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## Accepted Manuscript

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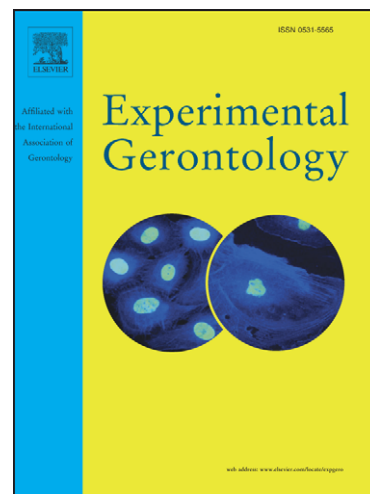
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**On the enigma of carnosine's anti-ageing actions**

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**Abstract**

Carnosine ( $\beta$ -alanyl-L-histidine) has been described as a forgotten and enigmatic dipeptide. Carnosine's enigma is particularly exemplified by its apparent anti-ageing actions; it suppresses cultured human fibroblast senescence and delays ageing in senescence-accelerated mice and *Drosophila*, but the mechanisms responsible remain uncertain. In addition to carnosine's well-documented anti-oxidant, anti-glycating, aldehyde-scavenging and toxic metal-ion chelating properties, its ability to influence the metabolism of altered polypeptides, whose accumulation characterises the senescent phenotype, should also be considered. When added to cultured cells, carnosine was found in a recent study to suppress phosphorylation of the translational initiation factor eIF4E resulting in decreased translation frequency of certain mRNA species. Mutations in the gene coding for eIF4E in nematodes extend organism lifespan, hence carnosine's anti-ageing effects may be a consequence of decreased error-protein synthesis which in turn lowers formation of protein carbonyls and increases protease availability for degradation of polypeptides altered postsynthetically. Other studies have revealed carnosine-induced upregulation of stress protein expression and nitric oxide synthesis, both of which may stimulate proteasomal elimination of altered proteins. Some anti-convulsants can enhance nematode longevity and suppress the effects of a protein repair defect in mice, and as carnosine exerts anti-convulsant effects in rodents, it is speculated that the dipeptide may participate in the repair of protein isoaspartyl groups. These new observations only add to the enigma of carnosine's real *in vivo* functions. More experimentation is clearly required.

## 1. Carnosine and ageing.

The naturally-occurring dipeptide carnosine ( $\beta$ -alanyl-L-histidine) has been described as forgotten and enigmatic (Bauer, 2005): the present paper is an attempt to rectify the former opinion, but, however only adds to the latter view. Amongst carnosine's enigmatic properties are its apparent anti-ageing actions; the dipeptide is one of a very few naturally-occurring compounds which suppresses cell senescence, induces rejuvenating effects (McFarland and Holliday, 1994 and 1999), and protects against telomere shortening (Shao et al., 2004) in cultured human fibroblasts. The dipeptide also extends the life-span of senescence-accelerated mice (Yuneva et al., 1999) and *Drosophila* (Yuneva et al., 2002). There are many possible mechanisms by which carnosine exerts these anti-ageing actions however, some of which were summarized a decade ago (Hipkiss, 1998). Meanwhile there have been a number of publications suggesting further possible routes by which carnosine could suppress additional processes associated with ageing at cellular and whole organism levels.

## 2. Carnosine and the metabolism of altered proteins

The most prominent biochemical symptom of ageing and many age-related conditions is proteotoxic stress resulting from the accumulation of altered proteins which arise from biosynthetic errors and/or deleterious post-synthetic polypeptide modifications (Rosenberger, 1992; Hipkiss, 2006a; Schoneich, 2006; Morimoto, 2008). While carnosine might suppress the post-synthetic generation of altered proteins, including anti-oxidant activity, toxic metal-ion chelation, anti-glycating and aldehyde/carbonyl-binding activities (Hipkiss, 1998), there is no unequivocal proof that any of these proposals can adequately account for all carnosine's effects on model ageing systems.

## 2.1 Carnosine, slowing protein synthesis and ageing

A number of recent studies have shown that ageing can be delayed by partial inhibition of protein synthesis. Studies in *Caenorhabditis elegans* have shown that senescence is delayed, stress resistance enhanced and life-span extended in mutants defective in the mRNA translation initiation factor eIF4E (Pan et al., 2007; Syntichaki et al., 2007). Similarly, *C. elegans* lifespan was extended and stress resistance enhanced when translation was inhibited when synthesis of eleven ribosomal proteins was suppressed using inhibiting-RNAs (RNAi) (Hansen et al., 2007). Furthermore, certain ribosomal protein defects have beneficial effects on yeast longevity (Chiocchetti et al., 2007). While the explanation of these effects is debated (Kaeberlein and Kennedy, 2007), it is likely that the lowered rate of bulk protein synthesis, resulting from the lowered translation initiation frequency, also decreases biosynthesis of erroneous proteins (Hipkiss, 2007a). This lowered production of biosynthetic error-proteins could directly lower the load that the chaperone and proteolytic apparatus must deal with: the chaperone/proteolytic apparatus is responsible for the elimination of altered protein generated both post-synthetically and those formed by biosynthetic errors. It should also be noted that, compared to normal gene products, error-proteins are more readily glycosylated and oxidatively-damaged by ROS (Dukan et al., 2000; Fredriksson et al., 2006) than the normal gene products, thus the mutant organisms would generate fewer protein carbonyls that normally characterize the senescent state (Stadtman, 1992). Consequently the decreased level of biosynthetic error-proteins would not only decrease formation of protein carbonyls, but also increase the relative availability of chaperone and proteolytic activities for the

recognition and elimination of altered proteins arising from deleterious post-synthetic modification (Hipkiss, 2007a).

Interestingly, methionine restriction (40% and 80%) has been shown to delay ageing in rats and mice (Miller et al., 2005; Naudi et al., 2007). Because methionine is the initiating amino acid in protein biosynthesis, this may again indicate that decreased translation initiation is an effective anti-ageing strategy. Therefore methionine restriction could also decrease biosynthetic formation of error-proteins, similar to the effects of the defective eIF4E initiation factor in nematodes outlined above (Hipkiss, 2008a).

A recent paper has revealed that carnosine can also exert suppressive effects on mRNA translation initiation. Son et al. (2008) showed that the dipeptide inhibits interleukin-8 mRNA translation by suppressing phosphorylation of initiation factor eIF4E in peroxide-activated intestinal epithelial cells and Caco-2 cells. Phosphorylation of eIF4E is required for effective mRNA translation, which explains the observed carnosine-mediated decreased synthesis of the pro-inflammatory cytokine. Carnosine was also shown to inhibit phosphorylation of other regulatory proteins ERK1/2 and p38 MAP kinase. Because defective eIF4E limits mRNA translation initiation and results in lifespan extension in *C. elegans* (as described above), it is possible that carnosine's beneficial effects on fibroblast senescence and lifespan are mediated via a similar mechanism in human cells. This should be relatively easy to test.

## **2.2 Carnosine and post-synthetic protein modification**

Carnosine has the potential to inhibit much deleterious post-translational polypeptide modification caused by oxidative, nitroxidative and glycoxidative agents.

There is much evidence, mostly obtained from model systems, suggesting that carnosine can suppress protein modification mediated by reactive oxygen species (ROS) (Kohen et al., 1988; Boldyrev 2005; Alhamdani et al., 2007a & 2007b), reactive nitrogen species (RNS) (Calabrese et al., 2005; Fontana et al., 2002), glycating agents (Hipkiss et al., 1995; Vinson and Howard, 1996; Seidler, 2000) and deleterious aldehydes such as malondialdehyde (Hipkiss et al. 1997 and 1998a and 1998b), hydroxynonenal (Aldini et al., 2002 and 2005), acrolein Carini et al., 2003) and methylglyoxal (MG) (Hipkiss and Chana, 1998). Carnosine also inhibited the cross-linking of MG-modified protein with normal polypeptides (Hipkiss and Chana, 1998), most likely by forming adducts with the MG-induced protein carbonyl groups (Brownson and Hipkiss, 2000).

Recent studies in mice have shown that decreased intake, or formation, of protein-AGEs can make major contributions to lifespan extension in mice (Cai et al., 2007; Uribarri et al., 2007a and 2007b). MG, a spontaneously-generated glycolytic by-product, induces senescence in cultured fibroblasts (Sejersen and Rattan, 2008), and is a primary source of intracellular glycation in both yeast (Gomes et al., 2008) and mice (Morcos et al., 2008). It is likely that MG generation is suppressed by caloric restriction and every-other-day feeding (Hipkiss 2006b and 2008b), both of which delay ageing. Carnosine's ability to react with MG and protect proteins against MG-induced damage may contribute to the dipeptide's apparent anti-ageing activity (Hipkiss, 2007b). The presence of carnosine-aldehyde adducts in muscle and urine (Orioli et al., 2005 & 2007) provides evidence for the occurrence *in vivo* of at least some of carnosine's putative aldehyde-scavenging actions. It should be pointed out that while no protein-carnosine adducts have been detected *in vivo*, NMR data has indicated the presence of carnosine-phosphatidylcholine adducts in human leg muscle,



possibly arising from oxidation of amino lipids and their subsequent reaction with the dipeptide (Schroder et al., 2004).

### 2.3 Carnosine and proteolysis of error-proteins

Carnosine may improve cellular ability to deal with altered proteins.

Decreased intracellular elimination of aberrant polypeptides by proteasomal activity and autophagy can contribute to altered protein accumulation, while upregulation of certain proteolytic functions is associated with increased longevity (Min et al., 2008; Yun et al., 2008; Bonelli et al., 2008; Cuervo 2008; Bergami et al., 2007). There have been three reports indicating that carnosine may stimulate intracellular proteolysis. Hipkiss et al. (1998a) showed that proteolysis was stimulated in carnosine-treated cultured human fibroblasts, and Bharadwaj et al (2002) found that carnosine stimulated degradation of HIF1- $\alpha$  in cardiomyoblasts. It has also been reported that carnosine stimulates neutral, non-lysosomal, proteolysis in cell-free extracts of rat brain (Bonner et al., 1995). Conceivably these effects could occur by the dipeptide stimulating synthesis of nitric oxide (Nicoletti et al., 2007; Tomonaga et al., 2005), which in turn may activate proteasomal-mediated protein degradation (Thomas et al, 2007).

Carnosine has also been shown to stimulate expression of vimentin (Ikeda et al., 1999), a cytoskeletal protein which turns out to be very readily glycosylated by deleterious aldehydes (Kueper et al., 2007). Although the significance of this observation is uncertain, the possibility arises that vimentin alongside carnosine may help to control protein glycation. However it has also been shown in COS cells that the enzyme oxidized protein hydrolase (OPH) is coexpressed with vimentin: as its name states, OPH degrades oxidized polypeptides in collaboration with proteasomes

(Shimizi et al., 2004). It should be possible to test whether carnosine stimulates expression of OPH in cultured fibroblasts or other cells.

#### **2.4 Carnosine and the stress response**

Stress/chaperone proteins participate in the recognition and proteolytic elimination of altered proteins and their upregulation is associated with increased organism longevity and suppression of some age-related diseases (Morimoto, 2008; Calabrese et al., 2008; Erjavec et al., 2007). Dietary restriction, which delays ageing in a variety of organisms and suppresses proteotoxicity, requires the action of a heat-shock transcription factor (Steinkraus et al., 2008). Carnosine-zinc complexes (poleprezinc) stimulate expression of certain stress (heat-shock) proteins (Odashima et al., 2002 and 2006; Ohkawara et al., 2006) which could improve cellular ability to deal with altered proteins and may again contribute to the dipeptide's apparent anti-ageing activity.

Some studies carried out 30-40 years ago showed that hydrocortisone or cortisone (Cristofolo and Kabakjian, 1975; Macieira-Coelho, 1966) have positive effects on the life-span of cultured human fibroblasts. These findings have recently been reactivated where the beneficial effects of glucocorticoids towards cultured human fibroblasts have again been demonstrated (Kletsas et al., 2007). It is possible that carnosine's effects on fibroblast lifespan (McFarland and Holliday, 1994) could be exerted via glucocorticoid upregulation because it has also been shown that intracerebroventricular carnosine administration stimulates corticosterone release in chick brain (Tsuneyoshi et al., 2007).

### **3. Carnosine, anti-convulsant activity and aging**

Studies using the nematode *Caenorhabditis elegans* have shown, somewhat surprisingly, that certain anti-convulsant agents suppress ageing and extend organism lifespan (Evason et al., 2005 and 2008; Kornfeld and Evason, 2006). The mechanism of anticonvulsant anti-ageing action is uncertain but may include histone deacetylase inhibition and/or effects on nematode nervous system (Leng et al., 2008).

Carnosine appears to possess anticonvulsant activity in rats (Wu et al., 2006; Kozan et al., 2008) and mice (Zhu et al., 2007). It is interesting to note that treatment of humans with anticonvulsants gabapentin and topiramate raise levels of homocarnosine, a carnosine homologue, in cerebrospinal fluid (CSF) (Petroff et al., 1998 and 2006), and that, while carnosine is absent from human CSF, the levels of CSF homocarnosine decrease markedly with age (Jansen et al., 2006; Huang et al., 2005).

Anticonvulsants have also been shown to suppress some of the deleterious effects of deficiency of the protein repair enzyme, protein isoaspartate methyl transferase (PIMT), in mouse brain (Kim et al., 1999): PIMT deficiency causes accumulation of aberrant proteins in mouse brain and induces of epileptic seizures. It may be significant that some anticonvulsants, e.g. ethosuximide, which suppress ageing in *C. elegans*, structurally resemble the succinimide intermediate generated following spontaneous asparagine deamidation. During PIMT-mediated repair of protein isoaspartate residues, methylation of the succinimide intermediate occurs. It has recently been shown that, following the methylation reaction, the isoaspartyl isopeptide bond can be selectively cleaved by hydroxylamine (Zhu and Aswad, 2007). The related compound, *N-t*-butylhydroxylamine, appears to suppress senescence of cultured human fibroblasts in a manner somewhat similar to carnosine (Atamna et al., 2000), and it was proposed that *N-t*-butylhydroxylamine and carnosine may mediate

their anti-ageing effects by acting similarly to each other, perhaps as protein carbonyl scavengers (Hipkiss, 2001). However, following the observations of Zhu and Aswad (2007), one speculates that carnosine might participate in protein repair, possibly by also cleaving the isopeptide bonds of protein isoaspartic acid residues, analogous to hydroxylamine. There is another theoretical position where carnosine could be involved in PIMT-mediated protein repair. PIMT catalyses the methylation of the succinyl intermediate forming a methyl ester which then undergoes spontaneous demethylation releasing methanol, the fate of which is likely to be formation of formaldehyde, which carnosine could scavenge.

Evidence for carnosine's potential to participate in asparagine and glutamine deamidation was shown by Kuroda et al., (2000) who detected  $\beta$ -aspartyl-carnosine- and  $\gamma$ -glutamyl-carnosine-adducts, respectively, when free asparagine and free glutamine spontaneously deaminate in the presence of dipeptide. Interestingly whilst  $\gamma$ -glutamyl-carnosine has been detected in muscle preparations,  $\beta$ -aspartyl-carnosine has not (Kuroda and Harada, 2002); perhaps this difference reflects that fact that isoaspartate residues, generated primarily by the spontaneous deamidation of asparagine residues, are repaired by PIMT, whereas there is no corresponding enzyme for the repair of  $\gamma$ -glutamyl peptide bonds formed following the spontaneous deamidation of glutamine residues.

#### **4. Carnosine and other known modulators of ageing: sirtuins, TOR, resveratrol, caloric restriction and aerobic exercise**

Control of ageing appears to be exerted by a variety of mechanisms mostly involving changes to energy supply and/or increased expression of agents which increase an organism's resistance to stress. It is totally unknown whether carnosine

participates in any of the proposed mechanisms or pathways thought to be responsible. A large number of papers have indicated that certain sirtuins, acting as histone/protein deacetylases, exert anti-ageing activity and that they may also be required when ageing is suppressed by caloric restriction (Leibiger and Berggren, 2006; Lin and Guarente, 2003; Longo and Kennedy, 2006). Although totally speculative, there are two theoretical possibilities where carnosine could be involved in protein deacetylation. Sirtuin-mediated protein deacetylation eventually generates acetyl-ADP-ribose as a product (Howitz et al., 2003). ADP-ribose and acetyl-ADP-ribose are powerful glycating agents which have the potential to damage proteins, DNA etc. Carnosine could act as an acetyl acceptor forming N-acetyl-carnosine, which has been isolated from various biological sources. Following loss of the acetyl group from the acetyl-ADP-ribose, the ADP-ribose could be scavenged by carnosine, thereby eliminating random glycation damage. It should be relatively easy to test whether any of these reactions occur by searching for the predicted carnosine adducts. Alternatively it is theoretically possible that N-acetyl-carnosine could behave as an acetyl donor in protein acetylation mediated by histone/protein acetylases.

Inhibition of the target of rifamycin (TOR) appears to extend life-span by stimulating respiration and upregulating mitogenesis (Bonawitz et al., 2007); these changes also occur when ageing is suppressed by dietary restriction and increased aerobic exercise. Again there is no evidence as to whether carnosine is directly involved in TOR signalling or sirtuin activity. It is possible that carnosine might be indirectly involved however; any resultant increase in mitochondrial activity and mitogenesis could decrease glycolysis together with generation of the toxic glycating agent, methylglyoxal. Carnosine's ability to scavenge methylglyoxal could therefore be additionally beneficial by augmenting elimination of the deleterious aldehyde.

Resveratrol is thought to behave as an anti-ageing agent by mimicking the effects of caloric restriction, i.e. inhibiting TOR and activating sirtuin activity (Orallo, 2008; Knutson and Leeuwenburgh, 2008). Resveratrol and carnosine seem to have a number of properties in common (they are anti-oxidants, and possess anti-inflammatory, anti-carcinogenic and platelet anti-aggregatory activity). Therefore one has to consider, admitted very speculatively, the possibility that carnosine could also activate SIRT1 in a manner similar to that proposed for resveratrol (Borra et al., 2005); at least both molecules are approximately the same size.

## 5. Future research

From the above suggestions it is clear that much more research is required to verify or eliminate the various mechanisms proposed to explain the anti-ageing effects of carnosine. Amongst the more obvious areas of investigation would be to search for some of the various carnosine adducts that have been predicted following the dipeptide's putative reaction with toxic aldehydes and carbonyl compounds. It should also be relatively simple to determine if carnosine stimulates proteolysis (proteasomal or autophagic) of altered proteins, and to test whether carnosine binds to SIRT1 and/or other sirtuins and stimulates their activity.

## 6. Conclusions.

Carnosine has been described as an enigmatic peptide (Bauer, 2005) and many recent observations certainly confirm this opinion in relation to its effects on ageing. Carnosine seemingly has the potential to intervene in most of the processes that are thought to contribute to the aged phenotype, as well as mimic, to some degree, the effects those manipulations (possibly including the effects of resveratrol) which

promote longevity. That carnosine can also interfere with mRNA translation by inhibiting phosphorylation of certain initiation factors, as well as upregulate synthesis of protective stress/chaperone proteins, reveal additional potential mechanisms of action. The unexpected role of anticonvulsants as anti-ageing agent, at least in nematodes, and the association of carnosine/homocarnosine with anticonvulsant activity in rodents and humans are further testament to the enigmatic pluripotency of this almost non-toxic peptide. Possible beneficial effects of the dipeptide on age-related pathologies such as diabetes (Hipkiss, 2005 & 2006c), Alzheimer's disease (Hipkiss, 2007c), Parkinson's disease (Boldyrev et al., 2008) and stroke (Dobrota et al., 2005) have therefore been proposed.

Finally perhaps the enigma of carnosine's "true" function(s) is merely a reflection of the fact that relatively little experimental work has been performed in some of the areas under discussion; much more research is required to determine whether any of these proposals are justified.

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