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REVIEW

Biosynthesis, Release, and Uptake of Carnosine in Primary Cultures

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Abstract—Biosynthesis, release, and uptake of carnosine (β-alanyl-L-histidine) in highly enriched primary cell cultures of skeletal muscle and CNS tissue have been investigated. The synthesis is restricted to muscle cells, oligodendrocytes, and ensheathing cells of olfactory bulb and increases during differentiation of these cells. Astrocytes, in contrast, do not synthesize carnosine but are equipped with a dipeptide transporter by which carnosine is taken up very efficiently.

Key words: carnosine, glia cells, astrocytes, oligodendrocytes, ensheathing cells

A century has already passed since Gulewitsch and Amiradzibi [1] first reported on the successful isolation of a crystalline substance from Liebig's meat extract, which they called carnosine. Subsequently, this substance was identified as β-alanyl-L-histidine [2, 3] and thus represents the first peptide ever isolated from natural material. Meanwhile, a variety of dipeptides structurally related to carnosine (ω-aminoacyl amino acids) have been identified as constituents of excitable tissues, brain, and muscle (for review see [4, 5]). In skeletal muscle, carnosine and anserine (β-alanyl-N^1-methylhistidine) represent the major non-proteinous nitrogen components that contribute as much as 0.2-0.5% to the net weight of some muscles.

While small amounts of the β-alanine-containing peptides are also found in brain, homocarnosine (γ-aminobutyryl-histidine) and other γ-aminobutyryl (GABA)-containing dipeptides are more abundantly and exclusively expressed in the central nervous system, presumably due to the restricted availability of the precursor GABA in this tissue.

In recent years, numerous studies have reported on the specific properties and possible therapeutic potential of ω-aminoacyl amino acids [6]. An interesting hypothesis about the protective effects of carnosine, N-acetylcarnosine, and homocarnosine against neuronal cell death is that all three peptides act as scavengers of reactive oxygen species, but carnosine has an additional anti-apoptotic effect because of its anti-glycating activity [7]. However, the biological roles of these peptides remain rather obscure. In order to get some clues as to their function in the CNS, some authors focused their studies on the distribution of these peptides in the brain of various species [8, 9, 11]. For a comprehensive review on this aspect and recent results provided by the excellent studies from Fasolo's laboratory, see [12]. Since all ω-aminoacyl amino acids are synthesized by carnosine synthase, an enzyme with broad substrate specificity, we chose an alternative approach: with primary cell cultures, we were interested in studying the synthesis, release, and uptake of carnosine by different cell types.

CHARACTERIZATION OF THE CELL CULTURES

Primary cultures established from muscle and CNS tissue consist of a mixed population of cells. Investigation of carnosine biosynthesis demands highly enriched cell cultures with purity and viability of about 95%.

Muscle cell culture. Embryonic chick pectoral muscle is a very rich source of carnosine and anserine [4]. Myoblasts could easily be isolated from 11-day-old chick embryos [13] and differentiated in serum-free media supplemented with transferrin and insulin [14, 15] or thyroid hormone [16]. The presence of cytosine arabinoside blocks DNA synthesis and removes contaminating fibroblasts almost completely.

Oligodendrocytes. Primary glia cell cultures prepared from newborn rat brain [17] grow as a mixed population of mainly astrocytes, oligodendrocytes, and

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microglia. The latter can be easily removed from the culture flasks by shaking at room temperature for 1 h. Oligodendrocyte progenitor cells could be obtained in sufficient amounts by a combination of the methods described by McCarthy and de Vellis [18] and Besnard et al. [19]. The cells differentiated in medium described by Barres et al. [20] are about 96% pure oligodendrocytes [21]. Oligodendrocytes from spinal cord could be prepared according to the method of van der Pal et al. [21, 22].

Astrocyte cultures. Highly enriched astrocyte cultures are obtained from the mixed primary glia cell preparations of rat brain after removal of the microglia and oligodendrocyte progenitor cells [21]. The residual oligodendrocytes are selectively removed by antibody mediated cell killing [23].

Olfactory bulb cultures. Mixed cultures of rat olfactory neurons and ensheathing cells could be prepared from new-born rats by a modification of a procedure described by Ronett et al. [24]. When switched to nerve growth factor (NGF) depleted medium containing dibutyryl cAMP (dBcAMP), the share of neurons decreases drastically and the cultures consist of cells expressing glia fibrillary acidic protein (GFAP) and galactocerebroside (GalC), typical markers of the ensheathing cells [25]. Glia cells prepared from whole olfactory bulbs of adult rats [26] could be maintained in oligodendrocyte differentiation medium supplemented with dBcAMP [28].

BIOSYNTHESIS OF CARNOSINE

The dipeptide carnosine is synthesized from its component amino acids β-alanine and histidine by the enzyme carnosine synthase. β-Alanine, a non-proteinogenic amino acid, is produced mainly by the liver as the final metabolite of uracil and thymine degradation [27]. Cells synthesizing carnosine should possess an efficient transport system for this amino acid. Investigations of such transport in some of the cell cultures described above show that muscle cells and oligodendrocytes are equipped with it and that the uptake of β-alanine increases with the differentiation of the cells *in vitro* [13, 21]. Carnosine biosynthesis in these cultures correlates in time with the morphological changes of the cells. In muscle, cell culture synthesis of carnosine increases and reaches maximum at the formation of thick multinucleated myotubes. With oligodendrocytes the highest rates of synthesis are reached when the cells strongly express myelin basic protein, a marker of differentiated oligodendrocytes.

Immunolocalization studies suggest that in the brain, except in the olfactory bulb, carnosine and related peptides are associated with glia cells [8]. These data are in agreement with studies on the biosynthesis of carnosine by primary cultures of glia cells from rodent brain [28]. Using highly enriched cultures of astrocytes and oligodendrocytes, it was possible to demonstrate that this ability is restricted to the oligodendrocytes [21]. In addition, a significant amount of newly synthesized peptide is found in the culture medium [21, 28].

It has been reported that primary cultures of glia cells show rapid uptake of radiolabeled carnosine by an energy dependent dipeptide transport system [30]. Subsequent investigations demonstrated that astrocytes effectively take up carnosine [21]. They express peptide carrier of the type PepT2 [30, 31]. Neither neurons [29] nor oligodendrocytes [21, 30] possess such a transport system. These observations should be taken into account for the interpretation of immunolocalization studies using antibodies against carnosine and anserine.

Concerning carnosine, olfactory mucosa and bulb take a special place in the CNS. Significant amounts of this peptide are found there [32]. Reported co-localization with glutamate in olfactory sensory neurons led to the suggestion that carnosine might have a neuromodulatory role in olfaction [10]. The question arises as to the cell type responsible for carnosine synthesis. Glia cells from adult rat olfactory bulb synthesize carnosine. The synthesis is enhanced by addition of dBcAMP to the culture medium. Doucette and Devon [33] have reported that this agent differentiates the ensheathing cells, which express GFAP and GalC. Carnosine synthesis correlates with the intensity of GalC staining and decreases after removal of these cells by antibody mediated cell killing [23].

Ensheathing cells from olfactory tissue of newborn rats produce carnosine after stripping the neurons from the flat cell layer [25]. The data suggest that this type of cell but not olfactory neurons synthesize carnosine.

The studies of carnosine biosynthesis *in vitro* demonstrated that the process is restricted to muscle cells, oligodendrocytes of brain and spinal cord, and ensheathing cells of the olfactory bulb, and the synthesis increases during differentiation of these cells. This fact puts forward the hypothesis that carnosine plays a role in mature tissue. Studies of its release as a response to stimulation could give an indication of its intra- or extracellular role.

CARNOSINE RELEASE

A significant amount of synthesized carnosine is released from oligodendrocyte cultures into the medium [21, 28]. This phenomenon raises the question as to whether the release is always accomplished at a basal rate or whether it could be stimulated by neurotransmitters, receptor agonists, or elevated potassium concentration. Oligodendrocytes provide a good model system for such investigations. They possess neurotransmitter receptors for glutamate, GABA, histamine, ATP, and

norepinephrine [34, 35]. Activation of glutamate and GABA receptors is followed by membrane depolarization [35], which correlates, in the case of glutamate, with an increase of intracellular Ca^{2+} [34] due to Ca^{2+} influx and intracellular Ca^{2+} release [36].

Treatment of oligodendrocyte cultures with glutamate or kainate stimulates carnosine and β-alanine release from the cells [37]. This stimulus-evoked release is observed only in the presence of cyclothiazide (CTZ), which blocks desensitization of AMPA-type receptors [38] and is abolished by a specific antagonist of this receptor: 6,7-dinitroquinoxaline-2,3(1H,4H)-dione (DNQX). Depolarization of oligodendrocytes with 55 mM KCl or 1 mM GABA has a minimal effect on the efflux, which again underlines the specificity of glutamate as stimulus [37]. The efflux caused by glutamate is enhanced by about 30% in the presence of 100 μM $ZnCl_2$. Zn^{2+} is released with glutamate at the excitatory synapses and increases the AMPA-receptor mediated transmission. However, rapid neuronal degeneration after exposure to 50 μM AMPA and 300 μM Zn^{2+} [39, 40] has been reported. Interestingly, carnosine is known as an efficient chelating agent of Zn^{2+} [6].

The release of neuroactive peptides by glial cells has been ascribed to several mechanisms (e.g., membrane depolarization, swelling of type 1 and 2 astrocytes due to high K^+, reversal of membrane transport). Based on our data, however, it seems unlikely that the release of carnosine from oligodendrocytes can be explained by such mechanisms. Instead, our observation that the Ca^{2+} selective ionophore A23187 effectively stimulates the efflux of carnosine is in favor of a vesicular release mechanism. Based on the identification of the synaptic proteins synaptobrevin II, cellubrevin, and syntaxin in astrocytes, a vesicular mechanism has been suggested to explain the bradykinin evoked release of glutamate from astrocytes [41]. Together with the very recent observation that soluble N-ethylmaleimide-sensitive fusion protein SNAP-23 (an analog of SNAP-25), syntaxin I, and cellubrevin are expressed in cultured astrocytes, oligodendrocytes, and microglia [42], the release of carnosine by A23187 strongly supports the new concept that the Ca^{2+}-dependent vesicular release mechanism may generally apply for exocytotic processes in glial cells that are involved in diverse biological functions.

A century after the isolation of carnosine we have learned many details about carnosine and related dipeptides. Nevertheless, the biological functions of these peptides in different tissues remain obscure, and a unifying concept to answer this question has not yet emerged. To gain some insight into the biological role of these peptides and closely related analogs thereof (e.g., β-alanylhistamine and carcinin, phylogenetically present throughout the animal kingdom), one approach should be to isolate the key enzyme, carnosine synthase, and to identify the cDNA encoding this protein.

Studies along these lines should provide new tools (e.g., antibodies and molecular probes to define the site of synthesis) and might open new avenues (e.g., generation of knock-out mutants) for future studies in the second century of carnosine history.

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