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REVIEW ARTICLE

Citrulline and nitrogen homeostasis: an overview

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Abstract Citrulline (Cit) is a non-essential amino acid whose metabolic properties were largely ignored until the last decade when it began to emerge as a highly promising nutrient with many regulatory properties, with a key role in nitrogen homeostasis. Because Cit is not taken up by the liver, its synthesis from arginine, glutamine, ornithine and proline in the intestine prevents the hepatic uptake of the two first amino acids which activate the urea cycle and so prevents amino acid catabolism. This sparing effect may have positive spin-off for muscle via increased protein synthesis, protein content and functionality. However, the mechanisms of action of Cit are not fully known, even if preliminary data suggest an implication of mTOR pathway. Further exploration is needed to gain a complete overview of the role of Cit in the control of nitrogen homeostasis.

Keywords Citrulline · Amino acids · Protein turnover · Muscle · Liver

For many years, citrulline (Cit) (Fig. 1), a neutral amino acid, was considered one of the amino acids that are only metabolic intermediates of little biological interest, although it appeared to play an important role in the plant world. Its name comes from watermelon (*citrullus vulgaris*), from which it was first identified in the 1930s (Curis et al. 2005). As Cit has no tRNA, it is not used for protein synthesis, but it can be found in proteins as it is a target for post-translational modifications (Curis et al. 2005): arginine in proteins can be catalyzed by arginine deiminase into Cit and this modifies protein function (a regulatory property that is highly important in histone metabolism) (Thompson and Fast 2006). The fact that this amino acid does not enter into protein composition explains why it was long considered just a simple intermediate of the urea cycle. However, Cit has recently garnered a lot of interest, with the discovery of its antioxidant effect (Akashi et al. 2001) leading to studies demonstrating many pleiotropic effects: a central role in the cardiovascular system (Romero et al. 2006), as regulator of immunity (Norris et al. 1995), and a key role in the regulation of nitrogen homeostasis (Osowska et al. 2004, 2006).

Metabolic role of CIT

Urea cycle

The urea cycle detoxifies ammonia, metabolizing it into urea. In this cycle, Cit is a mere intermediary. In the mitochondria of hepatocytes, Cit is synthesized from ornithine and carbamoyl phosphate (CP) by ornithine carbamoyl transferase (OCT). The CP is itself synthesized by CP synthase (CPS) I from ammonia and bicarbonate. This step is possible only in presence of an allosteric inductor, *N*-acetyl-glutamate (NAG) which allows a modification of conformation of the enzyme (Geng et al. 2011). CP is the limiting enzyme of Cit formation because OCT activity is 30–40 times more important than CPS I activity. OCT

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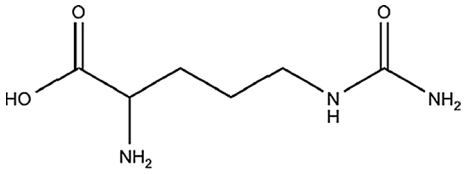

Fig. 1 Developed formula of L-Cit [From (Curis et al. 2005)]. Cit ($C_6H_{13}N_3O_3$) is an α-amino acid with a molecular weight of 175.19 g/mol. Because of its asymmetric carbon, Cit presents two enantiomers. In nature, it is found under the L form. At ambient temperature and pressure, Cit is a white crystalline powder with a melting point at 222 °C. Cit is well soluble in water but less in ethanol due to its polar lateral chain (Curis et al. 2005)

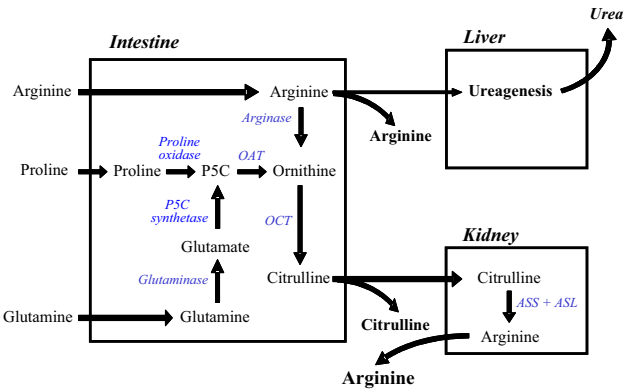

Fig. 2 Interorgan arginine–ornithine–Cit metabolism [Adapted from (Cynober et al. 2010; Wu and Morris 1998)]. *P5C* L-Δ^1-Pyrroline-5-carboxylate, *OAT* ornithine aminotransferase, *OCT* ornithine carbamoyltransferase, *ASS* argininosuccinate synthetase, *ASL* argininosuccinate lyase

deficiency, the enzyme synthesizing Cit, leads, most of the time, to hypocitrullinemia, hyperammonemia and higher glutamine, alanine and lysine plasma concentrations, and sometimes leading to coma and death.

Once synthesized, Cit is transported from the mitochondria to the cytosol. As the mitochondrial transporters for ornithine (in) and Cit (out), OCT and CPS are fully channelized, any Cit formed in the mitochondria is immediately re-used (Meijer et al. 1990). Cit, under the action of argininosuccinate (AS) synthase (ASS), binds to aspartate to give AS. In turn, AS is transformed into arginine, with catalysis by AS lyase (ASL) and releasing fumarate. Arginine is metabolized into ornithine by arginase, releasing a urea molecule. The ornithine formed by arginase gets into the mitochondria to be metabolized by OCT, and so on. The urea molecule leaves the liver and is captured by the kidney to be eliminated (Cynober 2013a).

It is important to note that the fact that Cit formed in the mitochondria is immediately re-used means that hepatic metabolism of Cit is in a closed circuit without any exchange with extra-hepatic metabolism of Cit, at least in the physiological state, as the picture can look different in certain pathological situations: Van de Poll et al. (2007a, b) have reported net uptake and release of Cit by the liver in patients undergoing upper gastrointestinal surgery and in patients bearing colorectal metastases in the liver. However, despite the interest of their work, the results must be taken with great caution, as the fact that these patients exhibit hepatic metastases makes it impossible to determine whether Cit was taken up by the liver or by tumor tissue (which is known to be able to metabolize Cit in some cases) (Selamnia et al. 1998; Delage et al. 2010) or even whether the tumor cells showed modified transporter expression in hepatocytes.

Nitrogen homeostasis

The importance of Cit in nitrogen homeostasis was demonstrated as far back as 30 years ago when Windmueller and Spaeth (1981) showed that Cit is released by the intestine. Circulating Cit comes mainly from endogenous synthesis from intestinal arginine and glutamine metabolism (Windmueller and Spaeth 1981; Blachier et al. 1991), but there is still controversy (Ligthart-Melis and Deutz 2011; Marini 2012; Marini et al. 2010) over which of these two amino acids is the main precursor. In enterocytes, a fraction of dietary arginine [approximatively 40 % (Castillo et al. 1993)] is metabolized by arginase into ornithine and a fraction of dietary and portal blood glutamine is catabolized by glutaminase and ornithine aminotransferase (OAT) into ornithine. The ornithine produced from these two amino acids is metabolized into Cit by OCT (Curis et al. 2005; Wu et al. 1994). Ornithine may also be a direct Cit precursor taken up from portal blood, especially when arginine availability is low (Marini et al. 2010, 2011). Moreover, Wu (1997), in a pioneering paper, showed that proline can also be a precursor of Cit in the small intestine in enterocytes of postnatal pigs. This latter has been confirmed in humans, in preterm neonates and healthy adults (Tomlinson et al. 2011a, b). Proline is metabolized in P5C (Δ1-L-Pyrroline-5-Carboxylate) then in ornithine by proline oxidase and OAT (Wu and Morris 1998) (Fig. 2).

Once that Cit is synthesized from ornithine by enterocytes, this latest is released in the portal vein where it escapes uptake by the liver but is then largely taken up by the kidneys (accounting for around 83 % of gut-released Cit, and 35 % of circulating Cit) (Windmueller and Spaeth 1981).

Once in the kidney, practically 100 % of Cit is metabolized by ASS and ASL to give arginine. Indeed, renal Cit uptake is tightly correlated to arginine release (Tizianello et al. 1980). This pathway is an important source of endogenous arginine, since it represents up to 15 % of circulating arginine and 60 % of synthesized arginine (Curis et al.

2005) and plays a role in maintaining arginine homeostasis (Lassala et al. 2009; Wu et al. 1997).

It is important to note that this arginine/glutamine-Cit-arginine cycle evolves with the age [For details about differences in development states, see the review of Wu and Morris (1998)].

Moreover, this arginine/glutamine–ornithine–Cit cycle is equally central to maintaining nitrogen homeostasis. Cit production from dietary arginine and glutamine, therefore, stems the flow of these two amino acids into portal blood to be taken up by the liver for urea formation, thus preventing the over-activation of ureagenesis that would lead to an inappropriate increase in amino acid catabolism (De Bandt et al. 1995) and thus a decrease in plasma amino acid concentrations that would blunt protein synthesis. As can be expected, Cit production also helps to maintain nitrogen homeostasis in low-protein diet settings. Indeed, plasma Cit concentration is increased during arginine-free or low-protein diets, almost certainly to limit the decrease in plasma arginine concentration observed in these situations (Castillo et al. 1993; Ventura et al. 2011). Recent studies point to this key role of the intestine in the control of liver ureagenesis. For example, a low-protein diet in rats is accompanied by an upregulation of OAT gene expression in the ileal mucosa (Ventura et al. 2011) that seems to be a direct effect of amino acid provision: in vitro, OAT gene expression in CACO-2 cells increases when the culture medium contains low amino acid concentrations (Ventura et al. 2011).

Therefore, Cit production from arginine and glutamine helps to maintain nitrogen homeostasis.

These factors explain why any intestinal failure is liable to affect Cit production and thus nitrogen homeostasis. Hypocitrullinaemia was particularly well demonstrated in a study on short bowel syndrome (SBS) (Crenn et al. 2008) that provided the first evidence for a connection between hypocitrullinaemia and SBS-related nitrogen impairment (Osowska et al. 2004). It was first proposed that, in this setting, arginine (synthesized from intestinal Cit) becomes an essential amino acid (Wakabayashi et al. 1994) and that this lack of a single amino acid should be limiting for protein synthesis. Supporting this hypothesis, Wakabayashi et al. (1994) showed that feeding massively resected (80 % of the small intestine) rats an arginine-free diet leads to weight loss and decreased nitrogen balance. However, arginine supplementation is inappropriate in SBS as arginine is known to induce ureagenesis (Kawamoto et al. 1985) and does not improve nitrogen balance, contrary to Cit as shown by Osowska et al. (2004). In this work (Osowska et al. 2004), rats underwent 80 % resection of the small intestine and gastrostomy to receive nutrition by enteral route, and were supplemented with Cit (1 g/kg/day), arginine (0.994 g/kg/day) or non-essential amino acids (all groups were isonitrogenous and isocaloric −200 kcal/kg/day) for 10 days. To note, dose of 1 g/kg/day of Cit for a rat corresponds to 12 % of its nitrogen supply, so, for a human who weights 75 kg and eats 1 g/kg/day of proteins, it corresponds to approximatively 9 g/day. Cit supply led to higher plasma Cit and arginine concentrations compared to arginine supply, but more remarkable was the fact that Cit supply also restored nitrogen balance from day 5 vs. controls and from day 7 vs. the arginine group, whereas arginine supply was unable to restore nitrogen balance.

To evaluate the effects of route of administration of these amino acids on nitrogen balance in SBS, the same team led a second study (Osowska et al. 2008) with strictly the same protocol except for route of administration (parenteral route instead of enteral route). As in the first study, Cit supply led to a better nitrogen balance compared to arginine supply. Interestingly, EDL (extensor digitorum longus) weight was higher in the Cit group than in the arginine group and controls.

Finally, in critically ill conditions with intestinal failure characterized by low plasma Cit concentration [see (Cynober 2013b) for a recent review], glutamate, glutamine or arginine supply cannot reverse the decrease of plasma Cit concentration (Boutry et al. 2012; Wijnands et al. 2012) while Cit administration increases citrullinemia, improves intestinal microcirculation and blunts the inflammatory response to stress (Wijnands et al. 2012).

Despite these encouraging results in animals, human studies are mandatory to confirm the interest of Cit supplementation to improve nitrogen homeostasis in intestinal failure.

Many works have underlined the metabolic superiority of Cit compared to arginine under some conditions, which are not limited to protein metabolism, either because Cit increases argininemia in a better extent than arginine itself, or because Cit has its own metabolic properties. Cit could be interesting in pregnancy-associated problems (Lassala et al. 2009), in cardiovascular diseases (Romero et al. 2006; Wu and Meininger 2000), in obesity and/or diabetes (Jobgen et al. 2006; Wu et al. 2007), in pathologies characterized by intestinal failure, like sepsis and SBS (see above) (Elwafi et al. 2012; Osowska et al. 2004; Wijnands et al. 2012) and in critically ill patients (Hao et al. 2004).

But all these data are preliminary results and need to be confirmed. Moreover, potential efficiency of Cit supplementation in other pathophysiological situations needs to be studied.

Consequences on muscle

Cit could find promising applications in the elderly, which is another population characterized by an impairment of nitrogen homeostasis tied to the splanchnic area (Osowska

et al. 2006). The first study to investigate the effect of Cit on nitrogen homeostasis in aging was conducted in a model of aged undernourished rats by restricting food to 50 % of spontaneous food intake for 12 weeks and then refeeding (at 90 % of spontaneous food intake) for a week in tandem with Cit supplementation at 5 g/kg/day or with an isocaloric and isonitrogenous standard diet. Protein synthesis in the tibialis muscle increased more significantly when refeeding included Cit supplementation. This increase in protein synthesis was accompanied by an increase in protein content in the same muscle. Moreover, using same model, it was recently shown (Faure et al. 2012) that the Cit-mediated increase in muscle protein synthesis is associated to increased muscle mass, strength and motor activity.

These effects of Cit on muscle protein synthesis were also found after a lower-dose Cit supplementation (1 g/kg/day) for 3 months in old healthy rats (Moinard et al. 2009), which suggests that Cit also has long-term effects. This could be crucial, since leucine, which is known as the major regulator of protein synthesis among amino acids (Garlick 2005), is potent on a short-term basis (Rieu et al. 2003) but not in the long-term (Verhoeven et al. 2009).

Results of human studies on Cit's effects on muscle mass in aging (Melchior 2014; Aussel 2014) are eagerly awaited to confirm the results of these pre-clinic studies.

The concept that Cit may be an important modulator of muscle protein synthesis was thus extended to other situations such as in a model of short fasting (Le Plénier et al. 2012b). Cit supplied as a bolus at 1.8 g/kg to rats submitted to a short fast (18 h, which triggers a twofold decrease in muscle protein synthesis) was able to restore muscle protein synthesis rates. Ventura et al. (2013) confirmed the ability of Cit to modulate muscle function in adult rats. Cit administration (1 g/kg/d) to female rats during food-restricted period (60 % of spontaneous intake for 2 weeks) preserved muscle protein synthesis rate at the same level as controls. Furthermore, the preservation of muscle protein synthesis was also associated with improved muscle strength via an action on contractile function.

Interestingly, in aged food-restricted rats, the Cit-induced improvement of muscle fiber contraction is accompanied by an increase proportion of proteins involved in muscle contraction increasing the expression of key components of myofibrils, including different isoforms of actin and myosin, while decreasing the expression of proteins involved in the depolymerization of actin filaments (Faure et al. 2013). These results are consistent with those of Ventura et al. (2013) in female rats submitted to a low-calorie diet (see above), where increased protein synthesis and muscle strength compared to food-restricted group not receiving Cit were associated to increased myofibrillar protein synthesis with no modification of sarcoplasmic protein synthesis.

Recent data showed that these effects of Cit on muscle function could be due to direct activation of mTORC1 in muscle (Le Plénier et al. 2012b), which was confirmed by two in vitro studies (Goron et al. 2013; Le Plénier et al. 2012a) in which Cit activated phosphorylation of mTORC1 pathway downstream molecules, i.e. Thr 389 S6K1 and Ser 65 4E-BP1 in cultured myotubes. In the same way, Cit is able to preserve protein synthesis in myotubes in catabolic conditions (Ham et al. 2013). To the best of our knowledge, it is unclear if Cit has a direct effect or if it acts though its metabolites in the cells.

Surprisingly, data of Cit's effect on protein synthesis begin to become consistent, but there are only very little data on the effects of this amino acid on proteolysis. At the whole body, Cit does not seem to have any effect on proteolysis. Indeed, in a study on healthy subjects on a weight-maintaining low-protein diet for 3 days, ingestion of Cit had no significant effect on phenylalanine and tyrosine flux when compared with NEAA group; indicating that citrulline did not alter whole-body protein turnover (Jourdan et al. 2014). In the same idea, in a study by Thibault et al. (2011), they failed to have any effects of Cit on leucine oxidation, reflect of proteolysis. In this work, healthy volunteers received Cit at 0.18 g/kg/d for 7 days and were then measured for proteolysis.

In a model of isolated perifused muscle, Cit addition in the medium did not lead to tyrosine flux changes (tyrosine is a good reflect of muscle catabolism) (Moinard et al. 2006). This is in accordance with the results obtained in the first study to investigate the effect of Cit on nitrogen homeostasis in aging with aged food-restricted rats (Osowska et al. 2006), where the 3-MH-to-creatinine ratio, the reflect of myofibrillar proteolysis was unaffected by Cit supplementation. Surprisingly, in the same model, Cit supplementation leads to a decrease in protein carbonylation in the *tibialis anterior* muscle and it is well known that protein oxydation is associated with proteolysis (Shang and Taylor 2011). This is consistent with the fact that, in the same study (Faure et al. 2013), Cit decreased the expression of a proteolysis activator protein (subunit 1 of the proteasome activator complex), resulting in a decrease in muscle protein catabolism. The apparent discrepancy could be related to the sensitivity of the parameters measured and this confirms that relationship between Cit and proteolysis remains to be elucidated.

Finally, concerning the effect of Cit on amino acid oxidation, to the best of our knowledge, there is no data available.

Part of the effects of Cit could be related to its antioxidant properties, since oxidative stress, inflammation and nitrogen homeostasis are tightly connected through NFκB activation and the resulting proinflammatory gene expression. Some vegetables are particularly resistant to stress

situations characterized by a high production of reactive oxygen species (ROS). One example is watermelon which grows in desert conditions and is particularly drought resistant and also contains a high quantity of Cit. The antioxidant power of Cit has been evaluated, and it has been demonstrated that Cit is a potent scavenger that protects DNA and enzymes from ROS attacks, especially °OH (constant rate for the reaction between hydroxyl radicals and citrulline was 3.9×10^9 M^{-1} s^{-1}, which is closed to those of glutathione 8.0×10^9 $M^{-1}.s^{-1}$ and ascorbic acid 7.2×10^9 M^{-1} s^{-1}, whereas it was limited to 5.4×10^8 M^{-1} s^{-1} for proline) (Akashi et al. 2001; Matsugo et al. 1995). More recently, the data found in animal model suggest that Cit has beneficial effects on oxidative stress, even if it is only indirect evidence. Indeed, as previously mentioned, Cit blunts protein carbonylation (Faure et al. 2013) which is a consequence of oxidative stress (Suzuki et al. 2010), in aged food-restricted rats. Protein carbonylation is an alteration of proteins that disrupts and ultimately disables the ability of muscle fibers to contract (Curtis et al. 2012). The decrease in protein carbonylation was associated to an improvement of muscle fiber contraction. Similar results were observed in brain: Cit supplementation in aged rats was able to reduce protein carbonylation and TBARS (end product of lipid peroxidation) production (Moinard et al. 2007). This protective effect against lipid oxidation has been also observed in plasma (Bonnefont-rousselot et al. 2010). If the antioxidant properties of Cit have been well characterized in different situations, both in vitro and in vivo, the precise mechanisms remain to be determined.

Finally, the action of Cit in aging conditions could result from fat mass–lean mass interaction in the muscle. Aging is characterized by fat mass infiltration within the muscle leading to a low-grade inflammation (Zoico et al. 2010) that accelerates the decrease in lean mass (Rieu et al. 2009). The administration of Cit (Moinard et al. 2009) or watermelon juice (Wu et al. 2007) has been shown to decrease fat mass with a direct effect on muscle lipid trafficking (Joffin et al. 2014).

Cit and protein homeostasis in humans: does it work?

Several studies have been led to assess the pharmacokinetics and tolerance of Cit in healthy humans (Moinard et al. 2008; Schwedhelm et al. 2008). The first study (Schwedhelm et al. 2008), using doses of 1.5–6 g/d, found that Cit is well tolerated and increases plasma argininaemia more than arginine itself, as is the case in rats (see above). The second study (Moinard et al. 2008), using twofold higher doses (2–15 g/d), found that the maximum concentration and area under the curve of Cit concentration increase with the Cit dose ingested. Moreover, this study clearly showed that Cit is well tolerated (no side effects), and did not induce gastrointestinal disorders at high dose (i.e. 15 g), which is surprising because a bolus (10 g) of related amino acids like arginine or ornithine usually causes diarrhea due to rapid saturation of the intestinal absorption of these amino acids (Grimble 2007). This difference suggests that intestinal absorption of Cit is not a limiting step, even at high Cit loads (i.e. 10 g). Unfortunately, very little is known about the Cit transport in enterocytes. Only one study has been performed on a culture cell model of enterocytes (Caco-2/TC7) and has concluded that the main transport systems for Cit in enterocytes seem to be systems $B^{0,+}$, L and $b^{0,+}$, with comparable activities (Bahri et al. 2008). The study by Moinard et al. (2008) has also shown that Cit supply leads to an increase in nitrogen balance. A similar result was also observed by Rougé et al. (2007) who showed that Cit supplementation at 0.18 g/kg/d, i.e. 12.5 g/d, split into 5 doses over 1 day results in an increase in nitrogen balance in healthy humans. However, these results cannot determine whether Cit increases protein synthesis, decreases proteolysis, or both. In another study (Jourdan et al. 2014), 10 healthy adult subjects were fed a low-protein diet (8 % of total intake) for 3 days and then received either Cit (10 g) or a mixture of isonitrogenous non-essential amino acids in a bolus every 30 min for 8 h. Ingestion of Cit resulted in a selective increase in mixed muscle protein synthesis in this low-protein intake situation.

The Cit effect appears muscle specific: whole-body protein synthesis is never improved, while muscle protein synthesis, when it is measured, is increased. Indeed, the study by Jourdan et al. (2014), described above, found an increased of muscle protein synthesis without difference in whole-body protein synthesis. Another study (see above) (Thibault et al. 2011) is in accordance with this idea: it failed to find any effect of Cit on whole-body protein synthesis. Nitrogen balance and whole-body protein synthesis were measured but Cit had no effect on these parameters. Unfortunately, muscle protein synthesis was not measured in this study. Third, in rats, Cit, compared to non-essential amino acids, activated muscle protein synthesis but not liver protein synthesis (Osowska et al. 2006), which explains why whole-body protein synthesis remained unchanged.

Conclusion

Cit plays a key role in nitrogen homeostasis. Recent studies have given us a clear picture of the mainspring of Cit. On one side, Cit curbs inappropriate activation of the urea cycle by arginine and glutamine. On the other, Cit is not taken up by the liver and so is available for muscles where it stimulates protein synthesis to increase protein content

and improve functionality. Cit supplementation could be promising in catabolic states as a way to limit the muscle wasting often associated with these situations, but human studies are needed to validate these encouraging data.

Conflict of interest C. Breuillard, L. Cynober and C. Moinard are shareholders of Citrage Company®.

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