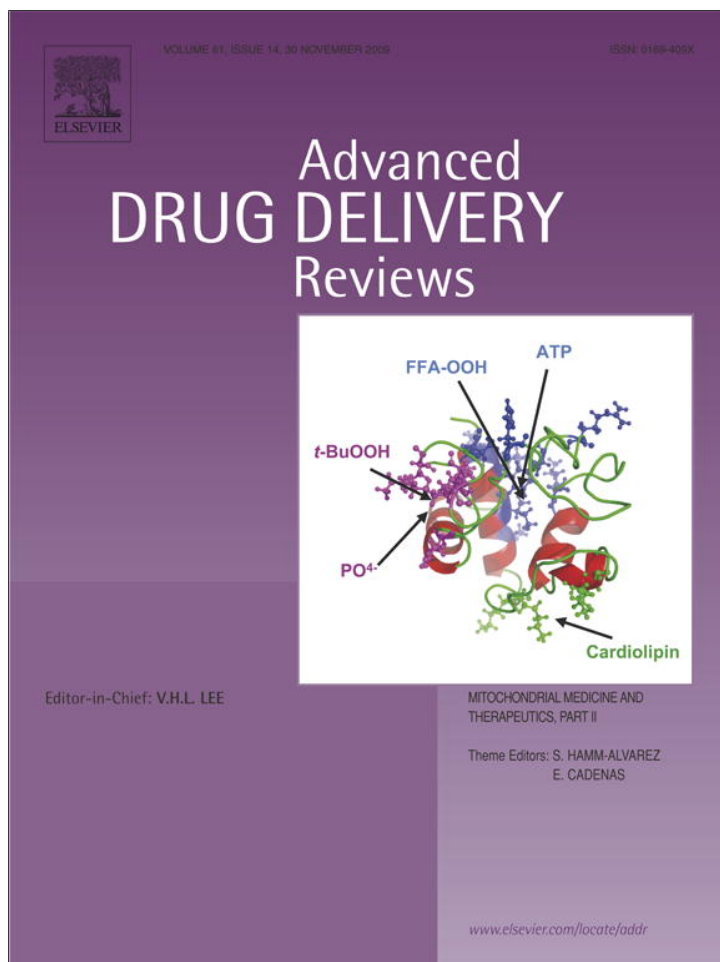


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journal homepage: www.elsevier.com/locate/addrCarnitine, mitochondrial function and therapy[☆]Victor A. Zammit^{a,*}, Rona R. Ramsay^b, Mario Bonomini^c, Arduino Arduini^{d,*}^a University of Warwick, Clinical Sciences Research Institute, Warwick Medical School, Coventry, England CV4 7AL UK^b Biomedical Sciences Research Complex, University of St Andrews, North Haugh, St Andrews, KY16 9ST Scotland, UK^c Department of Nephrology and Dialysis, University Hospital of Chieti, Chieti, Italy^d R&D Department, Iperboreal Pharma, Chieti, Italy

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ABSTRACT

Carnitine is important for cell function and survival primarily because of its involvement in the multiple equilibria between acylcarnitine and acyl-CoA esters established through the enzymatic activities of the family of carnitine acyltransferases. These have different acyl chain-length specificities and intracellular compartment distributions, and act in synchrony to regulate multiple aspects of metabolism, ranging from fuel-selection and -sensing, to the modulation of the signal transduction mechanisms involved in many homeostatic systems. This review aims to rationalise the extensive range of experimental and clinical data that have been obtained through the pharmacological use of L-carnitine and its short-chain acylesters, over the past two decades, in terms of the basic biochemical mechanisms involved in the effects of carnitine on the various cellular acyl-CoA pools in health and disease.

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Mitochondria are central to the maintenance of cell function and integrity. The generation of ATP, a process that is very sensitive to cellular energy demands, is regulated by multiple mechanisms to balance ATP use with ATP synthesis. The link between mitochondrial ATP formation and cytosolic processes involves not only the adenine

nucleotide transporter but also the mitochondrial kinases (e.g. creatine kinase, hexokinases, adenylate kinase) at the contact sites between the outer and inner mitochondrial membranes. In most mammalian tissues, mitochondrial oxidative phosphorylation is supported by one of two substrates: either pyruvate (derived from glucose, lactate, or amino acids) or fatty acids, especially long-chain fatty acids. The relative balance between the utilisation of these two substrates is very tightly regulated through a network of control mechanisms to ensure that metabolic processes within the cytosolic compartment and the mitochondrial matrix can interact to maintain cell function. These two compartments have quite different characteristics, such as metabolite concentrations, proteins, and redox status.

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These differences are achieved through the permeability characteristics of the mitochondrial inner membrane. In the present context the impermeability of the membrane (in common with most other cell membranes) to Coenzyme A and its esters is central to the function of many metabolic processes.

Coenzyme A (CoA) is a crucial cofactor in all living organisms, where it acts as an acyl group carrier and carbonyl-activating group in key biochemical reactions, including reactions in glucose and fatty acid metabolism. CoA is an obligate cofactor for many enzymes and it is involved in more than 100 different reactions in intermediary metabolism. Long-chain fatty acids (LC-FA) entering the cell are very rapidly esterified to CoA, to form acyl-CoA esters (FA-CoA); indeed this reaction may aid their uptake by trapping them inside the cell and lowering the concentration of unesterified LC-FA at their intracellular site of entry. Therefore, esterification to CoA acquires significance, not only in facilitating the transformations between intermediates that occur in metabolic processes, but also in compartmentalising them. This, in turn, necessitates the existence of specific mechanisms to enable the transfer of the molecular moieties esterified to CoA across intracellular membranes. Metabolic disorders in which this balance between different pools of CoA is disrupted from the normal pattern result in pathological conditions.

Specific acyl-CoA esters lie at important crossroads between metabolic pathways. Acetyl-CoA lies at the convergence of pathways leading to most routes of ATP formation within mitochondria. Similarly, LC-acyl-CoA esters, in addition to residing at the branch-point between fatty acid oxidation and glycerolipid synthesis, are biologically active molecules in their own right, and affect such parameters as gene expression, membrane trafficking and ion-channel activity (see [1]). Despite the need for free CoA for many energy-generating reactions, the intracellular, mostly mitochondrial, pool of esterified and non-esterified CoA is limited, so maintenance of the balance between 'free' and esterified CoA is important for normal cell function. One molecule in particular is of major importance in ensuring that such balance is maintained, and it can be used pharmacologically to modulate acyl-CoA-related functions in mitochondria and other cell compartments: L-carnitine. It permits maintenance of normal levels of free CoA by virtue of the existence

of: (i) a family of carnitine acyltransferases that catalyse the transesterification reaction between acyl-CoA esters and acylcarnitine esters, and the chain-length specificity of which covers the entire spectrum of acyl chain lengths; and (ii) the existence of a specific carnitine-acylcarnitine translocase (CACT) within the mitochondrial inner membrane [2] (and possibly other intracellular membranes [3]), which enables the transfer of acylcarnitines across this permeability barrier (Fig. 1). Through the action of these enzymes and translocases, the concentrations of acyl-CoA esters of various chain lengths, and of 'free' CoA itself can be modulated separately in each subcellular compartment, while effecting the net transfer of the acyl moieties between them, depending on the existing metabolic requirements of the cell.

1. Roles of the carnitine acyltransferases

The carnitine acyltransferases are a family of proteins that are widely distributed in the cell, and the properties of which are specifically tailored to their complementary roles in the above-mentioned functions of carnitine. Only one of the transferases has direct access to the cytosolic pool of acyl-CoA esters, the long-chain-specific carnitine palmitoyltransferase 1 (CPT 1). As LC-acyl moieties are a major substrate for mitochondrial ATP formation, CPT 1 occupies a central role in energy regulation [1,4]. Formation of LC-acylcarnitines enables the transfer of activated acyl groups into the matrix of mitochondria via the carnitine-acylcarnitine translocase located within the mitochondrial inner membrane (Fig. 1). The location of CPT 1 in the mitochondrial outer membrane, with its catalytic site exposed on the cytosolic aspect of the membrane is potentially significant. CPT 1 may have evolved as a protein of the outer membrane with its catalytic site extending into the cytosolic compartment, either because the effective substrate for CPT 1 is a complex of acyl-CoA with acyl-CoA binding protein (ACBP – see below), which would be too large to access the mitochondrial inter-membrane space [1,4], or because the voltage-dependent pore-forming molecule VDAC within the mitochondrial outer membrane is unable to translocate acylcarnitine esters [5], at least in the direction of the mitochondrial inter-membrane space. It has recently been

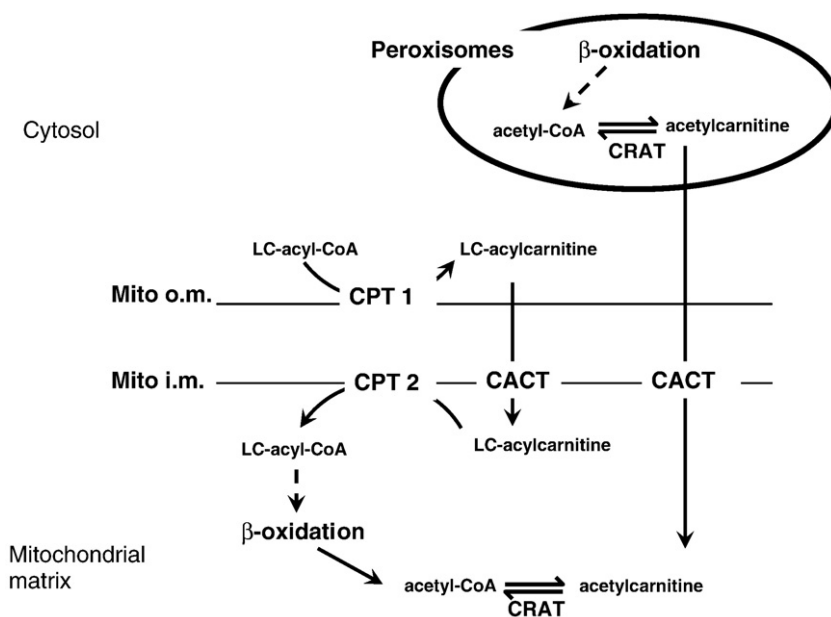


Fig. 1. The roles of the carnitine acyltransferases and mitochondrial inner membrane carnitine-acylcarnitine transferase (CACT) in the compartmentalisation of acyl-CoA and their conversion to the corresponding acylcarnitine esters. In the interests of clarity the role of carnitine octanoyltransferase (COT) in facilitating the formation of carnitine esters of chain-shortened fatty acids after peroxisomal β -oxidation is not shown. Abbreviations: CPT 1, CPT 2, carnitine palmitoyltransferases 1 and 2, respectively; CRAT, carnitine acetyltransferase; i.m. and o.m., inner and outer membrane of the mitochondria, respectively.

shown that CPT 1 exists as a hexamer *in vivo* [6,7]; the diameter of the space formed within this hexameric structure may be sufficiently large to accommodate molecules of the size of palmitoylcarnitine [7]. However, while it is attractive to suggest a directional transfer of the product of the CPT 1-catalysed reaction [6], the pore-forming characteristics of CPT 1 have not been experimentally demonstrated as yet.

Mammals express three separate genes for CPT 1 [8], and their protein products are expressed in a tissue-specific manner: CPT 1A is highly expressed in liver and kidney; CPT 1B in cardiac and skeletal muscle; and CPT 1C in the brain. Although CPT 1A and 1B are highly catalytically active, CPT 1C displays a very low catalytic efficiency [8,9] and its possible roles are only starting to be explored. Mice in which the CPT 1C gene is disrupted have a higher propensity for weight gain [10], and for insulin resistance accompanied by elevated hepatic gluconeogenesis [11], depending on the genetic background. Besides being targeted to the mitochondrial outer membrane [12], CPT 1 isoforms may also reside in other intracellular membranes, particularly the endoplasmic reticulum [3,8,9] where they may regulate the acyl-CoA/acylcarnitine ratio within specific microenvironments; in the case of CPT 1C this may be the main intracellular location [3,8,9]. In the case of CPT 1A, there is evidence that microsomal CPT 1 activity may be involved in the transfer of long-chain acyl moieties across the membrane of the endoplasmic reticulum for the synthesis of intralumenal triacylglycerol [13,14] from diacylglycerol by a lumen-facing diacylglycerol acyltransferase activity [15]. Therefore, CPT 1 isoforms may have a multiplicity of roles. However, it is the role of CPT 1 isoforms in the regulation of mitochondrial fatty acid oxidation that is by far the best studied.

The function of mitochondrial outer membrane CPT 1, catalysing what is effectively the first reaction that commits LC-fatty acids to mitochondrial fatty acid oxidation, is complemented by the activity of another LC-specific CPT, namely the CPT 2 loosely associated with the mitochondrial inner membrane that enables the transformation of acylcarnitine back to LC-acyl-CoA within the mitochondrial matrix. It is interesting to note that both CPT 1 and CPT 2 are highly enriched within the contact sites (areas of close apposition of the outer and inner membranes) of mitochondria [16], suggesting that protein composition of these structures may be specialised for the synthesis and transfer of fatty acids for oxidative formation of ATP. The most significant mechanism for the regulation of the transfer of fatty acids into the mitochondrial matrix (and, therefore, their oxidation) is the inhibitory action of malonyl-CoA on CPT 1. Although the CPT 1 isoforms differ in their intrinsic catalytic activities and other properties, they all bind malonyl-CoA with high affinity. In the case of CPT 1C, this may be its major physiological function [8].

Another member of the carnitine acyltransferase family, carnitine acetyltransferase (CRAT), is present both in the mitochondrial matrix and in the peroxisomal core. It has also been reported to be present in the endoplasmic reticular lumen and in the nucleus but it is absent from the cytosol (see [17]). In terms of its role in enabling carnitine to affect metabolism, its presence in the mitochondrial matrix and the peroxisomal core are the most important features. Within the peroxisomes it is involved in the export of acetyl moieties generated by peroxisomal β -oxidation out of the peroxisomes; it has been suggested that both acetylcarnitine and acetate are used to export the acetyl groups produced [18]. Although the nomenclature suggests that the enzyme catalyses primarily the interconversion of acetyl-CoA and acetylcarnitine, the optimum acyl chain length for human CRAT is C4 [19]. The specificity of CRAT for short-chain derivatives comes from steric hindrance to the binding of longer chains in the active site caused by Met564 instead of the Gly553 found in COT [20,21]. Informed by this structure of CRAT and that of COT [20], the chain-length specificity of specificity of COT and CRAT have been altered mutationally, proving the prediction that hindrance of the binding cavity for the acyl chain limits the length of chain that can bind at the

active site [20,22,23]. However, importantly, CRAT can also use a wide range of short and branched chain organic acids [24], and therefore its location mostly within the mitochondrial matrix is central to the export of organic acids in acyl-CoA dehydrogenase deficiencies (reviewed in [25]). The same gene codes for CRAT in both its major intracellular locations; alternative splicing results in variants that are targeted to the peroxisomal core and the mitochondrial matrix, respectively [26]. Thus, any mutations affecting activity will affect the short-chain acyl-CoA/carnitine equilibria in both compartments.

Carnitine octanoyltransferase (COT), found only in peroxisomes, exhibits optimum activity with medium-chain acyl-CoA esters, and, like CRAT, it is involved in the export of chain-shortened acyl groups from the peroxisomal pool. COT is also implicated in the breakdown of phytanic acid for the transfer from peroxisomes to the mitochondria which requires CACT but not CPT 1 [27]. We are unaware of any clinical deficiencies of COT alone but the absence of the enzyme accompanies general peroxisomal deficits. Like CPT 1 and CPT 2, COT is also increased by peroxisome proliferators [28].

2. Physiological consequences of equilibration between acylcarnitine and acyl-CoA esters

The intracellular distribution of the acyltransferases is integrative and related to the requirements of every cell to regulate carbohydrate and lipid metabolism co-ordinately. Therefore, the effects of changes in their catalytic activity are widespread and varied. The physiological effects that range from the selection of metabolic fuels by tissues, to the regulation of processes are dependent on the localised concentrations of acyl-CoA esters. These include the regulation of insulin secretion by the pancreatic β -cell, the determination of tissue insulin sensitivity, and the activity of appetite-controlling neuronal networks in the hypothalamus, to mention only a few of the myriad of processes that are already known to be affected (see [1] for review).

Due to the activities of the various carnitine acyltransferases, changes in the availability of L-carnitine in the cell affect acyl-CoA pools rapidly. This rapid equilibrium can also serve to mediate the efflux of acylcarnitine esters (e.g. the release of acetylcarnitine from hepatocytes under conditions of high rates of hepatic fatty acid oxidation). Thus, the carnitine acylation state in the plasma reflects the cytosolic acylcarnitine pool and serves as a diagnostic marker for the altered equilibria between acyl-CoA and acylcarnitine species. Conversely, deficiencies of specific carnitine acyltransferases or proteins that transfer carnitine between intracellular compartments result in pathological conditions accompanied by excretion of specific acylcarnitine esters.

3. Pathological consequences of deficiencies of carnitine carriers and the carnitine acyltransferases

Genetic and functional characterisation of the enzymes and carriers of the carnitine system (reviewed in [29,30]) has advanced considerably in the last 10 years. Identification of deficiencies in the carnitine system has been facilitated by reliable and now routine methods for the determination of urinary and plasma levels of individual acylcarnitines [31,32]. Changes in the metabolomic profile indicate which protein involved in carnitine metabolism is deficient. The particular defect is then determined by *in vitro* functional analysis and subsequent sequence analysis of cDNA to identify the mutation.

3.1. Uptake of carnitine into the cell – defects in OCTN2

Excess acylation of CoA in mitochondria is countered by equilibration with the ten-fold larger cytosolic carnitine pool through the action of the acyltransferases and carriers [33]. Increasing the cytosolic carnitine pool can increase the formation of intracellular

acylcarnitines which leave the cell down the concentration gradient into the plasma from which they are cleared through urinary excretion. The importance of carnitine for both oxidation of fatty acids and for the export of excess (deleterious) acyl groups is recognised not only in defects of fatty acid oxidation but also in primary carnitine deficiency associated with the plasma membrane transporter OCTN2. Several defects in OCTN2 are known, resulting in cellular carnitine deficiency and an inability to metabolise fatty acids [34]. OCTN2 is the Na⁺-dependent carrier that concentrates carnitine to millimolar concentrations inside the cell from 50 micromolar in plasma [35]. Bearers of truncation defects in OCTN2 present with hypoketotic hypoglycemia that develops into cardiomyopathy with age. However, a survey of cardiomyopathy patients found no difference in variant frequency in patients compared to controls [36].

3.2. Transfer of carnitine across the mitochondrial inner membrane: the mitochondria carnitine exchange carrier (CACT)

CACT exchanges carnitine and acylcarnitines across the mitochondrial inner membrane. Within the cell, the carnitine pools are assumed to be close to equilibrium. Through the action of the acyltransferases, the acylation state of the large cytosolic carnitine pool can also be equilibrated with the mitochondrial acyl-CoA pool as demonstrated for liver [33], but in muscle [37] an apparent lack of equilibrium suggests that this system is less able to export excess acyl groups, leading to potential functional deficits. Defects in CACT result in non-ketotic hypoglycemia similar to deficiencies of CPT 2 (reviewed in [38]). The range of clinical malfunction is likely to be related to the severity of the effect of the mutation on the function of the protein. For example, the mutation Q238R mutation has been shown to result in hypoketotic hypoglycemia and an excess of long-chain (C16 and C18:1) acylcarnitines in the plasma [39]. The rate of transfer of carnitine and acylcarnitines mediated across the mitochondrial inner membrane by CACT also enables the rapid export of organic acids when high levels of carnitine are made available in the plasma [40]. For infants in whom the activity of CACT is compromised by mutation, treatment consists of avoidance of prolonged fasting and the adoption of a low-fat diet. The equilibration of the acylation state of carnitine with the intracellular (cytosolic) acyl-CoA pools implies that supplementation with carnitine under these conditions may result in the net excretion of acylcarnitines rather than the promotion of the oxidation of fatty acids [38].

3.3. Control of LC-acyl-CoA concentrations in microsomal and mitochondrial microenvironments

As described above, each carnitine acyltransferase uses only the acyl-CoA derivatives in the specific cellular compartment to which it is localised. CPT 1 competes for the cytosolic pools of LC-acyl-CoA with the enzymes of lipid synthesis such as the respective glycerol-3-phosphate acyltransferases on the mitochondrial outer membrane and the endoplasmic reticulum. Recent work has demonstrated that acyl-CoA binding protein (ACBP) in the cytosol both keeps the 'free' LC-acyl-CoA concentration in the cytosol low and facilitates delivery of the LC-acyl-CoA to these membrane bound enzymes [41]. Transgenic mice with increased ACBP expression resulted in a 69% increase in hepatic LC-acyl-CoA. The increase was largest in the microsomal fraction and was accompanied by an increase in GPAT and phospholipids in liver. Mitochondrial acyl-CoA was not altered. Molecular association of ACBP and CPT 1 has not yet been demonstrated, but it has been suggested that the ACBP-acyl-CoA complex may be the effective substrate for CPT 1, and that this may be one reason why the latter has evolved as a mitochondrial outer membrane protein with its active site exposed to the cytosolic compartment [42].

3.4. Defects involving CPT 1A and 1B mutations

Homozygous deficiency is lethal in mice for both CPT 1A and 1B isoforms [43,44]. The lethality reported in mice (see above) and humans [45] and the current relatively few variants of CPT 1 associated with disease (e.g., in liver CPT 1A, [46,47]) is consistent with a high fetal lethality rate and with the key role of this enzyme in energy production during fasting [48]. Two mutations affecting the active site, G709E and G710E, abolish CPT 1A activity. The possible involvement of these residues in the catalytic function of the protein has been inferred from *in silico* homology modelling using the crystal structure of carnitine acetyltransferase as template [49]. Mutational analysis has shown that they contribute to the hydrophobic pocket in which the acyl moiety of the acyl-CoA substrate is accommodated, and thus influence the chain-length specificity of CPT 1 [50].

The P479L variant of CPT 1A [46] has recently been shown to be the predominant form (frequency 0.81) in the Inuit population a region of Canada [51]. The P479L mutation decreases the activity of liver CPT 1 (to less than 10% of control) but also makes it less sensitive to malonyl-CoA inhibition. The low sensitivity to the malonyl-CoA might have conferred an advantage to individuals consuming the high fat very low carbohydrate traditional diet of that population, avoiding the rapid and efficient shift to carbohydrate oxidation that must be followed by a slow transition back to the ketogenic state [51]. Despite the low CPT1 activity the rates of fatty acid oxidation were 70–80% of control values with palmitate but over 90% with myristate. This was surprising because CPT 1 exerts strong control over the rate of fatty acid oxidation [52], although substrate supply, cytosolic malonyl-CoA concentration, CPT 1A sensitivity its inhibitor, and energy demand (redox status) also influence the rate of fatty acid oxidation. Further cellular investigation is needed, including assessment of contributions from peroxisomal oxidation that is likely to be up-regulated.

3.5. Defects in CPT 2

CPT 2 uses the intramitochondrial pools of substrates and is particularly important not only for provision of substrates for fatty acid oxidation but also for transferring excess long-chain acyl groups to carnitine for export from the mitochondria. Defects in CPT 2 were identified early and are associated with a wide range of phenotypic severity. Where the activity is severely reduced, the deficiency is fatal in infancy. Milder forms are usually first noticed after excessive exercise during starvation; these forms usually cause muscle weakness and fatigue but can result in myopathy. A list of 64 disease-associated mutations are given in [53]. Of the mutations investigated, 19 were truncations and 42 were amino acid substitutions or deletions. The most common polymorphism, S113L, does not usually cause defects, but may be associated with insulin resistance. However, S113L is found in 66% of all variants of CPT 2 where activity is decreased, in contrast to 33% of variants with normal activity [54]. Another polymorphism P352C is found in 20% of the Japanese population without manifestation of defects, yet it is associated with myopathy and decreased CPT 2 activity in muscle in others. Carnitine binding to CPT 2 is affected by T120C and S588C, so that higher cellular carnitine concentration would be beneficial for patients with these mutations.

Mild CPT 2 deficiency has been treated with peroxisome proliferators, such as bezafibrate [55], because these agents upregulate the expression of the long-chain carnitine acyltransferases [56]. This reflects the role of PPAR-*alpha* in regulating gene expression for CPT 1, CPT 2, COT, and OCTN2 [57]. Indeed, acylcarnitine profiles demonstrated that PPAR-*alpha* knockout mice showed defects in fatty acid metabolism and carnitine levels, and that they benefited from supplementary carnitine [58]. An alternative management strategy is the feeding of medium-chain triglycerides [59].

4. Therapy through modulation of carnitine availability

The therapeutic potential of L-carnitine and some of its short-chain acyl-esters, namely, acetyl-L-carnitine and propionyl-L-carnitine, is still a matter of debate [60–66]. However, it is evident that in certain (rare) genetic disorders (e.g. those involving defects in the high-affinity plasmalemmal L-carnitine transporter) that L-carnitine treatment could represent a life saving therapy [67]. Indeed, these patients experience a severe plasma and tissue L-carnitine deficiency along with overt signs of cardiomyopathy and cardiac failure, which may be fatal unless they are promptly diagnosed and treated with high dose of L-carnitine. More controversial seems to be the beneficial action of L-carnitine treatment in patients presenting with a moderate carnitine deficiency (secondary) [63,64,66,68,69]. Although such uncertainty in the success of the use of carnitine and its short-chain acyl-esters may be due to various reasons (e.g. the variety of the diseases treated, different dosages used and the route of administration), it might be that, for certain pathological conditions, a successful and efficacious treatment with L-carnitine may require raising of plasma carnitine concentrations to supraphysiological levels in order to achieve an increase in L-carnitine in the target organs. This section focuses on the ability of sufficiently high plasma concentrations of L-carnitine to provide favourable pharmacological outcomes through modulation of the intracellular metabolic fluxes associated with the equilibria of the processes described above.

4.1. The acetyl-CoA pool size

As discussed above, mammalian cells contain sequestered pools of CoA that are functionally separated by intracellular membranes. Acetyl-CoA not only occurs at the crossroads of major anabolic and catabolic pathways (see above and Fig. 2) but also exerts significant metabolic control. For example, acetyl-CoA modulates metabolic flux by allosteric activation of pyruvate carboxylase (PC), a pivotal step in hepatic gluconeogenesis [70]. During prolonged fasting, neurones and red blood cells still rely to a large extent on glucose as the energy source. This glucose is synthesized from the non-carbohydrate precursors lactate, alanine, glycerol and glutamine in the liver [71], and to a lesser extent in kidney cortex and small intestine. PC is the enzyme responsible for the conversion of pyruvate to oxaloacetate, the

first committed step of gluconeogenesis, and it is activated allosterically by acetyl-CoA and ATP [72]. The oxaloacetate formed is reduced to malate for export to the cytosol where it is oxidised to generate oxaloacetate and NADH in the cytosol. This cytosolic oxaloacetate is decarboxylated to phosphoenolpyruvate and enters the gluconeogenic pathway. Thus, in the liver, when high amounts of other energy substrates are available for ATP production, pyruvate conversion to oxaloacetate is diverted into the gluconeogenic pathway rather than going through the TCA cycle [73]. In addition, acetyl-CoA activates pyruvate dehydrogenase kinases (PDHK) that catalyse phosphorylation of the pyruvate dehydrogenase complex thus inhibiting the utilisation of pyruvate to produce acetyl-CoA for the TCA cycle [74,75]. Indeed, in the fasted state, PDH is inactivated as a consequence of its phosphorylation which spares three-carbon compounds for gluconeogenesis. These actions of acetyl-CoA are important in the pathophysiology of type 2 diabetes, which is characterised by high rates of hepatic glucose production through gluconeogenesis [76].

If a supraphysiological increase of the acetyl-CoA pool in liver mitochondria is associated with accelerated gluconeogenic flux, an increase of acetyl-CoA in muscle mitochondria may also affect glucose utilisation. In skeletal muscle of diabetic subjects, insulin appears to be unable to mediate the switch from lipid to carbohydrate oxidation, a state described as “metabolic inflexibility” [77]. The fasting diabetic/insulin resistant muscle is characterised by a blunted suppression of glucose oxidation and lower than normal rate of fatty acid oxidation, and in the fed state insulin is less able to stimulate muscle glucose oxidation. One of the components of the pathogenetic mechanism responsible for the impaired muscle glucose disposal observed in diabetic/insulin resistant patients may be associated with PDK activation by an increase in the size of the relevant pool of intramitochondrial acetyl-CoA which would act synergistically with the increased expression of specific isoforms of PDHK, e.g. PDHK4 [78].

Increased rates of fatty acid oxidation associated with abnormal higher rates of glycolysis and lower rates of glucose oxidation are believed to be important pathogenetic determinants in the dysfunctional myocardium [79,80]. For example, in the ischemic heart, the uncoupling between glycolysis and glucose oxidation leads to the accumulation of deleterious by-products (lactate and protons) that contribute to the decreased efficiency of contractile proteins [81]. In addition, the increased proton production may result in the mediation of a greater influx of Na^+ and Ca^{2+} ions, and the diversion of ATP utilisation for the maintenance of ion homeostasis rather than for supporting cardiac contractile function [82]. Even in cardiac failure, a consistent body of evidence implicates impaired substrate metabolism as one of the main contributors to contractile dysfunction and to progressive left ventricular remodelling [83].

Recent findings raise the possibility that discrete hypothalamic centers control both energy balance and hepatic glucose production [84,85]. Specific neuronal cell-types in the arcuate nucleus sense the availability of peripheral nutrients, including glucose, and hormones/cytokines (e.g. insulin, adiponectin, ghrelin, and leptin). Thus, there is considerable experimental evidence that increased non-esterified fatty acids and/or glucose hypothalamic levels elicit neural activation that activate efferent neural circuits that in turn suppress endogenous (hepatic) glucose production [84,86]. In this regard, mounting evidence indicates that lipid and glucose metabolism in neurons play a key role in mediating the hypothalamic responses to fuel availability. Combined genetic and pharmacological manipulations have led to the identification of two biochemical sensors associated with the neuronal metabolism of lipid: malonyl-CoA/CPT 1 [87–89], and glucose/pyruvate/PDH [90]. In particular, pharmacological interventions aimed at increasing pyruvate entry into the TCA cycle (i.e., inhibition of PDHK and LDH) in the hypothalamus of conscious rats resulted in a lowered hepatic glucose production as measured in *in vivo* experiments involving pancreatic clamps [90].

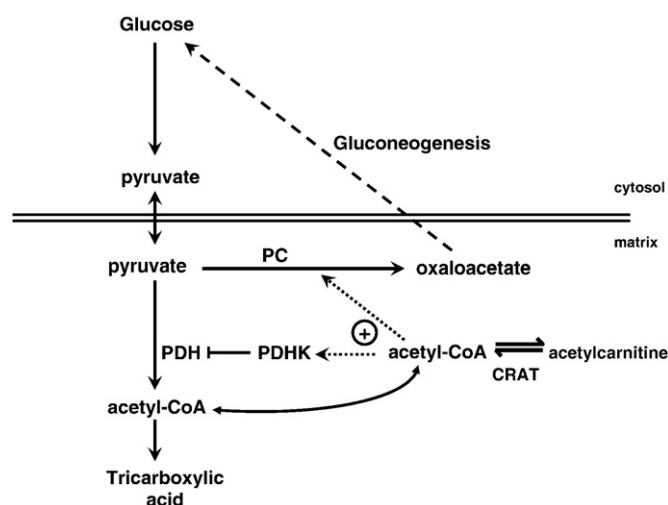


Fig. 2. Importance of mitochondrial acetyl-CoA in the integration of fatty acid and glucose metabolism in the liver, as a pathway intermediate and an effector of key enzymes. Abbreviations: as in Fig. 1; PC, pyruvate carboxylase; PDH, pyruvate dehydrogenase; PDHK, pyruvate dehydrogenase kinase; CRAT, carnitine acetyltransferase.

4.2. Effects of altering the acetyl-CoA pool size

Taken together, the examples above highlight the possibility of metabolic interventions designed to attenuate pathological consequences by restricting the expansion of the intramitochondrial acetyl-CoA pool. Different experimental approaches, such as the use of PDK and fatty acid oxidation inhibitors, and PDH complex activation, aimed at directly or indirectly attenuating the metabolic consequences of an increased intramitochondrial concentration of acetyl-CoA, have been shown to ameliorate the insulin resistant/diabetic condition [91–94] and to improve contractile function in cardiac failure and ischemia [79,95–98]. Among the various tools available to significantly affect the pool size of acetyl-CoA is the use of L-carnitine. Several *in vivo* studies conducted in animals and humans have shown that the administration of high doses of L-carnitine may favourably affect the course of such pathological conditions. The use of the euglycemic hyperinsulinemic clamp has demonstrated the ability of carnitine treatment to increase muscle glucose disposal in healthy and diabetic individuals. Ferranini et al. have shown that exogenous carnitine infusion led to a statistically significant increase in whole-body glucose utilisation in healthy subjects; this was quantitatively accounted for by a 50% increase in non-oxidative glucose disposal [99]. Using a similar treatment protocol [100,101], it has been demonstrated that type 2 diabetic individuals infused with L-carnitine showed a significant increase in muscle glucose disposal both in terms of glucose oxidation and glycogen synthesis. More recently, in a small randomized, double-blind, placebo controlled study, intravenous L-carnitine administration to uremic patients undergoing hemodialysis (HD) favourably affected insulin resistance and protein catabolism in non-diabetic HD patients [102]. This initial set of human studies suggests that if a sufficiently high L-carnitine muscle exposure is achieved, L-carnitine may affect the intramitochondrial acetyl-CoA pool by shifting the freely reversible CRAT-catalysed reaction towards acetyl-L-carnitine formation. Indeed, during euglycemic hyperinsulinemic clamp studies, plasma acetyl-L-carnitine levels significantly increased in L-carnitine infused subjects [99]. Moreover, by using an intravenous glucose tolerance test, analyzed according to the minimal model technique, together with indirect calorimetry, it has been shown that L-carnitine infusion in healthy subjects induced an increase of whole-body glucose disposal and oxidation [103] although this effect has not always been observed [104]. As discussed below, this negative finding is indicative of the difficulty in achieving a sufficiently high L-carnitine exposure, when it is administered orally. Additional biochemical findings seem to corroborate the ability of L-carnitine to relieve PDHK activation by acetyl-CoA: a significant increase of PDH activity was found in muscle biopsies of marathon runners treated with a high oral dose of L-carnitine for 4 weeks [105] as expected from the similar effects observed in human muscle mitochondria exposed to L-carnitine *in vitro* [106]. In the latter study, the presence of L-carnitine in the muscle mitochondria incubation mixture stimulated $^{14}\text{CO}_2$ production from $1\text{-}^{14}\text{C}$ -pyruvate. However, in [107] a significant decrease of PDH activity was found in muscle biopsy samples of healthy individuals exposed acutely (6 hours) to an L-carnitine infusion. Interestingly, at the end of L-carnitine infusion there was a 40% decrease of muscle lactate content and a 30% increase in muscle glycogen [107]. The authors suggested that these observations could have been the result of an increased fatty acid oxidative flux elicited by L-carnitine (presumably owing to a mass-action effect on the CPT 1-catalysed reaction), although experimental evidence for this is still required.

Although the clinical studies described above did not attempt to evaluate the potential action of L-carnitine on hepatic glucose production in the diabetic condition, some clues may be found in a number of preclinical studies conducted on diabetic experimental models. In a study originally designed to evaluate the beneficial action

of L-carnitine on diabetic heart [108], streptozotocin-treated diabetic rats were dosed for 6 weeks with a very high daily intraperitoneally dose of L-carnitine (3 g/kg); at the end of the treatment, the L-carnitine group showed a remarkable reduction of plasma glucose compared to the diabetic control group. Importantly, the lowering of plasma glucose was associated with the reversal of both glucosuria and polydipsia. L-Carnitine treatment of non-diabetic rats did not affect plasma glucose levels [108]. Taking into account that L-carnitine treatment did not affect the hypoinsulinemic state of streptozotocin-diabetic rats, and that in this diabetic model the severe hyperglycaemic state is mainly driven by an increased hepatic glucose production, it is possible that L-carnitine could have been acting, at least partly, through the inhibition of gluconeogenesis. Further evidence of the anti-gluconeogenic action of pharmacological doses of L-carnitine has recently been provided by the data of [109]. In this study, Wistar rats were fed for 1 month with fructose as the sole source of carbohydrate, an experimental model characterised by a severe impairment of insulin sensitivity, glucose intolerance, dyslipidemia and increased hepatic glucose production. Intraperitoneal L-carnitine administration (300 mg/kg/24 h) for the entire period of fructose feeding normalized the elevated plasma glucose and insulin levels, and liver TG and FFA content in fructose fed rats [109]. These authors suggested that the ability of L-carnitine treatment to mitigate the adverse effects of the fructose diet was mainly due to the correction of L-carnitine deficiency induced as a result of the fructose load. If their interpretation is correct, it is possible that L-carnitine depletion in the liver could have affected FFA oxidation and, hence, the metabolic partitioning of long-chain fatty acyl-CoA towards esterification, leading to steatosis and increased hepatic VLDL-triglyceride secretion. Amelioration of insulin-stimulated glucose disposal in an obese diabetic transgenic mouse model was obtained after a 3 week period of dietary L-carnitine supplementation (1 g/kg/day) [110]. Withdrawal of L-carnitine treatment for 6 weeks re-established the original diabetic state, though a further week of L-carnitine treatment reversed the loss of insulin sensitivity. After this second treatment period with L-carnitine, fasting glucose levels were strongly reduced, implying that L-carnitine therapy could have improved hepatic insulin sensitivity and, hence, the insulin-suppressive action on hepatic glucose production in this diabetic mouse model [110].

Given the importance of fatty acid oxidation for ATP formation by cardiac muscle, the physiological role of L-carnitine and the carnitine acyltransferases has been studied extensively in this tissue. The physiological relevance of L-carnitine in ensuring an adequate ATP supply for the contractile activity of the heart is probably best seen in severe cases of systemic L-carnitine deficiency in humans, which, if left untreated, are characterised by a lethal cardiomyopathy [67]. L-Carnitine has also been the subject of numerous investigations as potential pharmacological agent for the treatment of heart disease. One of the first convincing pieces of evidence that supraphysiological L-carnitine exposure may be beneficial in ischemic heart was provided by [111]. In their study, the mechanical function of ischemic pig hearts was remarkably preserved when they had been infused with an L-carnitine dose able to achieve a 200-fold increase of its plasma exposure. After this initial work, many other animal and human studies have addressed the therapeutic potential of L-carnitine and some of its short-chain esters, particularly propionyl-L-carnitine, on heart ischemia and failure [4,17,62,112,114,116,119–121]. The rationale behind the use of L-carnitine was mainly aimed at the correction of L-carnitine loss that occurs during the induction of ischemia in the heart of healthy or diabetic animals, and human failing heart [112,113]. Although there is little doubt that a significant lowering of the carnitine content of cardiac muscle may render the heart more vulnerable to the metabolic and contractile derangements in ischemia [114], it is not yet clear whether the human heart is prone to the development of a significant L-carnitine deficiency in the course of

ischemia, nor whether the deficiency would exacerbate or ameliorate the outcome of an ischemic attack.

Lopaschuk et al. were the first to address the metabolic effect of supraphysiological concentrations of L-carnitine in the intact normal working rat heart [115]. By maintaining an elevated L-carnitine concentration (10 mM) in their perfusion system, which allowed them to achieve an L-carnitine heart exposure two-fold higher than in control heart, they observed a marked increase in the rate of glucose oxidation, presumably as a result of a stimulation of PDH flux [115]. This illustrates how a significant increase in heart L-carnitine content may affect the intramitochondrial pool of acetyl-CoA by an efficient mass-action effect on the freely reversible reaction catalysed by CRAT in mitochondria. As discussed above, the consequent decrease in intramitochondrial acetyl-CoA would be expected to translate into a less active PDHK, and hence, a more active PDH. Interestingly this shows that intramitochondrial CRAT may be at a lower degree of saturation with respect to L-carnitine than the cytosol-facing CPT 1B because, despite the well known physiological role of L-carnitine in facilitating the mitochondrial oxidation of long-chain fatty acid at the CPT 1 step, supraphysiological exposure of L-carnitine in the heart resulted in a decrease rather than an increase of fatty acid oxidation rate. It has also been suggested that CPT 1 activity is attenuated under these conditions owing to an increased availability of malonyl-CoA.

When isolated working hearts were submitted to an ischemia/reperfusion injury (35 min of global ischemia) in the presence of 10 mM L-carnitine in the perfusion medium there was a significantly improved contractile function, as evaluated by heart rate-peak systolic pressure product, compared to control hearts in addition to the significant stimulation of glucose oxidation rate when perfused with high carnitine concentrations [116]. In a subsequent study, the same authors investigated the effect of supraphysiological L-carnitine concentrations in the perfusion media of isolated working hearts from streptozotocin-diabetic rats [112]. After a normoxic perfusion of 60 min, isolated working hearts were subjected to 60 min of low flow ischemia followed by a normoxic reperfusion for 30 min. Under these experimental conditions, control hearts were able to recover only 38% of their pre-ischemic contractile function, whereas hearts perfused throughout the study in the presence of 10 mM L-carnitine recovered almost 90% of their pre-ischemic function [112]. The L-carnitine treatment gave an almost two-fold increase in myocardial L-carnitine content and markedly increased glucose oxidation rates. These findings seem to indicate that an acute treatment with L-carnitine favours glucose utilisation and offers significant protection against the detrimental action of ischemia on the mechanical function of the heart upon reperfusion.

Chronic administration of L-carnitine has also been shown to protect the heart against the detrimental effect of diabetes. Streptozotocin-diabetic rats showed a remarkable deterioration in left ventricular developed pressure, contractility, and ventricular relaxation rates, whereas diabetic rats treated for 6 weeks with a very high daily intraperitoneally dose of L-carnitine (3 g/kg) were largely free of these effects [108]. Myocardial L-carnitine content in the treated group was almost three-fold higher than in the diabetic control group. Since L-carnitine treatment significantly reduced plasma glucose and triglyceride levels, part of its cardioprotective effect could also be related to a more systemic action on the dysmetabolic condition of the streptozotocin-diabetic rat. Using ^{31}P -NMR spectroscopy, Loster et al. [117] monitored the changes in adenine nucleoside phosphate, phosphocreatine (PCr) metabolism and intracellular pH in Langendorff rat heart preparations subjected to a no-flow ischemic period of 20 min followed by a reperfusion period of 60 min in the presence or absence of L-carnitine (5 mM). As expected, ischemia caused a rapid decrease in the PCr signal, followed by a decrease in the ATP and an increase in the inorganic phosphate (Pi) signals. A partial recovery of the ATP and PCr signals was observed in the reperfusion period. The presence of L-carnitine in the perfusion medium led to a marked recovery of the high

energy phosphates (e.g. increased PCr/Pi ratios) and counteracted drops in intracellular pH [117]. Interestingly, the presence of L-carnitine in the perfusion medium allowed the heart to tolerate better up to four cycles of ischemia/reperfusion in comparison to control hearts. Although the authors have shown that the presence of either acetyl-L-carnitine or propionyl-L-carnitine in the perfusion medium afforded a similar protection against the ischemia/reperfusion injury on the bioenergetic function of the perfused hearts, it is not clear to what extent their beneficial intervention was a direct effect of the L-carnitine moiety. When these two short-chain acyl carnitine esters are taken up by the heart they release L-carnitine via the enzymatic reaction catalysed by CRAT, although, in the case of propionylcarnitine, this is anticipated to have the additional metabolic advantage of providing intramitochondrial propionyl-CoA which feeds into the TCA cycle and promotes further formation of ATP [4,17].

Several small clinical trials on the effects of L-carnitine in patients affected by heart disease have provided both positive [118–121] and negative [122,123] indicators for a beneficial effect of the treatment. However, a large multicenter, randomized, double-blind clinical trial study has shown that both early- and long-term administration of L-carnitine attenuates progressive left ventricular dilatation after acute anterior myocardial infarction [119].

4.3. L-Carnitine: a conditional drug?

The potential limitation of L-carnitine-based “mitochondrial” therapy may be overcome through the attainment of supraphysiological concentrations of L-carnitine in plasma and target organs, so as to elicit the desired pharmaco-metabolic response. In target organs such as liver, heart, and skeletal muscle, the intracellular L-carnitine pool is in the high micromolar to low millimolar range, whereas in the plasma it is in the low micromolar range [124]. In addition, taking into account that physiological plasma levels of L-carnitine almost saturate the high-affinity L-carnitine transporters, relatively high L-carnitine plasma exposures are required to significantly achieve organ L-carnitine increases. Under these conditions, it is possible that L-carnitine moves into the intracellular milieu via passive diffusion and/or a low-affinity carnitine transporter [125]. However, the increase of L-carnitine plasma exposure upon L-carnitine oral administration, even when using high doses (e.g. more than 2 grams per day) [124], is quite modest, since L-carnitine has a very poor absorption and bioavailability, a very high renal clearance, and active uptake into tissues by a high-affinity transporter [124,125]. Intravenous administration of L-carnitine might overcome such a problem, particularly for acute/short-term treatment of hospitalized patients. However, this route of administration may present difficulties, particularly when kidney function is intact, because the efficient tubular reabsorption process ensures that more than 95% of L-carnitine filtered by glomeruli is retained [124,126]. Moreover, since renal tubular reabsorption occurs via an active transporter, once the transporter is saturated the excess of exogenous L-carnitine is readily excreted.

Thus, combination of a parenteral route of administration with impairment of the normally elevated renal clearance observed at high plasma L-carnitine concentrations might represent an ideal condition to achieve efficiently adequate L-carnitine exposures in both plasma and target organs. A patient population which may meet these criteria, particularly for longer periods of treatment, are uremic patients undergoing hemodialysis. Indeed, a large number of clinical studies conducted in hemodialysis patients treated with L-carnitine via the parenteral route have been published [127,128]. The rationale behind the use of L-carnitine treatment in hemodialysis patients is mainly related to the potential L-carnitine deficiency occurring during the hemodialysis procedure [129]. As a result of this and other causes of secondary L-carnitine deficiencies, L-carnitine has been described as a “conditionally essential nutrient” or “conditional vitamin” for these patients [126]. However, some controversy and misconceptions about

its use as a more general nutrient still persist [63–66]. The data reviewed here suggest that it would be interesting to evaluate clinically the possibility of a move from the paradigm of “conditional vitamin” to that of the “conditional drug”, particularly in hemodialysis patients affected by type 2 diabetes where there are not that many therapeutic tools available to improve the cardiometabolic syndrome.

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