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Focus on cyclo(His-Pro): history and perspectives as antioxidant peptide

Minireview Article

A. Minelli[1], **I. Bellezza**[1], **S. Grottelli**[1], and **F. Galli**[2]

[1] Dipartimento Medicina Sperimentale Scienze Biochimiche, Sezione Biochimica Cellulare, Università di Perugia, Via del Giochetto, Perugia, Italy
[2] Dipartimento Medicina Interna, Sezione Biochimica Applicata e Scienze della Nutrizione, Università di Perugia, Via del Giochetto, Perugia, Italy

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Summary. Cyclo(His-Pro) is an endogenous cyclic dipeptide structurally related to tyreotropin-releasing hormone that was originally discovered in brain. In the central nervous system it has been described to exert multiple biological activities, which seem to be related to a presynaptic dopaminergic mechanism and include among the others a leptin-like function. It can be found in several body fluids and in the gastrointestinal tract where it has been suggested to act as a gut peptide with influence on the entero-insular axis. The oral administration of cyclo(His-Pro) and zinc was described to improve with a synergistic mechanism the glycaemic control in diabetes.

The most intriguing function of this cyclic dipeptide is related with its neuroprotective role that was first reported in traumatic injuries of the spinal cord, and then confirmed in other models of experimental injuries of the nervous system. The mechanism that lies behind the neuroprotective activity of cyclo(His-Pro) remain poorly understood. Recent in vitro studies on rat pheochromocytoma PC12 cells have shown that it is a protective factor against stress stimuli and there is early pre-clinical evidence strongly suggesting that it enhances the expression of small heat shock proteins and antioxidant protection at the cellular level.

Future research is underway to better characterize the possible use of this cyclic dipeptide in the therapy of neurodegenerative and metabolic disorders.

Keywords: CHP – Presynaptic dopaminergic mechanism – Diabetes – ERK 1/2 – p-38MAPK – Small hsp – Neuroprotection

Introduction: general characteristics

Cyclo His-Pro (CHP) (Fig. 1), also known as histidyl-proline diketopiperazine, is formed as a metabolite of the hypothalamic tyrotropin-releasing hormon (TRH) by the activity of the enzyme pyroglutamyl peptidase (Prasad and Peterkofsky, 1976) and subsequent cyclization of the dipeptide His-Pro-NH_2 at 37 °C by a non enzymatic process that shows maximal velocity at pH between 6 and 7 (Perry et al., 1965).

The process of cyclization confers higher stability against the activity of peptidases and is a structural prerequisite for the active transport in the intestine (Mizuma et al., 1997, 1998). Accordingly, the linear form of the dipeptides is often less stable than the cyclic counterparts in vivo, and therefore cyclic dipeptides are far more promising in terms of therapeutic application using both parenteral and oral administration routes.

Diketopiperazines are readily formed from dipeptide esters (due to the presence of a good leaving group) and dipeptides (with alternate chirality) and it is a well-documented fact that dipeptide esters and amides readily undergo cyclization in vitro to the diketopiperazine with a rate that competes favorably with that of catabolism by hydrolysis (Goolcharran and Borchardt, 1998).

CHP is ubiquitous in the central nervous system, and it has been found in blood and in the gastrointestinal tract, as well as in several body fluids as semen, cerebrospinal fluid and urine. It is also abundant in the prostate (Prasad, 1995).

Distribution studies of the endogenous and exogenous CHP performed by immunological methods showed that CHP is not concentrated in synaptosomes, suggesting that it is not assimilable to a classical neurotransmitter. The structural similarities between TRH and CHP initially suggested the existence of a precursor/product relation-

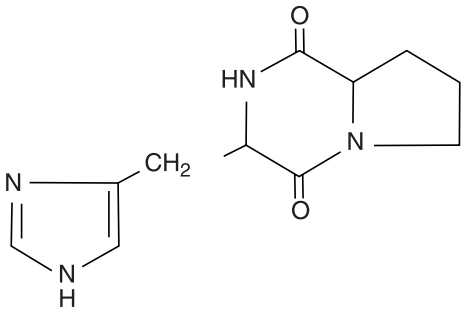

Fig. 1. Chemical structure of cyclo(His-Pro)

ship, but in subsequent years it was concluded that CHP can arise via multiple pathways and stimulate different biological processes as compared with the precursor (Prasad et al., 1987; Prasad, 1998). However, although new TRH receptors, detected in pituitary cells, were proposed, no specific receptors for CHP have been so far identified in brain. Nevertheless, the existence of a specific receptor for CHP could be postulated on the basis of a specific binding of CHP to adrenal cortex and liver membranes (Bataini et al., 1983; Mori et al., 1986). However, when two new subtypes of TRH receptors, i.e. TRHR1 and TRHR2, were discovered, no specific binding of CHP was observed thus excluding the possibility that TRHR are receptors of CHP (Itadani et al., 1998). More recently, studies of the signalling events triggered by CHP in PC12 cells have suggested that it acts via a receptor that is dually coupled to stimulatory and inhibitory G proteins (Minelli et al., 2006). However, the exact molecular identity and binding characteristics of this receptorial system remain undisclosed.

Biological roles

Since the discovery of CHP formation in brain in the 70s, a number of studies have been performed to define its biological roles. Administration of exogenous CHP to animals elicits a variety of biologic activities such as augmentation of pentobarbital-induced sleep, attenuation of ketamine anesthesia, and mitigation of some pharmacological effects of alcohol such as the ethanol-induced hypothermia (Prasad et al., 1977; Prasad, 2001). Moreover, these studies demonstrated that a continuous alcohol consumption can increase brain levels of CHP.

This cyclic-dipeptide plays also an important role in the perception of pain induced by physical, mechanical, thermal, and chemical stimuli. The administration of CHP to mice resulted in a significant dose-dependent increase in anti-nociception and the opioid antagonist naloxone incompletely antagonized the antinociceptive effects of CHP thus suggesting a simultaneous action at two sites that are opioid- and non opioid-dependent.

Both peripheral and intracerebral CHP lead to time- and dose-dependent hypothermia in rats. Attenuation of cold-induced hypothermia by passive immunization with rabbit anti-CHP antibody suggested that endogenous CHP may be involved in regulating core temperature in response to ambient temperature changes (Prasad, 1995).

The first observation of the anorectic role of CHP was made by Morley et al. (1981) and the observation that fasting elevates the hypothalamic CHP concentration, which returns to normal after feeding is consistent with the appetite-modulating role (Prasad, 1995). The first demonstration of an endocrine effect of CHP dates back to 1980 when Prasad and co-workers showed that CHP inhibits prolactin secretion. On the contrary, no effects have been reported on thyrotropin secretion (in vitro and in vivo) in the rat, monkey, and human and on in vitro growth hormone (GH) secretion in the rat (Prasad, 1995). Multiple mechanisms underlie these different effects, although most of the biological activities seem to share common dopaminergic mechanism. In fact, the thermoregulatory response to CHP is attenuated by dopaminergic antagonists and the same conclusions can be drown by analysing the effects of CHP on motor activity. A normoactive striatum requires a balance between excitatory glutamamtergic/cholinergic and inhibitory dopaminergic neurons and an imbalance of these antagonistic transmitters in the striatum results in motor activity dysfunction (Prasad, 1995). Therefore, all the existing data converge on a presynaptic dopaminergic mechanism of CHP (Imamura and Prasad, 2003).

As discussed more extensively in the next sections, the production of CHP in the GI tract has been associated with a role as gut peptide of the entero-insular axis (Prasad, 1995).

CHP in food

Perry et al. (1965) were the first to report the presence of CHP-like immunoreactivity (CHP-LI) in foods when they found the histidyl-proline diketopiperazine in the low phenylalanine dietary preparation, Lofenelac (Mead Johnson, New York, NY). Subsequently, high levels of CHP-LI were found in several common nutritional supplements (Hilton et al., 1990) and it was reported that the dietary intake of CHP-LI rich supplements in healthy volunteers can increase the levels of CHP in plasma far above the baseline values (829 ± 64 pg/ml) (Hilton et al., 1989,

Table 1. Concentration of CHP-LI in several common food

Food type	CHP-LI pmol/g food
Noodle	76
Potted meat	165
Nondairy creanmer	121
Hot dog	73
Ham	131
Egg	23
Bread	88
Tuna	2058
Whole milk	6
Chocolate milk	15
Fish sauce	5289
Dried shrimp	6576

From Hilton et al. (1992)

1990). Thus, this evidence in literature clearly demonstrated that dietary CHP can be absorbed from GI tract. In Table 1 is reported the CHP-LI of some types of food. Examples include dried shrimp, fish sauce, tuna, ham, potted meat, non-dairy creamer, white bread, and noodles.

The exact origin of CHP-LI in food is not clear. However, it seems to be formed by the thermal manipulation of hydrolysed proteins occurring during food processing (Prasad et al., 1991). In fact, the concentration of CHP-LI reported in several nutritional supplements is proportional to the degree of thermal manipulation during manufacture of partially hydrolysed casein-derived proteins (Hilton et al., 1990). Several lines of evidence suggest that CHP present in food is absorbed in quantities sufficient to alter plasma CHP levels. In humans, plasma CHP level rises to $182 \pm 29\%$ of the normal level 60 min after ingestion of nutritional supplements containing concentration of CHP similar to those found in food (Prasad et al., 1991).

Pharmacology and administration protocols

As endogenous molecule found also as food component, CHP has been considered in the context of ''non-conventional drugs'' or ''nutracueticals'' with possible application in the control of blood glucose and prevention of type II diabetes (Song et al., 1998). The fact that CHP is well absorbed when taken orally makes this the preferential administration route so far used in the clinical trials (see below). CHP has a long half-life in blood and high metabolic stability which suggest that, once administered, the cyclo dipeptide can reach several tissues at biologically relevant concentrations (Banks et al., 1992). Moreover, it can cross the blood–brain-barrier via a non saturable mechanism (Banks et al., 1993) and this is a key characteristic for delivery and specific targeting of CHP therapy in the CNS. The oral administration in adult mice of CHP radioactively labeled with ^{125}I showed that the radioactivity quickly appeared in blood at levels ranging from one half to one fourth of those previously found after IV injection. Between 25 and 32% of the radioactivity recovered from blood 30 min after feeding eluted on high-performance liquid chromotography in the position of the intact peptide (Banks et al., 1992). Powdered prostate gland or extracts from the prostate glands of various species of animals are used as sources of CHP which can be easily administered as capsules. Commercially available preparation show usually 1% final concentration of the dipeptide. Other formulations of CHP are available in combination with Zinc and have been patented in US, Asia and Europe. No evidence for toxicity and side effects associated with the oral administration of approx. 12 mg/day or higher has been reported. Some of the commercially available preparations have been approved for clinical trials by the FDA, but to our knowledge, a final approval has not provided so far to allow marketing, supporting its use as a drug for the treatment of diabetes.

Clinical applications

CHP and diabetes

The fact that plasma levels of CHP in humans are increased after glucose ingestion suggests a role in the entero-insular response to nutrient ingestion. The increased CHP levels in obese women might, at least partially, explain their hyperinsulinemia (Hilton et al., 2001). Moreover, the CHP-induced decrease in food intake mimics the action of leptin, a protein hormone with important effects in regulating appetite, body weight, metabolism and reproductive function (Friedman and Halaas, 1998). Therefore, CHP activity has been extensively suggested to closely relate to insulin and leptin sensitivity, so that it could at least partially contribute to the activity and pathophysiology of these hormones, particularly concerning the development of diabetes and obesity. Studies of the modulation of pancreatic hormones secretion by intraventricular administration of high doses of CHP-analogue or TRH showed inhibition of both insulin and glucagon secretion by rat pancreatic islets (Kato and Kanno, 1983) whereas CHP, ingested orally, did not affect blood glucose levels or parameters of insulin secretion (Mizuma et al., 1996). On the basis of the observation that administration of prostate extracts, containing very high amounts of zinc and agents that stimulate intestinal zinc absorption, pro-

duced antidiabetic activities in animal and human subjects (Song et al., 1998), studies were performed to determine whether the antidiabetic activity could be related to the high concentrations of zinc, CHP, and arachidonic acid in prostate tissue. It was concluded that CHP, arachidonic acid, and zinc synergistically affect blood glucose concentrations by stimulating intestinal zinc absorption and muscle zinc uptake (Song et al., 2001). Zinc deficiency critically affects diabetes because this oligoelement activates insulin receptor β-subunit and many of the vital genes involved in cell growth, thereby exerting an influence on glucose metabolism. Consequently, a defective zinc metabolism may critically affect the pathophysiology of diabetes. However, treatment of diabetic animals and human subjects with zinc alone is minimally effective in the control of blood glucose levels, probably because of a defective intestinal and muscle tissue zinc metabolism from normal dietary sources in diabetes (Kechrid et al., 2001). Also the metabolism of other trace element such as magnesium and chromium is tied to the clinical conditions of diabetes, although only the mechanisms by which zinc regulates glucose metabolism is known (Vaquero, 2002). Zinc is known to play an important role in the regulation of glucose uptake by cells (Davies, 1980), and several reports indicated that mineral and trace element absorption is decreased in diabetic animals and humans, while the absorption of other nutrients and non-nutrients, such as amino acids and carbohydrates, is either increased or unaffected. More recently it has been discovered that CHP is present in large quantities in ethyl alcohol-refluxed soy protein hydrolysate and, because of the protective effects against obesity and diabetes, soy proteins are becoming a very important component in the human diet (Friedman and Brandon, 2002; Song et al., 2005). It should be further noticed that inhibition of dipeptidase, which degrades CHP and/or its precursor, L-histidyl-proline, improves glucose tolerance in mice (Marguet et al., 2002).

CHP and neuroprotection

The study of the neuroprotective effects of CHP started in 1981, when Faden and co-workers (1981) showed that TRH improves neurologic recovery after spinal trauma. Since then, an extensive experimental literature on TRH and its derivatives has shown effectiveness across a variety of experimental models and species (McIntosh et al., 1989; Faden et al., 1990, 1999). Although such compounds have not been tested against all of the most effective neuroprotective agents, when directly compared, they have proved to be superior to many other experimental treatments such as corticosteroids, opioid receptor antagonists, certain *N*-methyl-D-aspartate antagonists, serotonin antagonists, and calcium channel blockers (Prakash et al., 2002; Faden et al., 2003a, b, 2005). TRH has a large therapeutic window since its neuroprotective effects have been demonstrated when the compound is administered 24 h or even longer after trauma. TRH and TRH-like compounds are well tolerated at even high doses in humans and a small pilot study showed considerable promise for the treatment of human spinal cord injury (Horita, 1998). There is also evidence that this class of drug may enhance cognitive function and act as nootropic agents. Nevertheless, TRH and traditional TRH analogs have certain potential limitations as clinical treatment for central nervous system trauma. TRH is rapidly broken down by endopeptidases therefore it has a very short biological half-life in humans, so that any therapeutic treatment requires a continous infusion. In addition, it has potent endocrine, analeptic, and autonomic actions that may limit chronic treatment. Although the endocrine effects might be desirable for its potential nootropic actions, its autonomic actions include a significant pressor effect that might exacerbate post-traumatic bleeding, an important contributing factor for mortality after traumatic brain injury. Under certain conditions, TRH might also increase body temperature: another undesirable effect following acute brain injury. In addition, its analeptic actions may serve to antagonize the induction of pharmacological coma treatment. All these effects limit the therapeutic use of TRH, whereas the metabolic product CHP retains all pharmacological activities without known side effects. The cyclic dipeptide has a marked neuroprotective activity in multiple rodent models of traumatic brain injury, and attenuates both necrotic and apoptotic neuronal cell death in vitro (Faden et al., 2004). Several well-established models, i.e. glutamate, maitotoxin and oxygen/glucose deprivation, trophic withdrawal, β-amyloid toxicity, and $FeSO_4$ have been used to test the neuroprotective effects of CHP and of several other dipeptides (Faden et al., 2005). Results from these studies suggested that these dipeptides may have utility in a variety of acute or chronic neurodegeneration, such as cerebral ischemia or Alzheimer's disease (Faden et al., 2004). High-density oligonucleotide based microarrays, used to examine effects of treatment on transcriptional events, showed an up-regulation of genes/proteins associated with endogenous neuroprotection, including brain-derived neural factor, hypoxia-inducible factor and heat-shock proteins (Faden et al., 2005).

More recently, the effects of CHP in PC12 cells stressed by serum deprivation have been investigated in

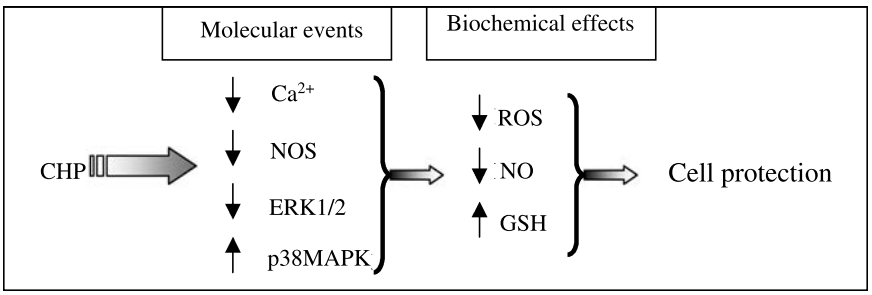

Fig. 2. Overview of proposed CHP effects in H_2O_2-injured cells. The effect of protection that CHP has been suggested to exert against cell oxidative stress is related with an increased content of GSH and a lower flux of ROS (both the oxygen and nitrogen derived species). These events associate with p-38MAPK activation, reduction in intracellular calcium levels, NOS down-regulation, and ERK1/2 inactivation. A possible cross-talk, still to be investigated, between ERK1/2 and p-38MAPK is proposed

our laboratory (Minelli et al., 2006). We have analysed the phosphoproteome of the response elicited by the treatment and found that the cyclic dipeptide affected cellular proliferation only under experimental conditions causing cellular stress and apoptosis. Neuroprotection was accompanied by ERK1/2 inactivation, an event that appeared to proceed through PKA and PKG, whereas it did not appear to involve either tyrosine kinase or calcium-calmoduline kinase as well as PKC. We have also found that the protective response to CHP was related to p-38MAPK activation which, in turn, was responsible for a remarkable phosphorylation of the small heath shock proteins, i.e. hsp27 and α-B-crystallin, capable of protecting the cells from different kinds of stress. The increased levels of phosphorylation were consistent with an overall protective effect of CHP at cellular level. ERK inactivation was initially a puzzling result since ERK activity is typically associated with cell survival, proliferation and differentiation. However it has been recently shown that ERK activation can contribute to neuronal cell death (Amadoro et al., 2006; Subramaniam et al., 2004, 2005; Levinthal and DeFranco, 2004; de Bernardo et al., 2004; Canals et al., 2003). Similarly to PC12 cells exposed to ischemia after treatment with homocarnosine (Tabakman et al., 2004), showing that the protective effect was related to a 40–50% lower activation of the survival kinase ERK 2, the observed ERK inactivation might be explained by the fine regulatory balance between the different MAPK members and/or between MAPKs and other cellular kinases. Although the inhibition of the survival kinase might suggest an anticancer effect, the only report on the effects of cyclo(His-Pro) in cancer cells shows that CHP has no significant effect on growth inhibition of HT-29 (colon carcinoma), MCF-7 (breast carcinoma) and HeLa(cervical carcinoma) cells (Brauns et al., 2004). Other Authors (McCleland et al., 2004; Lucietto et al., 2006) reported strong anticancer activity by cyclo(His-Ala) and cyclo(His-Gly) in HT-29, MCF-7, and HeLa proposing the histidine-containing cyclic dipeptides as ideal lead compounds for the rational design of an potential chemotherapeutic agent capable of preventing metastasis, inhibiting tumour growth.

Oxidative stress is thought to contribute to the aetiology of several neurodegenerative diseases by the toxicity of reactive oxygen and nitrogen species which cause damage and non-specific changes of lipids, proteins, and nucleic acids (Calabrese et al., 2005; Wang et al., 2006; Hidalgo et al., 2007). Accordingly, the most recent hypothesis suggested for the neuroprotective activity of CHP deals with an increased antioxidant protection and the control of redox signals that regulate basic developmental responses in eukaryotic cells such as cell differentiation and apoptosis (Taniyama and Griendling, 2003; Gutierrez et al., 2006), as well as protective gene and repair mechanisms that could be evoked in stressed neurons. Previous studies (Minelli et al., 2006) and further research work performed by our group show the effect of CHP on the increase of ROS flux following a stress condition in PC12 cells. Preliminary data obtained with PC12 cells exposed to serum starvation and hydrogen peroxide insult have shown that the oxidative stress, caused by increased intracellular generation of ROS and NO, enhanced cell calcium levels, and marked decrease of reduced glutathione, can be prevented by the pretreatment with CHP, thus confirming an indirect antioxidant role that can be described as shown in the scheme of Fig. 2.

Conclusions and future perspectives

The physiologic roles proposed for CHP on the bases of in vitro and some early in vivo studies suggest possible applications in the therapy of chronic and age-related dis-

eases such as diabetes and neurodegeneration. Evidence is available on the fact that CHP can be administered through dietary and therapeutic protocols, and it is obviously non-toxic at pharmacological levels in humans.

The possible application of CHP in the therapy of diabetes has been the most extensively investigated, but so far it remains still matter of speculation and a claim by the marketing of nutraceuticals. According with the most recent in vitro evidence examined in this review paper, the neuroprotective role appears to be one of the most relevant properties of CHP, promising future applications for the therapy of degenerative processes of CNS. Indeed, the considerable neuroprotective activity shown for CHP and several other derivatives was already reported by Prasad and Peterkofsky (1976) during their studies on the origin, pharmacology, and mechanism of action of TRH derivatives, and it has been further substantiated by the recent studies of Faden and collaborators (2005). Early in vitro results obtained in our laboratory suggested that CHP protects against the effect of oxidative stress. This may be due to an increase in glutathione levels and control of specific antioxidant cell signals (summarized in Fig. 2). More basic information would be needed to characterize the therapeutic applications of this dipeptide. In particular, molecular, cellular, and pharmacokinetic studies are required. However, the available results are encouraging, suggesting the use of CHP as a therapeutic agent against oxidative stress-based neurodegeneration.

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Authors' address: Alba Minelli, Ph.D., M.D., Dipartimento Medicina Sperimentale Scienze Biochimiche, Sezione Biochimica Cellulare, Via del Giochetto, 06123 Perugia, Italy,
Fax: +39 075 585 7442, E-mail: aminelli@unipg.it