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REVIEW

Carnosinase, diabetes mellitus and the potential relevance of carnosinase deficiency

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Abstract Carnosinase (CN1) is a dipeptidase, encoded by the *CNDP1 gene*, that degrades histidine-containing dipeptides, such as carnosine, anserine and homocarnosine. Loss of CN1 function (also called carnosinase deficiency or aminoacyl-histidine dipeptidase deficiency) has been reported in a small number of patients with highly elevated blood carnosine concentrations, denoted carnosinaemia; it is unclear whether the variety of clinical symptoms in these individuals is causally related to carnosinase deficiency. Reduced CN1 function should increase serum carnosine concentrations but the genetic basis of carnosinaemia has not been formally confirmed to be due to *CNDP1* mutations. A *CNDP1* polymorphism associated with low CN1 activity correlates with significantly reduced risk for diabetic nephropathy, especially in women with type 2 diabetes, and may slow progression of chronic kidney disease in children with glomerulonephritis. Studies in rodents demonstrate antiproteinuric and vasculoprotective effects of carnosine, the precise molecular mechanisms, however, are still incompletely understood. Thus, carnosinemia due to CN1 deficiency may be a non-disease; in contrast, carnosine may potentially protect against long-term sequelae of reactive metabolites accumulating, e.g. in diabetes and chronic renal failure.

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In 1900, Vladimir Gulewitsch identified carnosine (Gulewitsch and Amiradžibi 1900; Gulewitsch 1905). Carnosine (ß-alanyl-L-histidine), anserine (ß-alanyl-3-methyl-L-histidine) and homocarnosine (γ-aminobutyric acid-L-histidine) belong to the group of histidine-containing dipeptides and meanwhile, at least in experimental settings, it has been shown that these dipeptides exert a variety of protective functions (Boldyrev et al. 2013). Carnosine inhibits glycation (Alhamdani et al. 2007) and acts as ACE inhibitor (Hou et al. 2003; Nakagawa et al. 2006). Its function as antioxidant (Decker et al. 2000; Mozdan et al. 2005; Velez et al. 2008; Hipkiss 2011; Babizhayev et al. 2013) and its capacity to scavenge carbonyls (Negre-Salvayre et al. 2008; Barski et al. 2013; Brings et al. 2017), however, is debated. In vitro, anserine also scavenges carbonyls, may act as an antioxidant (Kohen et al. 1988; Aldini et al. 2005), and in rodents reduces renal sympathetic nerve activity and blood pressure (Tanida et al. 2010). Homocarnosine is a brain-specific dipeptide (Bauer 2005) and has been suggested as a precursor for the neurotransmitter γ-aminobutyric acid (GABA) and also acts as an antioxidant, free radical scavenger, and metal-chelating agent especially for copper(II) and zinc(II) (Grasso et al. 2014). The formation of carnosine and homocarnosine is catalyzed by carnosine synthase (CS; EC 6.3.2.11). CS belongs to the ATP-grasp family of ligases and the gene (*ATPGD1*) is mainly present in skeletal and heart muscle and certain brain regions (Drozak et al. 2010), but also in kidney (Peters et al. 2015a). The olfactory neurons display very high CS expression, which is in agreement with its very high carnosine content (Margolis et al. 1987). In other brain regions, however, homocarnosine rather than carnosine is the main dipeptide. Recombinant mouse and human CS catalyzes the ATP-dependent synthesis of carnosine and, with a lower affinity, homocarnosine and other related dipeptides (Drozak et al. 2010). The enzyme is localized in the cytosol (Ng and Marshall 1978; Harding and O'Fallon 1979). Little is

known about the regulation of CS expression and activity; a number of highly conserved cysteine residues indicates redox regulation (Drozak et al. 2010). The formation of anserine is more likely achieved through N-methylation of carnosine rather than enzymatic condensation of ß-alanine with N-methylhistidine (Bauer and Schulz 1994; Drozak et al. 2010). CS activity in diabetes and potential benefits of increased CS activity, e.g. under diabetic conditions, have not yet been studied.

Carnosinases

The carnosine-degrading enzyme carnosinase was first described and partially purified from porcine kidney (Hanson and Smith 1949) and in 1973, two electrophoretic forms of carnosinase in normal tissue extracts were identified, only one of which was lacking in a patient with carnosinaemia (Murphey et al. 1973). Lenny et al. confirmed two different metal-dependent porcine carnosinases, denoted homocarnosinase and carnosinase, with distinct differences in their substrate specificity (Lenney 1976; Lenney et al. 1977). In 1982, human serum carnosinase (EC 3.4.13.20) was isolated from human plasma (Lenney et al. 1983) and in 2003, two human carnosinase genes denoted CN1 (now *CNDP1*) and CN2 (now *CNDP2*) were characterized (Teufel et al. 2003). The term "carnosinase" is predominantly used for the enzyme also known as "serum carnosinase" or CN1 encoded by *CNDP1*. CN1 has a narrow substrate spectrum for histidine-containing dipeptides, such as carnosine, anserine and homocarnosine. In contrast, CN2 is a cytosolic nonspecific dipeptidase (EC 3.4.13.18) with a broader specificity of substrates, previously named prolinase (Lenney 1990). Both enzymes are members of the M20 family of metalloproteases (Teufel et al. 2003) and show 53% sequence identity in humans. *CNDP1* and *CNDP2* are located immediately adjacent on human chromosome 18q22.3 in a head-to-tail position. Although structurally related, CN1 and CN2 have quite different properties.

CN1

CN1 (EC 3.4.13.20) is the only dipeptidase with substrate specificity for carnosine (Fig. 1), anserine and homocarnosine. The pH activity curve of CN1 enzyme shows a broad maximum between pH 7.5 and 8.5. In humans, the CN1 gene *CNDP1* is expressed in the central nervous system, the liver (Teufel et al. 2003) and kidney (Peters et al. 2015a). Rat and mouse orthologues of human carnosinase are found in the kidney but are not expressed in the CNS, supporting previous results describing a homocarnosine-splitting enzyme activity in the kidney of these animals (Margolis et al. 1983; Teufel et al. 2003). In human serum and cerebrospinal fluid (CSF) CN1 activity increases with age (Lenney et al. 1982) and varies greatly between individuals (Peters et al. 2011), with higher activities in females compared to males (Bando et al. 1984). Lower CN1 activity in children is not due to lower CN1 protein concentrations but different allosteric conformations of CN1 in children and adults (Peters et al. 2010; Adelmann et al. 2012). The human enzyme is present as a monomer or dimer (Pavlin et al. 2016). Carnosine is the best substrate for CN1; hydrolysis rates for homocarnosine and anserine in human serum are 50-fold and 200-fold lower than for carnosine (Peters et al. 2011). Homocarnosine and anserine effectively inhibit carnosine degradation by CN1, whereas related compounds such as carcinine, (β-alanylhistamine), do not (Peters et al. 2011). Secretion of CN1 is influenced by a common leucine repeat polymorphism in the signal peptide region of *CNDP1* (exon 2) (Riedl et al. 2007) and by N-glycosylation (Riedl et al. 2010). CN1 has three N-glycosylation sites and its activity increases when all three sites are N-glycosylated (Riedl et al. 2010). Further, CN1 is a metal ion-dependent dipeptidase and its activity can be activated by addition of cadmium at low concentrations (0.1–3 μM) (Teufel et al. 2003).

Carnosinemia

Carnosinaemia as an inherited metabolic disease was first described in 1967 and confirmed to be due to serum carnosinase deficiency in 1968 (Perry et al. 1967; Perry et al. 1968; Jakobs et al. 1993; Scriver 2001). Since then several patients with this biochemical phenotype and a range of clinical symptoms have been reported (Fig. 2). In addition, reduced CN1 activity in serum may also cause a condition called homocarnosinosis with elevated concentrations of homocarnosine in cerebral spinal fluid (Gjessing and Sjaastad 1974; Sjaastad et al. 1976). Clinical features associated with serum carnosinase deficiency and carnosinaemia/homocarnosinaemia are highly variable (Bando et al. 1984, 1986; Duane and Peters 1988; Schoen et al. 2003; Balion et al. 2007), and a review of 23 patients in 1985 found no correlation between type and severity of neurological symptoms and residual serum CN1 activity (Cohen et al. 1985). This suggested that carnosinaemia may be an incidental finding of metabolic studies in children with symptoms with a different, independent cause (Cohen et al. 1985). The conclusion is indirectly supported by the report of a *CNDP1* frameshift null mutation c.48_49insTGCTG (p.Leu17fs*20, rs532358622) with a carrier frequency of 1–1.4% in Europeans (Zschocke et al. 2006). Homozygosity for this mutation, and consequently complete absence of CN1 function, is expected to have a prevalence of 1:20.000–1:40.000, and if harmful should have been found in a much higher number of individuals.

Dietary intake of carnosine usually results in rapid degradation upon absorption, yet this was assumed to be less

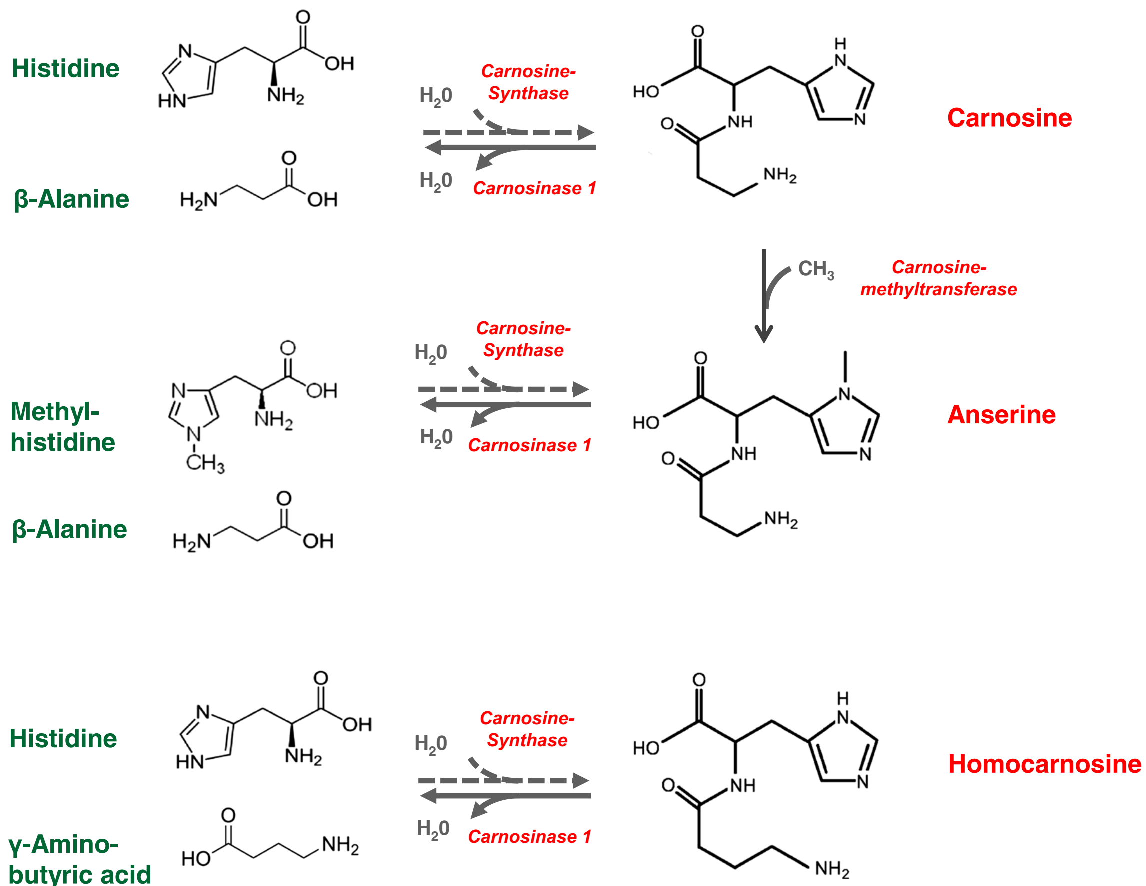

Fig. 1 Carnosine, anserine and homocarnosine pathway. CN1 degrades histidine-containing dipeptides, such as carnosine, anserine and homocarnosine. The formation of anserine by methylation is more likely than the enzymatic condensation of ß-alanine with N-methylhistidine by carnosine-synthase

pronounced in subjects with low CN1 activity (Everaert et al. 2012). However, the half-life of carnosine in the human circulation is minutes only, even in subjects with low CN1 activity/content (Baguet et al. 2014). In patients with liver cirrhosis, serum CN1 abundance and activity was found to be more than 10-fold lower than in healthy controls (Peters et al. 2011), most likely caused by reduced hepatic expression of CN1, but plasma carnosine levels were not increased in these patients (Peters et al. 2011).

CNDP1 and the risk of diabetic nephropathy

Susceptibility to diabetic nephropathy is strongly associated with a leucine repeat polymorphism in the signal peptide region of *CNDP1*. Homozygosity for five leucine repeats, the shortest allelic variant (denoted "Mannheim allele") is more common in patients with type 2 diabetes without nephropathy and associated with lower CN1 activities (Janssen et al. 2005). Mooyaart et al. showed that the effect may be gender-specific, with a stronger protective effect of the homozygous 5–5 genotype in women than in men (Mooyaart et al. 2010). This finding was recently reconfirmed in patients with biopsy-proven diabetic nephropathy (Albrecht et al. 2017). The association was also reported in European Americans (Freedman et al. 2007), South Asian Surinamese (Mooyaart et al. 2009) and in North Indians (Yadav et al. 2016) but not in African-Americans (Freedman et al. 2007; McDonough et al. 2009), Japanese (Kurashige et al. 2013) and in a small number of Scandinavian patients (Ahluwalia et al. 2011). In Japanese women with type 2 diabetes, a deep intronic SNP in *CNDP1* was associated with overt proteinuria but not with end-stage renal disease (Kurashige et al. 2013). In the Scandinavian cohort of type 2 diabetic patients, a haplotype comprising three SNPs in the 3′ untranslated region of *CNDP2* and the promotor region of *CNDP1* was associated with DN (Ahluwalia et al. 2011). In type 1 diabetes, a possible role of the *CNDP1* leucine repeat polymorphism is controversial (Bakker et al. 2008; Wanic et al. 2008; Craig et al. 2009; Alkhalaf et al. 2010). In non-diabetic children, the Mannheim allele is associated with slower progression of chronic kidney disease caused by glomerulonephritis but not by tubulointerstitial nephritis (Kiliś-Pstrusińska et al. 2010; Peters et al. 2016). On the other hand, women with the Mannheim allele

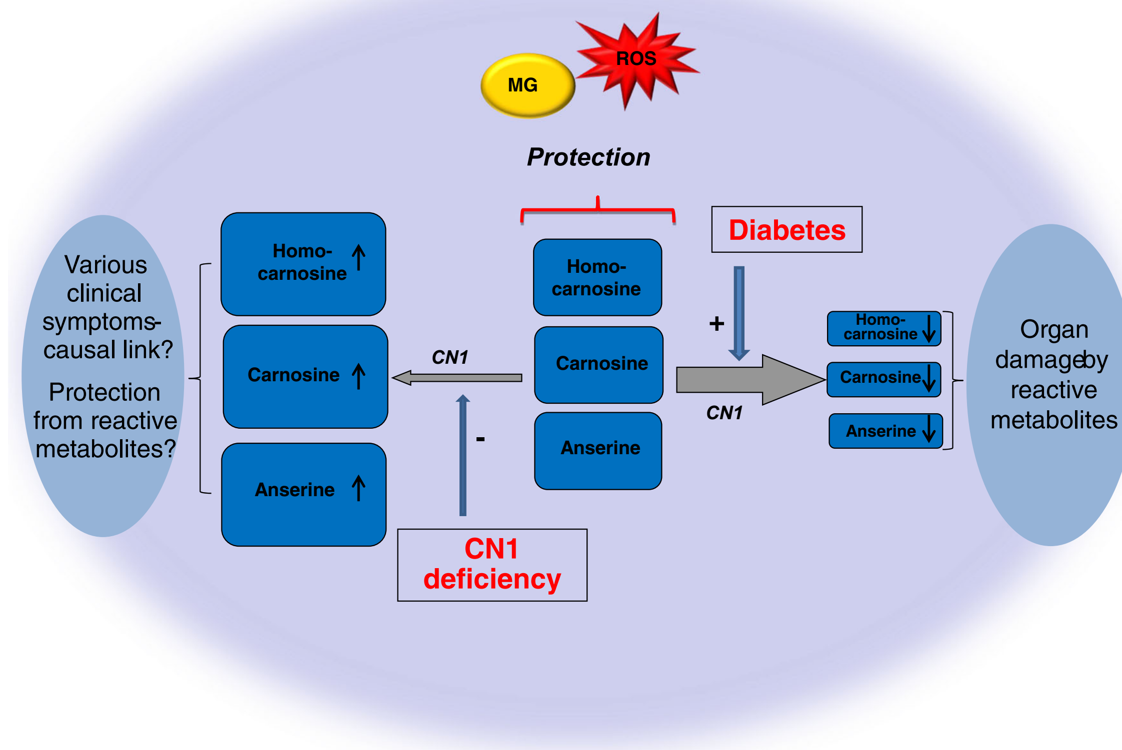

Fig. 2 Carnosine, anserine and homocarnosine have cytoprotective properties, i.e. scavenging carbonyls, such as methylglyoxal (MG), and act as antioxidants by scavenging reactive oxygen species (ROS). Increased CN1 activity under diabetic conditions may lead to organ damage by reactive metabolites due to decreased carnosine, anserine and homocarnosine tissue levels. Whether inborn deficiency of CN1 and subsequent carnosinaemia causes clinical symptoms is uncertain; experimental and clinical association studies suggest protection from reactive metabolites and diabetic nephropathy

(5 L–5 L genotype) were found to have a higher cardiovascular mortality risk (Alkhalaf et al. 2015).

Taken together, several association studies suggest renoprotection from lower CN1 activity in patients with diabetes mellitus and chronic glomerulonephritis. The molecular mechanisms underlying the association between *CNDP1* variants and renoprotection, however, remain to be clarified. The Mannheim allele is not associated with increased plasma carnosine levels (Peters et al. 2011). In diabetic mice, renal CN1 activity is increased due to post-translational modifications (Peters et al. 2015b), and histidine-containing dipeptide concentrations are decreased (Peters et al. 2012). We speculate that the *CNDP1* Mannheim allele may cause reduced CN1 activity and increased carnosine levels within the renal tissue; further work is required, e.g. in renal CN1 knock out models and respective human tissue.

In rodents, no serum CN1 is present and in diabetic mice, carnosine supplementation mitigates DN, reduces renal vasculopathy, normalizes vascular permeability (Peters et al. 2012) and improves wound healing (Ansurudeen et al. 2012) (Fig. 3). In diabetic rats, carnosine treatment prevents apoptosis of glomerular cells (Riedl et al. 2011; Peters et al. 2014), podocyte loss and vascular damage (Pfister et al. 2011). In humans, dietary supply of carnosine does not increase systemic histidine dipeptide concentrations due to rapid degradation by the serum CN1. An alternative approach to increase tissue carnosine concentrations is pharmacological inhibition of CN1 activity. We recently showed that cysteine-compounds inhibit CN1 activity by allosteric interactions through S-cysteinylation (Peters et al. 2017). The homocarnosine concentration in rodent renal tissue is below detection in health and diabetes, thus a role in protection from nephropathy is unlikely.

Under diabetic conditions, renal CN1 activity is increased (Fig. 2) due to post-translational modifications in diabetic mice and humans (Peters et al. 2015b). Reactive metabolites such as methylglyoxal (MG), reactive oxygen species (ROS) and nitrogen oxide (NO), increase CN1 activity by carbonylation and *S*-nitrosylation. Substitution of the two cysteine residues of CN1 by serine revealed the importance of cysteine at position 102 for enzyme activity, whereas cysteine at position 229 is irrelevant for CN1 activity. *S*-Nitrosylation of cysteine residue at position 102, but not at position 229, reduces CN1 activity (Peters et al. 2015b).

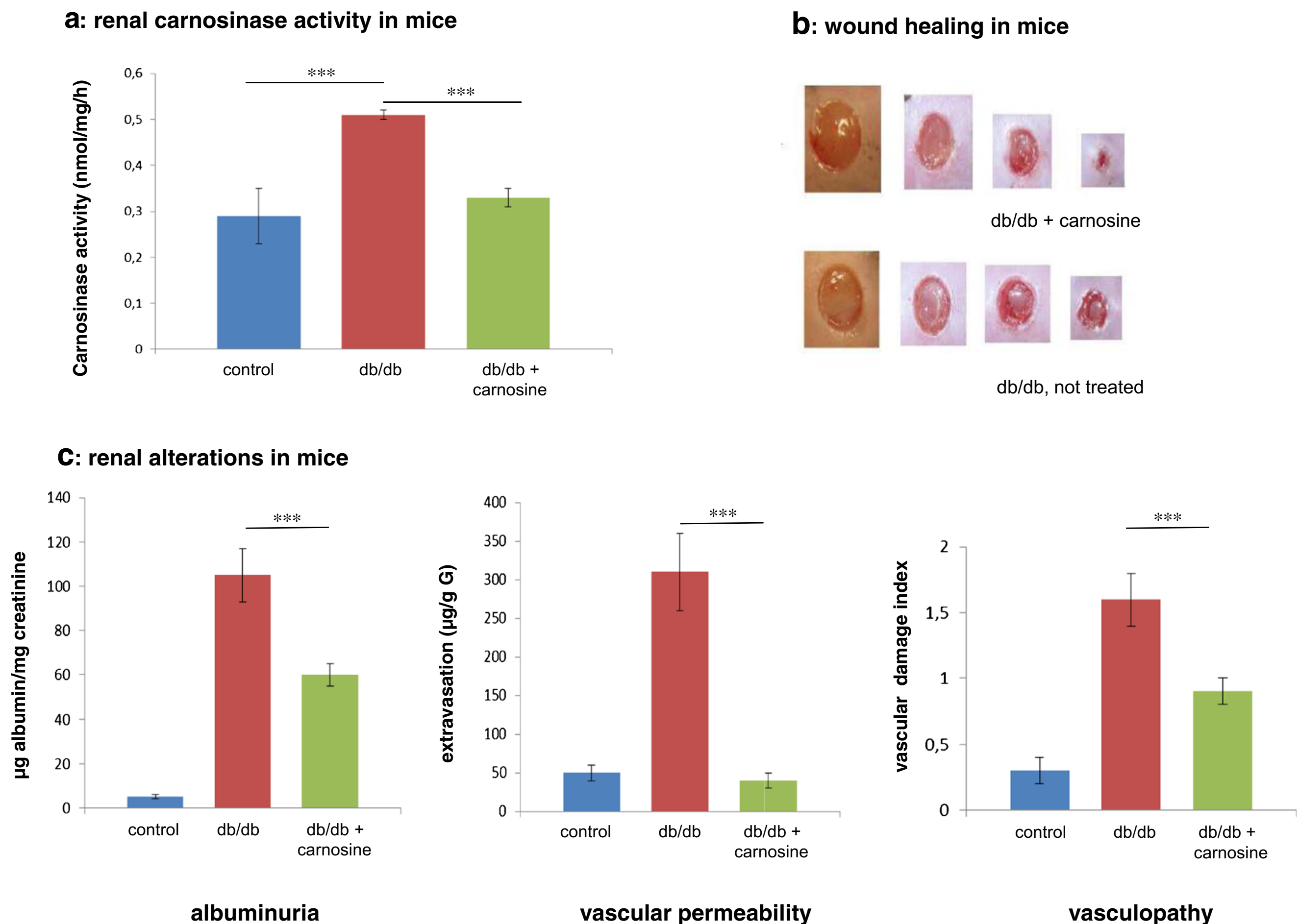

Fig. 3 Feeding of diabetic mice (db/db) with carnosine over 4 weeks (a) normalized increased renal CN1 activity, (b) improved wound healing (Ansurudeen et al. 2012, adapted with permission from publisher) and (c) improved albuminuria, normalized vascular permeability (measured by Evans blue injection intravenously into the tail veins of mice) and reduced vasculopathy (by morphological and stereological evaluation) (Peters et al. 2012). *** = $p < 0.01$

CN2

Whereas CN1 is well characterized, little is known about the regulation and function of CN2. The enzyme is a cytosolic nonspecific dipeptidase (EC 3.4.13.18), and as such not limited to histidine-containing dipeptides. It hydrolyzes carnosine only at alkaline pH with an optimum at pH 9.5 and does not degrade homocarnosine. CN2 RNA and protein are expressed in central and peripheral human tissues and in peripheral blood leukocytes (Lenney et al. 1985; Teufel et al. 2003). A recent crystallographic study of mouse CN2 revealed that each subunit consists of two domains, an A domain with catalytic and metal binding activity, and a B domain for dimerization (Unno et al. 2008). In contrast to CN1, CN2 can be inhibited by bestatin, a compound known to specifically inhibit various amino- and dipeptidases (Suda et al. 1976). Bound to domain A, bestatin interacts with several residues of domain B of the other subunit. These interactions are likely to be essential for enzyme activity (Unno et al. 2008). Furthermore, immunohistochemical staining of the rat hypothalamus with anti-CN2 antibody demonstrated high CN2 expression in histaminergic neurons of the tuberomammillary nucleus, suggesting that it may supply histidine to these neurons for histamine biosynthesis (Otani et al. 2008). Whether CN2 plays a role in carnosine metabolism remains unknown. Jansen et al. (2015) recently reported a different function of CN2, the formation of N-lactoyl-amino acids. N-lactoyl-aminoacids are rapidly formed by reverse proteolysis. The plasma levels of these metabolites strongly correlate with plasma levels of lactate and amino acids. In patients with phenylketonuria (PKU), N-lactoyl-phenylalanine (N-lac-Phe) plasma levels showed a positive correlation with plasma phenylalanine levels (Jansen et al. 2015).

Additional dipeptidases involved in carnosine metabolism

A cytosolic β-alanyl-lysine dipeptidase (PM20D2) activity was described in rodent muscle. This enzyme is an M20

metalloprotease which belongs to the same family as CN1 and CN2. It cannot degrade carnosine or homocarnosine but hydrolyzes β-alanyl-lysine, β-alanyl-ornithine, γ-aminobutyryl-lysine, and γ-aminobutyryl-ornithine. It assists carnosine synthase by degrading abnormal biosynthetic dipeptides and thus protects the physiologically relevant carnosine and homocarnosine from the accumulation of these compounds (Veiga-da-Cunha et al. 2014). In humans, PM20D2 is also present but less active. Another dipeptidase called anserinase is found in fish (EC 3.4.13.5); it also belongs to the M20 metalloprotease family and shows broad substrate specificity which includes carnosine, anserine and homocarnosine. In bacteria, two further carnosine-cleaving enzymes denoted peptidase V and peptidase D (EC 3.4.13.3) are known, for details see (Bellia et al. 2014).

Carnosine-methyltransferase

Anserine can be formed by N-methylation of carnosine in skeletal muscle (McManus 1962). Drozak et al. identified a carnosine N-methyltransferase in chicken named HNMT-like protein (CMT, EC 2.1.1.22), catalyzing the transfer of methylgroup from S-adenosyltransferase (SAM) onto carnosine (Drozak et al. 2013). Amphibian and mammalian genomes do not contain orthologues of the CMT gene, indicating another anserine-producing enzyme in mammals.

Carnosine transporter

Cellular uptake of carnosine and anserine occurs by proton-coupled oligopeptide transporters (H^+)(POTS). These are membrane proteins that translocate various small peptides and peptide-like drugs across the biological membrane via an inwardly-directed proton gradient and negative membrane potential (Daniel and Kottra 2004). At present, four members of the POT family — PEPT1, PEPT2, PHT1 and PHT2 — have been identified in mammals. The impact of carnosine transporter expression and carnosine transport activity on tissue and cellular carnosine homeostasis is largely unknown as are the potential clinical consequences. PEPT1 mediates intestinal absorption of luminal di/tripeptides from dietary protein digestion, while PEPT2 mainly allows for renal tubular reabsorption of di/tripeptides from the glomerular ultrafiltration. PHT2 and PHT1 possibly interact with di/tripeptides and histidine in certain immune cells (Verri et al. 2017). In rats, PHT1 is widely expressed and transports carnosine and histidine in brain and retina of rats (Yamashita et al. 1997). PEPT2 is responsible for the cellular uptake of exogenous carnosine in mice (Kamal et al. 2009) and mediates >90% of cellular carnosine uptake in the choroid plexus (Teuscher et al. 2004).

Conclusion

The biological relevance of carnosine and dipeptide metabolism is only partially understood. Carnosinaemia due to serum carnosinase deficiency has been suggested as an inherited neurometabolic disorder, but there is also evidence to suggest that this condition is clinically irrelevant. In contrast, low CN1 serum activity due to a functional leucine repeat polymorphisms in *CDNP1* is associated with a lower incidence of diabetic nephropathy and possibly a slower progression of chronic renal failure in children with non-diabetic glomerulopathies.

In diabetic mice, increased renal CN1 activity causes decreased histidine-containing dipeptide concentrations. Exogenous carnosine supply in rodents exerts a range of nephroprotective effects such as reductions of proteinuria, renal vasculopathy, and podocyte loss. Carnosine therefore may be a promising therapeutic target in patients with diabetes mellitus. While potential benefits of oral carnosine supplementation in humans are counteracted by a high serum CN1 activity, inhibition of CN1 or upregulation of CS activity might protect diabetic patients from damage exerted by hyperglycemia and accumulated reactive metabolites.

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Compliance with ethical standards This article is a review of previously published work and does not present any new previously unpublished studies with human or animal subjects performed by the any of the authors.

Animal rights The article does not contain animal subjects.

Conflict of interest V. Peters, J. Zschocke and C. P. Schmitt declare that they have no conflict of interest.

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