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REVIEW

In Search of New Pharmacological Targets: Beyond Carnosine's Antioxidant, Anti-Inflammatory, and Anti-Aggregation Activities

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ABSTRACT: Carnosine (β-alanyl-L-histidine) is a naturally occurring endogenous peptide widely distributed in excitable tissues, such as the heart and brain. Over the years, several beneficial effects of carnosine have been discussed well in scientific literature. In particular, this dipeptide is well-known for its antioxidant, anti-inflammatory, and anti-aggregation activities. It is of great interest in the context of numerous systemic and neurodegenerative diseases, besides performing important "side activities" such as metal chelation and pH-buffering. Despite a plethora of preclinical and clinical data supporting carnosine's therapeutic potential, researchers are still searching for new pharmacological targets that better highlight carnosine's overall multimodal mechanism of action and allow its disease-specific use. The aim of the present mini-review, after quickly summarizing the current knowledge of carnosine biological properties, is to pinpoint the role of some non-canonical factors/pathways positively modulated by this dipeptide, highlighting their perspective role as future pharmacological targets.

KEYWORDS: Carnosine; pharmacological targets; nitric oxide; TGF-β1; fractalkine; insulin-degrading enzyme; N-methyltransferase; energy metabolism

1 Introduction

Carnosine is a naturally occurring dipeptide composed of the amino acids β-alanine and histidine, synthesized by carnosine synthetase 1 [1], while its hydrolysis into its constituting amino acids takes place as a consequence of carnosinases' activity [2]. It was originally discovered in skeletal muscle, where it is present in a very high concentration, although it is widely distributed in different mammalian tissues, such as heart, gastrointestinal, and brain tissues [3,4]. Due to its involvement in many critical physiological functions, it can be considered an integral part of different tissues in all vertebrates, while it is not found in any plant foods [5]. Over the decades, the numerous beneficial effects of carnosine have been discussed well in scientific literature. Numerous studies have highlighted the pivotal involvement of the antioxidant,

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anti-aggregation, and anti-inflammatory activities in the neuroprotection exerted by carnosine, leading to an increase in the therapeutic value of this bioactive compound widely used not only in medicine, but also in cosmetics, and as a food supplement [6]. As a non-enzymatic free-radical scavenger, carnosine behaves as a natural antioxidant, exerting neuroprotective properties [7], ameliorating atherosclerosis conditions [8], and performing different additional effects, including pH-buffering [9], metal chelating, and antiglycation activity [10]. Moreover, carnosine is involved in the reduction of lipid peroxidation, perhaps enhancing the general antioxidant capacity of the tissue [11]. If, as stated, it is well-known the efficacy of the dipeptide on manifold biological activities, such as its critical role in brain-related disorders [12], and the potential to enhance muscle functionality due to contraction increase or lactate disposal [13], on the other hand, it has not been completely fully understood the multimodal mechanism of action by which carnosine exert its therapeutic potential in other multifactorial pathological conditions such as depression [14]. The increasing interest in the multimodal pharmacodynamic profile of carnosine leads researchers in the field to seek and investigate additional molecules, factors, and/or therapeutic pathways, responsible for the multiple effects exerted by carnosine in various districts. The present mini-review aims to summarize the current knowledge of carnosine biological properties, elucidating the role of some specific pharmacological targets of this dipeptide involved in oxidative stress, inflammation, and aggregation processes, all features characterizing numerous systemic and neurodegenerative diseases, moreover clarifying the therapeutical potential behind targeting those biological factors.

2 Potential Pharmacological Targets Modulated by Carnosine

2.1 Nitric Oxide Modulation

Nitric oxide (NO) is a diatomic, short-lived gas that regulates a wide range of homeostatic functions by transmitting both intracellular and intercellular signals mainly in the cardiovascular, immune, and nervous systems [15]. NO represents one of the critical components of the vasculature: in macro-vessels, it suppresses cell inflammation, inhibits thrombosis, and promotes blood flow; in microvessels, in the presence of specific growth factors, it promotes new vessel formation regulating the crucial process of angiogenesis [16]. NO is produced by three isoforms of an enzyme called nitric oxide synthase (NOS) through the oxidation of L-arginine to L-citrulline. Two of the isoforms, neuronal NOS (nNOS) and endothelial NOS (eNOS) are constitutively expressed, while the third one is inducible and is thus named iNOS [17]. iNOS, which is primarily present in microglia and macrophages, is usually overexpressed in case of pathological conditions, when the cell is induced or stimulated by pro-inflammatory cytokines or bacterial lipopolysaccharide (LPS), leading to a significant immune response [18]. When macrophages produce high levels of NO, it is common to detect an increase in reactive oxygen species (ROS) levels. In the presence of those molecules, such as superoxide, NO can react and generate highly reactive nitrogen species (RNS) such as peroxynitrite, capable of damaging different biological macromolecules and altering all the main biological functions [19], culminating in cell death. To guarantee a homeostatic condition, the cell must maintain an appropriate level of antioxidant molecules by regulating all the mechanisms provided by redox-activated signaling events [20]. In recent years, few works have discussed the activity exerted on NO modulation by carnosine. Numerous research studies have highlighted the ability of carnosine to decrease NO bioavailability [21,22] and/or negatively regulate the iNOS enzyme [21,23] (Fig. 1b). Only recently, a new mechanism of action, iNOS-independent, has been proposed, showing that carnosine can cause a dose-dependent suppression of NO production in RAW 264.7 murine macrophages treated with LPS and interferon-γ (IFN-γ), along with an important increase of intracellular nitrite (NO_2^-), representing NO low toxic end product [24]. This modulation of NO levels was not ascribed to iNOS activity affection, but rather to a direct interaction with

NO molecule favoring its oxidation to NO_2^-. Carnosine-NO adduct formation, depending on both β-alanine ($-NH_2$) and histidine (imidazole ring), allows the accelerated conversion of NO into NO_2^- (Fig. 1a).

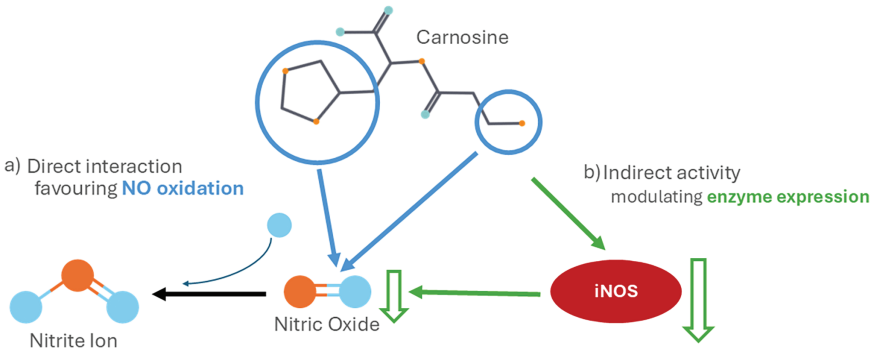

Figure 1: Carnosine modulates NO production directly and indirectly. (a) Directly by favoring NO oxidation to nitrite ion, thanks to its imidazole ring of histidine and $-NH_2$ group of β-alanine. (b) Indirectly by decreasing iNOS expression in neuroinflammatory conditions. iNOS: inducible nitric oxide synthase. NO: Nitric Oxide

For this reason, the authors stated that NO total levels were only apparently suppressed since the total NO production (NO + NO_2^-) was not modified. Behaving as an antioxidant agent, carnosine could be able to lead a faster removal of NO from the cell environment, reducing the possibility of RNS production (peroxynitrite) [24,25]. Moreover, since it is already known the beneficial effect of NO_2^- supplementation on the cardiovascular system [26], the carnosine efficacy on NO conversion (not implying any adverse effect) might represent an additional pharmacological effect for the dipeptide. The above-described mechanism of action could also help explain the increase in NO, measured through NO_2^-, observed in F-2 endothelial cells, reported in the work carried out by Takahashi et al. in 2009 [27].

2.2 TGF-β1 Signaling

Transforming growth factor-beta 1 (TGF-β1) is a polypeptide that plays central roles in different cellular functions, including cell growth, proliferation, differentiation, and apoptosis [28]. Produced by activated microglia, it is considered an anti-inflammatory cytokine that can act as a neurotrophic factor leading the neuronal differentiation and synaptic plasticity in the central nervous system (CNS) [29]. The involvement of TGF-β1 in the advancement of numerous neurological and mental health conditions has been reported, including schizophrenia, depression, and Alzheimer's disease (AD) [30]. The TGF-β1 neuroprotection activity, exerted within the AD context, is related to the ability to limit neurodegeneration induced by amyloid-beta1-42 (Aβ1-42) oligomers, considered an AD neuropathological hallmark. Indeed, a clear link between microglia activity supported by TGF-β1 and the reduction of Aβ-induced toxicity has been previously suggested [31,32]. On the other hand, experiments conducted on *in vitro* models of rat primary cells showed that Aβ1-42 oligomers could activate microglia thus inhibiting TGF-β1 gene expression [33]. Moreover, the impairment of the TGF-β1 pathway in the early phase of AD is strictly related to neuroinflammation and cognitive decline proper of the disease [34]. Based on this evidence, carnosine effects on Aβ1-42 oligomers through the TGF-β1 pathway have been investigated. Carnosine was able to increase both TGF-β1 gene expression and cytokine release in a rat model of microglia challenged with Aβ1-42 oligomers. Furthermore, TGF-β1 increased levels in the presence of carnosine were also detected in resting cells (not exposed to Aβ1-42), suggesting that carnosine treatment could enhance the neurotrophic factors pool in healthy cells, as well as recover from Aβ-induced toxicity [21] (Fig. 2a).

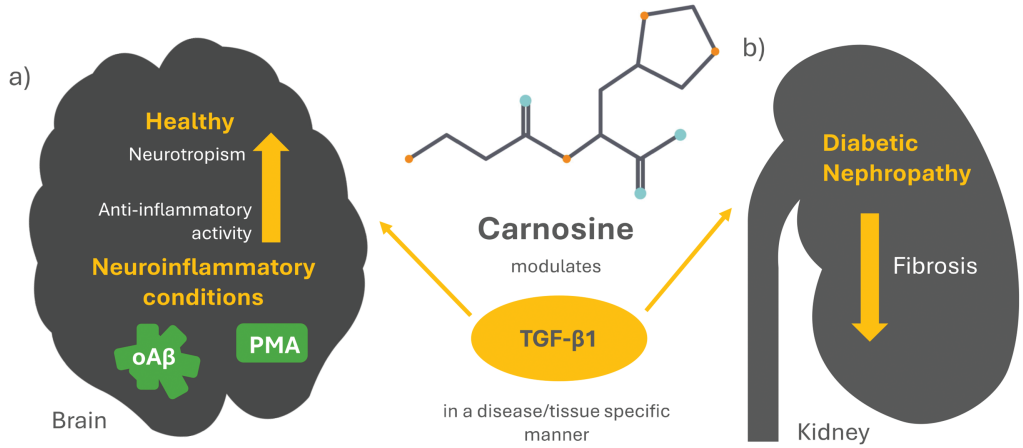

Figure 2: Carnosine modulates TGF-β1 in a tissue- and disease-specific fashion, to counteract its alterations due to disease conditions. (a) In a healthy brain, it increases neurotropism while neuroinflammation (related to Aβ or PMA) has anti-inflammatory activity. (b) In the kidney, it decreases fibrosis due to diabetic nephropathy; oAβ: Amyloid β oligomers; PMA: phorbol-12-myristate-13-acetate

Of note, carnosine's ability to prevent Aβ-induced neurodegeneration via the TGF-β1 pathway was also demonstrated in a different *in vitro* model consisting of primary neurons and glial cells. In a further *in vitro* model represented by murine macrophages stimulated with phorbol-12-myristate-13-acetate (PMA), carnosine was able to completely rescue the expression levels of TGF-β1, significantly decreased as a consequence of the treatment with this pro-oxidant stimulus [35] (Fig. 2a). Interestingly, carnosine modulatory effects of TGF-β1 levels are not limited to microglia. Contrary to what was discovered in microglia and macrophages, in which TGF-β1 was rescued or up-regulated in the presence of carnosine, the release of TGF-β1 has been demonstrated to be reduced by carnosine at a kidney level in specific disease contexts, such as diabetic nephropathy (DN) [36] (Fig. 2b), a common microvascular complication of diabetes and the main cause of end-stage nephropathy. A double-blind randomized controlled trial showed that urinary TGF-β significantly decreased as a consequence of oral carnosine supplementation; the study, conducted on adult type 2 diabetes mellitus (T2DM) patients with DN, indicated an additional reno-protective effect exerted by carnosine on urinary TGF-β level decrease, crucial to serving as a marker of renal injury within this disease [37]. Still, in the context of DN, when used on human mesangial cells, carnosine was able to inhibit matrix accumulation high-glucose-induced by interfering with TGF-β1 production and signaling; in particular, carnosine effects were related to the inhibition of the ALK5 (Smad2/3) pathway, while ALK1 (Smad1/5/8) signaling remained unchanged [36]. In conclusion, as stated by the literature, carnosine seems to be able to exert a stabilizing pharmacological activity on the TGF-β1 pathway, finely regulating its level depending on the pathological condition.

2.3 CX3CR1-CX3CL1 Axis (Fractalkine)

The chemokine fractalkine (CX3CL1) and its specific receptor, CX3CR1, play a pivotal role in neuroinflammation by affecting the signaling between neurons and microglia in a paracrine way [38]. CX3CL1 represents the only chemokine with a higher expression in the CNS than in the periphery: while neurons highly produce CX3CL1, the CX3CR1 receptor is exclusively expressed by microglia [39]. Differently than other chemokines, CX3CL1 can exist both as a static glycoprotein able to mediate cell adhesion and as a soluble isoform; the latter, exhibiting chemotactic features, is produced by proteolytic cleavage of disintegrins and metalloproteinases (ADAM10 and ADAM17) [40]. The reciprocal interaction between microglia and

neurons through CX3CR1-CX3CL1 binding allows effective communication between those cells in CNS, providing the coordination of many aspects of brain function, such as the increase of neuronal network, synapse maturation, and plasticity, besides regulation of cognitive function and immune processes [41]. Genetic variants for CX3CR1, resulting in an altered interaction between CX3CL1 and its receptor, have been reported in the literature, which is strictly associated with multiple CNS diseases, such as macular degeneration and neuroinflammatory disorders [42]. Some inflammatory stimuli and specific conditions, including Aβ extracellular accumulation, can modify CX3CL1/CX3CR1 signaling [43]. In this regard, within the AD context, some changes in the chemokine activity can occur depending on the stage of the disease [44]. Although different works have highlighted the involvement of the CX3CR1-CX3CL1 axis in AD models, such as the modulation of neuronal survival, plaque load, and cognitive processes [43,45,46], only one article is so far available regarding the activity of carnosine on the above-mentioned pathway. It has been reported that Aβ1-42 oligomers and LPS are both able to cause a down-regulation of CX3CR1 in RAW 264.7 macrophages: in those resting cells, CX3CL1 is normally not expressed despite it is possible to detect high levels of mRNA-related to the linked receptor [43]. Within this context, carnosine has been found to rescue the decreased CX3CR1 levels in macrophages, thus contributing to the full neuroprotective activity against Aβ1-42 oligomers toxicity [47] (Fig. 3).

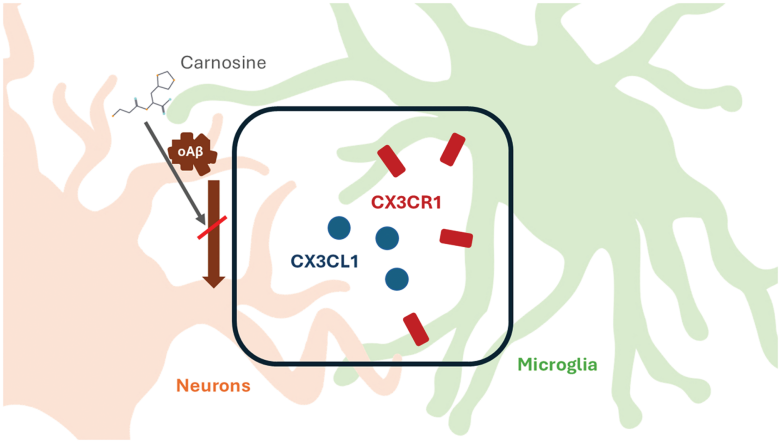

Figure 3: Carnosine restores the CX3CR1-CX3CL1 axis pathway, impaired by oAβ, thus modulating neuron-to-microglia communication

Since the lack of CX3CR1 has been shown to impair the internalization of toxic tau by brain macrophages, favoring an accumulation of extracellular tau, CX3CR1/CX3CL1 axis appears to play a critical role in AD progression, especially in late stages of the disease, in which the impairment seems to be enhanced [48]. Evidence suggested that carnosine affection of the CX3CL1/CX3CR1 axis might be considered a novel and interesting mechanism to be investigated to elucidate new aspects of AD pathology.

2.4 Enzymes and Other Proteins

Carnosine has shown the ability to modulate the activity of numerous enzymes as well as the levels of different proteins the dysregulation of which has been associated with inflammation and oxidative stress phenomena [4,49]. Despite this tremendous potential, a significant number of these targets, including ROS/RNS, pro-inflammatory cytokines (e.g., tumor necrosis factor-alpha (TNF-α)), and antioxidant enzymes (e.g., glutathione peroxidase 1 and superoxide dismutase) do not allow to target a specific pathological condition, being implicated in a plethora of neurodegenerative and systemic diseases, reason why finding a modulable

target related to a specific disease is of great interest. In this context, very recently, the ability of carnosine to modulate the activity of Insulin-degrading enzyme (IDE) has been demonstrated [50]. IDE, also known as insulysin, is a zinc-dependent metalloendopeptidase named after its ability to bind and degrade insulin [51]. In addition to insulin, IDE degrades a plethora of other different short peptides with similar conformation, including amylin, glucagon, somatostatin, insulin-like growth factor-2 (IGF-2), bradykinin, atrial natriuretic peptides, and Aβ, suggesting a multipotential role in pathophysiological processes modulated by these peptides [52–54]. Since the discovery of IDE activity against Aβ, it was demonstrated that this enzyme, highly expressed in the brain, is the major protease responsible for Aβ clearance in human hippocampal lysates and the degradation of Aβ in the cytoplasm and cerebrospinal fluid [55–57]. The double activity of IDE, both on insulin and Aβ, defined this enzyme as a candidate pathophysiological link between AD and T2DM [58]. Indeed, increased Aβ peptide accumulation has been found in the brain of IDE-deficient mice, along with a clear T2D phenotype [59]. Data reported in the literature suggested that IDE actively regulates Aβ levels *in vivo* and that its activity decreases during the early stages of AD, or with aging [60]. Moreover, the evidence demonstrated that high insulin concentrations would decrease IDE-mediated Aβ degradation due to competitive inhibition [61,62]. Even small genetic variation of IDE could be associated with the risk of AD onset: slight overexpression of IDE in transgenic mice strongly prevented AD-related Aβ plaque formation [63,64], while on the contrary partial loss-of-function mutation in IDE gene tested *in vivo* led to impaired degradation of Aβ [65–67]. Due to its multiple involvement in AD and T2DM, IDE-positive allosteric modulators are currently studied as potential drugs for both pathologies, including IDE inhibitors for T2DM and IDE activators for AD treatment [68–70]. Among all the papers published on new perspective drugs, only one is entirely focused on how IDE could affect or determine the neuroprotection exerted by carnosine, especially in the kind of cognitive deficit commonly induced by diabetes [71]. Experiments conducted on primary mixed neuronal cultures from rats, a well-known reliable *in vitro* model to assess Aβ harmfulness, showed that carnosine can reduce the toxicity induced by Aβ1-42 oligomers treatment [50] (Fig. 4a).

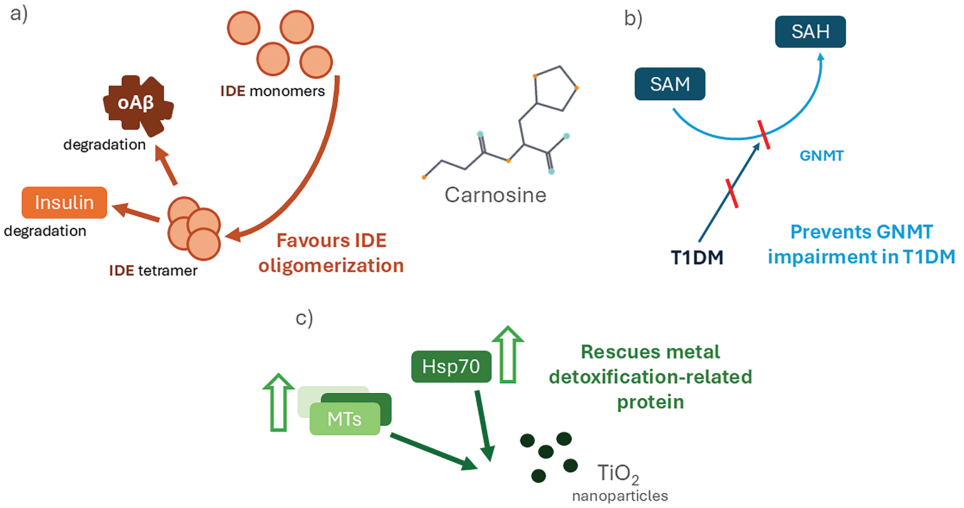

Figure 4: Carnosine biological activities include the modulation of different enzymes/proteins. Carnosine (a) Favors IDE oligomerization; (b) Prevents GNMT impairment in T1DM; (c) Rescues metal detoxification-related protein, in particular Hsp70 and MTs; IDE: insulin-degrading enzyme; T1DM: Type 1 Diabetes Mellitus; SAM: S-adenosylmethionine; SAH: S-adenosylhomocysteine; GNMT: Glycine N-methyltransferase; Hsp70: Heat shock protein; MTs: Metallothioneins

Since the co-treatment with a highly selective IDE inhibitor prevented the activity of carnosine, this neuroprotective mechanism appears to be somehow attributed to the implication of IDE [50]. Results demonstrated that carnosine enhances IDE activity by promoting its oligomerization, behaves as an IDE activator towards long substrates (such as insulin and Aβ1-40), and, most interestingly, affects the enzyme cooperativity by increasing the value of the Hill coefficient [50]. It was hypothesized that IDE modulation exerted by carnosine is due to its capability to bind the exosite of the enzyme, instead of the catalytic site, causing an improved interaction between IDE and long substrates, enhancing the reciprocal affinity and promoting the overall IDE catalytic activity [50]. Still, in the context of putative disease-specific targets, particularly diabetes, carnosine has shown the ability to inhibit glucolipotoxicity, a hallmark of T2DM, by scavenging glucolipotoxic free radicals. The authors showed that this activity of carnosine also resulted in beneficial actions on glucose homeostasis through both increased insulin secretion from isolated mouse islets or INS-1 β-cells and enhanced glucose uptake in C2C12 mouse myotubes [72]. Glycine N-methyltransferase (GNMT) represents the most abundant liver methyltransferase whose main role is to mediate methyl group availability in mammalian cells. It is involved in numerous processes, including the conversion of S-adenosylmethionine (SAM) into S-adenosylhomocysteine (SAH), folate cycle modulation, and liver regeneration [73,74]. GNMT also represents a key enzyme mediating renal inflammation and fibrosis, both playing a key role in the development and progression of DN. Liu et al., by employing web-prediction algorithms, cellular thermal shift assay, and molecular docking, identified GNMT as a possible target of carnosine [75]. The authors showed that GNMT was markedly down-regulated in the serum of Type 1 DM patients as well as in renal tissues obtained from DN mice. Of note, carnosine administration was able to rescue GNMT protein levels in the kidney of these mice, which was accompanied by the amelioration of inflammatory response and fibrosis (Fig. 4b). Importantly, suggesting carnosine as a promising therapeutic agent for DN and underlining as GNMT could represent a therapeutic target in the context of DN. Heat shock proteins (HSPs) represent the molecular chaperones that are mainly induced in cells both during the growth process and in the presence of various stimuli, including temperature variation, infections, mechanical insults, and stress conditions. Depending on their functions and molecular weights, HSPs are classified into five main families: Hsp90, Hsp70, Hsp60, and Hsp100 [76]. Metallothioneins (MTs), as low-molecular-weight sulfhydryl-rich proteins, can bind a plethora of metals (e.g., cadmium, mercury, platinum, and silver), and facilitate metal exchange in tissues. Despite their precise biological role not being fully characterized, MTs have been linked to heavy metal detoxification, metal ion homeostasis, and antioxidant defense [77]. Both HspSP70 and MTs represent two well-known markers of cellular stress. Very recently the expression levels are modulated by carnosine [78]. In particular, using a model consisting of zebrafish larvae challenged with TiO_2-nanoparticles, it was demonstrated the ability of carnosine to rescue the basal protein expression levels of Hsp70 and MTs, coupling this activity to a significant decrease of nanoparticles-induced stress (Fig. 4c). As previously mentioned, an enzyme whose enhanced expression has been associated with pathological condition is represented by iNOS. Despite that, it is worth mentioning that iNOS overexpression, and the related formation of reactive species such as peroxynitrite, has also been associated with phagocytosis [79], a complex process allowing the ingestion and subsequent elimination of pathogens, playing a pivotal role in tissue homeostasis [80]. Interestingly, differently from what was observed in numerous research studies, carnosine up-regulated iNOS expression in murine macrophages, in the presence or absence of Aβ1-42 oligomers, and this inductive effect was associated with enhanced phagocytic activity in these cells. These diametrically opposite effects of carnosine on iNOS expression are in line with the different regulatory activity exerted towards the TGF-β1 pathway described above, showing, once again, the differential modulation of a target as a consequence of a specific condition.

Glutathione peroxidase 4 (GPX4) represents a key regulator in the ferroptosis process, counteracting lipid peroxidation through the conversion of lipid hydroperoxides into non-toxic lipid alcohols [81]. Carnosine has shown the ability to mitigate renal ischemia-reperfusion injury by inhibiting GPX4-mediated ferroptosis and decreasing iron accumulation [82]. In the same context, the protective effect of carnosine on renal tubule cells has been evaluated in an *in vivo* model of cisplatin-induced acute kidney injury, in which the dipeptide significantly ameliorated both histopathological changes and different altered serum levels of renal tubular injury biochemical markers, including kidney injury molecule-1 and neutrophil gelatinase-associated lipocalin, by targeting Caspase-1 regulated pyroptosis [83]. Moreover, carnosine was also able to alleviate some specific disease-related symptoms, such as listlessness, reduced mobility, and oliguria.

Carnosine has shown the ability to positively modulate the expression of the translocase of outer mitochondrial membrane 20 (TOMM20), an enzyme involved in protein mitochondrial transport. This carnosine's activity along with its ability to reduce ROS levels and the expression of genes related to the apoptosis process have been associated with its cryoprotectant properties [84]. Cryoprotective characteristics of carnosine in the context of regenerative biology have also been described. In particular, carnosine has been considered a cryoprotectant for the cryopreservation of spermatozoa as well as for the nervous tissue of non-hibernating animals [85,86].

2.5 Energy Metabolism

Energy is essential for all biological structures as it allows indispensable cellular processes. For this reason, alterations in energy metabolism are considered strictly linked to the onset of numerous diseases. The metabolic systems responsible for the synthesis of ATP, the universal energy currency, are mainly based on the tricarboxylic acid cycle, oxidative phosphorylation, and glycolysis [87,88]. While the latter takes place in the cytoplasm, the former is carried out in the mitochondria. The cooperation between all the processes generates the crucial energy source, ATP, that reaches all the body districts, including high energy-demanding tissues from the heart, brain, and muscle. Energy-producing and storing processes are elaborately modulated by a variety of factors, including genetics, hormones, metabolites, lifestyle differences, and environmental alterations [89–91]. Impairment of these regulatory systems is related to the development of cellular abnormalities that result in various afflictions. Enormous efforts in studying the therapeutic potential of carnosine during the last three decades have been made by Alan Hipkiss, in his paper of 2013 [92] mentioned the possible importance of energy metabolism (and protein homeostasis) modulation in the protective effects exerted by this dipeptide. One year later, Shen et al. measured the oxygen consumption rates and extracellular acidification rates of cortical astrocytes under both normal and ischemic conditions, in the presence or absence of carnosine [93]. Carnosine decreased the energy metabolism of astrocytes under normal conditions with no negative effects on cell viability, while an opposite effect of the dipeptide was observed under the ischemic condition, with carnosine up-regulating the mitochondrial respiratory and cellular ATP content of astrocytes subjected to oxygen-glucose deprivation (OGD). Some years later, additional proof of the importance of considering energy metabolism as a pharmacological target and carnosine as a possible therapeutic agent was given by Heidari et al. [94]. Concentrations of carnosine ranging from 0.01 to 10 mM were able to improve mitochondrial membrane potential, promote mitochondrial ATP metabolism, also enhance mitochondrial dehydrogenase activity. Carnosine was also able to preserve mitochondrial indices of functionality in astrocytes incubated in a Ca^{2+}-overloaded environment. Numerous research studies have indicated that targeting microglia [95,96] and macrophages [97,98] could represent a potential treatment strategy for neurodegenerative and systemic disorders. During the last five years, lots of efforts have been devoted to the investigation of the carnosine activity of the above-mentioned cells, with a main focus on energy metabolism. When used to treat murine macrophages, carnosine was able to

lead to a generalized amelioration of the cell energy state, increasing the intracellular levels of nucleoside triphosphates as well as the sum of the pool of intracellular nicotinic coenzymes [35] (Fig. 5a). In the same study, in macrophages challenged with PMA, carnosine was able to decrease the $NADP^+$/NADPH ratio in a concentration-dependent manner, suggesting a significant inhibitory effect of this dipeptide towards the main source of reactive species production in these cells. In a different model represented by RAW 264.7 macrophages challenged with LPS + IFN-γ induction, the presence of carnosine counteracted the energy and redox metabolism imbalance in stimulated macrophages, with the restoration of nucleoside triphosphates that was accompanied by the counterbalance of the changes in phosphorylating capacity (ATP/ADP), NAD^+/NADH, and $NADP^+$/NADPH ratio [25] (Fig. 5b). The ability of carnosine to modulate energy metabolism has also been investigated in human microglial cells (HMC3). While the first, exploratory study demonstrated that carnosine *per se* is able to ameliorate energy state of microglia, measured in terms of ATP/ADP ratio and energy charge potential (ECP) [99] (Fig. 5c), a more marked modulatory activity was observed in cells challenged with a combination of LPS + ATP.

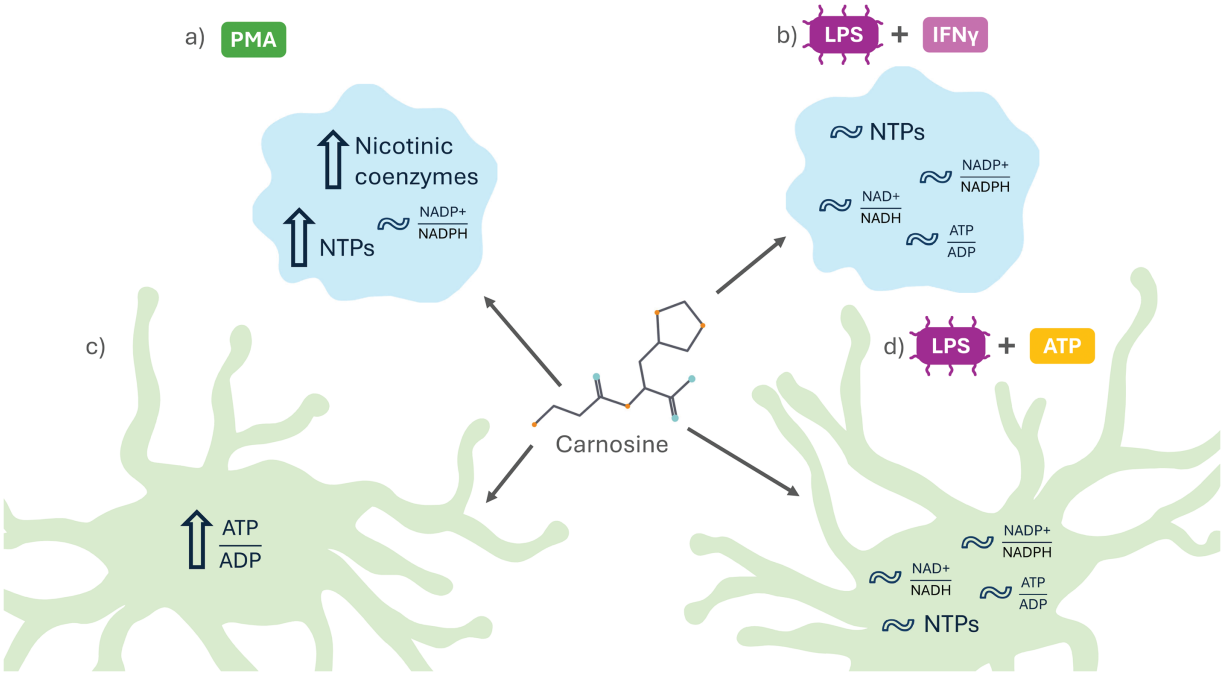

Figure 5: Carnosine regulates energy metabolism in macrophages and microglia. In macrophages: (a) in response to PMA, it increases nicotinic coenzymes and NTPs, generally ameliorating energy status, restores $NADP^+$/NADPH ratio, thus diminishing ROS production; (b) in response to LPS + IFNγ. It restores NTPs, ATP/ADP ratio, $NADP^+$/NADPH, and NAD^+/NADH ratio. In microglia: (c) in the absence of stimulation, it increases ATP/ADP ratio, generally ameliorating energy metabolism of the cell; (d) after LPS + ATP stimulation, it restores ATP/ADP ratio, NTPs, NAD^+/NADH ratio, $NADP^+$/NADPH. PMA: phorbol-12-myristate-13-acetate; LPS: Lipopolysaccharide; IFNγ: Interferon γ; ATP: adenosine triphosphate. NTP: nucleotides triphosphate. NAD(P)(H): Nicotinamide adenine dinucleotide(phosphate) (reduced)

This combined stimulation induced a drastic imbalance in the cell energy, as evidenced by the significant decrease of ATP/ADP ratio, ECP, and nucleoside triphosphates along with an imbalance of redox nicotinic coenzymes (decreased NAD^+/NADH ratio and increased $NADP^+$/NADPH ratio). Of note, carnosine was able to restore all the aforementioned parameters, rescuing the basal cell energy state (Fig. 5d). Still in the context of energy metabolism, a new target may be represented by uridine diphosphate (UDP)-derivatives,

the intracellular levels of which were down-regulated by LPS + ATP in HMC3 [100]. UDP-Gal, UDP-Glc, UDP-GalNac, and UDP-GlcNac are fully involved in the regulation of protein glycosylation, a process essential for protein trafficking (in/out) at cellular levels [101,102], making them an additional "carnosine target" worthy of further investigation.

Recently, carnosine has been shown to affect the heart's energy metabolism through its pH buffering activity. Evidence on cardio-specific carnosine transgenic mice, with a significant increase in the myocardial synthesis of histidyl dipeptides, including carnosine, demonstrated an enhancement of intracellular pH buffering, thus facilitating glucose utilization and then attenuating myocardial ischemia-reperfusion injury [103]. Enhanced buffering conditions in heart tissues, along with carnosine-induced pyruvate dehydrogenase over-expression, provide to maintain glycolysis during ischemia, allowing a high-rate generation of ATP. Moreover, lower levels of succinate were observed in transgenic hearts, compared to wild types. Being a crucial citric acid cycle intermediate, succinate is also recognized as a metabolic marker of ischemic injury, which accumulates in the ischemic tissues on reperfusion, with the ability to increase ROS production from mitochondria. The reduced levels of succinate in the transgenic heart could be potentially related to oxidative stress injury mitigation, also leading to an improved recovery of contractile activity of transgenic hearts after ischemia [104].

3 Conclusions and Future Perspectives

During the last decades, thousands of papers have been published describing carnosine biological activities also highlighting its multimodal mechanism of action. Its therapeutic potential has been described especially taking into consideration the anti-aggregation, antioxidant, and anti-inflammatory activities, which are all of great interest in the context of multifactorial systemic and neurodegenerative diseases, including AD and T2DM. Despite this, the main aim of the present mini-review was to describe some non-canonical factors/pathways, including CX3CR1, IDE, GNMT, nucleoside triphosphates, nicotinic coenzymes, and UDP-derivatives, that are "therapeutically" modulated by carnosine, underlying their perspective role as future pharmacological targets. It is worth recalling that all the mechanisms described must be considered given whether carnosine is expected to engage the different potential targets *in vivo*, the reason why increasing the bioavailability of carnosine in specific districts (e.g., development of human serum carnosinase inhibitors or route of administration bypassing the blood-brain barrier and first-pass metabolism) is essential to unveil the full potential of this molecule.

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