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ROLE OF SPHINGOLIPIDS IN MUSCLE ATROPHY

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Summary

Abstract

The sphingolipids were considered until recently as a family of membrane lipids with only a structural role, influencing lipid bilayer properties. However, advances in biochemical and molecular studies of their metabolism and function during the past two decades have revealed that they also act as effector molecules with essential roles in many aspects of cell biology.

Ceramide, sphingosine and sphingosine-1-phosphate are the most studied and have shown opposite effects in various cell functions: whereas ceramide and sphingosine usually inhibit proliferation and promote apoptotic responses to different stress stimuli, sphingosine-1-phosphate is known to stimulate cell growth, and promote cell survival. Ceramide, the central molecule of sphingolipid pathway can be processed into the others sphingolipids, which in turn can be re-metabolized to give again rise to ceramide. From this complex metabolic interconnection of bioactive lipids endowed with different properties arises the difficulty in studying this family of lipids.

Ceramide can be produced by two different ways: through the *de novo* synthesis pathway, and by membrane sphingomyelin hydrolysis catalyzed by sphingomyelinases. Both pathways can be activated by the pro-inflammatory cytokine TNFα. Because this cytokine has been shown to promote muscle loss and seems to be crucial in the development of cachexia, we hypothesized that the formation of ceramide, or a metabolite, can be involved in tumor-induced muscle wasting.

As a first step, we investigated the role of ceramide in the in vitro atrophic effects of TNFα on differentiated C2C12 myotubes, by using cell permeant ceramides and inhibitors of sphingolipid metabolism. We observed that TNFα atrophic effects, as evaluated by the reduction in myotube area, are mimicked by exogenous ceramides, supporting the idea that ceramide can participate in muscle atrophy. To verify if ceramide is a mediator of TNFα-induced atrophy, and to identify the metabolites potentially involved, we analyzed the effects of drugs able to block sphingolipid metabolism at different steps: the inhibition of *de novo* synthesis pathway was unable to restore myotube size in the presence of TNFα, whereas the inhibitors of neutral sphingomyelinases reversed TNFα-induced atrophy. Moreover, an accumulation of ceramide and sphingosine induced pro-atrophic effects, whereas sphingosine-1-phosphate had a protective effect.

These observations establish that in C2C12 myotubes, ceramide or other downstream metabolites such as sphingosine, produced by the neutral sphingomyelinase pathway in response to TNFα stimulation, participate in cell atrophy.

To evaluate the *in vivo* role of sphingolipids, we then used a well-established model of tumor-induced muscle atrophy, BalbC mice carrying C26 adenocarcinoma. Myriocin, an inhibitor of the *de novo* pathway of ceramide synthesis, that is able to deplete muscle tissue in all sphingolipids, was administered daily to the animals. This treatment partially protected animals against tumor-induced loss of body weight and muscle weight, without affecting the size of tumors. Moreover, myriocin treatment significantly reversed the decrease in myofiber size associated with tumor development, and reduced the expression of atrogenes Foxo3 and Atrogin-1, showing that it was able to protect against muscle atrophy.

These results strongly suggest that ceramide, or a downstream sphingolipid metabolite, is involved in tumor-induced muscle atrophy. The sphingolipid pathway thus appears as a new potential target of pharmacological interventions aiming at protecting muscle tissue against atrophy.

Résumé

Les sphingolipides ont été longtemps considérés comme une famille de lipides membranaires dotés d'un rôle structural, influant sur les propriétés de la bicouche lipidique. Cependant, plus récemment, les études de leur métabolisme et de leurs fonctions ont révélé qu'ils agissent aussi comme des molécules effectrices au rôle essentiel dans de nombreux aspects de la biologie cellulaire.

Le céramide, la sphingosine et la sphingosine-1-phosphate (S1P), les sphingolipides les plus étudiés, ont des effets opposés dans diverses fonctions cellulaires: tandis que le céramide et la sphingosine inhibent la prolifération et promeuvent la réponse apoptotique à différents stimulus de stress, la S1P est un stimulateur de la prolifération et de la survie cellulaires. Le céramide, molécule centrale de la voie des sphingolipides, et les autres médiateurs sphingolipidiques peuvent s'interconvertir, ce qui complique considérablement l'étude de leurs fonctions.

Le céramide peut être produit par deux voies différentes: la voie de synthèse de novo, et l'hydrolyse de la sphingomyéline membranaire catalysée par les sphingomyélinases. Ces deux voies peuvent être activées par la cytokine pro-inflammatoire TNFα. Comme cette cytokine est capable d'induire une perte musculaire, et semble jouer un rôle crucial dans le développement de la cachexie, nous avons fait l'hypothèse que le céramide, ou un de ses métabolites, peuvent être des médiateurs de la perte musculaire tumeur-induite.

Nous avons tout d'abord examiné le rôle du céramide dans l'atrophie induite in vitro par le TNFα chez les myotubes C2C12 différenciés, en utilisant des analogues cell-perméants de céramide et des inhibiteurs du métabolisme sphingolipidique. L'apport de céramides exogènes est capable de reproduire l'effet atrophique du TNFα, évalué d'après la surface des myotubes, ce qui suggère que le céramide peut participer à l'atrophie musculaire. Pour vérifier si les céramides sont les médiateurs de l'atrophie induite par le TNFα et identifier ses métabolites potentiellement impliqués, nous avons analysé l'effet d'inhibiteurs ciblant différentes étapes du métabolisme: l'inhibition de la voie de synthèse de novo est incapable de rétablir la taille des myotubes en présence de TNFα, alors que les inhibiteurs de sphingomyélinase neutre suppriment l'atrophie TNFα-induite. De plus, l'accumulation de céramide et de sphingosine augmente l'effet pro-atrophique, tandis que la S1P a un effet protecteur.

Ces observations montrent que, dans les myotubes C2C12, le céramide, ou un métabolite tel que la sphingosine, produits par la voie de la sphingomyélinase neutre en réponse à une stimulation par le TNFα, participent à l'atrophie des cellules.

Pour évaluer le rôle in vivo des sphingolipides, nous avons utilisé un modèle établi d'atrophie musculaire tumeur-induite, la souris BalbC porteuse d'un carcinome C26. La myriocine, inhibiteur de la synthèse de novo, capable d'induire une déplétion générale du muscle en sphingolipides, a été administrée quotidiennement aux animaux. Ce traitement protège partiellement les souris contre la perte de poids corporel et de poids des muscles induite par la tumeur, sans affecter la taille de celle-ci. De plus, la myriocine réverse significativement la perte de taille des fibres musculaires due au développement de la tumeur, et réduit l'expression des atrogenes Foxo3 et Atrogin-1, ce qui montre qu'elle protège le muscle contre l'atrophie.

Ces résultats suggèrent fortement que le céramide, ou un métabolite sphingolipidique en aval, est impliqué dans l'atrophie musculaire tumeur-induite. La voie des sphingolipides apparaît donc comme une nouvelle cible potentielle d'interventions pharmacologiques visant à protéger le tissu musculaire contre l'atrophie.

Chapter 1

Skeletal Muscle

1.1 Skeletal muscle organization

Skeletal muscle is a really amazing tissue for its many capabilities. It is indeed capable of generating force, allowing the movement of each part of the body, it contributes to body temperature regulation, and repairs itself when needed.

Skeletal muscles constitute about 40% to 50% of a person's total body weight. They are primarily attached to the bones and, unlike smooth and cardiac muscles, skeletal muscles are under voluntary control.

Skeletal muscle is composed of long cylindrical multinucleate cells called fibers, which lie parallel to each other. (Figure 1.1) Each cell is surrounded by a thin elastic membrane called *Sarcolemma* which encloses its contents. The sarcolemma encloses the *Sarcoplasm* of the cell, which contains *myofibrils*, mitochondria, and the *Sarcoplasmic Reticulum*, comprising a network of small channels and fluid-filled sacs. Pressed against the inside of the S*arcolemma* are the unusually flattened nuclei.

Overlaying the *Sarcolemma* of each fiber is a thin layer of connective tissue called the *Endomysium*, which also covers a dense system of vessels.

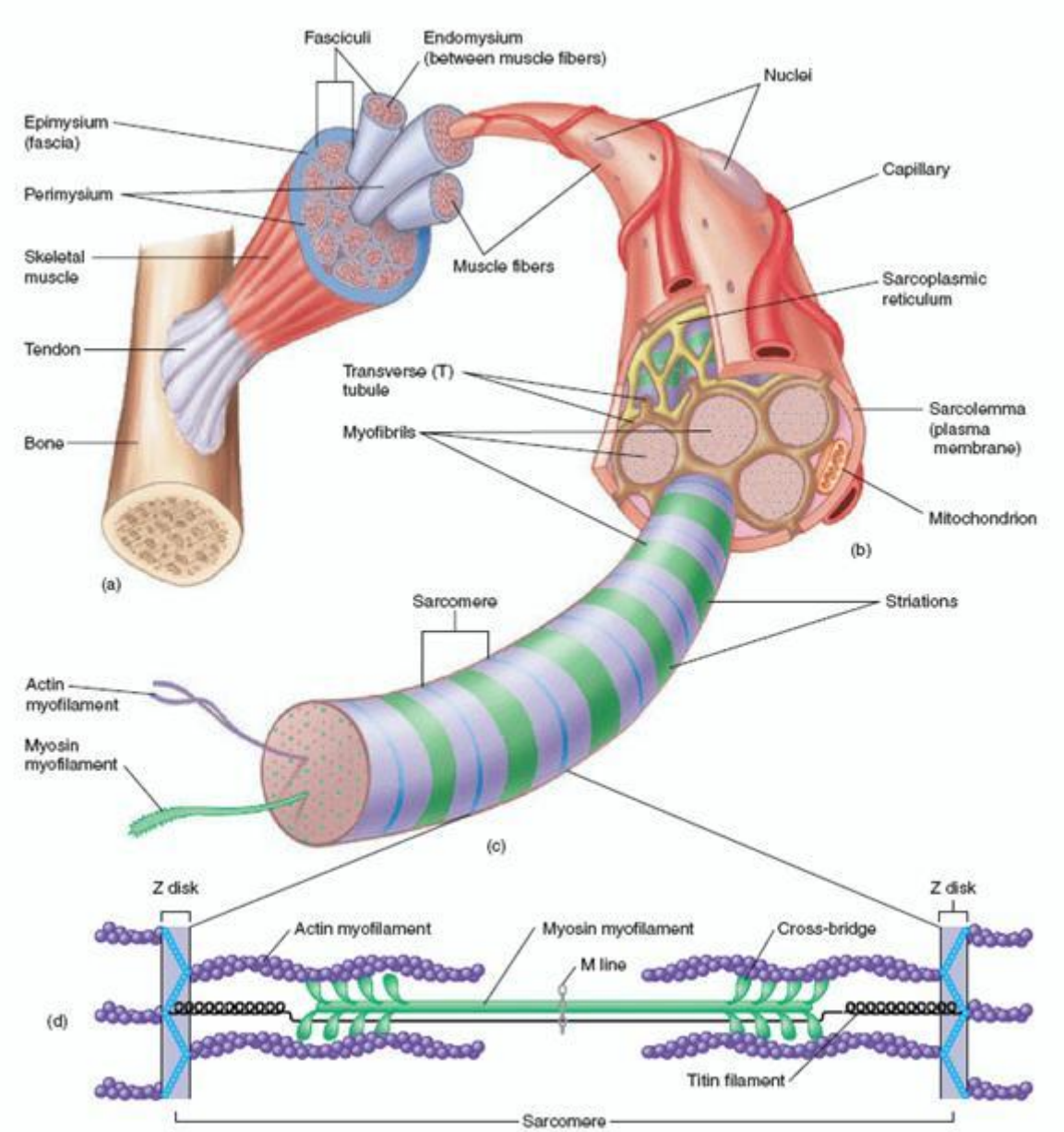


Fig. 1.1 The structure of skeletal-muscle fibre (Essentials of Anatomy and Physiology, Elaine Marieb, 8th Edition)

The fibers are grouped together into many individual bundles which are covered with a layer of connective tissue called the *Perimysium*. These bundles are known as *fasciculi* and together they form a muscle. Each *Fasciculus* contains between 10 and 100 muscle fibres, depending on the muscle. The entire muscle is covered by still another thin layer of connective tissue called the *Epimysium*, which itself is covered by connective tissue called *fascia*.

These two external protective layers are tapered at the ends to form the tendons which attach a muscle to bone, cartilage or connective tissue.

Each individual muscle fiber contains very fine, long protein strands called myofibrils, which are aligned side-by-side and extend the length of the fiber. They are the units which lengthen and contract the muscle. The myofibrils are chains of tiny contractile units, called *Sarcomeres*, which are aligned end-to-end along the length of the myofibrils. The *Sarcomeres* are formed by even finer strands known as *myofilaments* which give skeletal muscle its characteristic striated appearance.

There are two types of myofilaments: *actin filaments,* the thin filaments, that are primarily made of actin but also contain other proteins such as troponin and tropomyosin, and *myosin filaments,* the thick filaments formed from myosin.

The myosin filaments are very important because they contain both ATPase enzymes which split ATP to produce the energy for muscle contraction, and projections which, during muscle contraction, attach to binding active sites on actin filaments to produce movement. In resting muscle, troponin and tropomyosin cover the active sites and inhibit the myosin heads from bonding to the actin filaments, thereby preventing muscle contraction.

Skeletal muscle is stimulated to contract by impulses transmitted by specialized nerve cells called motor neurons. The cell body of a motor neuron resides in the central nervous system and its axon extends to the muscle. In the muscle, the axon is divided into numerous axonal terminals, each of which connects with individual muscle fibers. The intersection where the axonal terminals and muscle fibers connect is called the *neuromuscular junction*. A motor neuron and the muscle fibers to which it connects are together known as a *motor unit*.

When an action potential travels down the axonal membrane and reaches the axonal terminals, the neurotransmitter *acetylcholine* stimulates the release of calcium ions from the sarcoplasmic reticulum into the sarcoplasm. Calcium ions quickly attach to troponin in the actin filaments, which causes troponin to pull on tropomyosin, to which troponin is attached, thereby exposing the active sites of the actin filament and allowing it to interact with the myosin heads. As a result, the ATPase enzymes on the myosin heads become activated and split ATP, which energizes the link between the actin and myosin filaments and causes muscle contraction. Once a muscle has contracted, calcium is reabsorbed back into the sarcoplasmic reticulum, which allows troponin and tropomyosin to inhibit again the link between actin and myosin. As a result, the muscle returns to a relaxed state.

1.2 Skeletal muscle plasticity

Skeletal muscle is composed of different and specialized fibres and this variable muscle fibre composition provides several different motor performances in terms of strength, speed and durability.

According to the expression of specific isoforms of Myosin Heavy Chains (MyHC), the myofibers are classified as: type 1, type 2A, type 2B and type 2X (Pette and Staron, 2000).

The fibers of type 1 (slow) are characterized by a high content of mitochondria and by a predominantly oxidative metabolism, with a high resistance to fatigue and a slow speed contraction. The fibers of type 2B (fast), in contrast, have a mainly glycolytic metabolism, contract very quickly and get tired quickly. 2A and 2X fibers contractile and metabolic characteristics are intermediate between type 1 fibers and type 2B fibers. The slow fibers are used to maintain the posture, which requires prolonged contractile activity, whereas fast fibers are involved in movements which require strength and speed (Zierath and Hawley 2004).

During the life of each individual, the size and composition of muscle fibres is not constant, but can be modified through the extreme plasticity of skeletal muscle, adapting to any new physiological or pathological condition (Harridge, 2007).

In recent years, numerous studies have demonstrated the involvement of the myogenic regulatory factors (MRFs) in determination and regulation of muscle phenotype. Thus, the level of each MRF is different from a type of muscle fiber to another, and varies according to the change in muscle phenotype. In particular, the distribution of MyoD and myogenin in a single muscle is not homogeneous: MyoD appears more expressed in regions containing fast glycolytic myofibers, whereas myogenin is accumulated in regions characterized by slow oxidative myofibers (Hughes et al. 1993), and both are involved in specific isoforms of myosin expression.

Overexpression of myogenin in the muscle of transgenic mice leads to increased activity of oxidative enzymes such as succinate synthase, whereas the activity of glycolytic enzymes such as lactate or GADPDH dehydrogenase, decreases (Hughes et al.1999; Ekmark et al. 2003). The expression of myogenin is also related to the increase of citrate synthase in rats and results in muscle strength increase (Siu et al. 2004). Therefore, myogenin is specifically involved in the transition from fast to slow muscle phenotype.

The expression of MyoD, associated with a fast muscle phenotype, increases during denervation experiments or muscle discharge, in parallel with the expression of myosin 2X (Walters et al., 2000a; Hyatt et al., 2003; Ishido et al., 2004a).

Another MRF, MRF4, is highly expressed in all types of skeletal muscle suggesting an essential role in muscle function, but this factor plays only a minor role in determining muscle phenotype (Hughes et al., 1997).

However, MRF4 is expressed preferentially in slow oxidative myofibers, whereas in mixed or fast muscles this expression varies during muscle immobilization (Walters et al., 2000b).

Interestingly, electrical activity is essential for determining muscle phenotype (Bassel-Duby and Olson, 2006), promoting the establishment of a slow muscle phenotype (Schiaffino et al., 2007). These effects are transmitted by calcium, which affects many transcription factors. Indeed, calcium activates the pathway of calmodulin-dependent protein kinases, CaMK, which in turn activate calcineurin (Chin et al., 1998; Naya et al. 2000; Schiaffino et al. 2007) and another MRF, MEF2, a transcription factors that acts downstream of calcineurin, which is also able to stimulate the expression of myogenin (Friday and Pavlath 2001; Friday et al. 2003).

It is important to underline that muscle atrophy is characterized by a transition of muscle phenotype from the generally slow type to fast type (Bassel-Duby and Olson 2006; Harridge, 2007).

Myofibrillar proteins represent 85% of the volume of a muscle fibre, and any situation affecting the balance between protein synthesis and degradation will result in a gain or loss of muscle mass (Hoppeler 1986; Sandri 2008). Thus, hypertrophy is the result of a strong induction of protein synthesis, while atrophy is caused by a rapid decrease in the synthesis and a marked increase in protein degradation. Satellite cells are also involved in the process during hypertrophic growth, and in response to exercise, by fusing with the existing fibres, in order to maintain the ratio DNA / protein (Roy et al. 1999; Adams, 2006).

In addition to the essential sarcomeric proteins, actin and myosin, other proteins are expressed to ensure maintenance of the structure and function of myofibrills, including titin, a very long protein serving as a template for the formation of myosin filaments, troponins I, C and T, allowing the regulation of muscle contraction by calcium, nebulin, ensuring stability of actin filaments, and desmin, ensuring the stability of the sarcomere during contraction (Clark et al. 2002).

The expression of these proteins is also specifically regulated by the MRFs, e.g. MyoD regulates the expression of genes encoding the light chains of myosin, desmin, troponin I, but also creatine kinase and several subunits of the receptor for acetyl-choline (Piette et al. 1990; Lin et al. 1991, Wentworth et al. 1991, Li and Capetanaki 1993).

Even if at low levels, MyoD, Myf5 and myogenin are constantly expressed in adult skeletal muscle, suggesting that they continue to modulate the expression of muscle specific genes after birth and may contribute to muscle plasticity (Walters et al. 2000a; Hespel et al. 2001). In fact, their involvement in catabolic and anabolic processes in skeletal muscle has been shown (Favier et al. 2008; Legerlotz and Smith 2008).

The expression of MyoD, MRF4 and myogenin is increased in different models of hypertrophy, but with differences depending on the type of muscle studied (Loughna and Brownson 1996a; Loughna et al. 1996b; Carson and Booth 1998, Lowe et al. 1998; Adams and al. 2002; Haddad and Adams 2006; Bamman et al. 2007).

If the MRF are closely involved in controlling the size and muscle fiber phenotype as transcriptional regulators of muscle-specific genes and myofibrillar proteins, other pathways are critical in regulating muscle mass, controlling the balance between protein synthesis and degradation (Sandri2008). They will be discussed in the next chapter.

Chapter 2

Muscle atrophy

2.1 Muscle atrophy condition

Muscles maintain their mass and function because of a balance between protein synthesis and protein degradation, associated with equal rates of anabolic and catabolic processes, respectively. We can speak of muscles growth (hypertrophy) when protein synthesis exceeds protein degradation; conversely, of muscle loss (atrophy) when protein degradation dominates.

Muscle wasting can occur as a consequence of neuromuscular disorders such as Duchenne muscular dystrophy (DMD) or Amyotrophic lateral sclerosis (ALS) (Lynch, 2001), but also of several other chronic deseases such as cancer (cachexia), cardiac and renal failure, chronic and obstructive pulmonary disease (COPD), sepsis and HIV-acquired immunodeficiency syndrome (AIDS) (Doucet et al., 2007; Jackman and Kandarian, 2004; Jagoeand Goldberg, 2001; Lecker et al., 2006; Tisdale, 2004).

Similarly, aging is associated with a progressive loss of muscle, named sarcopenia, leading to increasing frailty, weakness, and loss of functional independence (Baumgartner et al., 2000). Other conditions of muscle wasting include denervation, disuse, inactivity, immobilization following acute injuries, unloading caused by microgravity or myopathy (Di Giovanni S, 2004).

We can thus conceive two distinct conditions, based on the origin of syndrome: *cachexia*, with a pathological origin, and *sarcopenia*, observed during normal aging, both resulting in muscle atrophy.

According to a recent new definition, "cachexia, is a complex metabolic syndrome associated with underlying illness and characterized by loss of muscle with or without loss of fat mass. The prominent clinical feature of cachexia is weight loss in adults. Anorexia, inflammation, insulin resistance and increased muscle protein breakdown are frequently associated with cachexia." (Evans WJ, 2008).

Cachexia affects from 50% to 80% of cancer patient, depending on the type of malignancy and is directly responsible for about 20% of all cancer deaths (Tisdale 1997).

This condition is associated with reduced mobility, impaired response to chemo/radio therapy, increased risk of complication in surgery, psychological stress, leading to overall reduction of quality of life (Dodson S et al, 2010). When the patient has lost 30% body weight, without therapeutic intervention, the outcome is often fatal (Tisdale 1997). This process is characterized by a loss of mass, but it is distinct from loss of muscle after starvation or ageing, because increased food intake does not change the condition (MacDonald N.2003). Clinical trials performed on cachectic patients showed that the preservation of body fat and skeletal muscle can decrease mortality risk (Schols et al., 2005; Habedank et al., 2009).

Sarcopenia is characterized not only by the age-related loss of skeletal muscle mass, but also by a gradual decline in muscle functional properties (Evans and Campbell, 1993; Newman et al., 2006). This condition has been even reported among healthy, physically active subjects and is highly prevalent, as it has been estimated that 25% of persons under the age of 70 years and 40% of those aged 80 years or older are sarcopenic (Baumgartner et al., 1998). This is a multifactorial syndrome due to disuse (Kortebein et al., 2007), chronic inflammation (Cesari et al., 2005), insulin resistance (Rasmussen et al., 2006), chronic disease, changing endocrine functions and nutritional deficiencies (Visser et al., 2003). All these causes may lead to alterations in both protein synthesis and degradation (Kayo et al., 2001; Welle et al., 2003; Giresi et al., 2005).

In addition, Giresi shows that it correlates well with age-related changes in gene expression, consistent with impaired oxidative defense, decreased activity of mitochondrial proteins, and differential expression in genes regulating energy

metabolism, DNA-damage repair, stress response, immune/inflammatory response, RNA binding and splicing, and proteasome-dependent proteolysis (Giresi et al., 2005). Sarcopenia represents a powerful risk factor for physical disability in older subjects. Moreover, mobility reduction resulting from muscle loss predicts major physical disability and mortality, and is associated with poor quality of life and social and health care needs.

Whereas cachexia may be a component of sarcopenia, these two conditions are not the same, and in Table 1 are compared the metabolic consequences of both.

Metabolic condition	Cachexia	Sarcopenia
Muscle protein synthesis	Decreased	Decreased
Muscle protein degradation	Increased	No change
Muscle mass, strength, and function	Decreased	Decreased
Fat mass	No change or decreased	Increased
Basal metabolic rate and total energy expenditure	Increased	Decreased
Inflammation	Increased	No change
Insulin resistance	Increased	Increased

Table 1 Comparison of metabolic consequences of cachexia and sarcopenia (from Evans W.J., 2010)

Since the progressive loss of muscle mass is the most prominent phenotypic feature of both cachexia and sarcopenia, causing major functional impairment, the stabilization of muscle loss and possible regain of lean tissue mass are the most important targets of intervention, but to achieve this goal, understanding the mechanisms behind muscle wasting is crucial.

2.2 Factors and molecular mechanisms regulating muscle mass

As mentioned above, muscle wasting results from a metabolic shift of mechanisms maintaining muscle homeostasis toward a simultaneous decreased anabolism and increased catabolism, and is believed to be mediated by different factors, which can differ between the various atrophic conditions.

In well defined situations, some factors are identified, such as pro-inflammatory cytokines and growth factors which regulate Ubiquitin proteasome pathway (UPP),

cell death and lysosome-mediated autophagy. The role of other factors, such as Myostatin, glucocorticoids, mechanical sensor of inactivity (DGC), and also satellite cell remodeling, is emerging.

2.2.1 Pro-inflammatory cytokines

Cytokines are important small polypeptide or glycoprotein mediators that regulate αvarious aspects of immunity and inflammation. These mediators are released by and/or act on most cells of the immune system, as well as other cells, and include several of the interleukins (IL), interferons, tumor growth factor β (TGF-β), tumor necrosis factors (TNF) and additional chemokines. The majority exert pro-inflammatory effects through the action on specific cytokine receptors on their target cells. (Arend and Gabay, 2000; Chen et al., 2001; Wahl and Chen, 2005; Chen and Goeddel, 2002).

Among the numerous cytokines known so far, TNFα, IL-1β and IL-6 are viewed as the most important due to their roles in the pathophysiology of several inflammatory responses (Borish and Steinke, 2003). TNFα, IL-6 and IFN-γ are implicated in loss of muscle tissue during muscle atrophy (Martin et al. 2000; Mantovani et al, 1999; Phillips and Leeuwenburgh, 2005) and in suppression of MyoD transcription, by activating the NF-kB pathway (Guttridge et al., 2000).

In particular *Tumor Necrosis Factor alpha* (TNFα) has long been recognized able to stimulate catabolism via different mechanisms: TNFα can stimulate the activation of the Ubiquitin-proteasome pathway (Llovera, 1998), induce cell death by enhancing the caspase cascade (Hengartner, 2000), but also alter circulating levels of hormones that regulate muscle growth, or inhibit myoblast differentiation, which could limit the regenerative response of injuried muscle (Li et al, 2000).

TNFα and Ubiquitin-proteasome pathway - In all tissues, the large majority of intracellular proteolysis occurs through the Ubiquitin-proteasome pathway (Cao et al. 2005) which is also the most important system of protein degradation occurring in muscle atrophy (Lecker et al. 1999) (Figure 2.1).

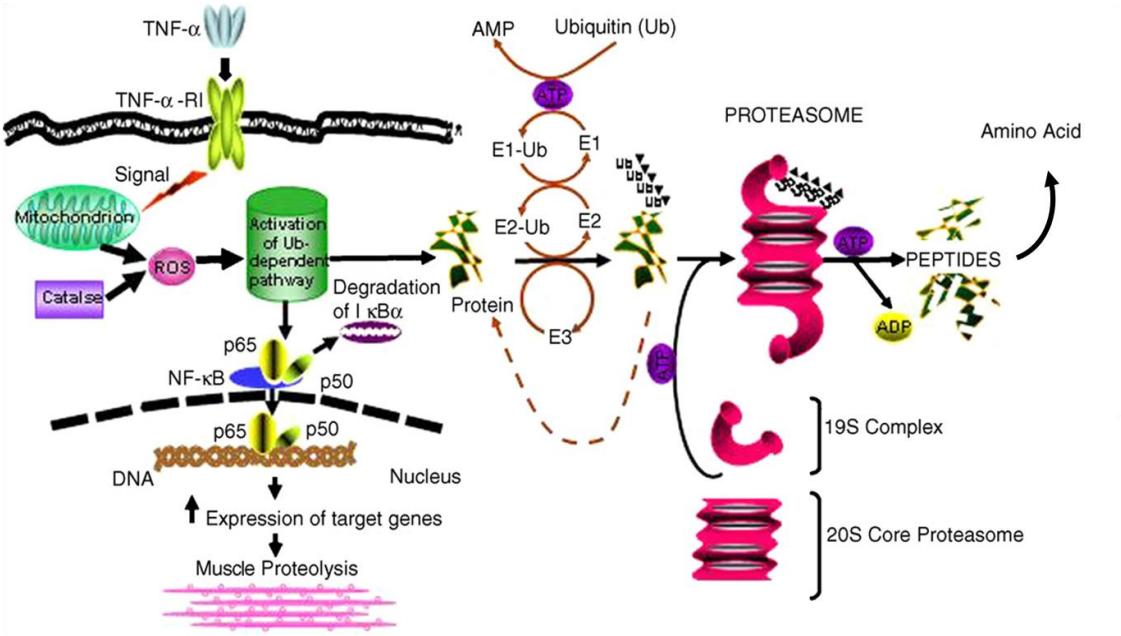


Fig. 2.1 - The activation mechanism of the Ubiquitin–proteasome pathway leading to intracellular protein breakdown by TNFα (adapted from Saini *et al*. 2006).

Transcription of the Ubiquitin–proteasome pathway is activated by the *Nuclear Factor kappa-light-chain-enhancer of activated B cells* (NF-kB) in response to a variety of physiological and pathological signals, including inflammatory cytokines, as well as oxidative and fluid mechanical stress. In inactive state, NF-kB is sequestrated in the cytoplasm by a family of inhibitory proteins named IkBα. In response to TNFα stimuli, through its receptor 1 (TNFR 1), the mitochondrial production of Reactive Oxygen Species (ROS) is activated. This response enhances IkBα phosphorylation by IkB Kinase (IKK), and subsequently its ubiquitination and proteasome-mediated degradation, thus leading to the nuclear translocation of NF-kB (Brown et al. 1995; Li et al., 1998). Nuclear NF-kB regulates the expression of genes involved in the induction of muscle proteolysis.

Proteins are targeted for degradation by proteasome, through an ATP-dependent process. Ubiquitin, that targets the different proteins, needs first activation by the ubiquitin-activating enzyme (E1) to be transferred, in a second step, by a family of carrier proteins (E2) to the ubiquitin ligases (E3), which are able to recognize the specific protein to be ubiquitinated, and catalyze the transfer of ubiquitin from E2 to this target protein.

The targeted proteins are recognized by the 19S complex of proteasome in an ATP-dependent binding step, and must then enter the 20S particle, the catalytic core of

proteasome, to be hydrolyzed in short peptides in this active site, and then in amino acids by direct action of cytosolic peptidases (Liu et al., 2006).

Interestingly, these last years, by comparing gene expression in different models of muscle atrophy, an important role has been recognized to two muscle-specific E3-ubiquitin ligases: atrogin-1/MAFbx and muscle RING-finger 1 (MuRF1) (Bodine et al., 2001; Gomes et al., 2001). MuRF1 has been shown to be directly regulated by the NF-kB transcription factor (Cai *et al.*, 2004).

The subset of genes that are markedly upregulated with atrophy resulting from cachexia (Bodine et al., 2001) and also disuse (Gomes et al., 2001), are called atrophy-related genes or "atrogenes" (Sacheck et al., 2007).

In atrophic conditions, biochemical and transcriptional adaptations of muscle are activated, leading to enhanced protein degradation which occurs by both proteasomal and lysosomal systems (Mammucari et al., 2007; Zhao et al., 2007); in particular, in skeletal muscle, the myofibrillar apparatus, which comprises at least 60% of muscle proteins, decreases in mass more than the soluble compartment (Munoz et al., 1993) and this degradation is mediated by the ubiquitin-proteasome pathway (Solomon and Goldberg, 1996).

Moreover, MuRF1 and the other members of the MuRF family have been found associated with the myofibrillar component titin at the M-line (Centner et al., 2001; McElhinny et al., 2004), with cardiac Troponin-I (Kedar et al., 2004) and myosin heavy chain (MyHC) (Clarke et al., 2007), but MuRF2 and MuRF3 are present in normal conditions and are implicated in contractile functions (Fielitz et al., 2007; Witt et al., 2008), whereas MuRF1 is expressed only from early stages of muscle wasting (Cohen et al., 2009).

These observations raised the possibility that MuRF1 might target components of the myofibrillar apparatus for degradation in atrophying muscle, via Ubiquitin-proteasome pathway. Indeed, a clear role of MuRF1 in the earlier step of thick filaments (myosin filaments) disassembly has been recently reported. By selectively ubiquitinating some important regulatory components, MuRF1 facilitates the breakdown of many resistant myofibril complexes by the proteasome (Cohen et al., 2009).

Furthermore, several groups have presented other evidences that a calpain (Tidball and Spencer, 2002) or caspase (Du et al., 2004) may initially cleave the myofibrillar

components, thereby accelerating disassembly and degradation by the ubiquitin–proteasome system.

TNFα and cell death - TNFα is also implicated in another important process in muscle wasting, cell death. Particularly, it is able to activate both caspase-mediated apoptosis (Hengartner, 2000) and autophagic cell death (Baregamian et al., 2009), in two complementary death pathways (Figure 2.2).

By binding trimeric extracellular death ligands to trimers of their death receptor TNFR1, TNFα recruits both the kinase receptor-interacting protein (RIP) and procaspases 8 and 10, through the adaptor proteins TNFR-associated death domain (TRADD) (Nagata, 1997) and Fas-associated death domain (FADD) (Shiokawa et al., 1997), to form the death-receptor signaling complex.

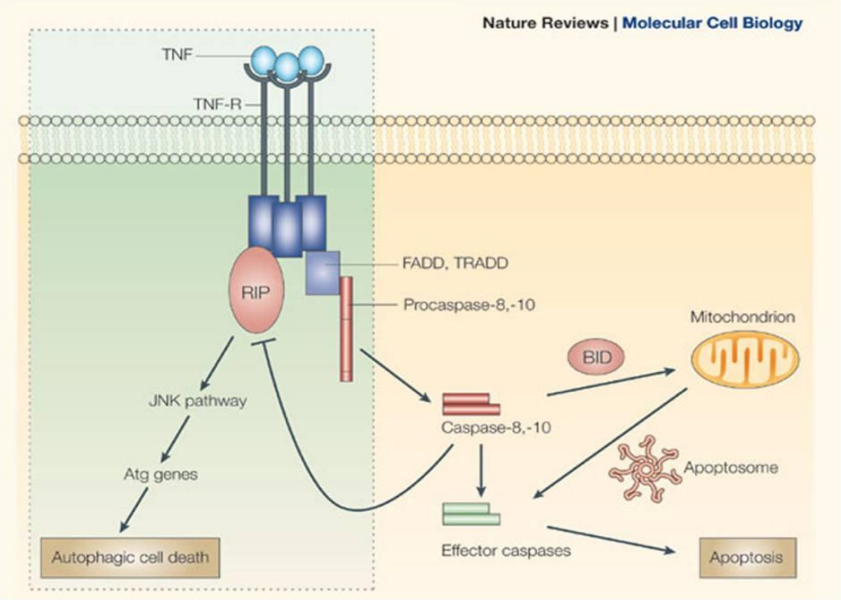


Fig. 2.2 - Tumour-necrosis factor (TNF) is able to activate caspases and autophagy mechanisms, involved in complementary death pathways in cells (from Baehrecke E.H., 2005).

The proximity of capase-8 and caspase-10 leads to the activation of either the mitochondrial cell death pathway involving BCL2 family members, such as BID, and the apoptosome (comprising caspase-9, apoptotic protease-activating factor 1 (APAF1) and cytochrome *c*) (Li et al., 1997), or the activation of effectors caspases and apoptosis (Shiokawa et al., 1997).

As caspases can be enhanced by TNFα, and have been proposed to have a role in early steps of myofibrillar proteolysis associated with pathological (Belizario et al., 2001; Du et al., 2004; Nakagawa et al., 2000) and certain physiological conditions (Dirks and Leeuwenburgh, 2002), there is sufficient evidence to suggest that apoptosis plays an important role in skeletal muscle loss.

If caspase-8 is inhibited, RIP, Jun N-terminal kinase (JNK) and Atg genes (autophagy related genes) regulate autophagic cell death. A model has emerged in which autophagic cell death and apoptosis might complement each other, as active caspase-8 cleaves RIP and prevents it from activating autophagic cell death (Devin et al., 2003).

Cytokines, through different proteolytic pathways seem to represent important regulatory molecules in the complex network of signals that control muscle protein breakdown. TNFα has been associated with activation of non-lysosomal ubiquitin-dependent proteases, and apoptosis via receptor–ligand mediated activation of the caspase cascades, but these pathways do not represent the unique effector pathways associated with this cytokine. Indeed, novel pathways have been explored, in which TNFα inhibits also muscle differentiation through activation of caspases in the absence of apoptosis (Coletti et al., 2002).

2.2.2 Growth factors

Growth factors are a family of signaling molecules positively involved in muscle homeostasis by promoting cell growth, proliferation and cell differentiation**.** Thus, when their action is suppressed or down-regulated, the disturbance of balance between anabolic and catabolic processes results in muscle atrophy.

Indeed, levels of Insulin-like growth factor (IGF), among the best characterized muscle growth-promoting factors, are extremely lowered (Szewczyk et al., 2005) in the same muscle wasting conditions in which high levels of TNFα are detectable (Ferrucci, 2003; Stewart, 2004).

The insulin-like growth factors (IGFs) constitute a complex regulation system produced mainly in the liver under the control of Growth Hormone (GH); however, its expression is located also in skeletal muscle under two forms, IGF-I and IGF-II, suggesting a paracrine/autocrine role of IGF in positively regulating muscle growth (Hameed et al. 2004).

The importance of these growth factors in growth and development is well established. Indeed, binding of IGF-I to its receptor induces a conformational change

in the associated tyrosine kinase, resulting in its trans-phosphorylation, the subsequent phosphorylation and activation of insulin receptor substrate 1 (IRS-1), and of downstream signaling molecules critical for hypertrophy, such as phosphatidylinositol 3-kinase (PI3K) and Akt (Stitt et al., 2004; Rommel et al., 2001; Glass, 2003).

An increase in muscle work stimulates the expression of IGF-I, which has been shown to be sufficient to induce hypertrophy through autocrine and paracrine mechanisms (Harridge, 2003).

Akt (also called PKB, protein kinase B) is a family of serine/threonine kinases consisting in three isoforms Akt1 (or PKBα), Akt2 (or PKBβ) and Akt3 (or PKBγ) with distinct functions. Indeed, Akt1 and Akt2 are highly expressed in skeletal muscle, whereas Akt3 is preferentially expressed in brain (Yang et al., 2004). Moreover Akt isoforms differ in their activators: Akt1 is preferentially stimulated by IGF-I, and Akt2 by Insulin (Sandri, 2008).

After translocation to the membrane, Akt can be phosphorylated/activated by phosphoinositide-dependent protein kinase 1 (PDK1) on Th308, and by the mTOR/Rictor complex (TORC2) on Ser473, for a complete activation (Kozma and Thomas, 2002; Sarbassov et al., 2005; Bodine et al., 2001).

After activation of PI3K/Akt intracellular signaling through the IGF-I receptor, two important targets involved in muscle hypertrophic response are regulated: mammalian target of rapamycin (mTOR) is activated, and glycogen synthase kinase 3β (GSK3β) is inhibited (Rommel et al., 2001). In addition, IGF-I receptor-mediated activation of PI3K/Akt leads to the inhibition of forkhead box O (FoxO) transcription factors and lowered expression of muscle specific ubiquitin ligases (i.e., atrogin 1/MAFbx-1 and MuRF1); this pathway is considered a crosslink between the protein synthesis machinery and the muscle atrophy response (Stitt et al., 2004) (Figure 2.3).

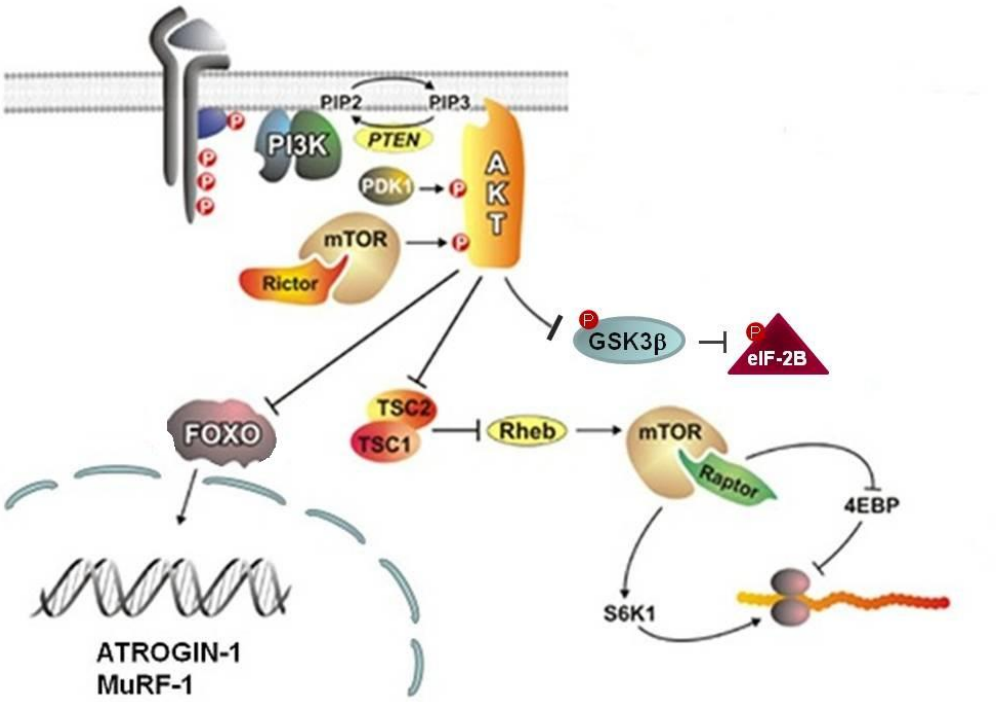


Fig. 2.3 – Insulin-like growth factor 1-mediated signaling pathways relevant for muscle mass maintenance (adapted from Plas and Thompson, 2005).

Akt and hypertrophy **–** Activation of Akt results in inhibition of Tuberous Sclerosis 2 (TSC2) function through direct phosphorylation (Figure 2.3) (Dan et al., 2002; Inoki et al., 2002; Manning et al., 2002; Potter et al., 2002), allowing Rheb to accumulate in a GTP-bound state. Rheb-GTP activates the protein kinase activity of mTOR in complex with the Raptor adapter protein (mTORC1). So, the activation of mTORC1 by Akt is an indirect activation.

mTORC1, in turn, phosphorylates downstream regulators of protein translation: 4E-BP1 and p70-S6-K1. Active S6K1 can in turn stimulate protein synthesis through activation of S6 Ribosomal protein, a ribosome component**,** and other components of the translational machinery. In normal conditions, 4E-BP1 binds to the translation initiation factor eIF4E, but, upon phosphorylation by mTORC1, it releases it, allowing its function (Bhaskar and Hay, 2007).

In recent years, a much more complex role is emerging for p70-S6K1. Indeed, knockout mice for this kinase have smaller muscle fiber size, and IGF-I/Akt pathway is down regulated (Ohanna et al., 2005); on the other hand the balance of protein synthesis and degradation is maintained (Mieulet et al., 2007).

In addition, p70-S6K1 is able to negatively regulate the IGF-I pathway, via phosphorylation/inhibition of insulin receptors substrate proteins (Um et al., 2004; Aguilar et al., 2007).

mTOR participates to the composition of two multiprotein complexes: mTORC1 the function of which is described above, and mTORC2.

mTORC2 kinase phosphorylates Akt on serine residue Ser473, thereby stimulating the phosphorylation of Thr308 by PDK1, resulting in full Akt activation (Guertin and Sabatini, 2007). This mTORC2-mediated activation seems to be related with the control of actin cytoskeleton organization (Sarbassov et al., 2004), and possibly with the control of cell size and cell cycle arrest (Foster and Fingar, 2010).

Moreover, p70-S6K1 is also able to negatively regulate mTORC2, by phosphorylating the Rictor adapter protein at Thr1135 (Treins et al., 2010).

Another important substrate of IGF-I-activated pathway, that has been shown to be involved in muscle hypertrophy (Glass, 2003), is Glycogen synthase kinase 3β (GSK3β). This protein normally inhibits eIF-2B factor, but its phosphorylation by Akt results in enhancing the protein translational process (Hardt and Sadoshima, 2002).

As mentioned above, the maintenance of skeletal muscle mass is determined by a fine balance between protein synthesis and protein degradation. As Akt activity is increased during skeletal muscle hypertrophy, many studies were performed to know its status in atrophy conditions.

Akt activity was reduced in several models of muscle atrophy, such as amyotrophic lateral sclerosis (ALS), sepsis induced by lipopolysaccharide (LPS), or denervation, as well as treatments with lovastatin, dexamethasone and serum starving (Léger et al., 2006; Crossland et al., 2008; Pallafacchina et al., 2002; Hanai et al., 2007; Sandri et al., 2004). In humans, Akt activity was reduced in both ALS and disuse atrophy conditions (Léger et al., 2006 a,b).

Akt and atrophy – Akt signaling, as we have already said, is involved not only in muscle hypertrophic pathways, but also in the inhibition of muscle atrophy signaling pathways.

Indeed, Akt-mediated phosphorylation of FoxO leads to its nuclear exclusion and inhibition of the Forkhead transcriptional program, which is necessary for the induction of both MuRF1 and atrogin-1/MAFbx atrogenes (Sandri et al., 2004; Stitt et al., 2004).

In mammalian skeletal muscle, there are three FoxO transcription factors: FoxO1, FoxO3 and FoxO4, all of them sharing the same "forkhead box" which contains the DNA-binding domain.

During muscle atrophy, a reduced Akt activity induces the dephosphorylation of FoxOs and their subsequent translocation to the nucleus, which results in "atrogenes" expression (Sandri et al., 2004; Stitt et al., 2004).

FoxO1 is sufficient to promote muscle loss without directly increasing the levels of atrogin-1, by blocking the inhibition of its upregulation exerted by IGF/PI3K/Akt signaling pathway (Kamei et al., 2004; Stitt et al., 2004). FoxO3 is a direct activator of atrogin-1 promoter in mouse muscles (Sandri et al., 2004). It was recently shown that FoxO4 is responsible for the increase in atrogin-1 following treatment with TNFα in mouse myotubes (Moylan et al, 2008).

FoxO factors mRNAs are upregulated in several conditions such as starvation (Kamei et al., 2003; Lecker et al., 2004), diabetes (Lee et al., 2004), heart failure (Schulze et al., 2005), cachexia and aging (Giresi et al., 2005; Kandarian and Jackman, 2006; Lecker et al., 2004). On the other hand, decreased levels of PI3K/Akt pathway activity are associated with skeletal muscle atrophy (Stitt et al., 2004).

Crosslinks between a purely hypertrophic pathway, IGF1/Aky/mTOR, and a purely atrophic one, FoxO pathway have been recently identified. Indeed, the activation of FoxO upregulates an effector of the mTOR pathway, 4E-BP1, and downregulates Raptor, the regulatory associated protein of mTOR, and thereby mTORC1 (Southgate et al., 2007). Therefore, if Akt is inactivated and FoxO transcriptional activity is induced in catabolic processes, we can observe in parallel a clear inhibition of protein synthesis. Moreover, FoxO can be activated by different regulation systems, most of which are Akt-independent (Huang and Tindall, 2007).

From the above, it is clear that considering two distinct processes, protein synthesis and, on the other hand, protein degradation, is misleading. It is important to emphasize that these two biochemical processes are not independent of each other, but finely coordinated by a complex signaling network, the core of which is constituted by Akt protein kinase.

2.2.3 Akt pathways and autophagy

Eukaryotic cells have developed some mechanisms to coordinately regulate the balance between hypertrophy and atrophy in response to cellular physiological conditions and environmental stress.

In atrophied muscles, the ubiquitin-proteasome pathway catalyzes the accelerated degradation of myofibrillar proteins (Lecker et al., 2006), but several evidences about the importance of another catabolic system, the autophagic/lysosomal pathway are emerging.

Autophagy is a catabolic process involving protein degradation through the lysosomal machinery that plays an important role in survival during unfavorable growth conditions, and for this reason it is tightly coupled to the regulation of cell growth.

Autophagy is negatively regulated by the phosphatidylinositol-3-kinase (PI3K) signaling pathway in many animal cells (Petiot et al., 2000; Melendez et al., 2003; Rusten et al., 2004). In particular, Akt is again the central negative regulator of this catabolic mechanism, through its downstream mTOR and FoxO pathways.

As mentioned above, Akt is known to activate mTORC1, the key regulator of translation and ribosome biogenesis in yeast and mammalian cells. As for mTORC2, it is involved in the regulation of phosphorylation/activation of Akt, protein kinase Cα, serum- and glucocorticoid-induced protein kinase-1 (Sarbassov et al., 2005; Sarbassov et al., 2006; Garcia-Martinez et al., 2008).

Akt positively regulates mTORC1, which has been shown to act as an inhibitor of autophagy in normal condition, but also an enhancer of autophagy in starvation conditions. mTORC2 is thus expected to act as a negative regulator of autophagy.

Despite significant progress in the autophagy study field, the mechanism by which mTORC1 regulates autophagy remains unclear, but it involves a protein complex that is associated with the autophagy-related genes, Atg, a family of kinases, which has been studied primarily in yeast (Kim et al., 2002; Kamad et al., 2000).

Autophagy induction by mTORC2 inhibition is mediated mainly by FoxO3, a transcription factor downstream of Akt, which is involved in autophagy gene expression (Mammucari et al., 2007; Zhao et al., 2007).

Thus, FoxO signaling is not only involved in the Ubiquitin-proteasome pathway by regulation of *Atrogenes*, but also in autophagy stimulation (Zhao et al., 2007). In particular, FoxO3 seems to be essential for activation of autophagy in C2C12

myotubes, in which lysosomal proteolysis is 3-fold higher than proteasomal degradation, and in mouse muscle (Zhao et al., 2007; Mammucari et al., 2007).

In confirmation, FoxO3 was shown to directly increase the transcription of several autophagy related genes, such as *LC3b*, *Gabarapl1*, *Atg12l*, *PI3KIII*, *Ulk2*, *Atg4b* and *Beclin1* (Mammucari et al., 2007), both in C2C12 myotubes and in mouse muscle after denervation or fasting (Zhao et al., 2007). Nonetheless, how FoxO3 exactly activates the autophagic pathway is still unclear.

All these data, taken together, demonstrate that the autophagic/lysosomal and the proteasomal pathways are coordinately regulated in muscle by FoxO action, and are both important in muscle atrophy occurrence.

An hypothesis suggested by Goldberg's group is that the UPP might be involved in myofibrillar degradation, carrying a loss of mass and strength, while autophagy would be involved in the loss of mitochondria and endurance (Zhao et al., 2008).

Interestingly this group has followed the fate of cell proteins, after appropriate labeling, through the two different protein degradation systems. It observed that, *in vitro*, using specific inhibitors of the degradation systems, the lysosomal system preferentially degrades the long-lived proteins, whereas the ubiquitin-proteasome system degrades the short-lived proteins.

Indeed, among the long-lived cell proteins, in control myotubes, 40% of degradation involves the lysosomal system, and 50% the ubiquitin-proteasome system. About 70% of FoxO3-induced protein degradation is thought to occur through the autophagic pathway. In contrast, *in vivo*, in adult muscle richer in myofibrils, the degradation mainly occurs by ubiquitin-proteasome pathway, both in growth and atrophy conditions (Mammucari et al., 2007; Solomon and Goldberg, 1996; Zhao et al., 2008). This suggests that the use of one of these two coordinately regulated systems depends on the type of cell, and varies in different physiological states.

2.2.4 Myostatin and Glucocorticoids

Myostatin, originally named "growth and differentiating factor 8" (GDF-8), is a secreted TGFβ family member functioning as a negative regulator of muscle growth and development.

Myostatin is produced primarily in skeletal muscle cells**,** circulates in the blood and acts on muscle tissue, by binding to tyrosine kinase receptors called the activin IIB receptors (Lee and McPherron, 2001). After activation of its receptors, the signal of

myostatin is relayed into the cytoplasm by phosphorylation of SMAD transcription factors that translocate into the nucleus to modify gene expression (Seuntjens et al., 2009). However, the downstream targets of myostatin pathway and their role in muscle regulation are still to be determined.

In murine skeletal muscle, this factor was found to be highly expressed during the later stages of development (Lee, 2004) and in adulthood (McPherron et al., 1997), and seems to be involved in the growth balance between fast and slow muscles (Roberts and Goetz, 2001).

Targeted disruption of myostatin gene expression in mice leads to dramatic increases in skeletal muscle mass due to muscle fiber hyperplasia and/or hypertrophy (McPherron et al., 1997; Grobet et al., 2003)

Moreover, natural inactivating mutations of the myostatin gene in cattle are associated with a double-muscling phenotype (Grobet et al., 1997; McPherron et al., 1997).

In atrophied mouse soleus (Type I fibers), myostatin mRNA levels were undetectable, whereas transient upregulation of myostatin mRNA was detected in atrophied fast twitch muscles. Thus, myostatin may modulate gene expression controlling muscle fiber type (Carlson et al., 1999).

In vitro studies on C2C12 demonstrate that myostatin treatment controls cell cycle progression and inhibits myoblast proliferation by upregulating p21 expression and decreasing the phosphorylation of retinoblastoma 1 protein (pRb). Myostatin treatment also perturbs myogenic differentiation events by decreasing MyoD, Myf5 and myogenin levels, whereas myostatin silencing leads to a considerable fusion index increment (Langley et al. 2002; Rios et al. 2002; Joulia et al. 2003).

In vivo gene delivery experiments on adult muscle showed that myostatin induces a severe atrophy probably due to a synergistic work of this factor with other pathways (Durieux et al., 2007).

In vitro, myostatin is shown to contribute to the FoxO-induced atrophic mechanism by blocking the IGF-1/Akt pathway and activating FoxO1, without requiring NF-kB action (McFarlane et al., 2006). The atrophic effect of myostatin is confirmed in another study performed on cardiac cells, in which it is shown to be able to inhibit Akt (Morissette et al., 2006), supporting the idea that myostatin pathway is closely related with Akt/FoxO signaling.

Because of its property of muscle mass modulation, myostatin has received great attention as a potential therapeutic target for treatment of muscle wasting and weakness.

Most important pathophysiological catabolic conditions are also associated with another factor, which is a high level of glucocorticoids (Hasselgren, 1999; Lang et al., 2002).

The experimental administration of high doses of glucocorticoids has been shown to induce muscle atrophy, mainly due to the stimulation of muscle proteolysis. This glucocorticoid-induced protein degradation is mediated by the activation of both the ubiquitin-proteasome and lysosomal pathways (Dardevet et al., 1998; Kanda et al., 1999). In particular, the muscle-specific E3-ligases atrogin-1 and MuRF1, and the lysosomal enzyme cathepsin L, are highly stimulated by glucocorticoids (Hasselgren et al., 1999; Auclair et al., 1997; Komamura et al., 2003).

Furthermore, the inhibition of glucocorticoid action by antagonists of the glucocorticoid receptor markedly reduces the loss of muscle mass encountered in several catabolic states (Lang et al., 2002; Schakman et al., 2008). Dexamethasone-induced muscle atrophy is associated with a dose-dependent induction of muscle myostatin mRNA and protein expression (Ma et al., 2003), and also with MyoD degradation by the ubiquitination pathway (Tintignac et al., 2005; Su net al., 2008), consistent with MyoD being an atrogin-1 substrate. Increased myostatin expression has been also reported in several models of muscle atrophy such as immobilization, microgravity, and burn injuries, in which glucocorticoids play a major role (Lalani et al., 2000; Kawada et al., 2001; Lang et al., 2001).

These observations indirectly suggest that myostatin plays a role in glucocorticoid-induced muscle loss. However, it is not yet directly established whether the presence of myostatin is required for the expression of glucocorticoid-induced catabolic effects. Moreover, the potential role of myostatin in the glucocorticoid activation of ubiquitin-proteasome and lysosomal proteolytic pathways is still unknown.

2.2.5 Dystrophin-glycoprotein complex (DGC)

The Dystrophin-glycoprotein complex (DGC) is a multicomponent complex with a mechanical role of connection between cytoskeleton, sarcomembrane and extracellular matrix. It includes different gene products: dystrophin, dystroglycans, four

sarcoglycans, sarcospan, syntrophins, nitric oxide synthetase (nNOS) and dystrobrevin.

This complex is important for correct transduction of myosin movements on actin to the extracellular matrix, in spite of cellular deformation and shortening resulting from continuous contractions in both heart and skeletal muscles. Throughout this process, the contractile machinery inside the myofibers must remain intimately connected with the membrane thanks to the binding of dystroglycan, and with extracellular matrix, thanks to laminin-2.

In the last years, in addition to this purely structural point of view, the idea of a possible role of this complex in signaling pathways has emerged. In fact, the breakage of laminin-2/dystroglycan binding has been shown to prevent Akt kinase activation, one of the master regulators of muscle mass (Langenbach and Rando 2002).

The mechanism through which this inhibition is possible is still unclear, but studies performed on animal models of tumor-induced cachexia showed a strong perturbation of the myofibrillar component connected with a loss of DGC integrity. Indeed, muscle wasting became more evident when the tumors were injected in the mdx animal model, lacking dystrophin expression, whereas, using an animal model overexpressing dystrophin, muscle loss was less pronounced compared to both wt and mdx mice, showing that dystrophin expression could help counteracting muscle wasting events. (Acharyya et al., 2005).

Furthermore, neuronal NOS (nNOS), which is normally bound to the DGC, dyslocates to cytoplasm when the DGC is disrupted, as it occurs in dystrophic muscle. Cytoplasmic free nNOS is able to activate the FoxO3-mediated transcription of atrogenes (Suzuki et al., 2007). This observation evidences a direct link between DGC and muscle atrophy.

2.2.6 Satellite cells and muscle remodeling

Satellite cells are multipotent cells presenting several characteristics of muscle stem cell (Zammit and Beauchamp, 2001; Dhawan and Rando, 2005; Zammit et al., 2006).

Satellite cells are characteristically located adjacent to the muscle fibers. They occupy a depression of the fiber between the plasma membrane and the basal lamina that surrounds each fiber. They are characterized by a very small nucleus, a high ratio nucleus-cytoplasm, few organelles and a high ratio of heterochromatin/euchromatin, suggesting a reduced mitotic and metabolic activity. While, after activation, the

volume of cytoplasm increases, the Golgi apparatus, the sarcoplasmic reticulum, ribosomes and mitochondria become visible and heterochromatin reduces (Schultz and McCormick, 1994).

During muscle development and growth, increase of muscle fiber size takes place through the inclusion of activated satellite cells in fibers, which maintain nucleus-cytoplasm ratio constant (Hawke et al., 2005). In adult skeletal muscle, satellites cells are quiescent, but they can be activated in response to specific diseases or injury, and fuse to form myotubes that differentiate into myofibers and regenerate lost muscle tissue.

The regeneration process activates an inflammatory response with a consequent release of many growth factors involved in the remodeling. Such factors, like hepatocyte growth factor (HGF), fibroblast growth factor 6 (FGF6), interleukin-4 (IL-4), interleukin 6 (IL-6), IGF1, play a key role during the activation, proliferation and differentiation events of the satellite cells (Allen et al. 1995; Husmann et al. 1996; Horsley et al. 2003). In particular, IGF1 seems to be the most important factor linking muscle hypertrophy and satellite cells. Satellite cells isolated from IGF1 overexpressing mice display an increased regenerative potential, mediated by FoxO1 and the activity of p27kip1 (Machida et al. 2003; Musaro et al.2001).

IGF1 and Notch are extensively studied pathways because, due to their effects on activation and proliferation of satellite cells, they could be important to slowdown age-related muscle wasting (Conboy and Rando 2002; Sherwood et al. 2004).

Specific proteins such as c-Met (Cornelison and Wold, 1997, Tatsumi et al., 1998), M-cadherin (Cornelison and Wold, 1997), MNF (myocyte nuclear factor) (Garry et al., 1997), VCAM-1 (Vascular Cell Adhesion Molecule-1) (Jesse et al., 1998), NCAM (Neural Cell Adhesion Molecule) (Covault and Sanes, 1986), CD34 (Beauchamp et al., 2000), Pax3 and Pax7 (Seale et al., 2000; Beauchamp et al. 2000; Reimann et al. 2004; Zammit et al. 2006) are expressed in satellite cells.

These proteins may be differently expressed in activated satellite cells, suggesting functional differences, or distinct stages of specification, in the myogenic cell lineage, in fact, the single satellite cells are characterized by proliferative (Beauchamp et al., 1999) fuse (Rouger et al., 2004) and differentiative intrinsic capacity (Zammit et al., 2004). Besides, some cells, part of the satellite cell compartment, may also generate non-muscular lines such as fibroblasts, osteocytes and adipocytes (Shefer et al., 2004, Asakura et al., 2001).

Chapter 3
Sphingolipids

3.1 Sphingolipids structure and metabolism

Sphingolipids are a family of ubiquitous membrane lipids that contribute to the regulation of the fluidity and the sub-domain structure of the lipid bilayers (Futerman and Hannun, 2004).

For a long time it has been thought that this was their only function, but in recent years it has become increasingly clear that these lipids are involved in signaling of important life processes such as growth, proliferation, cell death and cell differentiation (Futerman and Hannun, 2004; Kok and Sietsma, 2004; Ogretmen and Hannun, 2004; Reynolds et al., 2004; Fox et al., 2006; Modrak et al., 2006; Fernandis and Wenk, 2007; Eyster, 2007).

This family of bioactive molecules include Ceramide, Sphingosine, Sphingosine-1-phosphate, Ceramide-1-phosphate and other anphipathics molecules that have a similar structure (Figure 3.1), with an hydrophobic region consisting of a sphingoid long chain base (generally sphingosine or sphinganine) to which is attached, to carbon 2, a fatty acid.

Different Sphingolipids arise from the type of head group substituted at carbon 1, i.e. in the hydrophilic region. Attachment of phosphate forms sphingosine-1-phosphate

(S1P) and ceramide-1-phosphate, a simple hydroxyl group forms a ceramide, phosphorylcholine forms sphingomyelin, and a carbohydrate forms Glycosphingolipids (GSLs) (Lahiri and Futerman, 2007).

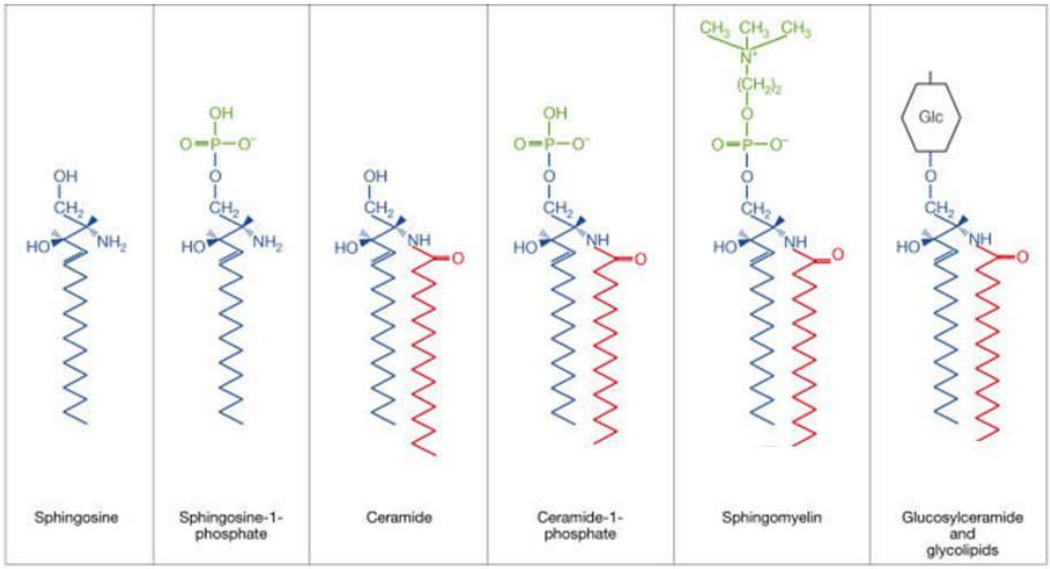


Figure 3.1 – The structure of some representative sphingolipids (adapted from Futerman and Hannun, 2004).

The complexity of Sphingolipids is thus based on three structural components, the sphingoid base, the fatty acid and the head group.

Ceramide is considered the backbone of all complex Sphingolipids and is at the centre of a complex network of highly regulated metabolic pathways (Figure 3.2).

This lipid can be mainly produced *de novo*, or from hydrolysis of sphingomyelin, but also from the salvage pathway.

In *de novo* pathway, the first step is the condensation of a serine and palmitate by action of serine palmitoyl-CoA transferase (SPT), and a series of subsequent steps which generate dihydrosphingosine, dihydroceramide and finally ceramide by the action of dihydroceramide desaturase (Hannun and Obeid, 2002; Linn et al., 2001; Dolgachev et al., 2004).

Sphingomyelin present in the plasma membrane can be cleaved by sphingomyelinase (SMase) in Ceramide (Cer) (Marchesini and Hannun, 2004; Clarke and Hannun, 2006 Clarke et al., 2006), which in turn can be then deacylated by ceramidase (CDase) to form Sphingosine (Sph) and fatty acid (Park and Schuchman, 2006).

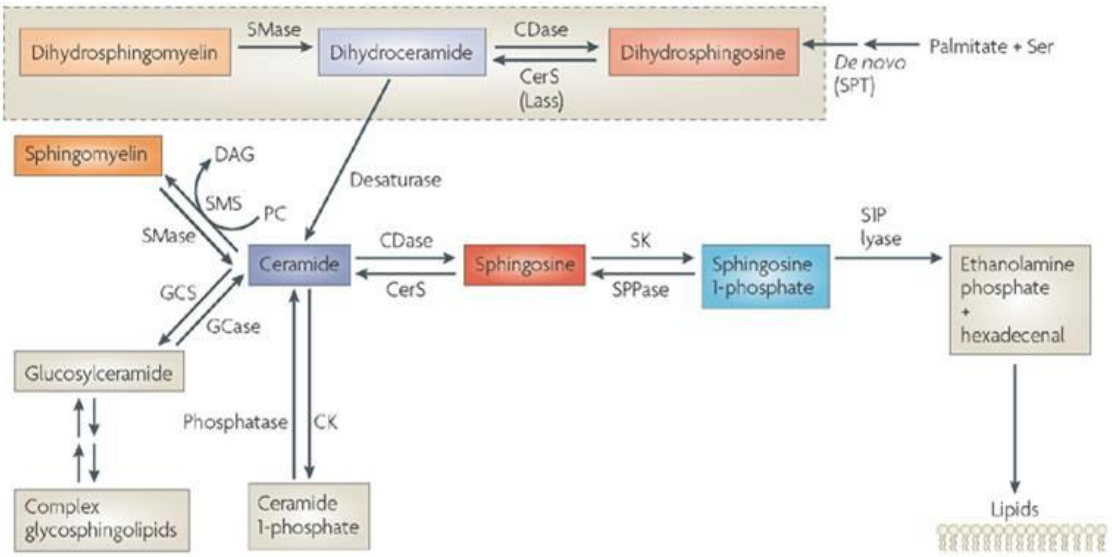


Figure 3.2 – Sphingolipid pathway (adapted from Hannun and Obeid, 2008).

The phosphorylation of sphingosine by sphingosine kinase (SphK), an ubiquitous enzyme present in the cytosol and endoplasmic reticulum, can generate sphingosine-1-phosphate (S1P). The inactivation of S1P may be ensured by S1P lyase, which irreversibly degrades it into ethanolamine-phosphate and a long chain aldehyde (hexadecenal), or by dephosphorylation performed by sphingosine 1-phosphate phosphatase (SPP), giving rise to sphingosine (Hait et al., 2006; Xu et al., 2006).

Ceramide can be also metabolized by the action of ceramide kinase (CK), or sphingomyelin synthase (SMS) to form respectively ceramide-1-phosphate and sphingomyelin (Sugiura et al., 2002; Van der Luit et al., 2007).

Another possibility for ceramide is to be converted into glucosylceramide (GlcCer), then used for the synthesis of more complex glycosphingolipids, which might essentially play crucial roles in determining the lipid composition of the plasma membrane (D'Angelo et al., 2007).

Ceramide, due to its hydrophobic nature, is unable to traffick autonomously through the cytosol (Venkataraman and Futerman, 2000), and to be delivered from its site of synthesis (ER or plasma membrane) to other subcellular compartments for further metabolism. It needs a facilitated mechanism which can be either vesicular or non-vesicular (Hanada et al., 2003; Kumagai et al., 2005; D'Angelo et al., 2007; Kudo et al., 2008). However, sphingosine and S1P have a sufficient acqueous solubility to move between membranes (Futerman and Hannun, 2004).

From the above, it is clear that the sphingolipid pathway is a complex and closely interconnected network of lipids, the amount of which can be balanced by a series of reversible reactions catalyzed by different enzymes, able to orchestrate cellular responses by regulating this metabolism.

Many different stresses, such as cytokines, environmental stresses or chemotherapeutic agents, can induce changes in ceramide levels by regulating different enzymes, which can be divided in two groups: enzymes inducing ceramide accumulation, and, on the other hand, enzymes promoting ceramide metabolization.

Sphingomyelinases **-** Ceramide, as mentioned above, can be produced by hydrolysis of SM, mediated by SMases. Five types of SMases are identified at present: the ubiquitous acid lysosomal SMase (A-SMase), the Zn^{2+}-dependent secreted form of A-SMase present in serum, the neutral, membrane-bound Mg^{2+}-dependent SMase (N-SMase), a neutral, Mg^{2+}-independent SMase, and an alkaline SMase (Levade et al. 1999; Andrieu-Abadie et al. 2001; Goni and Alonso 2002). Among these, A-SMase and Mg^{2+}-dependent N-SMase are activated in response to apoptotic and other stimulations to cause SM hydrolysis and subsequent ceramide generation (Chatterjee 1999; Goni and Alonso 2002).

A-SMase is located primarily in the endosomes an lysosomes, where it degrades SM in ceramide, and this specific ceramide can also be deacylated by the function of ceramidase, which appears to be physically associated with this SMase (Saad et al., 2007; Liu et al., 2008). Lysosomal A-SMase-produced ceramide seems to be involved in lysosomal cathepsins D regulation, proteolytic activation of BID protein, and subsequent apoptotic response (Heindrch et al., 2004). Furthermore, that A-SMase-dependent ceramide generation induces the recruitment of caveolin-1 to PI(3)K-associated receptor complexes within lipid raft microdomains, leading to inhibition of lipid kinase activity (Zundel et al. 2000).

The Mg^{2+}-dependent N-SMase is located primarily in plasma membrane (Marchesini and Hannun, 2004; Segui et al., 2001), and the best characterized targets of ceramide produced through this enzyme are the ceramide-activated protein phosphatases (CAPPs), which, when directly activated by ceramides, induce dephosphorylation of several substrates including Bcl-2 family proteins, caspases, c-Jun N-terminal kinase (c-JNK), Retinoblastoma gene product (Rb) (Pettus et al., 2002; Thon et al., 2005), Akt (Zhou et al., 1998) and protein kinases C (Lee et al., 2000; Kajimoto et al., 2001).

In addition, ceramide produced by this way, has been shown to activate a specific PKC, PKCζ, which is implicated in membrane potential regulation, Akt inhibition and pro-apoptotic functions (Bourbon et al., 2002; Wang et al., 2005).

Serine palmitoyltransferase - Another key mechanism emerging in eukaryotic cells for regulating the levels of ceramide and other sphingolipids is the *de novo* synthesis pathway, which generates ceramide in the ER.

This pathway is able to respond to changes in serine and palmitate concentrations by the action of serine palmitoyltransferase. Indeed, an accumulation of one of these two substrates results in a rapid activation of de novo synthesis pathway and consequently accumulation of ceramides (Zhou et al., 1998; Cowart and Hannun, 2007).

This excess of ceramide leads to activation of PP2 phosphatase and results in dephosphorylation/inactivation of Akt, a key mediator of Insulin signaling and metabolic control of skeletal muscle, as mentioned above (Cowart and Hannun, 2007).

Furthermore, ceramides generated in the ER can also act in the nucleus, since the nuclear membrane is a continuous structure of the ER membranes. Here, *de novo* produced ceramide can activates PP1, which then dephosphorylates serine/arginine-rich proteins (SR-proteins), inducing the alternative splicing of pro-apoptotic proteins Bcl-XS or caspase-9, and related apoptotic response (Chalfant et al., 2001, 2002).

Another recently identified nuclear target of ceramide includes a pro-survival protein telomerase, which catalyzes the elongation/maintenance of telomeres at the end of chromosomes and which is repressed by ceramide (Blackburn, 2005).

So, a very important thing to emphasize is that most enzymes of sphingolipid metabolism have a specific subcellular localization, and this, coupled with the poor solubility of sphingolipids, causes the compartimentation of signaling effects of the different generated sphingolipid.

Ceramidases (CDase) - Ceramidases comprise a heterogeneous family of enzymes the main function of which is to breakdown ceramides to sphingosine. These enzymes are critical in regulating not only ceramides levels, but also the generation of both Sph and S1P in cells. Indeed, the phosphorylation of Sph is the only pathway for the formation of cellular S1P, which is highly dependent on the availability of Sph generated by ceramidases. Therefore, the action of a ceramidase can lead to an alteration in the levels of ceramides, Sph, and S1P, thereby controlling cellular

responses mediated by these bioactive lipids (Nikolova-Karakashian and Merrill, 2000; el Bawab et al., 2002).

Five mammalian ceramidases have been cloned and biochemically characterized: Acid CDase, Neutral CDase, Alkaline CDase1, Alkaline CDase2, Alkaline CDase3, but the knowledge about this enzyme is still very limited.

Human Acid CDase (hAC) is localized to the lysosomes, but the tissue distribution of hAC mRNA remains unclear (Ferlinz et al., 2001). It can also be secreted extracellularly (Romiti et al., 2000). The mouse Acid CDase (mAC) mRNA is ubiquitous and highly expressed in kidney, lung, heart, and brain, but is at relatively low levels in spleen, skeletal muscle, and testes (Li et al., 1998). The human form of the enzyme has a pH optimum of 4.5, and its best substrates are the unsaturated medium-chain ceramides (Bernardo et al., 1995; He et al., 2003).

The human Neutral CDase (hNC) is ubiquitously espressed (El Bawab et al., 2000), whereas the mouse Neutral CDase (mNC) mRNA is expressed at high levels in kidney, liver, and heart, at medium levels in brain and lung, with low levels of expression in spleen, skeletal muscle, and testes (Tani et al., 2000). mNC is mainly located to the plasma membrane (Tani et al., 2003). The substrate specificity of hNC has not been established, but it is known to have a broad pH optimum, ranging from pH 7 to 9 (El Bawab et al., 2000), at variance with both mouse and rat enzymes, which have a pH optimum of 7.5. The NC activity does not require the presence of cations (El Bawab et al., 1999). mNC prefers long-chain ceramides as substrates (Tani et al., 2000).

Alkaline CDase 1 (ACER1) is located in ER, and is mainly expressed in skin, both in human and mouse (Sun et al., 2007; Mao et al., 2003). About human ACER1, no activity is reported with an acyl chain length less than C18, preferably unsaturated (Sun et al., 2007). This enzyme has a pH optimum of 8,5 and its activity is regulated by the content of Ca^{2+} in ER (Sun et al., 2007).

Alkaline CDase 2 (ACER2) has an high degree of similarity with ACER1; it is located in Golgi complex. The human form is ubiquitously expressed, but at higher levels in the placenta, while the murine form, nearly identical to human one, has still unclear cellular location and tissue distribution (Xu et al., 2006). Furthermore, human ACER2 prefers long- or very long-chain ceramides as substrates; it has a pH optimum between 7,5 and 8,5, and requires Ca^{2+} for its activity (Xu et al., 2006).

Human Alkaline CDase 3 (ACER3) is ubiquitously expressed at high levels, but mainly in the placenta, and is located in ER and Golgi complex (Mao et al., 2001). Similarly to the other two human Alkaline CDase, this enzyme prefers long-chain unsaturated ceramides as substrates. It has a pH optimum of about 9,5, and requires Ca^{2+} for its activity (Mao et al., 2001).

Due to differences in the substrate specificity, cellular location, tissue distribution, and expression levels, these ceramidases appear to have distinct roles in cellular responses. Indeed, current studies suggest that activation or upregulation of AC and NC mainly promote cell proliferation and survival, by attenuating ceramide signaling while augmenting S1P signaling, whereas inhibition or down-regulation of these enzymes induce cell growth arrest and/or apoptosis, by amplifying ceramide signaling while diminishing S1P signaling (Ehlert et al., 2007; Burek et al., 2001; Hara et al., 2004; Morales et al., 2007; Li et al., 2002; Osawa et al., 2005; Franzen et al., 2001; Tani et al., 2005; Kono et al., 2006).

ACER1 appears to have anti-proliferating and pro-differentiating roles in specific cell types, by controlling the generation of sphingosine and/or S1P (Sun et al., 2007; Houben et al., 2006). ACER2 has dual roles. Its activation or upregulation promotes cell proliferation and survival by generating S1P, while sphingosine is not aberrantly elevated by its action. On the other hand, ACER2 action may induce cell growth arrest and apoptosis by generating high cellular levels of sphingosine, the anti-proliferative and pro-apoptotic effects of which may exceed the mitogenic and anti-apoptotic effects of S1P (Mao et al., 2001). Therefore, the role of this ceramidase in cellular responses may be both cell type-specific and stimulus dependent. Unlike the other four ceramidases, ACER3 catalyzes the hydrolysis of unsaturated long-chain dihydroceramides, suggesting that it has a role in regulating the generation of dihydrosphingosine and its phosphorylated derivative, in addition to sphingosine and its phosphorylated derivative (Xu et al., 2006).

Sphingosine kinase (SphK) **-** Sphingosine kinase (SphK) converts sphingosine, produced from ceramide, into S1P, which promotes cell growth and survival. In mammals, two isoforms of SphK have been identified and cloned, SphK1 and SphK2, which have opposite biological functions. In fact, SphK1 promotes growth and cell survival (Olivera et al., 1999), while SphK2 promotes apoptosis (Maceyka, et al.,

2005). Interestingly, the location in the cell of the two isoforms seems crucial for their function.

SphK1 is mainly cytosolic, but in response to certain stimuli (hormones and cytokines), it is phosphorylated and translocated to the plasma membrane, where it can activate the signaling pathways associated with proliferation and cell survival (Pitson et al., 2005).

SphK2 is especially present in the inner leaflet of organelle membranes, but its presence has also been reported in the cytosol, nucleus and plasma membrane (Maceyka, et al. 2005). The level of SphK2 in the endoplasmic reticulum is critical for its pro-apoptotic function, whereas SphK1 artificially expressed in the ER switches from anti-apoptotic to pro-apoptotic factor (Maceyka et al., 2005). So, also in this case, the location of an enzyme is fundamental for it function: in the plasma membrane SphK produces a signal for growth and survival, while in the endoplasmic reticulum it activates a pro-apoptotic signal. *In vivo* studies conducted in knockout mice have also suggested that the two isoforms may have compensatory roles. SphK1/SphK2 double knockout mice do not survive, because of serious defects in the vascular and nervous systems development (Allende et al., 2004; Mizugishi, et al., 2005).

Sphingosine-1-phosphate phosphatase (SPP) **–** This enzyme converts S1P in sphingosine, which in turn can be N-acetylated into ceramide, according to what is called the "salvage pathway".

There are two isoforms of sphingosine-1-phosphate phosphatase, SPP1 and SPP2, but their expression pattern is largely overlapping (Johnson et al., 2003; Le Stunff et al., 2002).

The presence of both SPP1 and SPP2 in the ER suggests that they act by dephosphorylating intracellular S1P, then modulating S1P action within the cell. The expression of SPP1 increases the cellular levels of ceramide, but not of sphingosine, indicating a rapid conversion of this lipid in ceramide (Mandala et al., 2000; Le Stunff et al., 2002).

SPP1 seems to play an important role in the control of apoptosis, through the regulation of levels of S1P. Indeed, the inhibition of SPP1 expression leads to a resistance to apoptosis induced by different agents, such as TNFα (Johnson et al., 2003).

Sphingosine-1-phosphate lyase (S1PL) **-** This enzyme is responsible for the irreversible degradation of S1P in the only exit way of sphingolipid pathway.

Sphingosine-1-phosphate lyase is mainly located in the ER, although its expression in other organelles is not entirely excluded (Ikeda et al., 2004). The overexpression of S1P lyase is able to induce apoptosis in response to cellular stress stimuli, decreasing the amount of S1P, and then increasing the amount of ceramide (Reiss et al., 2004). This enzyme also regulates cell survival *in vivo*. Indeed, S1P lyase knockout Drosophila have important anomalies of apoptosis during both embryonic and postembryonic stages (Herr et al., 2003).

3.2 Role of principal Sphingolipids in cell biology

Ceramide, sphingosine and S1P are the best studied bioactive sphingolipids. They have been shown to be involved in many different cellular events, and interestingly, there are always more data to confirm that these three sphingolipids can have opposite roles in these processes (Figure 3.3).

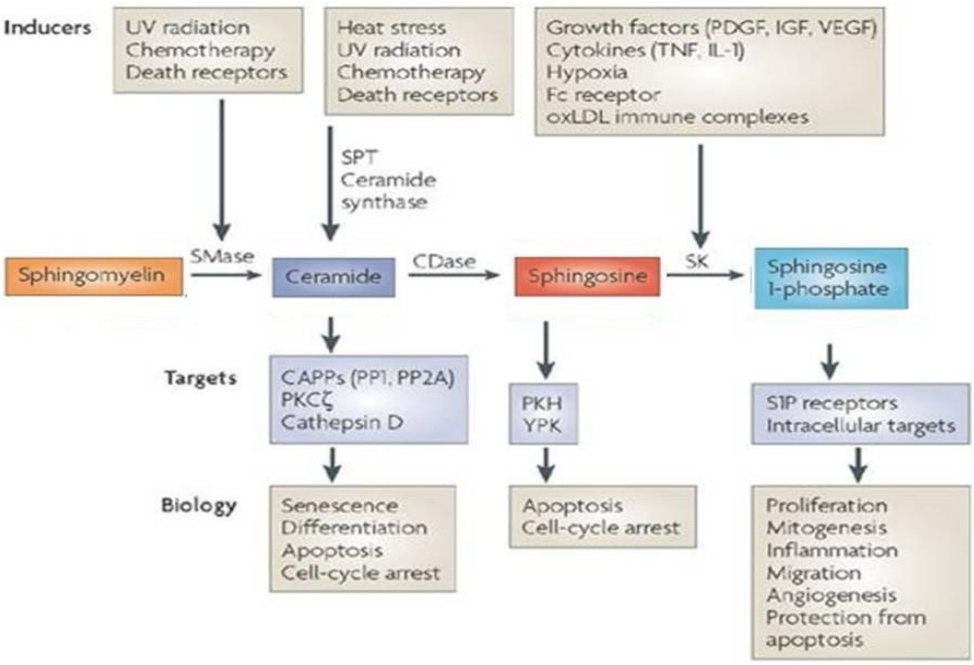


Fig. 3.3 – Role of Sphingolipids in cell biological responses (adapted from Hannun and Obeid, 2008).

In particular, ceramide and sphingosine are generally associated with growth arrest and cell death (Obeid and Hannun 2002; Kolesnick 2002). On the other hand, S1P is known to stimulate growth and survival in different cell types (Spiegel and Milstien, 2002).

It is now widely accepted that, since these lipids are metabolically interconnected, a dynamic balance between S1P and ceramide/sphingosine constitutes a "cellular rheostat", which can determine, among other things, cell survival or death (Spiegel and Milstien, 2002).

3.2.1 Ceramides

As mentioned above, ceramides, as bioactive lipids mediate various cell processes, such as apoptosis, cell growth arrest, differentiation and senescence.

The amount of these lipids can be increased, in mammalian cells, in response to different stimuli, such as pro-inflammatory cytokines (TNFα, IL-1β, FAS ligand, and interferon γ) ((Hanna et al., 2001; Rolz et al., 2003; Wakita et al., 1996), cancer chemotherapeutic agents (Charles et al., 2001), UV and ionizing-irradiation (Magnoni et al., 2002; Jaffrezou et al., 2001), vitamins or derivatives (vitamin D and retinoic acid) (Bektas et al., 2000; Geilen et al., 1996; Herget et al., 2000), and serum-deprivation (Colombaioni et al., 2002).

Many of these regulators of ceramide generation are inducers of apoptosis, suggesting a role for ceramide in apoptosis (Pettus et al., 2002).

The increase of intracellular levels of ceramide by administration of exogenous cell-permeable, short-chain ceramides (Obeid et al., 1993), by using inhibitors of ceramide metabolism enzymes, or by overexpression of ceramide-generating enzymes (Abe et al., 1995; Bielawska et al., 1996; Zhang et al., 1997), results in cell-growth arrest and/or apoptosis. In contrast, blocking increases in ceramide using inhibitors of de novo synthesis pathway prevents cell-growth arrest and/or apoptosis in response to these stimuli (Plo et al., 1999).

One of the mechanisms by which ceramide regulates apoptosis is via the induction of Fas capping, which involves the lateral segregation of cross-linked Fas ligand with its surface receptor at the SM-enriched plasma membrane of Jurkat T lymphocytes, necessary for its optimal function in cell death (Cremesti et al., 2001). Whereas, cells

resistant to ceramide and CD95/Fas-induced apoptosis, have a defect in apoptotic signaling upstream of the mitochondria (Raisova et al., 2000).

Recently, a new aspect of ceramide signaling in cell adaptation to stress stimuli has been revealed: the role of ceramide in induction of autophagy.

Ceramide has been shown to trigger autophagy by dissociation of Beclin-1/Bcl-2 complex in transfected MCF-7 and HL-29 cells. Bcl-2 protein act as an anti apoptotic factor by interacting with the autophagy protein Beclin-1 (Pattingre et al., 2005) and there are evidences that ceramide may use two strategies to perform this dissociation: by increasing the phosphorylation of Bcl-2 via JNK1 (Saslowsky et al., 2008; Kurinna et al., 2004), and by favoring the BNIP3-Bcl-2 complex rather than the Beclin 1-Bcl-2 complex (Daido et al., 2004; Pattingre et al., 2009).

The role of ceramide in induction of G0/G1 cell cycle arrest is well characterized and can be linked to the activation of the retinoblastoma gene product (Rb) (Dbaibo et al., 1995), or to a specific ceramide-mediated inactivation of cyclin-dependent kinase cdk2, through activation of a phosphatase (Lee et al., 2000). It has been shown *in vivo* that, using ceramide coated balloon catheters; growth arrest after stretch injury can be induced in vascular smooth muscle-cells (VSMC). This growth arrest of VSMC has been explained as ceramide-induced Akt inhibition, mediated through PKC-zeta (Charles et al., 2000; Bourbon et al., 2002).

Ceramide also plays a role as a regulator of cell differentiation. Indeed, in neuronal cell lines, it mimics nerve growth factor function, and induces differentiation in T9 glioma cells, Purkinje cells, and hippocampal neurons (Dobrowsky et al., 1994).

Moreover, in HL-60 and U037 human leukemia cells, it has been observed that vitamin D3-induced differentiation was accompanied by a progressive increase in SM hydrolysis by neutral-SMase (N-SMase), and consequent elevation of ceramide levels (Okazaki et al., 1989).

Finally, ceramide was also associated with the induction of senescence. Ceramide significantly increases as human fibroblasts enter the senescent phase. Through inhibition of phospholipase D, leading to the reduction of diacylglycerol (DAG) generation, ceramide accumulation results in the failure to translocate PKC to the membrane, a critical response in transducing mitogenic stimuli (Venable et al., 1995).

Another recently identified target of ceramide concerns the regulation of the elongation/maintenance of telomeres (Blackburn, 2005). The ceramide-mediated regulation of senescence also occurs by alteration in telomere length, which is

normally maintained by action of telomerase. Ceramide inhibits telomerase, mainly through increased ubiquitin/proteasome proteolysis and inactivation of c-Myc transcription factor, which activates the human Telomerase Reverse Transcriptase (hTERT) promoter (Ogretmen et al., 2001). In addition, ceramide also mediates rapid telomere shortening in a telomerase-independent way in A549 cells, by the inhibition of a nuclear form of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) involved in telomere binding and protection function (Sundararaj et al., 2004).

3.2.2 Sphingosine

Most studies about sphingosine agree on its role as a mediator of cell-growth arrest and apoptosis (Menaldino et al., 2003; Cuvillier et al., 2002; Cuvillier et al., 2003).

From *in vitro* studies on thymocytes (Lepine et al., 2004; Lepine et al., 2002) and 3T3/A3 cells (Suzuki et al., 2004), it has been shown that Dexamethasone treatment and serum deprivation induce sphingosine generation and apoptosis, while inhibition of sphingosine accumulation attenuate apoptosis.

Furthermore, in MCF-7 cells and in neutrophils, a treatment with, respectively, doxorubicin (Cuvillier et al., 2001) and TNFα (Sweeney et al., 1996) increased sphingosine-induced apoptosis, and the administration of exogenous sphingosine potently enhanced this response.

TNFα treatment markedly increases sphingosine generation in cultures of rat cardiomyocytes (Krown et al., 1996) and rat skeletal muscle cells (Dalla Libera et al., 2001), and exogenous sphingosine treatment increases apoptotic effects in both cases.

Interestingly, Ohta et al. (1995) have shown that, in HL60 cells, phorbol 12-myristate 13-acetate (PMA) induces the generation of sphingosine prior to apoptosis, and that administration of exogenous sphingosine potently induces apoptosis in the presence of fumonisin B, a ceramide synthase inhibitor which blocks the conversion of cellular sphingosine to ceramide, demonstrating that sphingosine induces apoptosis of HL60 cells on its own, and not by its conversion to ceramide.

In addition, in Jurkat cells, a treatment with anti-FAS antibody results in sphingosine generation, and also in cytochrome c release, activation of caspase-2, -3, -6, -7, -8, and apoptosis, and addition of exogenous sphingosine is able to induce apoptosis in these cells (Cuvillier et al., 2000), suggesting that this bioactive lipid may be involved in cell-mediated apoptosis.

Anti-tumor activity of sphingosine has also been reported in certain *in vivo* animal models, in which an increased generation of sphingosine is shown to inhibit tumor cell proliferation (Schmelz et al., 2001; Kohno et al., 2006).

An important mechanism of sphingosine action consists in the inhibition of different protein kinases, such as Ca^{2+}/calmodulin-dependent protein kinase, mitogen activated kinase (MAPK), ERK1/2, Akt, and protein kinase C (PKC) (Jarvis et al., 1997; Chang et al., 2001; Hannun and Bell, 1989; Smith et al., 1997) which results in an inhibitory effect on cell proliferation or survival.

Finally, the role of sphingosine in altering the integrity of the intracellular membrane system was revealed. In particular, it has been shown that sphingosine markedly increases the permeability of either lysosomes or mitochondria, and induces the release to the cytosol of lysosomal cysteine proteases, such as cathepsin B, D, or L (Kagedal et al., 2001), and of cytochrome c (Cuvillier et al., 2001), leading to activation of, respectively, lysosomal and intrinsic apoptosis pathways.

Sphingosine has also been shown to induce, in response to FAS, the fragmentation of Golgi complex, which results in cell apoptosis (Xu et al., 2006; Walker et al., 2004; Cuvillier et al., 2000). The integrity of Golgi complex is important for its function as a major organelle for protein trafficking and post-translational modifications, such as glycosylation. A protein that seems to suffer most of Golgi damage is β1 integrin, a major subunit of integrin receptors that mediate cell–cell or cell–extracellular matrix adhesion, and is highly glycosylated in ER and Golgi complex, before being transported to cell surface and act as a cell-adhesion receptor (Hu et al., 2005; Salicioni et al., 2004). The impossibility of integrin β1 to play its role leads to a defect of cell adhesion known as anoikis, a subtype of programmed cell death that is due to loss of cell contact with the extracellular matrix (Hu et al., 2005).

Therefore, to summarize, sphingosine can induces growth arrest, differentiation, apoptosis and anoikis, by acting on various intracellular targets

3.2.3 Sphingosine-1-phosphate

External stimuli, mainly growth factors and cytokines, are responsible for the increase of S1P level, as a direct result of rapid activation and/or translocation of SphK1 (Alvarez et al., 2007). The generated S1P can have a dual function: act as a second messenger inside the cell, or be secreted and act in autocrine and paracrine manner by

stimulating specific receptors located on the membrane of the same cell or neighboring cells (Meyer zu Heringdorf et al., 2001).

Although the intracellular targets of S1P are not yet fully known, it is known that S1P, as a second messenger, is able to mobilize intracellular Ca^{2+} from internal sources, independently of PI3 kinase (Ghosh et al. 1990), and to activate signaling pathways that stimulate proliferation and protect from apoptosis, such as the induction of ERK/MAPK and the inhibition of stress-activated protein kinase JNK (c-Jun N-terminal kinase) (Cuvillier et al., 1996).

As for the extracellular action of S1P, it is related to the presence of specific G protein-coupled receptors S1P1, S1P2, S1P3, S1P4 and S1P5, which are expressed differently in various tissues, and are associated with multiple types of G proteins, thus regulating multiple signaling pathways (Chun et al., 2002). S1P1, S1P2 and S1P3 activate ERK, but the S1P2 activation is less strong than S1P1 and S1P3 (Okamoto et al., 1999). In the same way, S1P3 was to be the strongest inducer of PLC/IP3/Ca^{2+} pathway (Ancellin and Hla, 1999).

The receptor-mediated effect of S1P is critically dependent on the level of expression of each receptor type, and S1P has thus been found to play an essential role in biological processes as different as cardiovascular development (Liu et al., 2000; Allende et al., 2002), angiogenesis (English et al., 2001; Kono et al., 2004), immunity (Goetzl et al., 2007; von Wenckstern et al., 2006; Rosen et al., 2005), reproduction (Mizugishi et al., 2007), and central nervous system development (Mizugishi et al., 2005).

S1P can induce profound cytoskeletal changes by controlling the activity of Rac and Rho GTPase proteins, thereby regulating cell migration, and communication between cells (Donati and Bruni, 2006). S1P1 and S1P3 signalings promote migration, while S1P2 stimulation activates Rho, inhibits Rac, and thus stops the migration. S1P2 stimulation is able to inhibit cell migration even in the presence of potent chemotactic factors such as IGF-I (Kon et al., 1999; Okamoto et al., 2000). So, depending on the relative level of expression of the different receptors, S1P may promote or inhibit migration. In melanoma cells, S1P inhibits migration by binding to S1P2, abundantly expressed in these cells (Donati and Bruni, 2006).

S1P has a key role in the growth, proliferation, survival of many cell types (Pyne and Pyne, 2000, Spiegel and Milstien, 2003; Cuvillier et al., 1998). It is considered to be a pro-survival lipid, because of its involvement in malignant transformation, cancer

proliferation, inflammation, vascularogenesis, and resistance to apoptotic cell death (Maceyka et al., 2002; Hla, 2004; Taha et al., 2006a). For exemple, increased S1P levels promote proliferation and survival in human glioma and breast cancer cells (Nava et al., 2002; Sarkar et al., 2005), and, importantly, in ovarian cancer patients, in whom elevated S1P levels are found in the serum (Tilly and Kolesnick, 2002).
Recently it has been also suggested that S1P also regulates autophagy (Lavieu et al., 2006).

3.3 Sphingolipid metabolism modulation

Most of the biochemical pathways of synthesis and degradation of sphingolipids have been currently determined, and most of the involved enzymes are characterized. Several specific inhibitors of these enzymes have been described, which provides accurate tools to analyze the role of different metabolites of the sphingolipid pathway in relevant experimental settings. Among these inhibitors, some are natural sphingolipids that have been modified synthetically, others are non-natural analogs that, because of their structural similarity with the natural parent compound, inhibit various metabolic steps. Others are structurally unrelated synthetic inhibitors.

C_2, C_6, and C_8 *Ceramides* - These compounds are cell-permeant short chain anologs of ceramides used to induce an increase of cellular ceramide (Dagan et al., 2003) and mimic the effects of naturally occurring ceramides in signal transduction.
C_2 (N-Acetylsphingosine) is a biologically active, cell-permeable ceramide analog. This compound inhibits cell proliferation and induces monocytic differentiation of HL-60 cells; it induces apoptosis, stimulates protein phosphatase 2A4, activates MAP kinase and SAP kinase, and induces PKCδ and ε translocation. Physiological levels of C_2 ceramide, produced by the action of a PAF:sphingosine CoA-independent transacetylase, have been detected in HL-60 cells. (Bielawska et al., 1992;)
C_6, is a biologically active, cell-permeable, but non physiologic ceramide analog. It is able to activate protein phosphatase 2A and MAP kinase (MAPK/ERK) (Dobrowsky et al., 1993), it suppresses insulin-induced tyrosine phosphorylation and inhibits glycoprotein traffic by the secretory pathway (Dobrowsky and Hannun, 1992). This compound inhibits diacylglycerol accumulation and phospholipase D activation in

fibroblasts (Venable et al., 1994). It induces an arrest and causes apoptosis in Molt-4 leukemia cells (Raines et al., 1993; Jayadev et al., 1995; Jarvis et al., 1994).

C_8 is a biologically active, cell-permeable, but non physiologic ceramide analog. It is able to induce phosphorylation of epidermal growth factor receptor on Thr-669 in A-431 cells by stimulation of ceramide-activated protein kinase (Mathias et al., 1991; Mathias et al., 1992) and induces apoptosis (Jarvis W.D., et al. (1994).

Myriocin **-** This drug is a fungal metabolite of the Chinese herb *Iscaria sinclarii* (Im, 2003; Fujita et al., 1994). It is an inhibitor of serine palmitoyl-CoA transferase (SPT), the first enzyme in sphingolipid biosynthesis (Miyake et al., 1995). Initially studied as a potential antibiotic drug, myriocin was found to be an immunosuppressant in mice (Kiuchi et al., 2000).

Myriocin blocks sphingolipid biosynthesis, and is therefore useful as a tool to deplete cells of sphingolipids. This depletion results in disruption of substratum adhesion of melanoma cells (Hidari et al., 1996), suppression of cell proliferation via inducion of apoptosis in the murine cytotoxic T cell line CTLL-2 (Nakamura et al., 1996), suppression of replication of the hepatitis C virus (HCV) in a murine model (Umehara et al., 2006), and increased plasma phosphatidylcholine (PC) levels and decreased atherosclerotic lesions in apoE knock-out mice (Hojjati et al., 2005).

Fumonisin B1 **–** This is a fungal toxin which inhibits ceramide synthase, leading to an inhibition of ceramide biosynthesis and an increase of free sphinganine and sphingosine, which result in disturbances of cellular processes such as cell growth, cell differentiation, cell morphology and apoptosis.

Fumonisin treatment has been shown to inhibit protein serine/threonine phosphatases (PP1, PP2A, PP2B, PP2C, and PP5/T/K/H) (Fukuda et al., 1996). Inhibition of PP5 may play an important role in the toxicity and carcinogenicity of fumonisin B1. Fumonisin has also been shown to induce the activation of mitogen-activated protein kinase (MAPK) (Wattenberg et al., 1996). In fact, high concentrations of Fumonisin B1 seem to work synergistically with insulin in the induction of mitogenesis in Swiss 3T3 cells (MAPK) (Wattenberg et al., 1996).

Moreover, it has been shown that FB1 administered to different animal species is able to produce increased apoptosis in various tissues (Soriano et al., 2005).

In vivo Fumonisin B1 is poorly absorbed and rapidly excreted (Soriano et al., 2005).

***GW4869* –** This compound is a cell-permeable non-competitive inhibitor of neutral sphingomyelinases (N-SMase).

Neutral sphingomyelinases mediate the release of ceramide from sphingomyelin in cellular membranes, and, as mentioned earlier, they can be activated by certain stresses. This inhibitor specifically affects the action of neutral sphingomyelinases, without affecting acid sphingomyelinase activity (Luberto et al., 2002). GW4869 inhibits TNFα-mediated cell death in MCF7 cells without interfering with activation of NF-κB or glutathione depletion in response to TNF (Luberto et al., 2002; Marchesini et al., 2003). It also reduces the inhibitory effect of oxidized phospholipid products on lipopolysaccharide-mediated induction of interleukin-8 in human aortic endothelial cells (Watson et al., 2006) and prevents hypoxia-induced pulmonary vasoconstriction in rats in vivo (Cogolludo et al., 2009).

The GW4869-mediated inhibition of N-SMase prevents the activation of some effector caspases, and then the formation of apoptosome (Adrain et al., 2001; Daniel et al., 2000).

***3-O-Methyl-Sphingomyelin (3-O-MSM)* –** This drug is a novel selective inhibitor of neutral sphingomyelinase, devoid of effects on acid sphingomyelinase (Lister et al., 1995).

Pretreatment with 3-*O*-MSM protected 1c1c7 cell cultures against the pro-apoptotic effects of TNFα and CHX co-treatment (Caruso et al., 2006), and prevented TNFα- and CHX-mediated disruption of lysosomes (Caruso et al., 2005).

Treatment of glial cells with vitamin E or 3-O-methyl sphingomyelin can downregulate the expression of inducible Nitric Oxide Synthases (iNOS), as well as production of NO, induced by cytokine and Amyloid beta (Aβ), indicating a role of ceramide in Aβ(25–35)-mediated induction of iNOS (Ayasolla et al., 2004; Sheng et al. 1998).

***DL-threo-Dihydrosphingosine (DHS)* -** This is a non natural isomer of dihydrosphingosine (sphinganine), which acts as a potent, cell permeable, and competitive inhibitor of sphingosine kinase (Buehrer and Bell, 1993). This drug can therefore prevent the formation of S1P from sphingosine, which is itself exclusively formed from ceramide (Rother et al., 1992). According to the sphingolipid rheostat

concept, the DHS-induced imbalance in favor of ceramide at the expense of S1P should direct the cells to apoptosis (Spiegel and Milstien, 2002).

This inhibitor is of particular interest due to its anticancer activity. It was shown to synergistically increase the toxicity of established chemotherapeutic agents in several cancer cells in vitro (Schwartz et al., 1995), as well as in preclinical animal studies (Kedderis et al., 1995) and in a phase I clinical trial (Schwartz et al., 1997). The anticancer properties of DHS can be explained by its inhibitory effect on the activity of either protein kinase C or sphingosine kinase. The competitive interaction of DHS with the regulatory phorbol binding domain of PKC could be correlated with partial inhibition of the multidrug resistance phenotype of certain tumor cells (Sachs et al., 1995).

N,N-Dimethylsphingosine (DMS) **-** This is a potent and specific inhibitor of sphingosine kinase (Cuvillier et al., 1996; Kono et al., 2007) which blocks conversion of sphingosine to sphingosine-1-phosphate.

DMS has been shown to induce apoptosis in cancer cells of both hematopoietic and carcinoma origin (Sweeney et al., 1996; Park et al., 1995; Ohta et al., 1994), as well as in transformed rat mesangial cells (Sweeney et al., 1996). However, DMS did not induce apoptosis in primary cultures of human umbilical vein endothelial cells, or rat mesangial cells (Sweeney et al., 1996). DMS also inhibits the growth of various human tumor cell lines in vivo in nu/nu mice (Endo et al., 1991) and blocks neurite extension (Edsall et al., 1998; Uemura et al., 1993) and platelet aggregation (Okoshi et al., 1991). Cell surface expression of crucial selectins which promote adhesion of Lex or sialosyl-Lex expressing cells on platelets, or on endothelial cells, was also inhibited by DMS (Handa et al., 1991). DMS induces apoptosis, but it is not an inhibitor of protein kinase C (Edsall et al., 1998).

D-erythro-2-(N-Myristoylamino)-1-phenyl-1-propanol) (D-MAPP) **–** This molecule is an analog of ceramide that specifically inhibits alkaline ceramidase, inducing ceramide accumulation by blocking its hydrolysis to sphingosine and free fatty acid.

This compound enhances the apoptotic response to ionizing radiation by enhancing the ceramide signal. Moreover, D-erythro-MAPP suppresses growth of HL-60 cancer cells in culture, and elevates the levels of endogenous ceramide, resulting in growth suppression and G_0/G_1 cell cycle arrest (Bielawska et al., 1996).

Sphingosine-1-phosphate (S1P) **-** This is the exogenous sphingolipid, which can act extracellularly through five specific G protein-coupled receptors $S1P_1$, $S1P_2$, $S1P_3$, $S1P_4$, and $S1P_5$, triggering various signalling pathways (Spiegel and Milstien, 2003). The different downstream proteins Gi/o, Gq, $G_{12/13}$, are associated with activation of Ras GTPase and ERK, leading to cell proliferation, PI3K/Akt pathway, which promotes survival and growth, Rac GTPase, which is involved in cytoskeletal organization, protein kinase C and phospholipase C (PLC) that increase intracellular Ca^{2+}, and inhibition of adenylate cyclase (AC) with a consequent reduction in cAMP, resulting in various biological responses (Brinkmann, 2007).

FTY720 is an immunosuppressive drug derived from *Myriocin.* It induces a rapid internalization of S1P receptors, and as such it is used as an inhibitor of S1P signaling. FTY720 has been considered as a structural analog of sphingosine. In fact, it can be phosphorylated by sphingosine kinase, and then acts as a potent agonist at four of the S1P receptors ($S1P_1$, $S1P_3$, $S1P_4$, and $S1P_5$) (Brinkmann et al., 2002). Down-regulation of $S1P_1$ receptors induced by FTY720 in T and B lymphocytes results in defective egress of these cells from spleen, lymph nodes, and Peyer's patch, causing immunosuppression (Matloubian et al., 2004).

As an immune modulator, this drug prolongs allograft transplant survival in numerous models (Brinkmann et al., 2001). FTY720 also enhances the activity of the sphingosine transporter Abcb1, and the leukotriene C_4 transporter Abcc1, and inhibits cytosolic phospholipase A_2 activity (Honig et al., 2003; Payne et al., 2007).

Aims of the Research Project

Atrophy of skeletal muscle, is a condition characterized by a progressive decline of muscle mass and strength. It is a very important component of cachexia, the pathological state which occurs at the endpoint of several diseases such as cancer or chronic infections.

In many cases (cancer, aging, inactivity), the pro-inflammatory cytokines such as Tumor Necrosis Factor alpha (TNFα) or Interferon-gamma (IFN-γ) seem to play an important role in inducing muscle atrophy. For example, circulating levels of TNFα, usually undetectable in healthy subjects, can increase, in case of cancer, to several nanograms per millilitre, a concentration able to induce muscle loss in experimental models (Li and Reid, 2000).

Signalling triggered by TNFα is very complex and involves a family of receptors, including TNF-R1 which can activate neutral sphingomyelinase, through recruitment of the adapter protein FAN to the NSD domain of the receptor. This is of particular interest because hydrolyzing membrane sphingomyelin gives rise to ceramide, a bioactive sphingolipid known to be a messenger for many cellular funcions. TNFα can also induce the activation of acid sphingomyelinase via the signaling cascade of death domain of TNF-R1, and stimulate the synthesis of ceramide by *de novo* pathway (Wajant et al, 2003).

A role of ceramides in muscle atrophy process is suggested by many observations. Indeed, this messenger inhibits myogenic differentiation induced by IGF-I in C2C12

myoblasts (Strle et al, 2004), and differentiation induced by vasopressin in L6 myoblasts, as shown by our team (Mebarek et al, 2007). Ceramide could thus oppose the regeneration of muscle tissue. It is also involved in the reduction of protein synthesis induced by TNFα in C2C12 myotubes (Strle et al, 2004), and inhibits the A-transport system of amino acids and protein synthesis in L6 myotubes (Hyde et al, 2004). Moreover, ceramide was shown to be an activator of transcription factor NF-kB (Wu et al, 2007), which promotes protein catabolism by inducing the expression of various components of proteasome, the main way of intracellular proteolysis (Eley and Tisdale, 2007). Ceramide is an activator of the other important pathway of protein catabolism, autophagy. This pathway that involves lysosomal acid hydrolases is widely involved in muscle atrophy (Zhao et al, 2007). Ceramide has been shown to activate autophagy by inducing the expression of Beclin-1, an activator of PI3 kinase III, and inhibiting the Akt kinase (Meijer and Codogno, 2006).

Since TNFα is well known to affect muscle function, and ceramide is involved in important aspects of the etiology of muscle atrophy, we hypothesized that ceramide, or a metabolite, could be an important actor of tumor-induced muscle wasting process. We aimed to delineate in vivo the role of ceramide in a model of cachexia, and to confirm this role in *in vitro* models of atrophy, to identify which sphingolipid metabolite(s) is or are involved in muscle loss, and also elucidate the molecular mechanisms of protective effects of ceramide inhibition.

Very few data were available about a possible *in vivo* role of Sphingolipids in the occurrence of muscle atrophy. We thus set out to establish whether ceramide participates in muscle loss using a well-established model of tumor-induced atrophy, Balb/C mice carrying C26 adenocarcinoma, and treatments with drugs able to modify sphingolipid metabolism, first of all Myriocin, an inhibitor of the first enzyme of *de novo* Sphingolipid synthesis pathway. In parallel, we established an in vitro model of TNFα-induced myotube atrophy, so as to mimick the pathophysiological situation. We studied in this model the effects of drugs targeting various steps of the sphingolipid pathway, and the involvement of major signaling pathways related to the control of muscle size regulation.

These approaches might help identifying the sphingolipid pathway as a possible target of therapeutic interventions aiming at protecting against muscle loss in various pathological situations.

Chapter 4
Materials and Methods

4.1 Materials

Products: Dulbecco's Modified Eagle medium (D-MEM), Fetal Calf Serum (FBS), Dulbecco's phosphate buffer (***PBS***) and Horse Serum (HS) were supplied by Euroclone; Meyer's Hematoxylin and Schiff's reagent by Sigma-Aldrich; Anti-myosin *MF20* antibody by Developmental Studies Hybridoma Bank, Iowa City, IA; anti-mouse *Alexa Fluor*® 546 by Invitrogen; DAPI and Hoechst33342 by Sigma-Aldrich; Vectashield® Mounting Medium by Vector Laboratories; TRIzol® reagent by Invitrogen; SuperscriptII Reverse Transcriptase by Invitrogen; ABsolute ™ QPCR SYBR® Green Mix by Thermo Sciences; Proteinase inhibitor cocktail Complete ™ by Roche Diagnostic, Germany; TritonX 100 by Sigma-Aldrich; Bradford Assay by Bio-Rad Laboratory, California; Hybond N+membrane by Amersham Bioscences; Akt/mTOR pathway antibody by Cell Signaling Technology®; Prestained Protein Ladder from 10kDa to 170kDa by Euromedex; Secondary anti-rabbit and anti-mouse antibodies coupled to horseradish peroxidase by Bio-Rad Laboratory, California; ECL chemiluminescence system by Amersham Bioscences. BALB/c mice were from Charles River Italia SPA (Milan).

4.2 Cell cultures

Myogenic C2C12 cells, a subclone of the C2 mouse myoblast cell line, were maintained as proliferating myoblasts in Dulbecco's Modified Eagle medium (D-MEM) - high-glucose medium supplemented with 10% Fetal Calf Serum (FCS), 4 mM L-glutamine, 1 mM pyruvate, and 100 U/ml penicillin-100 mg/ml streptomycin at 37°C in the presence of 5% CO_2. The seeding density used for the experiments was $1x10^4$ cells/cm^2 on 35mm plastic dishes. To induce fusion of myoblasts, 90–100% confluent cultures were switched to D-MEM supplemented with 2% adult Horse Serum (HS). Myoblast growth and myotube formation were examined using phase-contrast microscopy and the medium was changed every 48h.

After the differentiation, chemicals were added to serum- and antibiotic-free medium and renewed every 24 h, for 3 days:

a) TNF-alpha (mTNFα recombinant (*E.Coli*) by Roche Applied Science) was added to medium at a final concentration of 1ng/ml, 5ng/ml or 10ng/ml;
b) Cycloheximide (High Purity by Calbiochem®) was added to the medium at a final concentration of 0.3 µg/ml;
c) Ceramides (C_2, C_6 and C_8 cell-permeant short chain Ceramides by BioMol® International) were added to the medium at a final concentration of 5µM or 10 µM.
d) Myriocin (BioMol® International) was added to the medium at a final concentration of 100 nM.
e) Fumonisin B1 (FB1, Sigma-Aldrich) was added to the medium at a final concentration of 20 µM.
f) GW4869 (Calbiochem®) was added to the medium at a final concentration of 10µM.
g) 3-O-Methyl-Sphingomyelin (3-O-MSM, BioMol® International) was added to the medium at a final concentration of 0.5 µM.
h) DL-threo-Dihydrosphingosine (DHS, BioMol® International) is added to the medium at a final concentration of 10 µM.
i) N,N-Dimethylsphingosine (DMS, BioMol® International) is added to the medium at a final concentration of 10 µM.

j) D-erythro-2-(N-Myristoylamino)-1-phenyl-1-propanol) (D-MAPP, BioMol® International) was added to the medium at a final concentration of 40 µM.

k) Sphingosine-1-phosphate (S1P, Cayman Chemicals) is added to the medium at a final concentration of 1µM.

l) FTY720 (Cayman Chemicals) was added to the medium at a final concentration of 0.5µM.

TNF-alpha was added to the plates 1h after drug addition, at a final concentration of 1ng/ml.

4.3 Myotube Morphological Analysis

PAS staining – After treatements, myotubes from C2C12 were fixed in 3.7% formaldehyde buffered in PBS, stained with PAS (Periodic Acid-Shiff) stain: after undergoing further PBS washing, cells were incubated in 1% periodic acid for 20 min and washed three times for 5 min in distilled water. Samples were then incubated for 20 min in Schiff's reagent, followed by three incubations with 0.5% potassium bisulfite-0.05 M HCl for 5 min each. Nuclei were counterstained with Meyer's Hematoxylin (Sultan *et al.*, 2006).

Cells were mounted in an acqueous medium including 70%Glycerol in PBS, and then photographed using a digital camera coupled to a Zeiss Axioskop2plus microscope using the Axiovision software (Carl Zeiss, Göttingen, Germany).

The area of individual myotubes was measured on 100 myotubes in each condition, in four different experiments, using the Scion Image Beta 4.02 (Scion) software.

Immunofluorescence – After treatments, myotubes from C2C12 cells were fixed by 3.7% formaldehyde for 10 minutes at 4°C, permeabilized with 0.1% Triton X-100 for 15 minutes at 4°C, and aspecific _abelling was blocked in 1% BSA for 20 minutes. Anti-myosin MF20 antibody was added 1/50 diluted, and incubated for 1h at room temperature. After extensive washing with PBS, anti-mouse-AlexaFluor546 1:1000 diluted in 0,1% BSA was added, and samples were incubated 1h at room temperature. The nuclei were detected with DAPI (1µg/ml) incubated for 3 minutes at room temperature.

Samples were mounted with Vectashield and coverslip to perform image acquisition by fluorescence microscopy, with an Axiovert 200 microscope, Axiocam MRm camera and Axiovision 4.1 image acquisition software (Carl Zeiss, Göttingen, Germany).

The percent area occupied by myotubes was measured in 10 fields in each condition, in three different experiments, using the ImageJ software, developed by the National Institutes of Health of the United States.

4.4 Morphological detection of apoptosis

After treatment of myotube cultures with drugs, morphological analysis of apoptosis was performed according to *Cannavò et al*. (2003): at the end of treatment, the cells were washed and then incubated with Hoechst33342 10µg/ml solution, for 5-10 min at 37°C. Thereafter the cells were repeatedly washed and fixed in 3.7% Formaldehyde solution for 30 min at 4°C. Stained nuclei were then visualized by fluorescence microscopy on an Axiovert 200 microscope coupled with an Axiocam MRm camera and Axiovision 4.1 image acquisition software (Carl Zeiss, Göttingen, Germany).

Apoptotic cells are usually defined, using this method, by the condensation of nuclear chromatin, its fragmentation, or its margination to the nuclear membrane.

4.5 Gene Expression Analysis

The RNA expression levels of different genes were measured by Real Time Quantitative-PCR.

Extraction - The extraction of total RNA from murine muscular tissue or from murine muscular cells in culture was performed with TRIzol® reagent, according to the recommendations of the supplier.

Reverse Transcription - Total RNA was then quantified using NANODROP ND-1000 spectrophotometer.
The cDNA was obtained from 1µg of total RNA by means of reverse transcriptase reaction with Superscript II Reverse Transcriptase (Invitrogen), using random hexamer primers and an oligodT mix.

qPCR - 5 µL Aliquotes of 1/60-diluted cDNAs were processed for quantitative PCR by mixing in 15 µL ABsolute ™ QPCR SYBR® Green Mix (Thermo Sci.), using Rotor-Gene 6000 ™ (Corbett Research, Mortlake, Australia).
Couples of oligonucleotides derived from mouse cDNA sequences were designed to be used as primers:

Atrogin1	*forward primer* 5'- CTCTGCCAGTACCACTTCTC -3'
	reverse primer 5'- ATGGTCAGTGCCCTTCCAGG -3'
MuRF1	*forward primer* 5'- TGCATCTCCATGCTGGTGGC -3'
	reverse primer 5'- CTTCTTCTCGTCCAGGATGG -3'
FoxO1	*forward primer* 5'- CCTGTCGCAGATCTACGAGT -3'
	reverse primer 5'- CTCTGGATTGAGCATCCACC -3'
FoxO3	*forward primer* 5'- GAGAGCAGATTTGGCAAAGG -3'
	reverse primer 5'- CCTCATCTCCACACAGAACG -3'
PLD1	*forward primer* 5'- GAAGCGAGACAGCGAAATGG -3'
	reverse primer 5'- GGTCAGAAAGATAACCCAGG -3'

4.6 Immunoelectrophoretical Analysis

Protein extraction - Frozen muscle tissues samples were homogenized directly in lysis buffer containing 20 mM Tris HCl pH 7.6, 100 mM NaCl, 50 mM Sodium Fluoride, 10 mM Sodium Pyrophosphate, 1.5 mM Sodium Orthovanadate, 10 mM Glycerophosphate, 1% Triton-X 100 and Protease Inhibitor Cocktail.
Samples were rotated 3x10"- in Precellys 24 (Bertin Technologies), 2x2' at 10000rpm and 15' at 13000rpm at 4°C.
Proteins from the cell samples were extracted at 4°C using the same lysis buffer.
The supernatants were collected and total protein concentrations were measured using Bradford assay.

Western Blot - Protein samples were denaturated at 100°C for 5 minutes in Laëmmli Buffer 1X: Tris-HCl 50mM , 12% Glycerol, 1% SDS, 4% β-mercaptoethanol, 0.01% Bromophenol Blue, pH 6.8.
Aliquotes of approximately 50µg of each sample were separated by SDS-PAGE. The separating gel was composed of either 10% or 15% polyacrylamide, 1.5M Tris-HCl pH 8.8, 10% SDS, 10% Ammonium persulphate and TEMED. The stacking gel consisted of a 4% polyacrylamide, 1M Tris-HCl pH 6.8, 10% SDS, 10% Ammonium persulphate and TEMED. The running buffer was a Tris-Glycine-SDS 1X solution.
The electrophoresis was initially set at 60 V to allow the protein to cross the stacking gel and create the front, and then at 100 V to proceed through the separating gel. The protein of interest was identified by comparison with a relevant molecular weight marker. Proteins were then transferred from the gel to a nitrocellulose membrane.
The transfer was carried out at 4° C at 300mA constant for 1h30 with a Tris-Glycine 1X solution with 20% methanol as transfer buffer.
After verifying the successful transfer of proteins, with Red Ponceau staining, the blotted membranes were blocked in TBS-T (20 mM Tris-HCl pH 7.5 + 137 mM NaCl + 0.1% Tween-20) containing 5% nonfat dry milk for 45-60 min at RT, and then incubated with specific primary antibodies diluted in the same buffer, overnight at 4°C. We used the following primary antibodies from Cell Signaling Technology®:

a) Phospho-Akt (Ser473) XP™ Rabbit mAb (1:2000);

b) Akt Rabbit mAb (1:1000);

c) Phospho-p70 S6 Kinase (Thr389) Rabbit mAb (1:1000);

d) p70 S6 Kinase Rabbit mAb (1:1000);

e) Phospho-4E-BP1 (Thr37/46) Rabbit mAb (1:1000).

To normalize our results we used: Anti-α-Tubulin Mouse mAb (1:1000) or Anti-Actin Rabbit mAb (1:1000) both purchased from Sigma-Aldrich.

Membranes were washed several times with TBS-T and then probed with secondary antibodies coupled to horseradish peroxidase (anti-rabbit IgG-horse radish peroxidase conjugated, 1:10000 or anti-mouse IgG-horse radish peroxidase conjugated, 1:10000) in TBS-T at room temperature for 1h.

The immunoreactive bands were visualized by chemiluminescence (ECL or ECL Plus kit, Amersham), autoradiographed on X-ray films, and the densitometric analysis of protein expression was performed on digital images obtained by scanning of the film using ImageJ software (NIH).

4.7 Animals

6 week-old male BALB/c mice weighing from 23g to 25g were obtained from Charles River Italia. Animals were housed in the animal facility under conventional conditions with constant temperature and humidity, and fed a standard diet. Tumor implantation was performed using cubic pieces of ~ $1mm^3$ of solid C26 tumor kept in liquid N_2. Tumor pieces were injected, with a trocart, under the skin of mice anesthetized with sodium pentobarbital (30 mg/kg). Control mice were injected only with saline. At day 8, some mice displayed a palpable tumor; at days 10-11, most of the tumor-injected mice carried a palpable tumor. The animals were weighted every day.

Starting on day 8, half of control mice and half of mice with tumors were injected intraperitoneally at 0.1 mg/kg, with a 2 mg/ml Myriocin solution in 0.5% methanol. Myriocin was injected daily until the day of sacrifice, while control mice received 0.5% methanol in saline solution.

When the tumor-injected mice had lost 3g of weight, they were sacrificed by cervical dislocation. At the same time, a matched control was sacrificed (tumor-uninjected, same dose of Myriocin). The tumor-injected mice and matched controls were sacrificed between days 13 and 17. All the surviving mice were sacrificed on day 17, whatever their weight loss.

Mice were sacrificed according to the standard approved procedure in order to minimize any unjustified suffering. Blood was collected via intracardiac puncture.

The muscles were dissected from both hind-legs (tibialis, gastrocnemius, soleus), frozen in liquid N_2-cooled isopentane, transferred to a cryotube, shifted to dry ice and stored at -80° for histology and molecular analyses.

4.8 Muscle fibre Analysis

Muscle transverse sections (8µm) of *Tibialis* and *Gastrocnemius* were cut in a cryostat at -20°C, collected on slides and kept refrigerated at -20°C.

Subsequently, these sections were stained with classic protocol for Hematoxylin and Eosin to measure fibre Cross-Sectional Area (CSA).

Each sample was photographed using a digital camera coupled to an Axioskop2plus Zeiss microscope using the Axiovision software (Zeiss) and fibres were traced with imaging software (Scion Image Beta 4.02, USA).

4.9 Morphological detection of inflammation

Nonspecific esterase (α-naphthyl butyrate esterase) staining was used to identify macrophage infiltration. Briefly, 8 μm muscle cryosections were stained for 5 minutes at room temperature, after rehydration of frozen sections, in a staining solution containing 100 mM phosphate buffer, pH 7.4, 4% α-naphthyl acetate, and 4% azotized pararosanilin. After washing of tap water, were dehydrated in a graded ethanol series: 50%, 70%, 80% (1 min each) and 95%, 100% (5 min each). Finally these sections were cleared in toluene, for at least 5 minutes and mounted with an organic mounting medium.
Macrophages stained dark brown in the interstitial region.

4.10 Statistical Analysis

Individual *in vitro* experiments were replicated three to five independent times, and the results presented as means ± SEM. Student's *t*-test was used to determine whether differences existed between results from different cells and experimental conditions. The acceptable level of significance was set at $P < 0.05$.
For the *in vivo* experiments, we performed a statistical analysis with ANOVA-test or 'Analysis of Variance between groups'. The acceptable level of significance was set at $P < 0.05$.

Chapter 5

Results

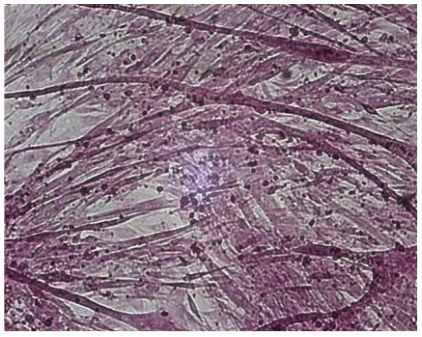
5.1 TNFα and ceramides induce in vitro atrophy.

5.1.1 Morphometric analysis.

In our study, as an *in vitro* experimental model we used C2C12 murine myogenic cells differentiated and then submitted to pro-atrophic treatments. The atrophic response was evaluated by a morphometrical quantification of mature myotubes under the different experimental conditions tested.

As a first step, we set up an in vitro system to determine and quantify size changes of large myotubes derived from C2C12 cells using digital quantification of the area of individual Periodic Acid Schiff (PAS)-stained myotubes (Figure 5.1) according to (Sultan *et al*., 2006).

Fig.5.1 - Periodic acid Schiff (PAS) staining of C2C12-derived myotubes.



To induce atrophy, myotubes were treated with the pro-inflammatory cytokine TNFα. Cycloheximide, an inhibitor of protein biosynthesis in eukaryotic organisms, was used as a positive control.

The atrophic effect (Figure 5.2) of TNFα on C2C12 cells (from 49% to 37% reduction in myotube area) is clearly evidenced, although slightly smaller than the effect of Cycloheximide (-55%).

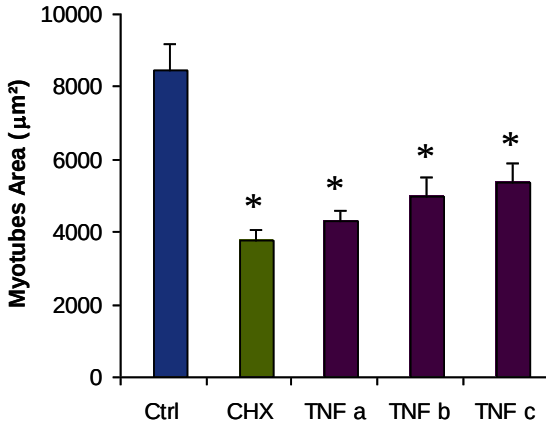


Fig. 5.2 - Evaluation of the area of individual C2C12 myotubes after PAS staining. Reduction in myotube area (μm^2) by a nontoxic dose of Cycloheximide (CHX, 0.3mg/ml) and TNFα treatment (TNF a=1ng/ml; TNF b=5ng/ml; TNF c=10ng/ml). Values are means ± SEM; * = significantly different from control (p <0.05).

In agreement with several reports of the literature *(Alvarez et al., 2002a; Alvarez et al.*, 2002b), we observed that the dose of 1ng/ml TNFα was the most efficient, the effect decreasing for higher doses.

To evaluate the implication of ceramides in muscle atrophy, we performed the same quantification treating the C2C12 myotubes with three different cell-permeant short-chain ceramides, with a C2, C6, or C8 acyl chain.

All three tested ceramides, especially the C6 species, induced a reduction of myotubes area comparable to that induced by TNFα (Figure 5.3), supporting the idea that ceramides can participate to muscle atrophy.

We can conclude that both TNFα and ceramides affect myotube size, and hypothesize that ceramides may participate to the atrophic effect of TNFα.

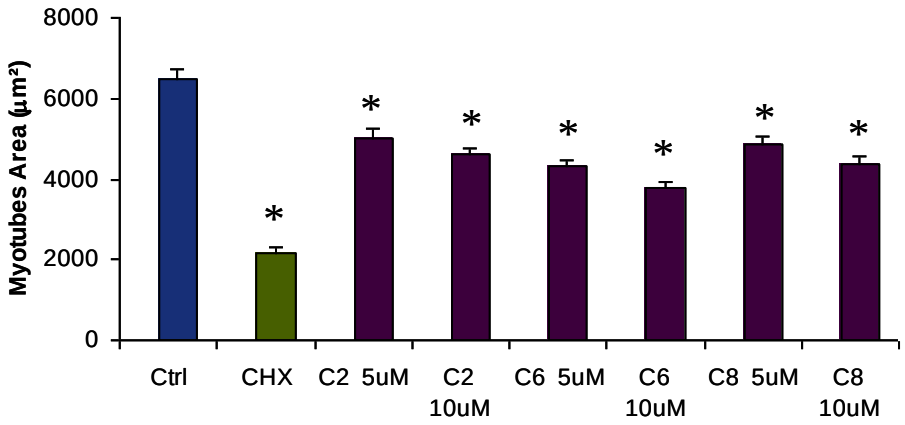


Fig. 5.3 - Evaluation of the area of C2C12 large myotubes after PAS staining. Reduction in myotube area (μm^2) by a non-toxic dose of Cycloheximide (CHX; 0.3µg/ml) and C2-Ceramide, C6-Ceramide, C8-Ceramide treatment. (n = 3; Values are means ± SEM; * = significantly different from control with $p < 0.05$).

5.1.2 Apoptosis detection.

Muscle atrophy may result from: a reduction in protein content, an increase in cell death and a reduction of muscle regeneration.

TNFα is involved in reduction of proteins and increase of cell death (Carbo et al., 2002) and ceramide can also induce apoptosis (Dirks-Naylor and Griffiths, 2009).

We thus considered important to evaluate if TNFα and ceramide induce an in vitro apoptosis at the doses we used in myotube cultures.

For this purpose, we performed a morphological analysis of apoptosis after fluorescent labelling of nuclei with Hoechst 33342 (Cannavò *et al.* 2003), but we did not detect any difference in the number of apoptotic nuclei between myotubes untreated and treated with TNFα, ceramide or the other studied drugs (not shown).

5.2 In vitro TNFα- induced atrophy involves sphingolipid metabolism.

5.2.1 Effect of sphingolipid metabolism inhibitors on the atrophic response of C2C12 myotubes to TNFα..

Although very complex, sphingolipid metabolism is presently well known.

Most of the biochemical pathways of synthesis and degradation have been determined, and most of the enzymes involved are characterized. A number of specific inhibitors of these enzymes have been described, which gives us the opportunity to analyze the role of sphingolipid pathway in our *in vitro* model of atrophy.

By blocking different steps of sphingolipid metabolism with specific inhibitors (Figure 5.4), we can expect to identify the metabolite(s) potentially involved in TNFα–induced atrophy. C2C12 myotubes were then treated by TNFα in the presence of the tested inhibitor and their surface was evaluated.

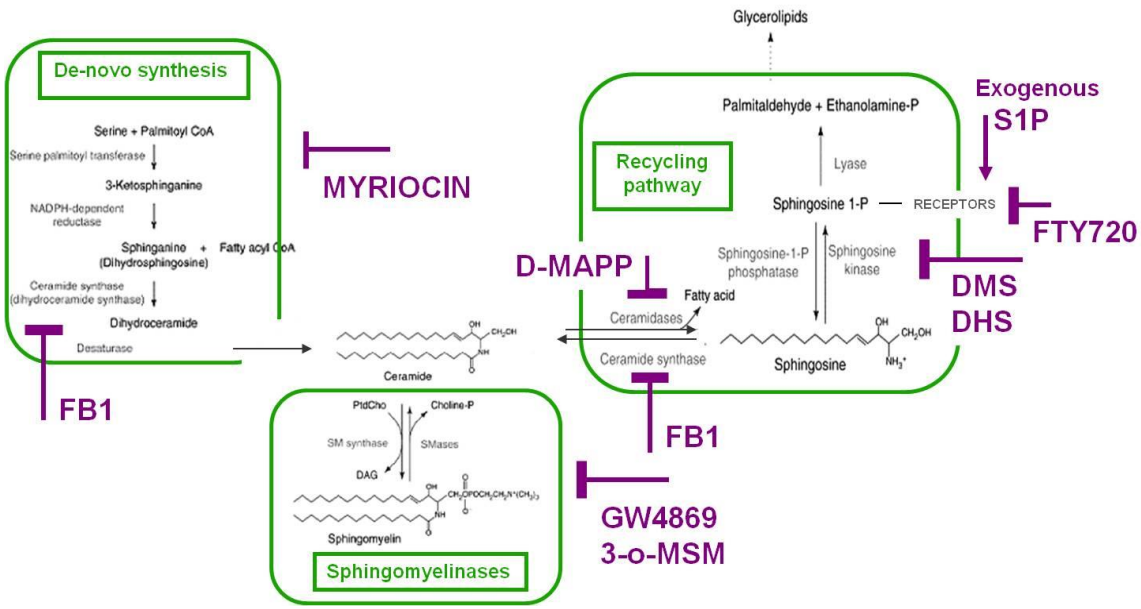


Fig. 5.4 – Sphingolipid pathway.

First of all we tested Myriocin, a potent and selective inhibitor of Serine palmitoyl transferase, the first enzyme of the *de novo* pathway of ceramide synthesis.

We performed the quantification of myotube area (Figure 5.5), with two different methods: 1) by Periodic Acid of Shiff (PAS) stain and direct quantification of the area in μm^2 of individual large myotubes, using the Scion Image Beta 4.02 software, or 2)

by Immunofluorescence labelling of Myosin and quantification of the percentage of area taken up by all myotubes in an entire image field, using ImageJ software.

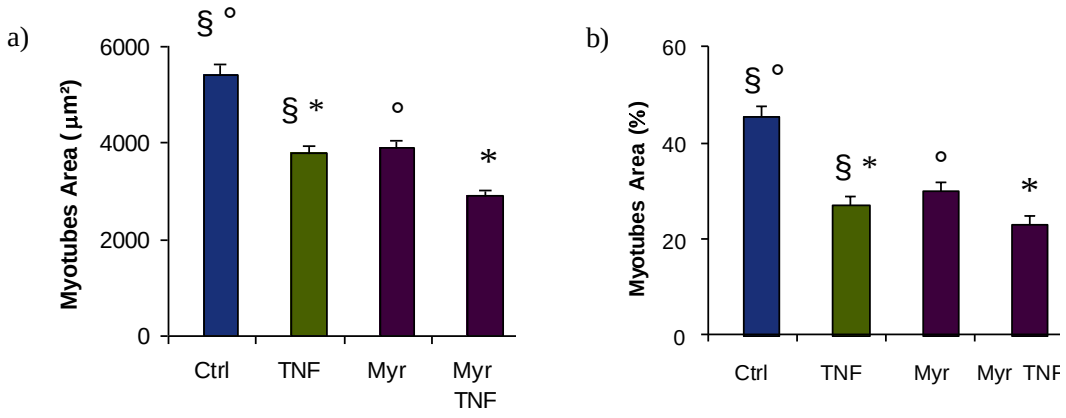


Fig. 5.5 – Effect of sphingolipid inhibitors on myotube size, in presence of TNFα: MYRIOCIN treatment. (a) Evaluation of individual C2C12 myotubes area (µm^2) after PAS staining. (n=4; Values are means ± SEM; §: p<0.0001; *: p<0,0001;°: p<0,0001). (b) Evaluation of C2C12 myotubes area (%) after Immunofluorescence labelling of Myosin labelling. (n=3; Values are means ± SEM; §: p<0.0001; *: p<0,05; °: p<0,0001). Statistical analysis performed with ANOVA-test.

As expected, TNFα significantly affected myotube size according to both morphometric quantification methods. Unexpectedly, Myriocin had, *per se*, an atrophic effect and was unable to restore myotube size in presence of TNFα.

We can assume, to explain this effect of Myriocin, that, by acting upstream of the sphingolipid synthesis pathway, this inhibitor not only depletes the cell of ceramides, but also of sphingolipid metabolites that have a positive role on myofibre size, such as sphingosine-1-phosphate (S1P) (Lahiri and Futerman, 2007), or that are essential for basic cell functions, such as sphingomyelin or glycosphingolipids, for example.

In contrast, experiments performed in our group using another myogenic cell line, the L6 rat cells, (data not shown) indicated that, in myotubes treated with TNFα, Myriocin administration, while still inducing a limited negative effect *per se*, reverted the atrophic effect of TNFα, restoring myotube size to that of untreated cells.

This result, in agreement with our hypothesis that blockade of the main way of ceramide biosynthesis induces a protective effect against TNFα-induced atrophy; therefore ceramide seems to play, at least in L6 model, an important role in TNFα-induced atrophy.

Considering that both myotube surface quantification methods gave similar results, the following studies were performed using immunofluorescence labeling of myotube surface, and quantification of surface by ImageJ software.

We tested another inhibitor of *de novo* Sphingolipid synthesis pathway, Fumonisin B1 (FB1) (Figure 5.6), a fungal toxin which inhibits ceramide synthase. This enzyme is involved in both de novo ceramide synthesis, converting sphinganine into dihydroceramide, and in the recycling pathway, catalyzing the formation of ceramide from sphingosine.

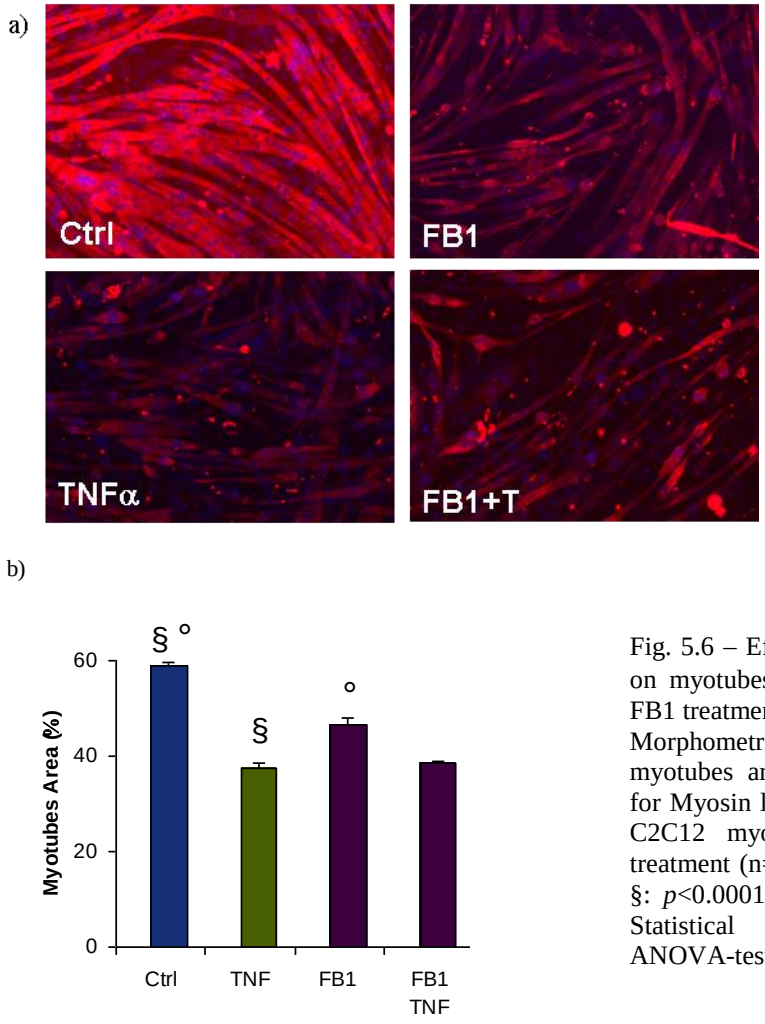


Fig. 5.6 – Effect of sphingolipid inhibitors on myotubes size, in presence of TNFα: FB1 treatment.
Morphometrical analysis of C2C12 myotubes area after Immunofluorescence for Myosin labelling (a). (b) Evaluation of C2C12 myotubes area (%) after FB1 treatment (n=3; Values are means ± SEM; §: $p<0.0001$; *: $p<0,0001$; °: $p<0,0001$). Statistical analysis performed with ANOVA-test.

Fumonisin B1 treatment of C2C12 myotube cultures, as we have seen for Myriocin, was not able to revert the effect of TNFα on myotubes size, although it had a less evident effect *per se*, as compared with Myriocin.

Compared to Myriocin, Fumonisin B1 acts on a more downstream step of sphingolipid pathway, and, by its action on the recycling pathway, it is able to induce an accumulation of sphingosine.

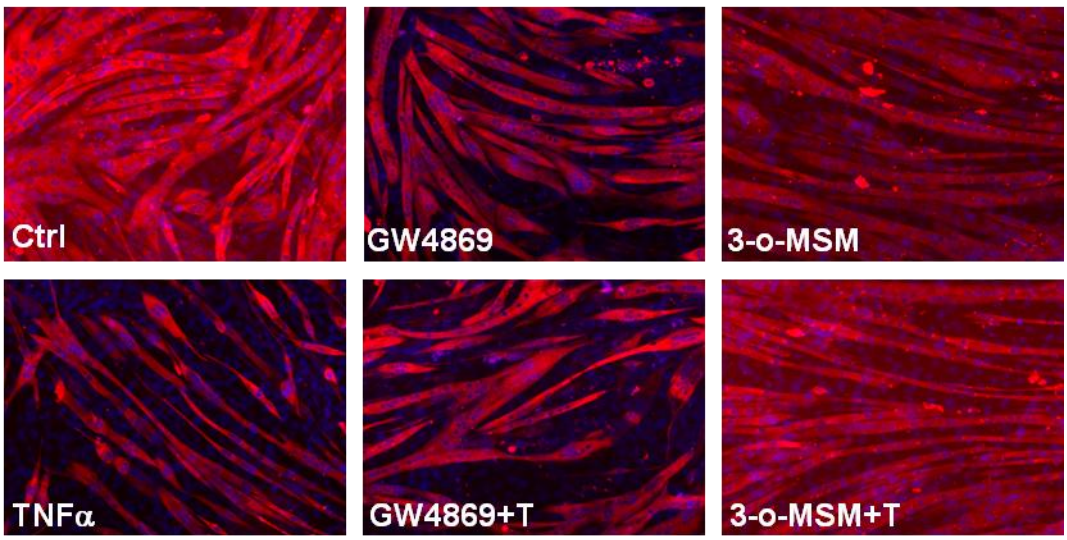
It is thus conceivable that it has a less drastic depleting effect on putative sphingolipid metabolites required for normal cell functions, and in particular that it does not prevent S1P formation. In addition, sphingosine itself is a bioactive compound known to induce cell apoptosis and blockade of differentiation (Menaldino et al., 2003; Cuvillier et al., 2002).

It is thus possible that sphingosine accumulation counteracts the beneficial effect of ceramide depletion, explaining why FB1 did not revert TNFα effects.

Ceramides can be produced both through the de novo synthesis pathway, and through hydrolysis of sphingomyelin, the most copious Sphingolipid in plasma membrane, by the activation of neutral sphingomyelinase (N-SMase). The action of this Sphingomyelinase can be inhibited by *GW4869* and *3-O-Methyl-Sphingomyelin*.

We tested both compounds in C2C12 myotubes cultures, in TNFα-induced atrophic condition (Figure 5.7).

a)



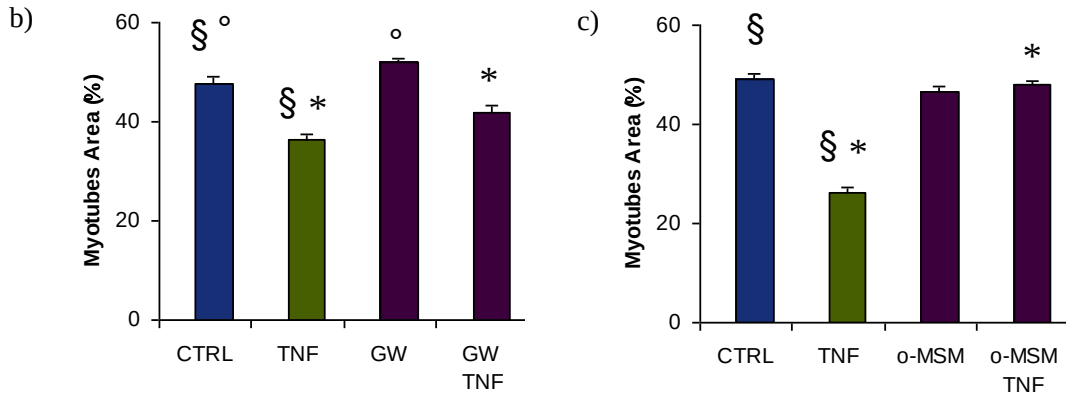


Fig. 5.7 – Effect of sphingolipid inhibitors on myotubes size, in presence of TNFα: GW4869 and 3-O-MSM treatments. Morphometrical analysis after Immunofluorescence for Myosin labelling (a).
(b) Evaluation of C2C12 myotubes area (%) after GW4869 inhibitor treatment (n=3; Values are means ± SEM; §: $p<0.0001$; *: $p=0,01$; °: $p=0,01$). (c) Evaluation of C2C12 myotubes area (%) after 3-o-MSM treatment (n=3; Values are means ± SEM; §: $p<0.0001$; *: $p=0,01$). Statistical analysis performed with ANOVA-test.

As shown in Figure 5.7b, *GW4869* had a slight but significant positive effect *per se* in non treated cultures. TNFα treatment had a confirmed atrophic effect that was significantly reverted by *GW4869* treatment.

3-O-MSM treatment had no effect by itself on myotube size, but it completely reverted TNFα effect, restoring myotubes size at the level of untreated cultures.

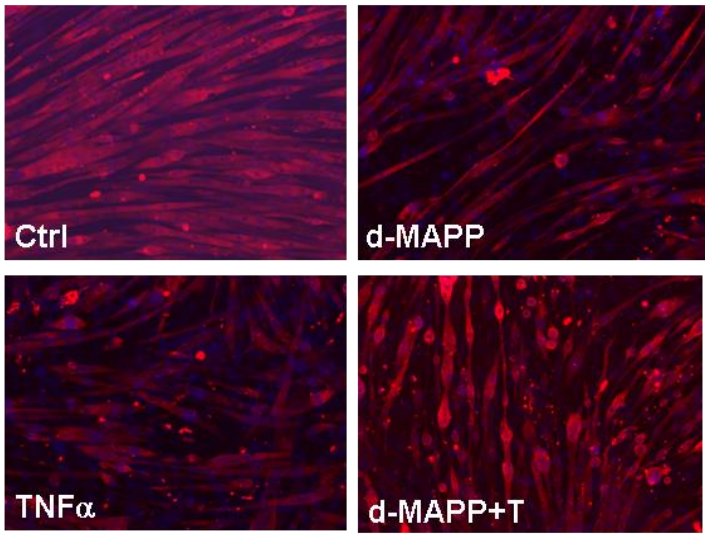
Because both inhibitors of neutral sphingomyelinases *GW4869* and *3-O-Methyl-Sphingomyelin* reverted TNFα-induced atrophy in C2C12 differentiated myotubes, we can conclude that ceramides, or some other downstream metabolite produced by the N-SMase pathway in response to TNFα stimulation, have a negative effect on C2C12 myotube size.

To better understand the role of ceramides in muscle atrophy, we evaluated the effect of inhibitors that target downstream steps of Sphingolipid pathway.

We thus tested, in our model of differentiated C2C12 myotubes, *D-erythro-2-(N-Myristoylamino)-1-phenyl-1-propanol* (d-MAPP), a specific inhibitor of alkaline ceramidase able to induce ceramide accumulation, and at the same time, to prevent the formation of Sphingosine-1-phosphate.

This inhibitor had a significant negative effect on C2C12 myotube size, by itself. When used alone, it induced a reduction in myotubes comparable to that induced by TNFα (Figure 5.8) and it aggravated the negative effect of TNFα.

a)



b)

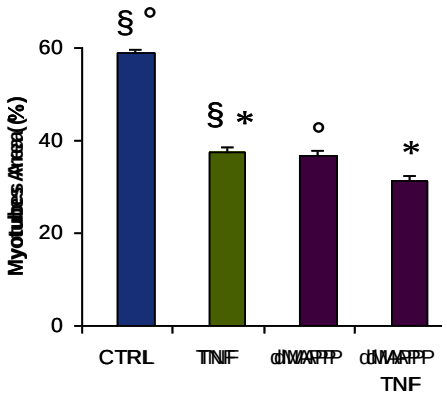


Fig. 5.8 – Effect of sphingolipid inhibitors on myotubes size, in presence of TNFα: D-MAPP treatment.
Morphometrical analysis of C2C12 myotubes area after Immunofluorescence for Myosin labelling (a). (b) Evaluation of C2C12 myotubes area (%) after D-MAPP treatment (n=3; Values are means ± SEM; §: p<0.0001; *: p<0,0001; °: p<0,0001). Statistical analysis performed with ANOVA-test.

This result can be interpreted as a confirmation of the negative effect of TNFα–induced ceramide accumulation on myotube size.

However, part of the effect of D-MAPP may results from its inhibitory action on the formation of S1P, since sphingosine is produced from ceramide by ceramidases action, and is then phosphorylated to Sphingosine-1-phosphate by sphingosine kinase.

Sphingosine generally has, within the cell, the same negative effect as ceramides (Hannun and Obeid, 2008). To evaluate the effect of sphingosine in our cell model, we analyzed the effects of *DL-threo-Dihydrosphingosine* (DHS) and *N,N-Dimethylsphingosine* (DMS), which induce sphingosine accumulation by inhibiting sphingosine kinase and Sphingosine-1-phosphate formation.

The inhibition of sphingosine kinase by both DHS and DMS in our differentiated C2C12 cultures, lead to a significant reduction of myotube size (Figure 5.9).

This suggests either that sphingosine has pro-atrophic properties, or that sphingosine-1-phosphate has protective effects.

In the presence of TNFα, the two compounds had different effects: whereas DMS potentiated the negative effect of TNFα, DHS slightly, but significantly, reverted TNFα effects.

A possible explanation is that DHS can be metabolized in cells into the rather stable and inactive *DL-threo-Dihydroceramide*, which could compete with endogenous ceramide for its targets, and thereby limit its pro-atrophic effects.

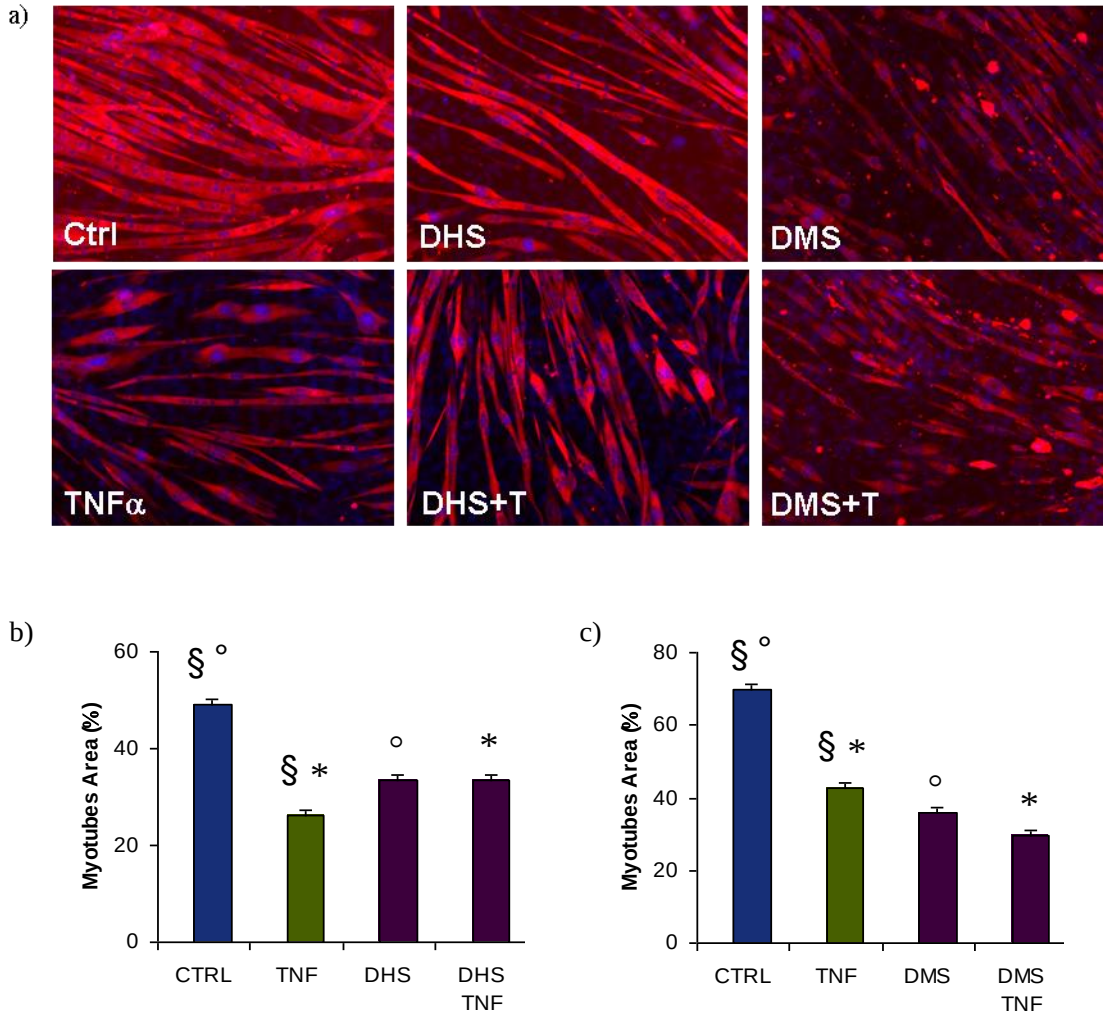
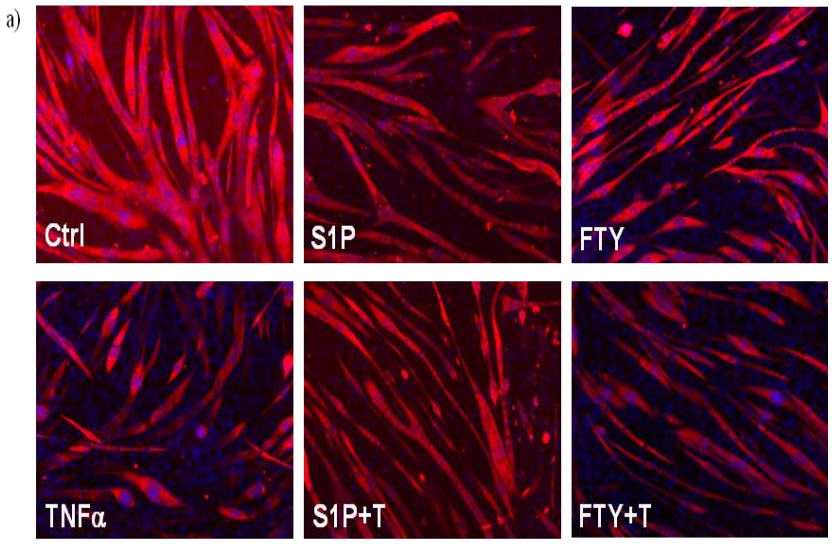


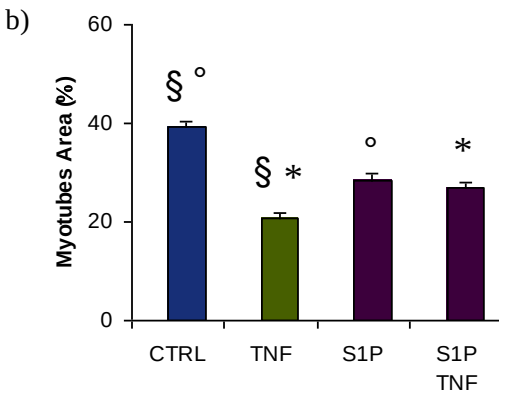
Fig. 5.9 – Effect of sphingolipid inhibitors on myotubes size, in presence of TNFα: DHS and DMS treatments. Morphometrical analysis of C2C12 myotubes area after Immunofluorescence for Myosin labelling (a). (b) Evaluation of C2C12 myotubes area (%) after DHS treatment (n=3; Values are means ± SEM; §: $p<0.0001$; *: $p<0{,}0001$; °: $p<0{,}0001$). (c) Evaluation of C2C12 myotubes area (%) after DMS treatment (n=3; Values are means ± SEM; §: $p<0.0001$; *: $p=0{,}0001$; °: $p<0{,}0001$). Statistical analysis performed with ANOVA-test.

In an attempt to delineate the role of S1P in our cell model, we evaluated the action on myotube size of other two drugs interfering with S1P signaling pathway (Figure 5.10). Exogenous *Sphingosine-1-phosphate* (S1P) had a negative effect, by itself, on myotube size. However, it significantly reverted atrophy in the presence of TNFα. Conversely, *Fingolimod* (FTY720), a general inhibitor of S1P receptors, increased TNFα-induced atrophy in C2C12 differentiated myotubes.

These data are consistent with a positive effect of S1P on myotube size, although the negative effect of S1P alone is difficult to explain in this regard.

In view of the complex signalling induced by S1P in muscle cell, which involves three different types of receptors (S1P1, S1P2, S1P3) (Brinkmann, 2007), it can be hypothesized that the response to S1P is dependent on the agonist concentration, and that this parameter is modified in the presence of TNFα.





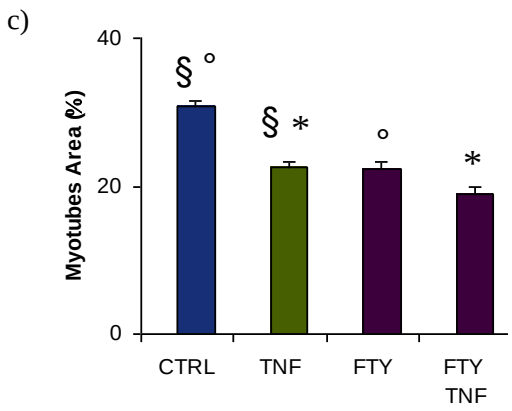


Fig. 5.10 – Effect of sphingolipid inhibitors on myotubes size, in presence of TNFα: exogenous S1P and FTY720 treatments. Morphometrical analysis of C2C12 myotubes area after Immunofluorescence for Myosin labelling (a). (b) Evaluation of C2C12 myotubes area (%) after Exogenous S1P treatment (n=3; Values are means ± SEM; §: $p<0.0001$; *: $p<0,0001$; °: $p<0,0001$). (c) Evaluation of C2C12 myotubes area (%) after FTY720 treatment (n=3; Values are means ± SEM; §: $p<0.0001$; *: $p=0,006$; °: $p<0,0001$). Statistical analysis performed with ANOVA-test.

In conclusion, in our *in vitro* model, ceramide mimicked TNFα-induced atrophy. Conversely, inhibitors of sphingomyelinase, a major pathway of ceramide synthesis, counteracted TNFα effect, confirming that ceramide is involved in the negative effect of TNFα on myotube size.

The fact that two inhibitors of the *de novo* synthesis pathway of ceramides had a negative effect may appear contradictory; however, several explanations can be put forward to solve this discrepancy.

First, as general inhibitors of Sphingolipid pathway, these compounds induce a depletion of several components essential for cell functioning.

A complementary explanation is based on the subcellular compartments where drug-induced ceramide accumulation takes place. *De novo* ceramide synthesis is known to take place in the Endoplasmic Reticulum (van Meer and Lisman, 2002), whereas sphingomyelinase-induced ceramide formation occurs in plasma membrane (Marchesini and Hannun, 2004).

It is thus conceivable that the pro-atrophic ceramide pool is only plasma membrane related, the de novo synthesized ceramide pool being unrelated to regulation of cell size.

However, *Sphingosine-1-phosphate* (S1P), a sphingolipid mediator deriving from ceramide metabolism, had a positive effect on TNFα-treated myotubes, whereas inhibitors of S1P synthesis or of S1P receptors had a negative effect on myotube size. The Sphingolipid pathway has thus a complex effect on muscle cell size, with both a negative influence of ceramide and a positive effect of S1P.

5.2.2 Effects of sphingolipid inhibitors on the expression of genes involved in the control of muscle atrophy.

We set out to investigate the possible impact of sphingolipids on the expression of genes involved in proteolysis. We thus quantified by RT-qPCR the expression of Atrogin-1, MuRF1, FoxO and PLD1 in myotubes treated with the inhibitors discussed in the previous paragraph.

Atrogin-1 and MuRF1 are two muscle-specific ubiquitin ligases (E3) that are part of a set of genes called "atrogenes" the expression of which increases or decreases coordinately when muscles atrophy (Bodine et al., 2001; Gomes et al., 2001).

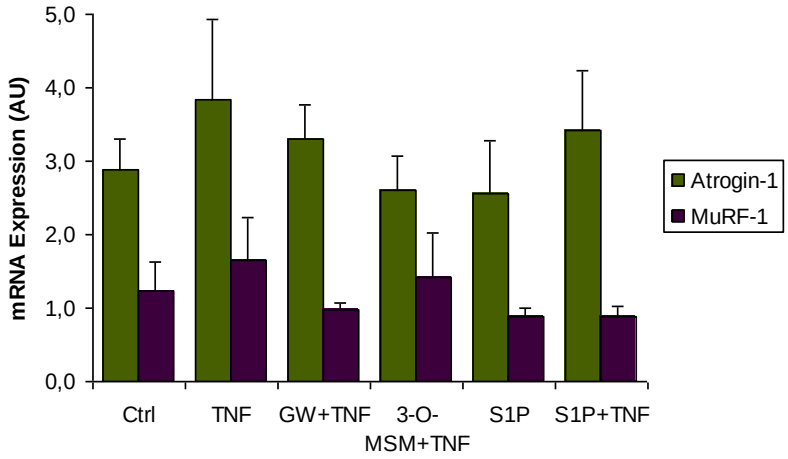


Fig. 5.11 – mRNA expression of Atrogenes in C2C12 myotubes cultures treated with sphingolipid pathway inhibitors in presence or absence of TNFα. qRT-PCR analyses of mRNA expression of Atrogin1 and MurF1. qRT-PCR values are normalized by TBP (TATA-box binding protein). Values are means ± SEM.

We observed (Figure 5.11) that TNFα slightly increased, as expected, the expression of these atrogenes. Compound GW4869 decreased the expression of MurF1, while 3-O-MSM had no effect, showing that the two inhibitors, although sharing a common target, sphingomyelinase, may have different mechanisms of action. Among the other tested drugs, only S1P had a significant effect, a reduction of MurF1 expression in the presence of TNFα, which is consistent with a protective effect of S1P against atrophy.

Atrogin-1 expression is directly controlled by FoxO transcription factors, which are in turn inhibited by the PI3K/Akt/mTOR pathway. Previous studies showed FoxO1 (Kamei et al., 2004) and FoxO3 (Sandri et al., 2004) to be involved in muscle atrophy. We did not observe significant changes in either FoxO1 or FoxO3 expression under

the influence of the various drugs. This may reflect the fact that these factors are in a large part controled at a post-translational level, by phosphorylation.

Phospholipase D (PLD) is an enzyme able to catalyze the hydrolysis of phosphatidylcholine to form phosphatidic acid (PA) a well recognized activator of mTOR signalling pathway (Foster, 2007). Moreover, ceramide is considered a general down-regulator of PLD (Mebarek *et al.*, 2007), able to prevent its activation and reduce its gene transcription (Venable and Obeid, 1999);(Nakashima and Nozawa, 1999). We thus considered important to assess PLD expression in myotubes treated with different inhibitors. None of the considered drugs induced significant changes in PLD1 expression. This ruled out an effect on myotube size mediated by this mechanism, but an effect of the compounds on enzyme activity remains possible.

5.2.3 Effect of sphingolipid inhibitors on the status of the Akt /mTOR pathway.

The signaling pathway that plays the most important role in regulating muscle mass is the Akt/mTOR pathway (Glass, 2003).

Protein catabolism in muscle is tightly controlled by the activity of Akt, which is reflected by its phosphorylation status. As for protein synthesis, it directly depends on the activity of the mTORC1 complex, which can be confidently evaluated by the phosphorylation status of its substrate S6K1. We thus evaluated by Western Blotting the phosphorylation of both proteins in TNFα and sphingolipid inhibitor treated myotubes.

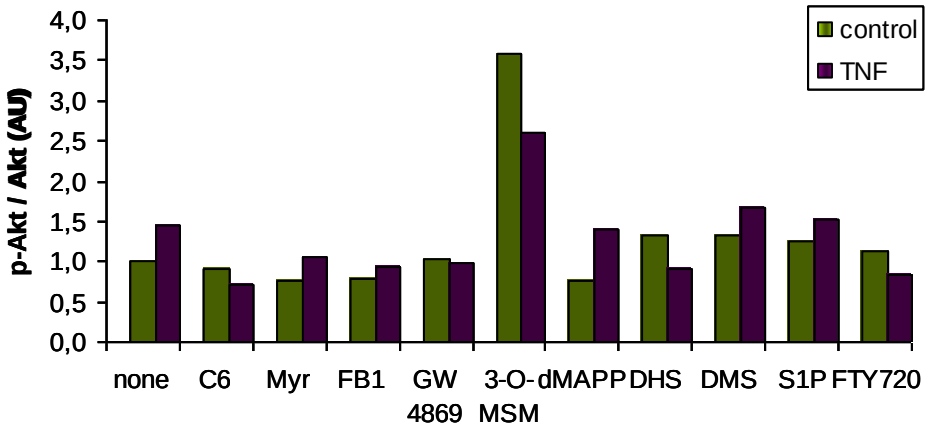
Akt phosphorylation was evaluated using an anti-phospho-Akt (Ser473), which detects the fully activated form of this kinase.

Statistical significance was blurred by rather high variations between the triplicates. However, major trends could be observed (Fig. 5.12a). We observed that, as described in literature, the stimulation of C2C12 myotubes with TNFα increased the activation of Akt. On the other hand a reduction of the activation of Akt was clear after C6 ceramide addition to myotubes cultures, in presence or absence of TNFα. An interpretation is that the complex signalling triggered by TNFα globally results in an activation of Akt, whereas the particular ceramide-related component of the response has a negative effect, in agreement with the reported inhibition of Akt by ceramide.

Except 3-O-MSM, none of the tested compounds had a clear effect on Akt phosphorylation. 3-O-MSM strongly enhanced basal and TNFα-induced Akt

activation, which is consistent with a negative effect of ceramide on Akt. However, surprisingly GW4869 rather had a negative effect, which is hard to reconcile with the ceramide hypothesis. To ascertain the above results, the amounts of phospho-Akt were also normalized with respect to tubulin (Fig. 5.12b), to eliminate the possible influence of changes in Akt protein expression. In this case too, 3-O-MSM strongly increased phospho-Akt amount, whereas GW4869 decreased it. Although these results would need confirmation on a higher number of experiments, they tend to suggest that the effects of the drugs on myotube size are not related to ceramide-induced changes in Akt activation.

a)



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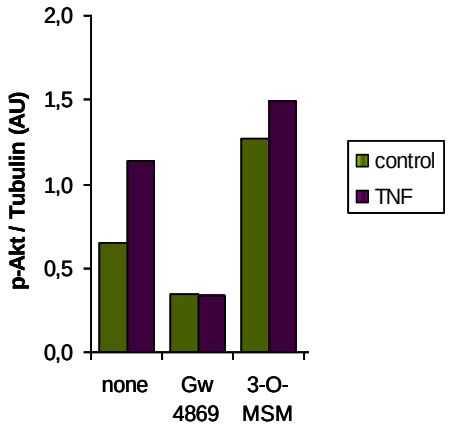

Fig. 5.12 – Immunoblot analysis of activation status of Akt kinase in C2C12 myotubes cultures treated with sphingolipid pathway inhibitors in presence or absence of TNFα
(a) Evaluation of the activation of Akt by quantification of phosphorylated Akt normalized by total Akt. (b) Evaluation of the total amount of phosphorylated Akt (normalized by tubulin).
Cell extracts were analyzed by immunoblotting using anti-Akt, anti-phospho-Akt (Ser473), anti-Tubulin.

The phosphorylation status of S6K1 was also evaluated by Western blotting using an antibody recognizing S6K1 phosphorylated on Thr389, a position targeted by mTORC1. Whereas TNFα had little effect (5.13a), C6-ceramide decreased S6K1

activation. The compounds inducing the most prominent changes in S6K1 phosphorylation were myriocin, 3-O-MSM, D-MAPP, S1P, all of them enhancing phospho-S6K1. The positive effects of these compounds were still observed when phospho-S6K1 was normalized by tubulin (5.13b). Here again, there does not appear to exist a correlation between the expected effects of the drugs on ceramide levels, their effect on myotube size, and their effect on S6K1 activity. Nevertheless, the marked enhancement by 3-O-MSM of both Akt phosphorylation and S6K1 phosphorylation suggests that this compound may reduce proteolysis, favouring protein synthesis, and present interesting therapeutic potentialities.

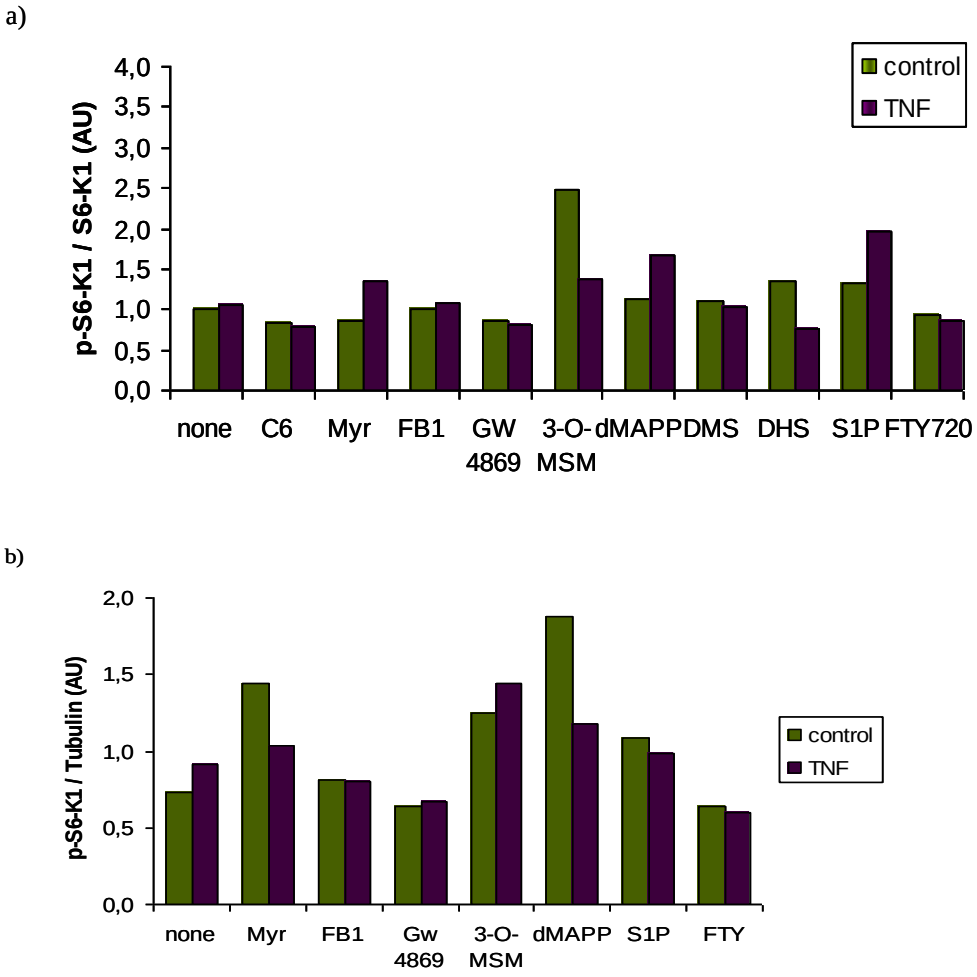

Fig. 5.13 – Immunoblotting analysis of activation status of mTORC1 pathway in C2C12 myotube cultures treated with sphingolipid pathway inhibitors, in presence or absence of TNFα. (a) Evaluation of S6-K1 activation by quantification of phosphorylated-p70-S6K1 normalized by total S6-K1. (b) Evaluation of the amount of phosphorylated-p70-S6K1, normalized by tubulin. Cell extracts were analyzed by immunoblotting using anti-S6-K1, anti-phospho-70 S6-K1 (Thr389), and anti-Tubulin antibodies.

5.3 In vivo effect of ceramide synthesis inhibition on tumor-induced atrophy.

5.3.1 Morphometric analysis of cachexia.

Because very few data are available about a possible role of ceramide, or a metabolite, in the *in vivo* process of muscle atrophy, we decided to study the effects of drugs able to block ceramide synthesis on cachexia, using a well-established model of muscle atrophy: BalbC mice carrying C26 Adenocarcinoma.

We inoculated a solid tumor under the skin, and after eight days we started daily treatment with Myriocin, a potent and selective inhibitor of Serinepalmitoyl-CoA transferase, the first enzyme of the *de novo* synthesis pathway of ceramide.

We began in vivo experiments with Myriocin because this drug is able, by acting upstream of ceramide synthesis, to deplete the cells in all Sphingolipids.

This experimental model leads to the development, within 6-10 days, of a palpable tumor on the back of mice, and a consequent muscle atrophy evaluated by body weight loss, muscle weight loss, and decrease in cross-section area of myofibers.

By analyzing the body weights of control mice (Figure 5.14), we deduced that Myriocin had no toxic effects, because animals treated with this drug had a weight-curve similar to that of untreated animals.

In tumor-injected mice, we observed a loss in body weight, as compared to control animals, but this decrease was significantly smaller in tumor-injected and myriocin-treated mice.

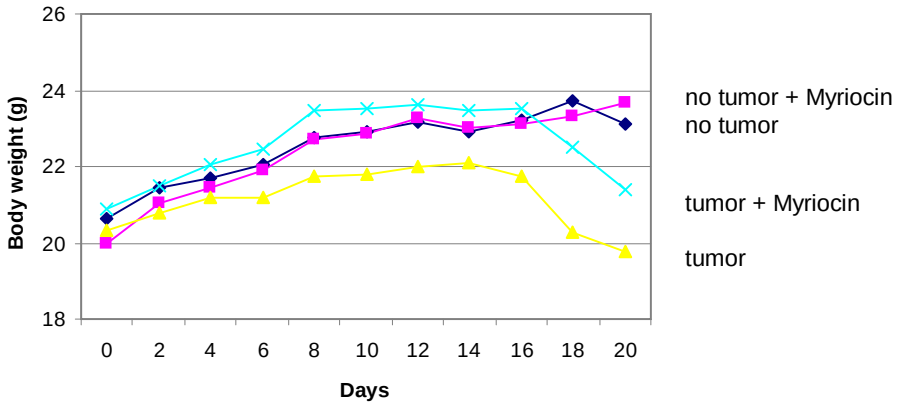

Fig. 5.14 – Mice body weights. Mice injected or not with C26 Adenocarcinoma were daily treated with Myriocin or saline solution and were weighed every day.

Moreover, we observed that muscle weight tended to be higher in tumor-injected and Myriocin-treated mice, as compared to tumor injected mice (Figure 5.15).

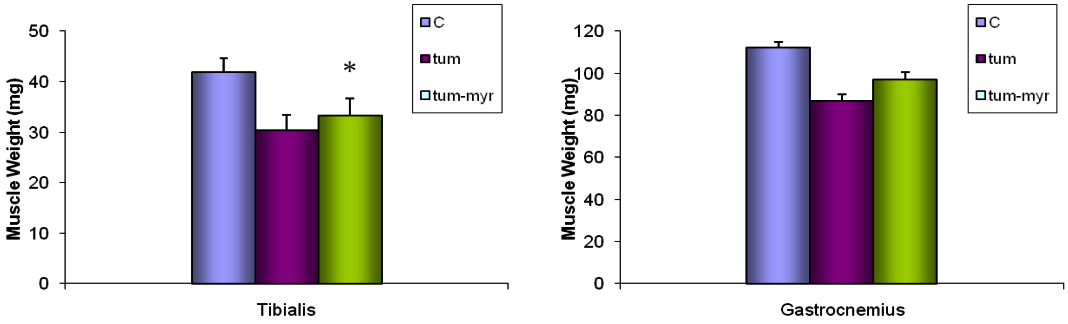

Fig. 5.15 – Muscle weights (*: 'tumor+Myriocin' different from 'tumor', p <0.05).

We also analyzed another important parameter of muscle atrophy: the Cross-sectional Area of myofibers (Figure 5.16a). We quantified the CSA of *Tibialis* (Figure 5.16b) and *Gastrocnemius* (Figure 5.16c) muscle and we observed that, as expected, tumor induced a marked reduction in myofiber size for both muscles. Importantly, Myriocin treatment significantly reduced this decrease.

This observation suggests a protective effect of Myriocin against tumor-induced atrophy, presumably due to the depletion of ceramides in treated mice.

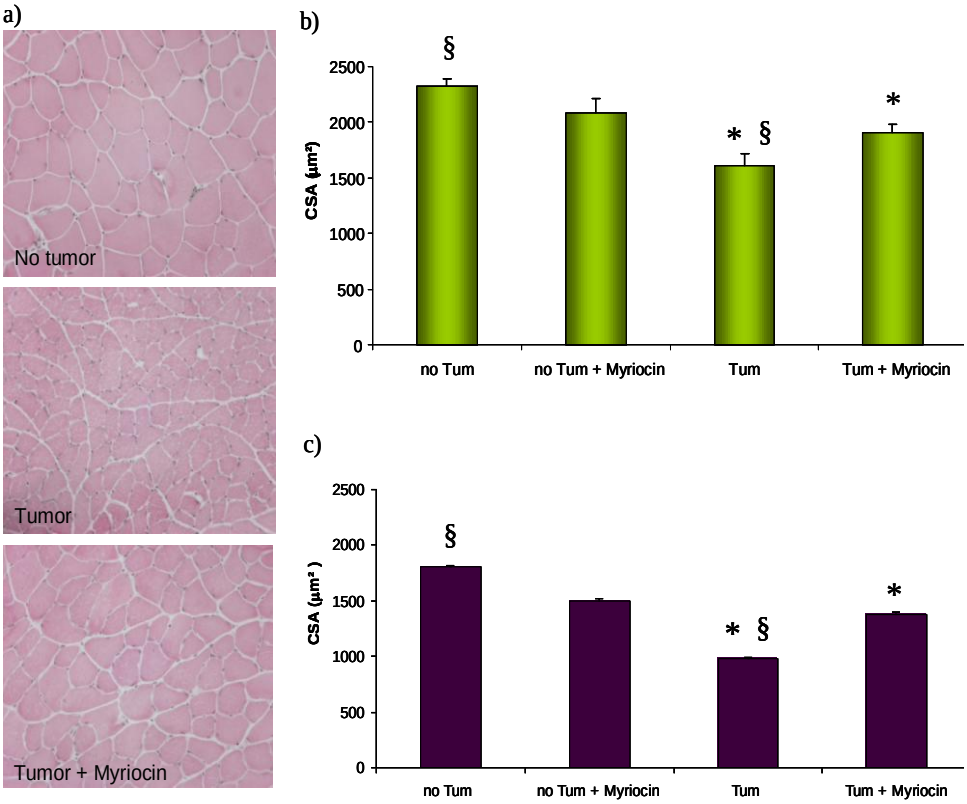

Fig. 5.16 – Histological analysis of mouse muscles. (a) Cross-sections of muscle stained with Hematoxylin-Eosin. (b) CSA quantification of myofibres from Tibialis muscle (Values are means ±SEM; §: p <0.0001; *: p<0,05). (c) CSA quantification of myofibres from Gastrocnemius muscle. Values are means ± SEM; §: p<0,001;*: p =0.0002. Statistical analysis with ANOVA-test.

However, Myriocin *per se* had a slight negative effect on fibre size, probably due to depletion in cell, besides ceramide, of sphingolipidic metabolites involved in a positive regulation within the cell, such as Sphingosine-1-Phosphate, that is known to promote cell growth and hypertrophy in certain cell types (Robert *et al.*, 2001).

Furthermore, the analysis of myofibre size distribution (Figure 5.17) provided important information. As expected, the tumor shifted the sizes towards small fibres, and Myriocin treatment restored a distribution similar to that of tumor-devoid mice.

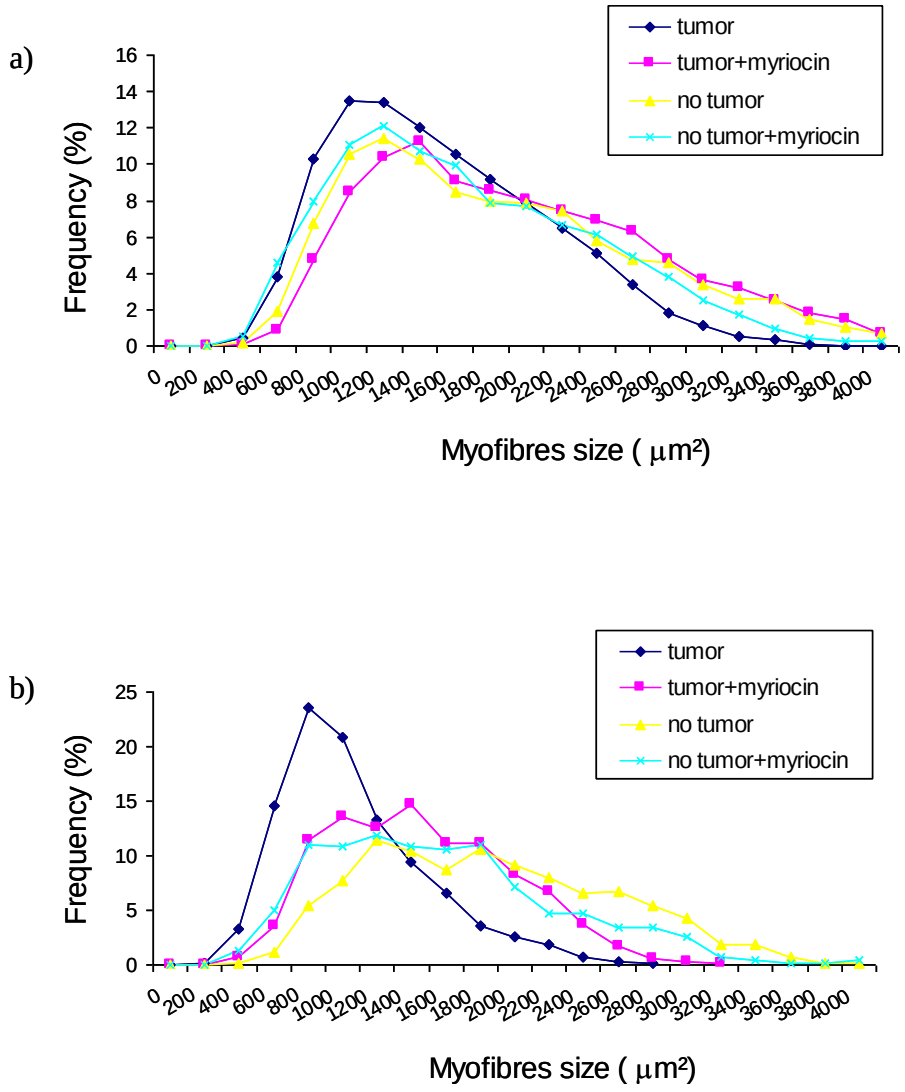

Fig. 5.17 – Myofibre size distribution. (a) Distribution of myofibres from Tibialis muscle. (b) Distribution of myofibres from Gastrocnemius muscle.

In Gastrocnemius muscle (Figure 5.17b), myofibre sizes from non tumor-injected mice are widely distributed, with a plateau in the curve at 1200-2000 μm^2 of CSA.

In tumor-injected mice, the distribution curve is drastically shifted to lower sizes, and shows a peak at 800-1200 μm^2, reflecting a major cachexia, with a loss of large fibres (1800-3600 μm^2).

Treatment with Myriocin (tumor-injected and Myriocin-treated group) brings back the curve to the right, indicating a partial preservation of large fibres, and the almost absence of very atrophic fibres formation (<800 μm^2). The curve shows a plateau at 800-2000 μm^2. So, there is a clear anti-atrophic effect of Myriocin.

The treatment of non tumor-injected mice with Myriocin leads to a distribution very similar to the distribution obtained with the tumor-injected and Myriocin-treated animals. This suggests that Myriocin *per se* has a small reducing effect on the size of myofibres, although it does not affect the heterogeneity of their distribution.

In *Tibialis* muscle (Figure 5.17a), the effects of the tumor and Myriocin are similar, although less marked, presumably because of the greater heterogeneity of this muscle, richer in type fibres I considered more resistant to atrophy (Zierath and Hawley 2004), than *Gastrocnemius*.

5.3.2 Expression of genes related to muscle atrophy.

To better define the atrophic status of the muscle samples we also performed molecular analysis of several atrophy markers. Under atrophic conditions, it is well known (Cao et al. 2005) that protein degradation processes are largely dependent on ubiquitin-proteasome-mediated proteolytic pathway. We thus assessed, by quantitative PCR, the expression levels of FoxO factors, Atrogin1 and MuRF1 (Figure 5.18), which are involved in this pathway.

All these specific markers were, as expected, more expressed in tumoral samples. Interestingly, the expression of FoxO3, Atrogin-1 and MuRF1 was reduced following Myriocin treatment. Only FoxO1 expression was not affected by myriocin.

This reduction of mRNA expression could contribute to decreased proteolysis as a result of treatment with Myriocin, and participate in the anti-atrophic effects of the drug.

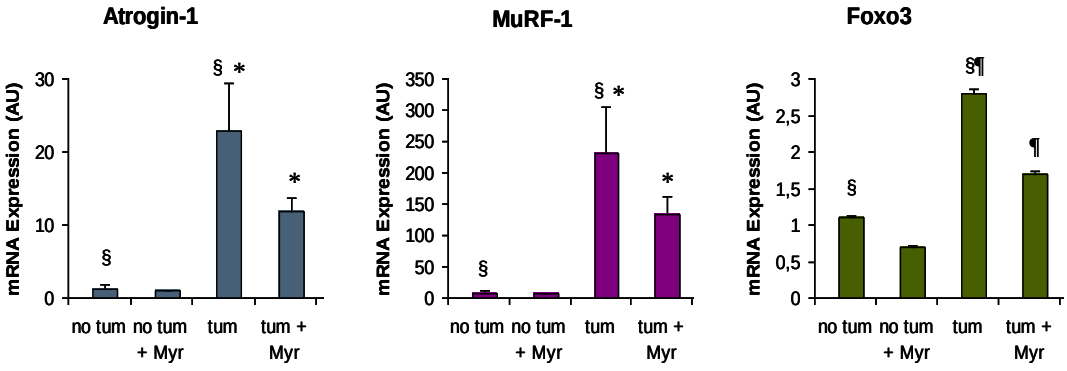

Fig. 5.18 – mRNA expression of Atrogenes. qRT-PCR analyses of mRNA expression of Atrogin1, MurF1 and FoxO3 in Gastrocnemius muscle of the different groups of mice: 'no tumor', 'no tumor+Myriocin', 'tumor' and 'tumor+Myriocin'. qRT-PCR values are normalized by Cyclophilin B. Values are means ± Sd; §: p <0.05; *: p <0,05; ¶: p = 0,05.

We also analyzed by quantitative PCR the mRNA expression of other genes directly or indirectly involved in muscle atrophy (Tab.1).

Gene	no tumor	no tumor + Myriocin	tumor	tumor + Myriocin
FoxO1	0,14±0,08	0,11±0,05	0,28±0,15*	0,26±0,08
Myostatin	0,91±0,40	0,49±0,08	0,82±0,62	0,78±0,55
MyoD	0,22±0,12	0,2±0,01	0,26±0,14	0,05±0,03 §
MEF2	2,21±1,1	1,65±0,24	1,24±0,98	0,28±0,07 §
Myogenin	ND	ND	ND	ND
PLD1	0,024±0,01	0,02±0,002	0,04±0,024	0,015±0,01 §
PLD2	0,037±0,01	0,038±0,002	0,068±0,03*	0,016±0,002 §
SREBP-1c	0,02±0,009	0,014±0,002	0,002±0,001*	0,0018±0,001
SREBP-1a	0,03±0,014	0,022±0,004	0,042±0,02	0,022±0,008

Tab. 1 – mRNA expression levels. qRT-PCR analyses of mRNA expression of different genes in Gastrocnemius muscle of the different groups of mice: 'no tumor', 'no tumor+Myriocin', 'tumor' and 'tumor+Myriocin'. qRT-PCR values are normalized by Cyclophilin B. Values are means ± SD; *: 'tumor' different from 'no tumor', p <0,05; §: 'tumor+Myriocin' different from 'tumor', p <0.05 and ND = not detectable.

The expression of Myostatin, a negative regulator of muscle mass, is neither affected by the tumor nor by Myriocin, so we can deduce that this factor is not involved in the observed effects.

The expression of myogenic regulatory factors MyoD and MEF2 was not affected by the tumor. However, it was significantly lowered by treatment with Myriocin, which appears rather paradoxical. As for Myogenin, it was undetectable in any sample.

The expression of Phospholipase D (PLD) was increased by the tumor, significantly for the isoform PLD2, and Myriocin treatment significantly decreased the expression of PLD2 in tumor-injected mice. PLDs are involved in multiple cellular functions, including inflammation, so we hypothesized that inflammation induced by tumor increased the expression levels of PLD, and that this inflammation was decreased by Myriocin treatment, because the activation of macrophages is known to induce an increase in PLD activity (Melendeza and Allen, 2002).

We thus tested the possibility that an increased infiltration of macrophages occurred in muscle of tumor-injected mice that would be suppressed by Myriocin treatment. Muscle sections were stained by the Esterase method (Figure 5.19), and macrophages quantified. This analysis did not confirm our hypothesis.

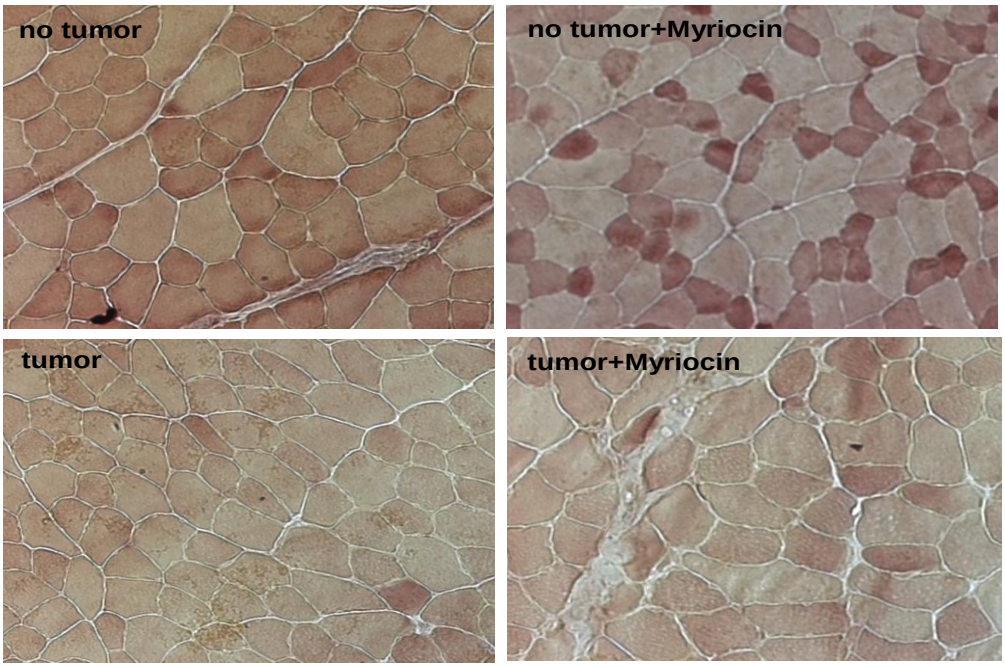

Fig. 5.19 – Esterase staining of muscle sections.

Transcription factors SREBP-1 are known to regulate the expression of several enzymes implicated in fatty acids and cholesterol synthesis. They also have a role in muscle atrophy, in fact their over-expression leads to *in vivo* and *in vitro* muscle atrophy (Lecomte *et al.*,)2010).

We observed a drastic reduction of SREBP-1c under the effect of the tumor. This might be understood as a reaction of cells to limit atrophy. Myriocin had no effect on SERBP-1, and thus probably does not act on this pathway.

5.3.3 Activation status of the Akt/mTOR pathway.

The transcriptional activity of FoxO is regulated by insulin through the phosphoinositide-3 kinase (PI3K)/Akt signaling pathway (Stitt et al., 2004). In normal conditions, FoxO is repressed by phosphorylated Akt, the active form, but when atrophy occurs, the non-phosphorylated form of Akt becomes predominant, leading to nuclear exclusion and eventual ubiquitination-dependent proteasomal degradation of FoxO factors.

The activation status of Akt was evaluated, in muscles samples from the different groups of mice by Western Blot, using an anti-phospho-Ser473-Akt antibody.
We assessed both the relative amount of phosphorylated Akt (normalized by actin amount), and the fraction of phosphorylated Akt reported to total Akt (Figure 5.20).

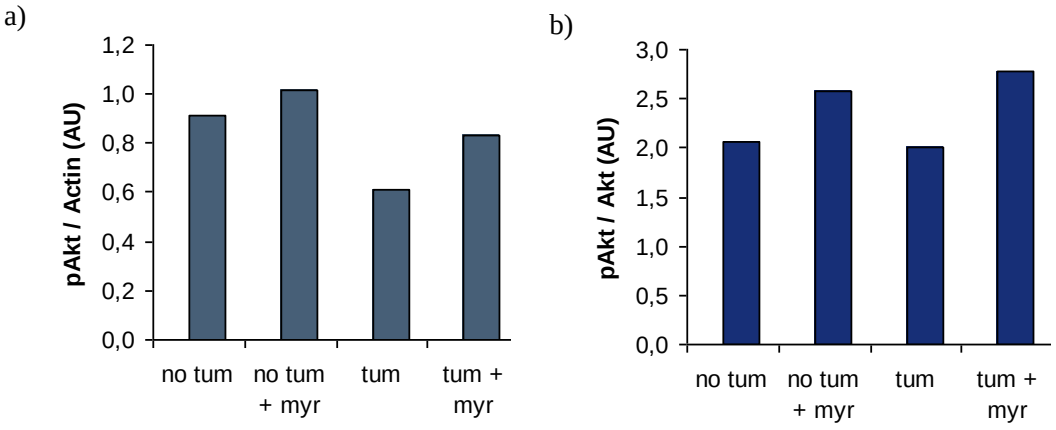

Fig. 5.20 – Immunoblot analysis of activation status of Akt kinase in Gastrocnemius muscle of mice from the different groups: 'no tumor', 'no tumor+Myriocin', 'tumor' and 'tumor+Myriocin'. a) Evaluation of the amount of phosphorylated Akt, as normalized by actin b) Evaluation of the fraction of phosphorylated Akt vs. total Akt.

The active form of Akt kinase, phospho-Akt, was reduced in tumor-injected mice compared to uninjected mice, and, as expected, Myriocin treatement tended to revert this reduction (Figure 5.20a).
If we analyze the ratio of phosphorylated Akt / total Akt (Figure 5.20b), we do not observe a reduction after tumor injection, possibly because the presence of tumor decreased also the amount of total Akt. However, we still observe an increase after Myriocin treatment.
This result shows that ceramide inhibition seems to restore in part tumor-reduced Akt activity in skeletal muscle *in vivo*.
Akt kinase is closely related, both as an activator and as an effector, to one of the signaling pathways most important in regulation of muscle growth and myofiber size: the mTOR pathway. In particular, Akt activates mTORC1 complex, which in turn regulates protein synthesis and transcription through phosphorylation/inactivation of the repressor of mRNA translation 4E-binding protein 1 (4E-BP1), and by the phosphorylation/activation of ribosomal S6 kinase (S6K1) (Bhaskar and Hay, 2007). The activation status of mTORC1 in muscle samples of mice was assessed by Western

Blot using anti-phospho-Thr389-p70 S6K1 (Figure 5.21a) and anti-phospho-Thr37/46 4E-BP1 (Figure 5.21b) antibodies.

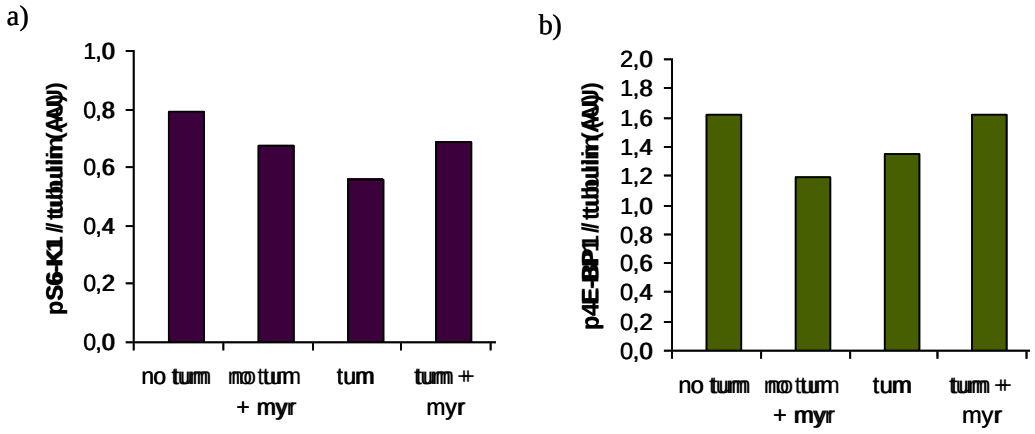

Fig. 5.21 – Immunoblots analysis of activation status of mTOR pathway in Gastrocnemius muscle of mice in all our condition: 'no tumor', 'no tumor+Myriocin', 'tumor' and 'tumor+Myriocin'. (a) Evaluation of expression of phosphorylated p70 S6K1. (b) Evaluation of expression of phosphorylated 4E-BP1. The densitometric values are normalized by the amount of Tubulin.

The presence of tumor tended to decrease both phospho-S6K1 and phospho-4E-BP1 amounts. Although it had a negative effect *per se*, Myriocin treatment tended to restore the phosphorylation of both mTORC1 substrates in the presence of tumor.

These results suggest that tumor-induced cachexia is accompanied by an inhibition of the Akt/mTORC1 pathway. Interestingly, in cachectic mice, Myriocin treatment restores the activity of the Akt/mTOR pathway, with expected positive repercussions on protein synthesis and negative on proteolysis, which can participate in the protective effect of myriocin on muscle.

Chapter 6
Discussion

In mammalian cells, ceramide are a major precursor of complex Sphingolipids, which are important constituents of the cell membrane and bioactive lipids that mediate various cellular processes, such as cell growth arrest, differentiation, and apoptosis (Futerman and Hannun, 2004 ; Hannun and Obeid, 2008).

Studies from various laboratories indicate that cellular ceramide are increased in mammalian cells in response to various stressful stimuli, such as pro-apoptotic cytokines (TNF-α (Hanna et al., 2001), IL-1β (Rolz et al., 2003), FAS ligand, and interferon γ (Wakita et al., 1996), cancer chemotherapeutic agents (Charles et al., 2001), UV (Magnoni et al., 2002) and ionizing-irradiation (Jaffrezou et al., 2001), vitamins or derivatives such as vitamin D (Bektas et al., 2000; Geilen et al., 1996), and retinoic acid (Herget et al., 2000) and serum-deprivation (Colombaioni et al., 2002).

Blocking increases in ceramide inhibits cell-growth arrest and/or apoptosis in response to these stressful stimuli. In contrast, addition of exogenous cell-permeable, short-chain ceramides (C2-ceramide, C6-ceramide or C8-ceramide) induces cell-growth arrest and/or apoptosis (Hannun and Obeid, 2008).

Moreover, ceramide, has been shown to participate in affecting of all the factors involved in the etiology of muscle atrophy such as the inhibition of myogenic

differentiation and muscle regeneration (Strle et al., 2004; Mebarek et al., 2007) or muscle protein reduction by blockade of protein synthesis (Strle et al., 2004) and activation of different catabolic pathways (Meadows et al., 2000).

All these observations suggest that an increased generation of ceramide can mediates muscle wasting and, in this thesis, we set out to investigate the potential role of ceramides in tumor-induced cachexia.

To study the effect *in vivo* of Sphingolipids in the occurrence of muscle atrophy, we used Balb/c C26-bearing mice a well characterized experimental model for cachexia (Aulino et al., 2010).

In vivo, we showed that inhibition of *de novo* Sphingolipid synthesis by Myriocin significantly reduced the atrophic effect of C26 adenocarcinoma on skeletal muscle.

This suggested that ceramide, the central metabolite of sphingolipid pathway, could participate in tumor-induced muscle loss.

The effect of Myriocin could not be ascribed to an anti-tumoral action, because the drug did not affect the size of tumors. It remains to be verified that Myriocin had no effect on pro-inflammatory cytokine levels, and that it actually lowered ceramide levels in plasma and muscle tissue.

The protective effects of Myriocin are reflected by a significant reduction in the expression of FoxO3 factor, a major positive regulator of the atrophic process, and of the Ubiquitin ligases Atrogin-1 and MuRF-1, which are involved in myofibrillar protein degradation, and whose expression is induced by FoxO3 (Sandri et al., 2004; Stitt et al., 2004).

By examining the phosphorylation status of Akt and mTORC1 substrates, S6-K1 and 4E-BP1, we could observe that the presence of the tumor was linked with a decreased activation of these factors.

Because Akt regulates both protein synthesis, through mTORC1/S6-K1/4E-BP1 activation (Stitt et al., 2004; Terada et al., 1994), and proteolysis, through the cytosolic sequestration of FoxO factors, which are the major inducers of proteasome and autophagy proteolytic systems (Zhao et al., 2008), the muscle loss induced by tumors could result from both a decrease in protein synthesis, and in protein degradation (Bodine at al., 2001; Mammucari et al., 2008).

Interestingly, Myriocin treatment in tumor bearing mice restored the activation of both Akt and mTORC1, showing that Myriocin suppressed an inhibition taking place upstream the Akt/mTOR pathway.

Our working hypotheses were that the tumor-induced atrophy resulted from the activation by pro-inflammatory cytokines which are highly expressed in cancer (Li and Reid, 2000), leads to ceramide formation by various pathways, and that accumulated ceramide could affect the Akt/mTOR pathway in various ways.

One possibility was that ceramide inhibited Phospholipase D (PLD), as reported in several cell systems (Venable and Obeid, 1999; Nakashima and Nozawa, 1999; Singh et al., 2001; Mansfield et al. 2004; Nakamura et al., 1996).

PLD is an activator of both mTORC1 complex, the regulator of protein synthesis, and mTORC2 complex, responsible of full Akt activation and inhibition of proteolysis (Fang et al, 2003; Hornberger et al, 2006).

However, we did not detect a positive effect of Myriocin on the expression of PLD1 and PLD2 isoforms, which rules out the involvement of PLDs in the observed phenomena.

Other ceramide targets are known to interfere with mTOR signalling, such as amino acid transport, which is necessary for mTOR activity (Hyde et al, 2005).

Furthermore, ceramide is known to activate the NF-kB pathway (Wu et al, 2007), and the autophagic process (Pattingre et al., 2009; Scarlatti et al., 2004), which are involved in proteolysis.

It remain to be determined if these systems are affected in our *in vivo* model.

Nevertheless, our results show that Sphingolipid metabolism participates in tumor-induced muscle wasting, and suggest that drugs affecting this pathway may present a therapeutic interest in the management of cancer-associated troubles.

To study in more details the involvement of Sphingolipid metabolism in the atrophy of muscle cells and to understand if other drugs, able to modulate the Sphingolipid metabolism at different stages, could have an effect on muscle wasting, we set up an *in vitro* model, by using C2C12 differentiated myotubes treated with TNFα.

At doses present in cancer patient circulation, TNFα is known to induce muscle atrophy (Li et al., 2003; Argiles et al., 1999; Li et al., 1998; Tisdale et al., 2009; Mantovani et al., 2000; Saini et al; 2006; Phillips and Leeuwenburgh, 2005).

In vitro, we observed a marked reduction of myotubes size under TNFα treatment and this effect could be mimicked by cell-permeant ceramides.

However, inhibition of ceramide synthesis by Myriocin was unable to counteract TNFα effect in C2C12 myotubes, in contrast with what observed, in our group, with myotubes deriving from another myogenic cell line, L6 myoblast.

The result obtained in L6 myotubes was consistent with the *in vivo* effect of Myriocin and confirmed that this drug acted directly in muscle cells to prevent ceramide-mediated atrophy.

In C2C12 myotubes, the lack of positive Myriocin effect might be ascribed to a strong negative effect of Myriocin *per se* on cell size, this compound is indeed able to extensively deplete the cells in all sphingolipid metabolites, by blocking the first step of their synthesis. It is likely that Myriocin induced a deprivation in a component necessary for normal cell functions, e. g. sphingomyelin and glycosphingolipids, which are important components of cell membrane.

The results were similar with Fumonisin B1, which also targets *de novo* ceramide synthesis, confirming that C2C12 myotubes require a certain level of sphingolipid synthesis to maintain their size.

However, by blocking the other major pathway of ceramides synthesis, the sphingomyelin hydrolysis neutral sphingomyelinase-mediated (N-SMase), we could prevent the loss in myotubes size induced by TNFα, with both GW4869 and 3-O-methylsphingomyelin compounds (3-O-MSM).

This result confirms that TNFα-induced ceramide formation participates in the atrophy of myotubes.

These two compounds, contrary to Myriocin and Fumonisin B1, are not expected to block the general sphingolipid synthesis pathway and to deplete the cell in all sphingolipid metabolites, which could explain their positive effects.

Another explanation is that *de novo* ceramide synthesis takes place in the Endoplasmic Reticulum, whereas neutral sphingomyelinase produces ceramides in the inner leaflet of plasma membrane. It is thus conceivable that only the plasma membrane pool of ceramide is involved in protein regulation. Fractionation of subcellular compartments and measurement of localized ceramide pools might help to solving this question.

One difficulty encountered in the study of sphingolipid signalling is that ceramide can be efficiently converted in others metabolites. It is thus hydrolyzed by ceramidase into sphingosine, which can be itself phosphorylated by sphingosine kinase to give sphingosine-1-phosphate (S1P). S1P, secreted in the intracellular space or in the bloodstream can act as an agonist on a set of G protein-coupled receptors, three of which ($S1P_1$, $S1P_2$ e $S1P_3$) are expressed in muscle cells (Brinkmann, 2007). These receptors induce several signalling pathways likely to interfere with cell metabolism regulation.

We thus attempted to delineate the role of the various sphingolipid metabolites, by blocking the pathway at different steps, downstream ceramide.

D-MAPP, an inhibitor of ceramidase which is thus expected to potentiate the TNFα-induced ceramide accumulation, aggravates the atrophic effect of this cytokine, which confirms that ceramides are accumulated in response to TNFα- induced atrophy.

The effects of sphingosine kinase inhibitors in the presence of TNFα were not clearcut, and did not allow to conclude about the role of S1P.

However, the effect of the more stable inhibitor DMS tended to suggest that inhibition of S1P formation had negative effects on myotube size, and thus that S1P is positively involved in myotubes size regulation.

Accordingly, exogenous S1P is able to partially counteracted TNFα- induced atrophy, and the general S1P-receptor inhibitor FTY720 potentiated TNFα effect.

On the whole, these *in vitro* studies confirm that ceramide participates in the atrophic effects of TNFα, and that inhibition of its synthesis presents therapeutic potentialities.

The possible positive involvement of S1P in the regulation of myotubes size would deserve more detailed studies, in particular by selective blockade of the various S1P receptors presents.

The *in vitro* protective effects of two sphingomyelinase inhibitors are a strong incentive for studying their *in vivo* effects in animal models of cachexia.

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Prénoms : ALESSANDRA
Titre : RÔLE DES SPHINGOLIPIDES DANS L'ATROPHIE MUSCULAIRE / ROLE OF SPHINGOLIPIDS IN MUSCLE ATROPHY
Nature : DOCTORAT Numéro d'ordre : AAAAISALXXXX
Ecole doctorale : EDISS (ECOLE DOCTORALE INTERDISCIPLINAIRE SCIENCES-SANTE)
Spécialité : BIOCHIMIE

Resumé : Les sphingolipides ont été longtemps considérés comme une famille de lipides membranaires dotés d'un rôle structural, influant sur les propriétés de la bicouche lipidique. Cependant, plus récemment, les études de leur métabolisme et de leurs fonctions ont révélé qu'ils agissent aussi comme des molécules effectrices au rôle essentiel dans de nombreux aspects de la biologie cellulaire.
Le céramide, la sphingosine et la sphingosine-1-phosphate (S1P), les sphingolipides les plus étudiés, ont des effets opposés dans diverses fonctions cellulaires: tandis que le céramide et la sphingosine inhibent la prolifération et promeuvent la réponse apoptotique à différents stimulus de stress, la S1P est un stimulateur de la prolifération et de la survie cellulaires. Le céramide, molécule centrale de la voie des sphingolipides, et les autres médiateurs sphingolipidiques peuvent s'interconvertir, ce qui complique considérablement l'étude de leurs fonctions.
Le céramide peut être produit par deux voies différentes: la voie de synthèse de novo, et l'hydrolyse de la sphingomyéline membranaire catalysée par les sphingomyélinases. Ces deux voies peuvent être activées par la cytokine pro-inflammatoire TNFα. Comme cette cytokine est capable d'induire une perte musculaire, et semble jouer un rôle crucial dans le développement de la cachexie, nous avons fait l'hypothèse que le céramide, ou un de ses métabolites, peuvent être des médiateurs de la perte musculaire tumeur-induite.
Nous avons tout d'abord examiné le rôle du céramide dans l'atrophie induite in vitro par le TNFα chez les myotubes C2C12 différenciés, en utilisant des analogues cell-perméants de céramide et des inhibiteurs du métabolisme sphingolipidique. L'apport de céramides exogènes est capable de reproduire l'effet atrophique du TNFα, évalué d'après la surface des myotubes, ce qui suggère que le céramide peut participer à l'atrophie musculaire. Pour vérifier si les céramides sont les médiateurs de l'atrophie induite par le TNFα et identifier ses métabolites potentiellement impliqués, nous avons analysé l'effet d'inhibiteurs ciblant différentes étapes du métabolisme: l'inhibition de la voie de synthèse de novo est incapable de rétablir la taille des myotubes en présence de TNFα, alors que les inhibiteurs de sphingomyélinase neutre suppriment l'atrophie TNFα-induite. De plus, l'accumulation de céramide et de sphingosine augmente l'effet pro-atrophique, tandis que la S1P a un effet protecteur. Ces observations montrent que, dans les myotubes C2C12, le céramide, ou un métabolite tel que la sphingosine, produits par la voie de la sphingomyélinase neutre en réponse à une stimulation par le TNFα, participent à l'atrophie des cellules.
Pour évaluer le rôle in vivo des sphingolipides, nous avons utilisé un modèle établi d'atrophie musculaire tumeur-induite, la souris BalbC porteuse d'un carcinome C26. La myriocine, inhibiteur de la synthèse de novo, capable d'induire une déplétion générale du muscle en sphingolipides, a été administrée quotidiennement aux animaux. Ce traitement protège partiellement les souris contre la perte de poids corporel et de poids des muscles induite par la tumeur, sans affecter la taille de celle-ci. De plus, la myriocine réverse significativement la perte de taille des fibres musculaires due au développement de la tumeur, et réduit l'expression des atrogenes Foxo3 et Atrogin-1, ce qui montre qu'elle protège le muscle contre l'atrophie.
Ces résultats suggèrent fortement que le céramide, ou un métabolite sphingolipidique en aval, est impliqué dans l'atrophie musculaire tumeur-induite. La voie des sphingolipides apparaît donc comme une nouvelle cible potentielle d'interventions pharmacologiques visant à protéger le tissu musculaire contre l'atrophie.

Mots-clès : SPHINGOLIPIDS – CERAMIDES – MUSCLE ATROPHY - TNFα - CACHEXIA

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