle atrophy encountered in human diseases. Indeed, this gene transfer method induces some damage to the muscle electroporated and transfection is limited to few muscles [50**]. In contrast, systemic administration of specific rAAV serotypes (S6) offer a more promising therapeutic way to prevent muscle atrophy [62**]. Indeed, a single intravenous injection of rAAV enables muscle-specific expression of the transgene in widely dispersed skeletal muscles. This method might be therefore applicable for systemic treatment of a wide variety of genes to the skeletal muscles of adult mammals including humans.

CONCLUSIONS

Catabolic states are characterized by an accelerate loss of lean body mass mainly skeletal muscle. Despite recent better understanding of the metabolic and molecular derangements leading to muscle wasting, therapy of muscle atrophy has still a poor success rate. Given their opposite action and regulation, the overexpression of IGF-I or the inhibition of Mstn by overexpression of its binding proteins appear to be a promising therapeutic way to prevent muscle atrophy. Although IGF-I gene therapy has been successfully tested in different

muscular atrophic conditions, now it seems more tantalizing to test the inhibition of Mstn action, seeing the huge hypertrophic effects observed on muscle mass of transgenic mice overexpressing Mstn binding proteins (Mstn propeptide and follistatin). Overexpressing IGF-I and inhibiting Mstn locally by gene therapy may provide a clinically relevant avenue for the attenuation or reversal of disease-related muscle loss.

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