The Role of Carnitine System in Maintaining Muscle Homeostasis

Menotti Calvani, Gianfranco Peluso[(1)], Paola Benatti, Raffaella Nicolai and Emilia Reda

Scientific Department, Sigma Tau S.p.A., Pomezia, Rome and (1) Department of Experimental Oncology, National Cancer Institute, Naples, Italy

Abstract

Carnitine-dependent metabolic processes provide skeletal muscle with the ability to gain and to use energy from the environment, and relate mainly to the fatigue-resistant type I fibers, which are more dependent on oxidative metabolism than type II fibers, and are much higher in mitochondrial content. Functional specificities of carnitine and its system of proteins to intervene in the mitochondrial beta-oxidation pathway of long-chain fatty acids in the skeletal muscle account for the partitioning of lipids towards oxidation in skeletal muscle rather than storage in adipose tissue or skeletal muscle. Abnormalities at the control and regulatory steps of this function disrupt muscle homeostasis and impact on resistance to insulin, and body composition changes consisting in obesity. Lipid accumulation in type I fibers, reduced contractile efficiency, weakness, and fatigue are underlying features.

Conditions in which carnitine system is at the center of skeletal muscle changes, e.g. fatigue, obesity, drugs exerting toxicity through carnitine system, microgravity, denervation-induced atrophy are viewed from their ability to cause a state of functional insufficiency of carnitine system leading to the skeletal muscle loss of its shape, size and force output. Abnormal coordination of the expression of both metabolic and contractile properties of type I myofibers underlie a role in metabolic diseases.

Key words: carnitine insufficiency, carnitine system, insulin resistance, skeletal muscle.

Basic Appl Myol 13 (3): 105-120, 2003

The skeletal muscle has not only the important role in structure and motility but also a critical role in metabolic homeostasis, it being considered the "repository" of the major portion of the non-lipid fuels, i.e. muscle protein, that can be used for metabolic balance [1]. Muscle mass and cellular and subcellular composition are sensibly influenced by the extent and type of contractile activity, while the contractile machinery devoted to transduce chemical energy into mechanical work also respond to systemic regulation of protein metabolism, i.e. flow of amino acids between muscle and other organs with feeding and fasting, in health and disease. The mechanical role of muscle also impinges metabolically upon the regulation of glucose tolerance, the partition of the fat and lean body masses, and the maintenance of the lean body mass throughout middle and old age [2]. Thus, the metabolic and contractile activity of muscle plays a predominant role in metabolic homeostasis, with a major and extreme contribution during periods of starvation or severe wasting diseases when the muscle proteins can be metabolized to amino acids.

Muscle, itself, has an active metabolism. Resting muscle primarily maintains itself on the energy provided by fatty acid oxidation, and can be one of the major contributors to homeostasis by the provision of amino acid carbon for gluconeogenesis, whereas in periods of exercise the flux of energy demands turn about with muscle becoming a predominant user of the body's metabolic fuels.

Muscle capacity for dynamic responses to altered functional demand has evolved with a complex metabolic machinery of which carnitine system is part. Muscle growth and differentiation, atrophy and regeneration capacity, aging, disease and injury, and responses to hormones and nutrients all involved control and regulatory steps at the carnitine system level. The intracellular carnitine system composed of free carnitine and acylcarnitines (i.e. natural esters of carnitine) includes a family of membrane-bound carnitine acyltransferases (CPTs) able to synthesise acylcarnitines of different chain lengths for their utilization or elimination during cell metabolic processes [3-5]. In humans, the mitochondrial beta-oxidation pathway of long-chain fatty acid, ubiquitously dependent on carnitine, has been the most investigated role of carnitine [5-7].

Upon the premise that each tissue or cell does not simply look after its own future energy needs, the particular case of skeletal muscle is viewed from the standpoint of control and regulatory mechanisms of the mitochondrial carnitine-dependent metabolism as determinant both to maintain muscle architecture and function and to subserve homeostatic mechanisms for metabolic balance at a systemic level of orchestration.

Carnitine Physiological Roles

The function of carnitine in metabolism refers to its physiological roles in intermediary metabolism as a carrier of carbon chains (Table 1), more specifically, of long-chain fatty acids for their beta-oxidation in mitochondria, and of endogenous and exogenous toxic metabolites for their elimination.

Carnitine roles result from peculiar biological properties of this molecule and the existence of an intracellular network of carnitine-dependent proteins, and strategical tissue distribution of carnitine transporters underlying differential tissue concentrations, kinetics of uptake, and regulatory mechanism of transport.

Carnitine, gamma-trimethyl-beta-hydroxybutyrobetaine, is a water soluble organic compatible solute with different biological functions. Ubiquitous in nature, in simple prokaryotes carnitine is either a sole source of carbon and nitrogen or an osmolyte, whereas in the complex eukaryotes it acquires a distinct function in relation to fatty acid signal recognition and transduction pathways as a carrier of acyl moieties, and exclusively of long-chain fatty acids for mitochondrial beta-oxidation in mammals [4, 20]. The trimethylated nitrogen group composing the molecule provides carnitine with osmolyte properties as seen for other components (the osmolytes glycine betaine, trimethylamine N-oxide, sarcosine) of the family to which this molecule belongs, i.e., the methylamines family [20]. Since life builds gradually not excluding what has been previously build but rather building upon it, carnitine in mammals has been hypothesised to maintain osmolytic properties due to the fact that its concentration in various tissues does not consistently correlate with tissue energy requirements or lipid metabolism. Indeed, in the lens whose main source of energy is glucose absorbed from ocular fluids [22], carnitine has been shown to protect the molecular chaperone activity of lens alpha-crystallin and to decrease the post-translational protein modifications induced by oxidative stress (compatible osmolyte strategy) [21]. In this case, carnitine may represent among methylamines, the special osmolyte endowed with an essential role in cell energy metabolism and caloric homeostasis. In addition, in models of acute hyperammonemia the administration of carnitine has prevented against ammonia-precipitated encephalopathy [23-25]; this experimental condition suggests a counteracting effect of carnitine that offset the protein destabilizing effects of ammonia, while cell functioning is not disturbed by the increased carnitine availability (counteracting osmolyte strategy).

Table 1. Carnitine physiological roles.

- beta-oxidation of long-chain fatty acids in mitochondria, beta-oxidation of very long-chain fatty acids in peroxisomes [3];
- transfer of acetyl- and other short-, medium-chain acyl groups from peroxisomes to mitochondria [8, 9];
- control of ketone bodies production through the mitochondrial enzymes carnitine palmitoyltransferase (CPT) II and 3-hydroxy-3-methylglutaryl coenzyme A synthase; this shifting the regulation of ketogenesis to step(s) beyond CPT I (seen in response to fasting and mediated by the peroxisome proliferator-activated receptor alpha (PPARalpha) [10];
- re-esterification of triacylglycerol in the endoplasmic reticulum before secretion as very-low density lipoproteins [11];
- stimulation of pyruvate and branched-chain amino acid oxidative metabolism [3];
- scavenger system for acyl groups, whether originated as xenobiotic- or as endogenous carboxylic acids [12-14];
- deacylation and reacylation to remodel erythrocyte membrane phospholipids [15];
- partner in the pathway of phospholipid and triglyceride fatty acid turnover in neurones [16];
- synthesis and elongation of polyunsaturated fatty acids [3, 17-19];
- stabilization of proteins and membranes, and counteraction of denaturing solute effects, e.g. ammonia [20, 21].

Cell Carnitine-Dependent Protein Network

Carnitine itself is not metabolized in humans. It is found in most cells at millimolar levels after uptake via carnitine transporters (Table 2). To exert its metabolic function it forms esters with a wide range of fatty acyl groups (i.e. acylcarnitines) [5] depending on an intracellular system of membrane-bound carnitine-dependent enzymes, carnitine acyltransferases (CPTs), able to synthesise acylcarnitines of different chain lengths for their utilization during cell metabolic processes [3, 5] (Table 2). Carnitine and carnitine acyltransferases were thought to be simply mechanisms for the rapid transfer of activated long-chain fatty acids into the mitochondria for beta-oxidation, instead, eight different carnitine acyltransferases have been characterized which are localized in subcellular organelles other than mitochondria, i.e. peroxisomes, endoplasmic reticulum [5, 30, 31]. The carnitine/acylcarnitine translocase protein, first characterized in mitochondria, has now been demonstrated immunologically in peroxisomal membranes too. This finding suggests that acylcarnitine transport into peroxisomes, as in mitochondria, is also mediated by carnitine/acylcarnitine translocase [31]. Peroxisomes are a major route for fatty acid oxidation (not ending in ATP production) and generation of regulatory molecules involved in cell signalling [33, 34]. Interestingly, a third

Table 2. Cell carnitine-dependent protein network.

- high affinity, sodium-dependent plasma membrane carnitine transporter OCTN2 [26];
- low affinity, sodium-independent plasma membrane carnitine transporter OCTN1 [27];
- intermediate affinity, sodium-independent carnitine transporter (CT2) localized in the luminal membrane of epididymal epithelium and within the Sertoli cells of the testis [28];
- intermediate affinity OCTN3 localized in peroxisomes [29];
- carnitine palmitoyltransferase I (CPT I), located on the outer mitochondrial membrane, peroxisomes, and endoplasmic reticulum catalyzes the transfer of acyl groups from acyl-CoA to carnitine to produce acylcarnitine [5, 30, 31];
- carnitine/acylcarnitine translocase (CT) located in the inner mitochondrial membrane exchanges cytoplasmic acylcarnitine for mitochondrial free carnitine (antiport modality; ping-pong mechanism), and transports short-chain acylcarnitine out of mitochondria with the same modality. CT is also located in peroxisomes. A CT unidirectional transport of carnitine is also demonstrated which is slow (generally concentration- and gradient-driven) (uniport modality) [32];
- carnitine palmitoyltransferase II (CPT II), located in the matrix side of the inner mitochondrial membrane, peroxisomes, and endoplasmic reticulum catalyzes a reaction that is the reverse of that of CPT I, reconverting acylcarnitine to acyl-CoA conveyed to ß-oxidation in mitochondria and peroxisomes, and to triacylglycerol re-esterification in the endoplasmic reticulum [5, 30, 31];
- carnitine octanoyltransferase (COT), located in peroxisomes catalyzes the transfer of medium-chain acyl moieties from CoA to carnitine; COT appears to be involved in the transfer of chain-shortened fatty acyl groups from the peroxisomes to the mitochondria for further oxidation [5, 30, 31];
- carnitine acetyltransferase located on the matrix side of the inner mitochondrial membrane and in peroxisomes transfers acetyl groups from acetyl-CoA to carnitine, forming acetylcarnitine that can be exported out of these organelles or utilized for ATP production and elongation of polyunsaturated fatty acids [5, 17, 30, 31].

carnitine organic cation transporter, OCTN3, has been identified and the protein localized in peroxisomes, suggesting a unique role for OCTN3 in the maintenance of intracellular homeostasis [29], and dictating the molecular basis for the understanding of carnitine system as a distinct mechanism of signal transduction. Several signal pathways have been identified (cyclases, kinases, phospholipases) but the complexity of the functions and processes monitored by cells suggests the existence of an enormous number of distinct mechanisms of signal transduction [35]. Among these distinct mechanisms is the carnitine-dependent metabolic machinery with its main involvement in cell lipid metabolism and role in integration of cell responses to environmental stimuli.

Carnitine System-Dependent Metabolism in the Skeletal Muscle: Homeostatic Features

Carnitine system physiology in the skeletal muscle is at the basis of a metabolic phenotype in which type I muscle fibers predominate. Contractile properties of the fatigue resistant, slow-twitch oxidative type I fiber is molecularly distinguished by the myosin heavy chain (MHC) I isoform. This impacts on the partitioning of lipids towards oxidation in skeletal muscle rather than storage in adipose tissue or skeletal muscle (i.e. intramuscular triglycerides). Under physiological conditions, this participation in fat balance involves the control of mitochondrial beta-oxidation flux predominantly by the muscle carnitine palmitoyltranferase system [36]. In fact, the special feature related to systemic or muscle carnitine deficiency is a lipid storage myopathy predominantly affecting type I fiber associated with a decrease of muscle strength, hypotonia, and type II fiber atrophy including myolysis, along with hepatic steatosis, hypoketonemia, hyperglycemia, hyperammonemia in the systemic form [37-39]. Skeletal and cardiac muscle cells exhibit ragged-red appearance, and electron microscopy evidences an increased number of mitochondria and lipid droplets [40].

From deficiency to insufficiency of carnitine

In between the state of extreme carnitine deficiency disrupting metabolic homeostatic mechanisms and the state of normality (eucarnitinemia) is that one of carnitine insufficiency with poorly characterized clinical features. Efforts to search for these features are in the recognition of abnormal carnitine metabolic patterns with relevant signs and symptoms, and the consequent strategies to adopt for maintaining organ constant physiology and homeostasis.

From a general point of view, the critical issue of differential tissue carnitine concentration, and major changes in tissue metabolism sensibly responding to variations in carnitine concentration account for the influence of this substance in fatty acid oxidation, ketone body formation, ATP/ADP potential, whether the fatty acids are directed to oxidation or to esterification, degradation of branched chain amino acids in liver and muscle, and gluconeogenesis. Then, the concept that changes in these parameters may highlight states in which carnitine insufficiency arises, under variable and specific organ functional/dysfunctional demands. Some ideas came from healthy subjects who served as a model of carnitine insufficiency induced by pivalic acid treatment. Metabolic adaptive strategies were investigated under exercise (or fasting) stress conditions in comparison to untreated subjects [41, 42]. Cycloergometer testing (maximal effort exercise) did not result in apparent

changes in physical performance (i.e., unchanged VO_2 max); whereas, the same subjects under a submaximal effort test had muscle metabolic changes indicating differences to afford exercise (Table 3). As muscle functionality is to be viewed under the inter-organ carnitine dialogue, the patterns for each organ to manifest carnitine insufficiency are mandatory involved (Table 3).

Skeletal muscle fatigue during exercise performance may center a relevant physiological state of muscle carnitine insufficiency and highlight the physiological duties of carnitine in generating diversity in cell responses.

Worthy of note is that insufficiency of carnitine is not only a "simply" matter of insufficient amount of carnitine but also a less intuitive concept of insufficiency which falls into the insufficient functionality of the carnitine-dependent proteins (e.g., activity and expression CPT I, CPT II, and carnitine/acylcarnitine translocase), resulting almost in similar abnormalities in skeletal muscle phenotype and contractile efficiency. Disease states such as obesity, diabetes, hyperthyroidism, renal failure, dialysis, cancer, belongs to this concept of carnitine insufficiency, where pathophysiological events related to carnitine insufficiency may manifest and underlie relevant risk factors worsening the course of disease.

From the organ point of view, to participate in the coordination of metabolic homeostasis carnitine relies on critical variables that involve the entire system determining organ-specificity: ratio between free carnitine and carnitine esters (acylcarnitines); tissue carnitine transporters; isoforms of carnitine specific enzymes; transcription of genes coding for carnitine enzymes and transcription of genes resulting from carnitine system activities which will depend on tissue-specific fatty acid metabolism and abundance of tissue specific transcription factors involved in gene expression [4]. Among these transcription factors are peroxisome proliferator-activated receptors (PPARs) [47], HNF-4 alpha receptors [48], and thyroid hormone receptors [49, 50].

Table 3. Parameters expressing skeletal muscle, liver, and heart changes in homeostasis induced by carnitine insufficiency.

Skeletal muscle [41, 42]
• marked reduction of muscle glycogen concentration indicating that glycolysis works efficiently; • slightly reduced performance in few subjects
Liver [41]
• reduced capacity to produce ketone bodies following fasting
Heart [43-46]
• predisposition to cardiac hypertrophy; • increase of heart weight (body weight normalized); • reduction of heart total carnitine content by about 60%; • increase of oxidative utilization of glucose in parallel with an increase of hexokinase activity, this indicating that glycolysis works efficiently; • reduction of fatty acid beta-oxidation; • no reduction of cardiac performance at increasing work load; • no changes in myosin isoform distribution.

Nutritional status, hormones, pathological conditions, and pharmacological agents affect carnitine system dynamics [51] leading to changes in skeletal muscle capacity to gain (and convert) energy from the environment, and to use this energy to maintain itself, repair and reproduce.

Skeletal muscle is a major site of fatty acid oxidation in humans and in terms of regulation of fat metabolism it relates to the development of insulin resistance, obesity, diabetes, cardiovascular disease, nutrition [52-57]. Changes in qualitative and quantitative properties of muscle are shown to be associated with increased risk of morbidity, mortality and disability [58-65].

Skeletal muscle carnitine pathway of fatty acid oxidation, control, and regulatory steps

Most studies have concentrated their aims at investigating the role of carnitine system at the mitochondrial level for two reasons: 1) skeletal muscle energy metabolism centers on carnitine to oxidize long-chain fatty acids; 2) long-chain fatty acids are involved in all aspects of cellular structure and function [36, 66]. Many factors, including the availability of fatty acids and the abundance of fatty acid transporters, may influence their rate of oxidation in muscles. However, the predominant point of control appears to be the rate at which fatty acids are transported into mitochondria by the carnitine system [36].

The skeletal muscle is characterized by the presence of: 1) two well described membrane carnitine transporters, the sodium-dependent high affinity OCTN2 and the sodium-independent low affinity OCTN1 [26, 27]; an active and not strictly sodium-dependent transport of carnitine into rat skeletal muscle membrane vescicles which is distinguishable from that of OCTN2 suggests that OCTN2 is not the only high-affinity carnitine transporter in muscles [67]; 2) a high concentration of carnitine (e.g. 4.10 ± 0.82 micromole/g wet tissue in the vastus lateralis muscle; 3.54 ± 1.06 micromole/g wet tissue in the gastrocnemius) [68, 69]; 3) the muscle isoform of the enzyme CPT I (M-CPT I) which shows a lower affinity for carnitine than the liver isoform (L-CPT I), and a higher sensitivity to malonyl-CoA inhibition than L-CPT I [70]; 4) a step regulated by insulin in a tissue-specific manner [71, 72] (Table 4). The malonyl-CoA/CPT I interaction, activated by insulin, regulates cell fuel "cross talk" allowing for appropriate changes in the direction of both glucose and fatty acid carbon flux [5, 73-75]. Clinically relevant is that long-chain acyl-CoAs which depend on carnitine for their

metabolic destiny determine insulin sensitivity as shown by prolonged inhibition of CPT-I promoting insulin-resistance because of increased muscle lipid accumulation [83]. In humans, it is also shown that long-chain acyl-CoA content in muscles provides a direct index of intracellular lipid metabolism and its link to insulin sensitivity [56].

One Way for Carnitine System to Sense Cell Energy Level and Adapt to Increased Skeletal Muscle Demand

Despite many findings on carnitine fatty acid oxidation pathway, control and regulatory steps involving malonyl-CoA (Table 4) some question remain still unsolved. First, the surprising fact that in muscle, a nonlipogenic tissue, a CPT I isoform is much more sensitive to malonyl-CoA inhibition than in liver, a lipogenic tissue [84]; second the fact that because of the concentration of malonyl-CoA measured in muscles, only a small fraction of this malonyl-CoA must be accessible to CPT I for fatty acid oxidation not to be inhibited at all times; third, the hypothesis that malonyl-CoA is confined within mitochondria since malonyl-CoA carboxylase the enzyme degrading malonyl-CoA is thought to be a cytosolic or at least an extramitochondrial enzyme [74]. Therefore, the interest is focused on other determinants controlling carnitine metabolism. How does carnitine system dynamics change if muscles change their state of activity, e.g. exercise?

During exercise or electrically induced contractions or even nutritional stress, when the need of muscle cell for fatty acid oxidation rises, increases in the AMP:ATP ratio and/or decreases in the creatine/phosphate to creatine ratio activate an isoform of an AMP-activated protein kinase (AMPK), which phosphorylates acetyl-CoA carboxylase beta (ACC beta), and inhibits both its basal activity and activation by citrate [74, 85]. The AMPK signalling activated by increases in the AMP:ATP ratio, monitors cellular AMP and ATP levels, and switches off ATP-consuming anabolic pathways and switches on ATP-producing catabolic pathways, such as fatty acid oxidation [85]. The stimulation of fatty acid oxidation seems to rely on the activation of the carnitine enzyme network by mechanisms of M-CPT I deinhibition induced by depletion of intracellular metabolic factors, i.e., malonyl-CoA levels due to the AMPK-induced phosphorylation of ACC beta. In addition, there is mounting evidence that AMPK can also phosphorylate and activate malonyl-CoA decarboxylase, an enzyme that is thought to function in concert with ACC for the turnover of malonyl-CoA in muscle cells [86].

By viewing skeletal muscle dynamics of carnitine system in relation to metabolic balance, there are recent papers showing that AMPK mediates the effects of leptin [87] and adiponectin (a hormone secreted by adipocytes) [88] on fatty acid oxidation both *in vivo* and *in vitro*. In particular, early activation of AMPK occurs by leptin act-

Table 4. Control and regulation of skeletal muscle carnitine pathway of fatty acid oxidation.

- Malonyl-CoA, formed from acetate units deriving from glucose and fatty acid metabolism, is the natural inhibitor of CPT I [5, 73-75]. This inexorable link involves another mitochondrial carnitine-dependent enzyme, carnitine acetyltransferase, which modulates intramitochondrial acetate levels by reversibly forming acetylcarnitine, which is exported out of mitochondria by carnitine/acylcarnitine tranlocase. The removal of acetate (acetyl-CoA) units as acetylcarnitine stimulates the oxidative utilization of pyruvate [76] by relieving acetate-induced inhibition of pyruvate dehydrogenase, an enzyme located in the inner mitochondrial membrane. Otherwise, glucose is shunted into non-oxidative glycolysis (lactate/alanine production or glycogenosynthesis) [77, 78].
- A regulatory step of the malonyl CoA/CPT I partnership relies on insulin. Malonyl-CoA is generated through a reaction catalysed by acetyl-CoA carboxylase (ACC). ACC activation by insulin is a crucial step influencing the malonyl CoA/CPT I partnership [70, 75, 79], and a special isoform, ACC beta, is primarily expressed in the skeletal muscle (and in heart), where it may play a pivotal role in the insulin-mediated regulation of mitochondrial long-chain fatty acid uptake and oxidation. Another ACC isoform, ACC alpha, is primarily found in lipogenic tissues, such as fat tissue and liver and plays a role in fatty acid synthesis, it being also activated by insulin [71, 72].
- In humans, it is shown that an infusion of insulin and glucose during a sequential euglycemic hyperinsulinemic clamp is associated with an increase in the concentration of malonyl-CoA in the vastus lateralis muscle, compatible with CPT I inhibition, which correlates both with increases in the concentrations of citrate (an allosteric activator ACC) and malate (an antiporter for citrate efflux from the mitochondria) and a decrease in whole-body and, presumably, skeletal muscle fatty acid oxidation [80].
- Increases in malonyl-CoA concentrations occur also acutely in rat muscle in response to insulin and glucose or inactivity by denervation [81]. Increases in malonyl-CoA occur also very rapidly in muscle (soleus and gastrocnemius) during refeeding after a fast, in association with increases in whole body respiratory quotient, and decreases in plasma free fatty acids and muscle long-chain fatty acylcarnitine concentration; interestingly, under this situation the increase in muscle malonyl-CoA is not due to changes in the assayable activity of either ACC or malonyl-CoA decarboxylase (enzyme which degrades malonyl-CoA) or an increase in the concentration of citrate. The most likely cause is a decrease in the cytosolic concentration of long-chain acyl-CoA, an allosteric inhibitor (which counters the action of citrate) of ACC [82].

ing directly on muscle, whereas later activation depends on leptin functioning through the hypothalamic-sympathetic nervous system axis (the alpha-adrenergic, but not the beta-adrenergic pathway being involved) [87].

Biochemical, molecular and morphological studies evidence the modality by which the metabolism of fatty acids efficiently support mechanical work to sustain prolonged physical exercise. Changes in transcriptional activity during exercise accounting for carnitine-induced shift in muscle fiber type are observed. Increases of transcriptional activity of M-CPT I gene are followed by increases in M-CPT I mRNA levels during recovery from 60-90 min of exhaustive one-legged knee extensor exercise after a 5-day exercise protocol [89]. In rats, a pretranslational regulation of CPT II mRNA by contractile activity has also been demonstrated [90]; an increased transcription, an increased mRNA processing or an enhanced stability of CPT II mRNA may result from treadmill running and chronic electrical stimulation.

During exercise, muscle carnitine contents and carnitine enzymes activity, blood carnitines concentration, and urine elimination of carnitines evidence the intense activation of the system [91]. In particular, it is shown that during submaximal dynamic exercise, the concentration of free carnitine decreases significantly in type I fibers at the end of exercise [92].

A link with muscle contraction through the carnitine system

Normal muscle function is dependent on the concentration gradients of Na^+ and K^+ across the sarcolemma and on the intracellular concentration of Ca^{2+}, which in turn are regulated by membrane-bound cation pumps/ATPases. In birds, it has been demonstrated that long-chain acyl metabolites (palmitoylcarnitine and other long-chain acylcarnitines, and palmitoyl-CoA) but not fatty acids and short-chain acylcarnitines are able to modulate sarcoplasmic reticulum ATP-dependent Ca^{2+}-dependent Ca^{2+} cycling in vivo [93]. This by stimulating Ca^{2+} release channel at concentrations lower than those observed for inhibition of the Ca^{2+}-ATPase. These results are similar to those obtained in mammalian skeletal muscle Ca^{2+} release channel [94].

In humans, total levels of long-chain acyl CoA in skeletal muscle are reported to be within the range of 4-10 nmol/g [95], whereas acylcarnitines within the range of 10-80 nmol/g [96]. In myocytes 20-40% of the total long-chain acylcarnitines was shown to be associated with sarcoplasmic reticulum membranes [97].

The above data indicate the participation of long-chain acylcarnitine principally in the Ca^{2+} release, thus in the sarcoplasmic reticulum Ca^{2+} cycling. The meaning of this condition is to provide the way for lipid catabolism to control sarcoplasmic reticulum Ca^{2+} cycling, a pathway that account in part for muscular non-shivering thermogenesis [98].

Modulation of Ca^{2+} cycling by fatty acid metabolites is highly relevant in some metabolic diseases such as malignant hyperthermia. Instead, during prolonged exercise [99] fatty acids are important substrates for ATP production, thus no increase in fatty-acid-mediated Ca^{2+} cycling would be expected since it would uselessly increase the energetic cost of contraction.

Regulation of Carnitine System Enzymes Expression

Different metabolic conditions are known to affect carnitine-dependent beta oxidation of long-chain fatty acids: fasting, fed state, (high carbohydrate or high fat diets), hyperthyroidism, hypothyroidism, diabetes, cancer [4]. In recent years, molecular studies on carnitine proteins are delucidating regulatory mechanisms for the expression of genes coding for these proteins.

It has been shown that the human M-CPT I gene is a target gene for the action of peroxisome proliferator-activated receptors (PPARs), since a PPAR responsive element (PPRE) upstream of the first exon of the gene has been localized, which is able to confer PPARalpha and PPARgamma responsiveness [47]. Even though PPARdelta is able to bind the M-CPT I PPRE *in vitro*, it does not activate the expression of the chimeric gene even in the presence of linoleic acid as activator [47]. At the same time, other authors have demonstrated that the M-CPT I gene promoter contains a fatty acid response element (FARE-1) localized to a hexameric repeat sequence upstream the initiator codon that, cotransfection experiments with expression vectors, demonstrated to be a PPARα response element [100].

Human skeletal muscle expresses PPARgamma in low amounts under basal conditions, and PPARgamma-1 is the dominant isoform present in this tissue [101, 102]. The expression of PPARgamma is found to correlate significantly with the expression of M-CPT I and other two important genes in lipid metabolism, i.e. fatty acid-binding protein and lipoprotein lipase, but not with the expression of carnitine/acylcarnitine translocase and glucose transporter-4 [103].

The relevance of PPAR nuclear receptors in the transcriptional control of mitochondrial fatty acid oxidation is confirmed by a recent study showing that the CPT II promoter is also occupied *in vivo* by PPARalpha [104]. In addition, the orphan receptors COUP-TF I and ERRalpha also bind to this element, modulating the PPARalpha *trans* activation of CPT II promoter. A single isoform for CPT II is known to be present in all tissues [105].

Regulation of M-CPT I has been more investigated in heart than in skeletal muscle tissue. It is demonstrated that the M-CPT I promoter contains a myocyte enhancer factor-2 (MEF2)/DNA binding site, and mutational analysis reveals a 46% depression in M-CPT I gene expression when MEF2 is mutated [106]. A requirement for MEF2 in M-CPT I gene expression is consistent with the known role for the MEF2 proteins in the differentiation of muscle cell lineages. Promoter deletion analysis reveals, 20 base pairs downstream of the MEF2 binding element, the presence of an E box that acts as a suppresser of M-CPT I gene expression. Binding of the

upstream stimulatory factors (USF1 and USF2) to two E box sites within M-CPT I promoter is shown. The consensus E box binds basic helix-loop-helix leucine zipper regulatory proteins and are contained in the regulatory regions of most developmentally controlled, muscle-specific genes. MEF2A and the PPARgamma coactivator-1alpha (PGC-1alpha) synergistically activate M-CPT I gene promoter activity, and overexpression of the USF proteins in myocytes depresses M-CPT I activity by significantly reducing MEF2A and PGC-1 alpha synergy [107]. Moreover, the transcriptional regulation of M-CPT I is coordinated with contractile gene expression through the cardiac-enriched transcription factors, GATA4 and serum response factor [106].

PGC-1alpha is also expressed in skeletal muscle and powerfully induces mitochondrial biogenesis [108]; it is also critically involved in other aspects of mitochondrial energy metabolism [109]. Because of this and the fact that mitochondrial metabolism is viewed as a critical part of the muscle-fiber-type phenotype, many authors focus on the potential role of PGC-1alpha in the control of specification of fiber type through changes in gene expression, involving carnitine-dependent protein CPT I. PGC-1alpha is preferentially expressed in muscle enriched in type I fibers. When PGC-1 alpha is expressed at physiological levels in transgenic mice driven by a muscle creatine kinase (MCK) promoter (a promoter that is preferentially activated in type II fibers), a fibre type conversion is observed: muscles normally rich in type II fibres are redder and activate genes of mitochondrial oxidative metabolism [110]. Furthermore, putative type II muscles from PGC-1 alpha transgenic mice also express proteins characteristic of type I fibres, such as troponin I (slow) and myoglobin, and show a much greater resistance to electrically stimulated fatigue.

PGC-1alpha is demonstrated to activate transcription in cooperation with MEF2 proteins and to serve as a target for calcineurin signalling, which has been implicated in slow fiber gene expression. In fact, at a transcriptional level, an important role of MEF2 proteins is indicated in the activation of slow-fiber-selective myofibrillar proteins [110]. Thus, PGC-1alpha is a principal physiological regulator (a switch) for type I fiber specification, and may act as the transcriptional component which could potentially integrate calcium signalling, mitochondrial biogenesis, and myofibrillar protein regulators (such as MEF2).

PGC-1 is also capable of coactivating PPARalpha in the transcriptional control of genes encoding fatty acid oxidation enzymes as shown in experiments of mammalian cell cotransfection. PPARalpha and PGC-1 cooperatively induce the expression of PPARalpha target genes, i.e. CPT I, long-chain acyl-CoA dehydrogenase, medium-chain acyl-CoA dehydrogenase and increased cellular palmitate oxidation rates. Separable PPARalpha interaction and transactivation domains within the PGC-1 molecule are shown, and certain features of the PPARalpha-PGC-1 interaction are distinct from that of PPARgamma-PGC-1 [111].

Conditions with Compromised Skeletal Muscle Carnitine Homeostasis

Fiber composition of a given muscle can change significantly under the influence of physiological and pathological factors (mechanical demand, neural activity, hormonal output and pathological conditions such as diabetes and muscular diseases) [112]. This property is referred to as muscle plasticity. A constant state of flux concerns a cycle of molecules entering and leaving every muscle structure. Therefore a sarcomere today will not be made of the same molecule as tomorrow. Conditions affecting muscle physiology are extremely determinant for the efficient replacement of proteins at the level of the contractile machinery. The contractile proteins in vivo are among the longest to live of known proteins (actin's and MHC's half life are around 20 days and 7-10 days, respectively); however, if a molecule leaves the protection of the intact filament a rapid degradation follows, as it occurs either in response to altered work in terms of removal of the activity or load (e.g. tenotomy of skeletal muscle, space flight, separation of tissues into cells to culture them) [113].

Under this concepts, conditions affecting the integrity of carnitine system which may lead muscles to the loss of (or not) its shape, size, and force output are viewed following a premise on fatigue, the shared symptom.

Fatigue

Fatigue results from abnormalities in carnitine metabolism either inherited or acquired (Table 5). Patients referring this symptom are objectively characterized by impaired performance. The muscle functional phenotype related to carnitine, i.e. type I fiber, deals with resistance to fatigue, and dictates the rate of ATP hydrolysis and the shortening velocity [112]. In this case muscle fatigue is measured as a decrease of force development which follows repeated contractions. This is exemplified in a study with dogs receiving carnitine and minimal electrical stimulation at the left muscle latissimus dorsi "in situ" [114]. In addition to the higher percentage of type I fibers, a significant decrease in contraction speed and increase in cytochrome c oxidase activity are shown, while no changes occurred in the contralateral non electrically stimulated *right latissimus dorsi.*

Obesity

In obesity-related insulin resistance there are significant correlations between insulin resistance and several markers of skeletal muscle fatty acid metabolism, notably, M-CPT I and plasmamembrane fatty acid binding protein (FABPpm) [52, 54]. M-CPT I activity is lower in obese than in normal subjects and correlates positively to insulin sensitivity, while FABPpm is higher and correlates negatively to insulin sensitivity. This suggest that the metabolic capacity of skeletal muscle

Table 5. Carnitine metabolism and fatigue.

Medical conditions that may present fatigue induced by altered carnitine metabolism
• primary carnitine deficiency, systemic and myopathic (carnitine transporter defects)
• secondary carnitine deficiency (i.e. organic acidurias, inborn errors of fatty acid beta-oxidation)
• endocrine: diabetes, hyperthyroidism
• cardiovascular: chronic heart and peripheral arterial disease
• nephrological: chronic renal insufficiency, dialysis
• gastrointestinal. short bowel and celiac disease
• general: cancer, surgery
Screening tests
• blood chemistry (including free carnitine and acylcarnitine levels/search for diagnostic acylcarnitines)
• urine test (including for 24-h carnitine elimination)
• thyroid function
• skeletal muscle function (creatine kinase, exercise testing)
• liver function (including ketone bodies after prolonged fasting)

appears to be organized toward fat esterification rather than oxidation. Dietary-induced weight loss does not correct this disposition [54], while glucose metabolism improves. Thus the concept that mitochondrial bioenergetic dysfunction in human obesity may contribute to the expression of insulin-resistant patterns of glucose metabolism and in partitioning of fat toward esterification within muscle. Other findings in obese women have also shown reduced M-CPT I activity and depressed long-chain fatty acid oxidation [55]. Moreover, decreased skeletal muscle carnitine/acylcarnitine translocase (enzyme localized in the inner mitochondrial membrane for the transport of acylcarnitines inside mitochondria) at transductional and translational level in the insulin resistant obese patients has been demonstrated [57]. Consequently, the low level of carnitine/acylcarnitine translocase or M-CPT-I in insulin resistant muscle may contribute to the elevated muscle concentrations of triglycerides (intramyocellular triglyceride), diacylglycerol, and fatty acid-CoA characteristic of insulin resistant muscle. Muscle insulin resistance, which is found in 85-95% of non-insulin dependent diabetic (NIDDM) patients, exists before adiposity and is likely to induce it. Actually, muscles of subjects at risk for NIDDM exhibit a very early defect in both glycogen storage ability and free fatty acid oxidation capacity that can impair fuel utilization and increase fat storage [53].

Iatrogenic factors

Diverse drugs interact with carnitine either affecting its transport, synthesis, and intracellular-dependent protein network [115]. For instance, drugs which activates PPARgamma and/or PPARalpha (Table 6), thus involving carnitine dependent fatty acid oxidation, are shown to adversely affect myogenesis and induce abnormal expression of muscle cell-specific genes [116, 117].

Table 6. Muscle injury and repair: PPAR-gamma activation by drugs influencing myogenesis and adipogenesis.

NO EFFECT Muscle cell differentiation
• ciglitazone
• naproxen
INHIBITION Muscle cell differentiation
• prostaglandins
• thiazolidenediones
• phenoxyacetic acids
• flurbiprofen
• piroxicam
INHIBITION Muscle protein synthesis
• ibuoprofen
• prednisolone
• paracetamol
• flurbiprofen
• piroxicam
TRANSDIFFERENTIATION Muscle cell into adipocyte
• prostaglandins
• thiazolidenediones
• phenoxyacetic acids
• dexamethasone
• insulin
TRANSDIFFERENTIATION Fibroblast into adipocyte
• indomethacin*
• fenoprofen*
• ibuprofen*
• flufenamic acid*
* drug which also activates PPARalpha receptors.

This strongly affect muscle plasticity during processes of repair following injury. Indomethacin and other non-steroidal anti-inflammatory drugs bind and activate PPARgamma. Indomethacin, fenoprofen, ibuprofen, and flufenamic acid activate both PPARgamma and alpha. Specifically, PPARgamma in muscle cell attenuates the expression of MyoD, a key myogenic factor [117]. Statins, i.e. simvastatin, in *in vitro* studies using L6 myoblasts induce the tyrosine phosphorylation of sev-

eral cellular proteins followed by apoptosis [118], whereas in cultured neonatal rat skeletal muscle cells exposed to increasing concentrations of pravastatin or lovastatin, it has been shown that statins-induced myotoxicity involves post-translational modification of specific regulatory proteins (low-molecular-weight proteins) by inhibiting their geranygeranylation [119]. These drugs are not direct ligand of PPARalpha receptors, but may generate PPARalpha ligands (downstream products of the mevalonate pathway) or may increase the activity of the DNA-binding domain of PPARalpha [120]. It was found that geranylgeranyl-modified intermediates may antagonize PPARalpha. Farnesyl-pyrophosphate and geranylgeranyl-pyrophosphate are implicated in membrane translocation, leading to the activation of a variety of proteins, including Ras and Rho GTP-binging proteins, respectively. This process has been shown to be inhibited by statins using rabbit kidney13 cells [120], and smooth muscle cells [121], and, more specifically, the effects of statins on PPARα are mediated by Rho A.

Microgravity

A requirement for MEF2 in M-CPT I gene expression is shown with experiment of muscle atrophy under microgravity (14 days spaceflight) in rats. Type I muscle atrophy is observed, and expression of MEF2C and MEF2C-related genes including M-CPT I, Aldolase A and muscle ankyrines decreased [122]. In fact, muscle fatigue increases during work in space are due to a reduced capacity to oxidize long-chain fatty acids resulting from a reduced ability to activate and carnitine-dependently translocate fats from the cytosol to the mitochondria [123, 124]. After 9 days ground recovery, expression of MEF2C increases and it is mainly located on the satellite cells in the muscle regeneration state [122].

Using the hindleg immobilization model to mimick some aspect of spaceflight conditions, it has been observed that after 3 weeks of immobilization there is a 50% decrease in muscle carnitine (wet weight) and a slight loss, about 10%, of non-collagen protein (unpublished data). CPT and carnitine acetyltransferase (CAT) activities appeared less affected by immobilization. Carnitine supplementation to rats prevented the loss of non-collagen proteins and cause a significant faster regain of muscle wet weight during remobilization, while more interestingly there was an amplification of the loss of CPT and CAT activities during the immobilization period in contrast with increases observed during the remobilization period. This clearly indicates the importance of carnitine system as a distinct mechanism of signal transduction by regulating the level of bioactive molecules, e.g. long-chain fatty acyl-CoA, where length and unsaturation of the acyl chain as well as binding to CoA or not are determinant for specificities in nuclear receptor activation, thus gene transcription [34].

Denervation-induced atrophy

It has been demonstrated that components of the Bcl-2 family are expressed within regions characterized by intense anabolism of muscle fibers during recovery after denervation-induced atrophy, and in the regenerating muscle after eccentric exercise (the most potent stimulus for functional hypertrophy) which is known to cause severe injury to muscle fibers [125]. Recovering muscle fibers show strong reaction with antibodies specific for Bcl-2 family proteins, both anti- and pro-apoptotic ones. This reaction was concentrated within the subsarcolemmal regions of the fibers. In parallel, it was shown that the reaction with antibodies specific for activated caspases is always negative in the muscles reactive for the Bcl-2 family proteins. In addition, no morphological features of apoptosis is detected in recovering muscles. Diversely, denervated atrophying muscle fibers are stained by anti-caspases antibodies; in the same fibers also some staining with antibodies specific for Bcl-2 family proteins is evident [125]. Thus, Bcl-2 proteins, known modulators of apoptosis, may also act in other cell processes.

Mitochondrial CPT I and Bcl 2 have been identified as an interacting pair of proteins, raising the possibility that one of them controls a function of the other [126, 127]. Direct protein interaction was confirmed in a glutathione-S-transferase binding assay and in co-immunoprecipitations using two different kinds of anti-Bcl 2 antibodies. Relevant to observe is that Bcl 2 [128] and proteins with CPT activity [6] resides not only in mitochondria but also in the nuclear envelop and parts of the endoplasmic reticulum (ER).

Krajewski et al. [1993] [128] demonstrate that Bcl 2 resides primarily in the nuclear envelope, endoplasmic reticulum, and outer mitochondrial membrane in a patchy nonuniform distribution participation in protein complexes perhaps involved in some aspects of transport. Since the Bcl 2 protein lacks any obvious organelle-specific targeting sequences, one possibility is that Bcl 2 enters into large protein complexes in these intracellular membranes, and is held there through protein-protein interactions.

Bcl 2 binding to CPT I may be important in the modulation of sphingolipid metabolism in a way yet to be defined. Sphingolipid metabolism has been shown to play an important role in the regulation of apoptosis, and CPT I can protect from palmitate-triggered cell death by down-regulation of sphingolipid synthesis [129]. CPT I association with programmed cell death was initially discovered based on enhanced expression of its mRNA in LyD9 cells deprived of IL-3 [130].

Note of worthy is that palmitate-mediated mitochondrial effects are independent of macromolecule synthesis, caspase activation, and ceramide generation, yet are inhibited by Bcl 2. Bcl 2 may inhibit the mitochondrial permeability transition pore (PT) via maintenance of relatively reduced pyridine nucleotides based on the finding of Ellerby et al. [1996] [131] that Bcl 2 shifts

the redox potential of several cell types to a reduced state. Bcl 2 either directly or indirectly enhances H^+ efflux to maintain mitochondrial permeability (*mitochondrial transmembrane potential*) in the presence of apoptotic stimuli and even under conditions that do not allow PT. This suggests that the functional target of Bcl 2 is regulation of mitochondrial membrane permeability rather than PT [132]. Interestingly, carnitine prevents the PT induced by anoxia and rotenone [133]. Rotenone is a respiration inhibitor which blocks NADH dehydrogenase (complex I) in the respiratory chain but has no effect on the oxidation of succinate. Carnitine may be considered the naturally abundant regulator of mitochondrial permeability transition [127, 133]. Irrespective of its exact exact composition, the PT contains multiple targets for pharmacological interventions and is regulated by numerous endogenous physiological effectors. If a function of the PT could be that of a fatty acid sensor and a principle of modulation are long-chain fatty acids able to induce PT, i.e. palmitate and stearate, then carnitine is hypothesised to prevent PT [127].

Thus, Bcl 2 may serve as an enhancer of the efficiency of mitochondrial energy coupling by decreasing the endogenous PT activity [134], the Bcl 2 binding to CPT I being one among other protein-protein interactions to protect mitochondrial function.

Normal innervation is shown to influence carnitine muscle concentration as evidenced by the early marked decrease of total carnitine and acylcarnitine in rat denervated soleus, extensor digitorum longus (EDL), and anterior tibial muscles after sciatic nerve lesion; in controls but not in denervated muscles, the carnitine concentration was age-dependent with the highest concentration observed at 14 weeks of age [135]. Denervation is also shown to cause a decrease of both CPT and CAT activities, with a greater CPT activity decrease in the soleus muscle than in the EDL [136].

In humans, during development carnitine is shown to be high in the skeletal muscle and liver relative to other tissues during preterm gestation (from 14-30 weeks of gestation), whereas the skeletal muscle carnitine concentration increases with growth [137].

Carnitine levels and CPT activity investigated in muscles of patients with infantile and juvenile spinal muscular atrophy and polyneuropathies were significantly decrease in the infantile spinal muscular atrophy, but not in the other neurogenic muscle atrophies. These findings when compared with the experimental effect of denervation and reinnervation upon the lipid metabolism in soleus and extensor digitorum longus (EDL) of adult and newborn rats showed that i) twenty-one days after denervation free and total carnitine decreased significantly in the EDL and soleus of adult animals, ii) CPT activity was significantly decreased in the soleus 50 days after denervation, iii) denervation in newborn rats influenced carnitine concentration in soleus and EDL to a lesser extent, while reinnervation restored carnitine level within 50 days [138].

A study investigating carnitine uptake in human skeletal muscle growing in culture for up to 30 days revealed a saturable specific process with two distinct components: a high affinity uptake at carnitine concentration between 0.5 and 10 microM, and a low affinity uptake at carnitine concentration between 25 and 200 microM. The high affinity did not change during muscle maturation in culture, whereas the low affinity uptake did not change in the various stages of muscle differentiation. Thus, a muscle-specific system operates at physiological carnitine concentration, which gradually develops during muscle maturation in culture [139]. Finally, using of C2C12 myoblasts carnitine uptake was shown to be sodium-dependent and partly inhibited by a Na+/K+ ATPase inhibitor, while removal of carnitine induced growth inhibition of cultured C2C12 myoblastic cells. This suggesting that myoblast growth and/or differentiation is dependent upon the presence of carnitine [140].

Conclusions

Carnitine system functioning in muscle homeostasis provides the understanding of carnitine system as a distinct mechanism of signal transduction supporting complexity of the functions and processes monitored by cells. Thus, when carnitine is introduced as an exogenous signal, changes allowing for improved control of muscle fiber types, physiology and metabolism are to be observed.

For instance, an experimental study shows that normal dogs treated with carnitine and receiving minimal electrical stimulation “in situ” at the left muscle latissimus dorsi, presented with both a higher percentage of type I muscle fibers and a significantly lower contraction speed than untreated dogs while the right muscle latissimus dorsi did not [114].

A specific trophic effect on type I fibers was evidenced in muscle biopsies from the deltoid of hemodialysed subjects [141], it being a human model that better exemplifies the many ways skeletal muscle (and not only) carnitine metabolism may be perturbed. The patients receiving L-carnitine intravenously at the end of each dialysis session had a marked increase in serum and muscle carnitine together with hypertrophy and predominance of type I fibers. This phenotype reversed by discontinuation of therapy.

With the use of carnitine derivatives diverse intracellular signals arise in relation to carnitine. The derivative acetylcarnitine, the most abundant natural occurring ester, is also shown to influence fuel partitioning but it exhibits differential effects as compared to carnitine. Noteworthy evidences with this substance in models of nerve injury or aging complete the scenario of muscle phenotype affected by carnitine system. Greater plasticity in terms of muscle morphology and function are associated with greater complexity of neuromuscular junction structure, and better nerve recovery from injury with increased capacity for nerve regeneration [142].

Achieving metabolic homeostasis involves the participation of many organs that serve as the reservoirs or site of synthesis of key metabolites, thus metabolic effects induced by carnitine administration at the skeletal muscle level do not leave out of consideration new metabolic balances in tissues other than muscle.

Address correspondence to:

Prof. Menotti Calvani, Scientific Department, Sigma Tau S.p.A., via Pontina Km 30,400, 00040 Pomezia, Rome, Italy, Email menotti.calvani@sigma-tau.it, tel. +39 06 91393269, fax +39 06 91393940.

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