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Upregulation of Endogenous Neurotrophin Levels in the Brain by Intranasal Administration of Carnosic Acid

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Abstract

The potential of intranasally administered carnosic acid to enhance the endogenous levels of neurotrophins [nerve growth factor and brain-derived neurotrophic factor] in the brain was investigated. Hydroxypropyl- β -cyclodextrin (HP- β -CD) was used to enhance the aqueous solubility of carnosic acid. The effect of different concentrations of chitosan on the permeation of carnosic acid was investigated across the bovine olfactory mucosa using Franz diffusion cell setup. The formulations were administered [intranasal (i.n.)/subcutaneous route] in Sprague–Dawley rats, and the neurotrophins were sampled from the brain by microdialysis after the treatment period and measured by enzyme-linked immunosorbent assay. Phase solubility studies revealed that the solubility of carnosic acid was enhanced significantly with increase in the concentration of HP- β -CD. The neurotrophin levels were enhanced significantly upon i.n. administration of carnosic acid with chitosan, which was approximately 1.5–2-fold more over the parenteral route. Nose-to-brain delivery of carnosic acid along with chitosan is a potential approach for treating disorders associated with depletion of neurotrophins.

Keywords

carnosic acid; solubility; cyclodextrin; nose to brain; chitosan; NGF; BDNF; microscopy; microdialysis

INTRODUCTION

Neurotrophins are proteins, which play an important role in the survival and generation of neurons during development. Depletion of the endogenous neurotrophin levels is known to cause neurodegenerative disorders and several other central nervous system (CNS) disorders (schizophrenia, epilepsy, and Huntington disease) as well.^{1,2} Therefore, restoration of the

depleted neurotrophin levels is one of the potential approaches to treat neurodegenerative disorders.

Neurotrophic factors, nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF), are primarily synthesized in the hippocampus and basal forebrain regions of the brain.²⁻⁴ They play a vital role in promoting growth, differentiation, and function of both peripheral and central cholinergic neurons. Neurotrophic factors (NGF and BDNF) are hydrophilic, dimeric proteins of molecular weight of approximately 30 kDa.¹ Currently practiced methods (intracerebroventricular, intrathecal, and intraparenchymal) of treating neurodegenerative disorders are invasive.^{5,6}

One of the unique approaches of restoring the endogenous neurotrophin levels is to administer small molecules that can upregulate the expression of neurotrophins in the brain. It is a challenging task to identify the therapeutic molecules that are effective in inducing the expression of neurotrophins in the brain. The second challenging task is to deliver them into brain at effective levels so as to derive the benefit of their upregulation property. Several molecules that can successfully enhance the neurotrophin expression in cell culture experiments may fail *in vivo* due to their poor bioavailability at the target regions in the brain.

Carnosic acid [molecular weight (MW) ~330 Da, p.Ka 4.9] is a phenolic diterpene isolated from rosemary (*Rosmarinus officinalis*) leaves extract (Fig. 1). It has been reported to promote the synthesis of NGF in the human glioblastoma cells and enhance BDNF production in dopaminergic neuronal cell lines.⁷⁻⁹ In addition, the carnosic acid is already known for its neuroprotective effect, antioxidant, anti-inflammatory, antibacterial, anticancer, and antiplatelet activity.^{8,10-14}

The carnosic acid is poorly soluble in aqueous vehicles. Hydroxypropyl- β -cyclodextrin (HP- β -CD) was used because it has been reported to enhance the solubility of lipophilic drugs by forming inclusion and/or noninclusion complexes without changing their intrinsic ability to permeate lipophilic membranes.¹⁵ Moreover, HP- β -CD is reported to be safe as compared with other derivatives of β -CD.¹⁶ Hence, HP- β -CD was used to enhance the solubility of carnosic acid in this project.

The objective of the present study was to explore the plausibility of delivering carnosic acid to the brain *in vivo*, via nose-to-brain pathway, in turn to enhance the endogenous levels of neurotrophins (NGF and BDNF). Brain targeting of drugs by intranasal (i.n.) route is a safe and noninvasive method of drug delivery, which allows frequent administration and is certainly more patient compliant than the currently followed invasive routes of treating neurodegenerative and CNS disorders. Nose-to-brain pathway has been investigated by many research groups as an alternative, potential route for targeting drugs to brain via the olfactory pathway.¹⁷⁻¹⁹ However, the olfactory epithelial barrier limits the delivery of drugs via this pathway. Chitosan, one of the barrier modulating agents, was found to improve the delivery of drugs by safe and transient permeabilization of the olfactory mucosa.¹⁷

MATERIALS AND METHODS

Materials

Carnosic acid, Krebs ringer bicarbonate (KRB) buffer (premixed powder), phosphate buffered saline (PBS), and chitosan (MW 250 kDa) were procured from Sigma chemicals (St. Louis, Missouri). Acetonitrile and phosphoric acid were purchased from Fisher Scientific (Pittsburgh, Pennsylvania). HP- β -CD (mean degree of substitution 0.8) was procured from Roquette Pharma (Keokuk, Iowa). All solutions were prepared in deionized water. BDNF and NGF $E_{\max}^{\circ}$ ImmunoAssay systems were procured from Promega Corporation (Madison, Wisconsin). The bovine olfactory mucosa was purchased from Pel-Freez Biologicals (Rogers, Arkansas). Microdialysis probes (CMA 12) were procured from CMA Microdialysis Inc. (North Chelmsford, Massachusetts). The MW cutoff of the probes (membrane length 4mm, membrane diameter 0.5 mm) used in this study is 100 kDa.

Methods

Analytical Method—The amount of carnosic acid permeated across bovine olfactory mucosa (*in vitro*) was quantified by measuring the samples collected from the receiver compartment using high-performance liquid chromatography (HPLC) system (1525; Waters, Milford, MA) with an autosampler (717 plus; Waters) consisting of a Phenomenex C18 (2) 100 R analytical column (4.6 $\times$ 150 mm; Luna, 5.0 μ m, Torrance, CA) and a dual λ absorbance detector (2487; Waters). Mobile phase consisted of acetonitrile and 0.1% phosphoric acid solution (55:45, v/v).¹⁴ Elution was performed isocratically at 25°C at a flow rate of 1.5 mL/min. Injection volume was 100 μ L and the column effluent was monitored at 210 nm. The range for the calibration curve was 5–1000 ng/mL ($R^2 = 0.99$).

Phase Solubility Studies of Carnosic Acid—The phase solubility study was carried out to determine the stoichiometry according to the method reported by Higuchi and Connors.²⁰ An excess amount of carnosic acid was added to the solution of HP- β -CD prepared in KRB buffer at various concentrations (0.002–0.143 M). The contents were stirred for 24 h at room temperature. After equilibrium, the samples were filtered and analyzed by HPLC.

In Vitro Permeation Studies—The *in vitro* permeation studies of carnosic acid were carried out across bovine olfactory mucosa. The olfactory mucosa was sandwiched between two compartments of Franz diffusion cell (Logan Instruments, Somerset, New Jersey) such that the olfactory mucosa side is in contact with the upper donor compartment and the ventral side with the receiver compartment. The temperature of the chamber was regulated at $37 \pm 1^\circ\text{C}$ by water circulator. In both the donor and receiver compartments, Ag/AgCl electrode wires of 0.5 mm in diameter (obtained from Alfa Aesar, Ward Hill, Massachusetts) made in the form of concentric rings were placed 2 mm away from the olfactory mucosa. In order to determine the effect of chitosan on permeability status of the olfactory mucosa, the transolfactory epithelial electrical resistance (TEER) was monitored in presence and absence of chitosan using an electrical setup consisting of a wave form generator and multimeter (Agilent Technologies, Santa Clara, California). The initial TEER values were recorded by taking 500 μ L and 5 mL of KRB buffer with 20% HP- β -CD in the donor and receiver

compartments, respectively. The alternating current electrical resistance of the mucosa was measured by placing a load resistor (100 k Ω) in series with the mucosa. Voltage drop across the whole circuit and across the mucosa was measured using an electrical setup consisting of a waveform generator and multimeter (Agilent Technologies). For measuring the resistance, a small voltage of 100 mV was applied at 10 Hz and the olfactory epithelial resistance in k Ω cm² was approximated.¹⁷ The olfactory mucosa that had a resistance greater than 2k Ω cm² only was used for the permeation studies.

The donor and the receiver compartments were filled with carnosic acid solution (500 μ L of 1 mg/mL) and 5 mL of KRB buffer + 20% HP- β -CD, respectively. KRB buffer was used as the receiver medium in order to maintain sink condition and to keep the tissue viable throughout the study period.¹⁷ The effect of different concentrations of medium viscosity grade chitosan [0.1% or 0.25% or 0.5% (w/v)] on the permeation of carnosic acid across bovine olfactory mucosa was studied. Control set of experiments were run without incorporation of chitosan in the donor solution. Samples were collected at 1-h intervals for 6 h and analyzed by HPLC.

In Vivo Studies—Brain Microdialysis—*In vitro* calibration of microdialysis probes (CMA 12) was carried out according to the method reported by Vaka et al.¹⁷ *In vivo* studies were carried out in male, Sprague–Dawley rats (250–300 g; Harlan Company, Indianapolis, Indiana) under anesthesia [ketamine (80 mg/kg) + xylazine (10 mg/kg), intraperitoneal injection]. The experimental procedures were approved by the Institutional Animal Care and Use Committee at the University of Mississippi (Protocol #10-017). The rats were divided into five groups ($n = 3$). To the first four groups of rats, formulations were administered by i.n. route, whereas to the fifth group of rats, formulation was administered by subcutaneous (s.c.) route as stated below.

To group 1 rats, 0.25% (w/v) chitosan in HP- β -CD solution (vehicle control group, i.n. route); to group 2 rats, carnosic acid (4 mg/kg) in HP- β -CD solution (i.n. route); to group 3 rats, carnosic acid (2 mg/kg) in HP- β -CD solution with 0.25% (w/v) chitosan (i.n. route); to group 4 rats, carnosic acid (4 mg/kg) in HP- β -CD solution with 0.25% (w/v) chitosan (i.n. route); to group 5 rats, carnosic acid in HP- β -CD solution with 0.25% (w/v) chitosan by s.c. route (dose equivalent to that administered by i.n. route to group 4).

After 4 days of administration of different formulations to five groups of rats via i.n./s.c. route, the brain microdialysis was carried out by securing the rats in the stereotaxic frame (Harvard Instruments, Holliston, Massachusetts) and inserting the probe (CMA 12) into the hippocampal region (anterior–posterior = 5.6 mm, mediolateral = 5 mm, and dorsoventral = 7 mm from bregma).¹⁷ Intranasal formulations were administered to the rats by placing them on their back after giving mild anesthesia and inserting a microsyringe connected with a soft polymer capillary directly into posterior segment of the nose. The microdialysis probes were equilibrated by perfusing KRB buffer at the rate of 2 μ L/min, using a microinjection pump for a period of 1 h. The microdialysis samples were collected after 4 days of administration (i.n./s.c. route) of formulations and assayed for NGF and BDNF by enzyme-linked immunosorbent assay.

Microscopic Studies—In order to determine the toxicity of the formulations, rats treated with i.n. carnosic acid and/or chitosan solutions for 4 days were euthanized and the brains were collected and fixed for 16 h at 4°C using freshly made, prechilled (4° C) PBS-buffered 4% paraformaldehyde. The brains were then washed in PBS for 20 min for three times at room temperature, transferred into 65% ethanol (30 min), and finally transferred to 70% ethanol for storage. Brains were then trimmed and embedded in paraffin, sectioned, and stained with hematoxylin and eosin for routine histological examination. Tissue sections were evaluated histologically using an Olympus BX41 light microscope (Olympus America, Inc., Center Valley, Pennsylvania), and images were captured with an Olympus digital camera (model DP20; Olympus America, Inc.) using appropriate software supplied by the manufacturer.

Data Analysis—Statistical analysis was carried out using Graph Pad Prism 5 software (GraphPad Software, Inc., La Jolla, CA). The unpaired *t*-test/analysis of variance with Dunnett's multiple comparison test was selected as the test of significance, and *p* value less than 0.05 was considered as level of significance.

RESULTS AND DISCUSSION

Phase Solubility Studies of Carnosic Acid

The inherent aqueous solubility of carnosic acid was 25 ng/mL, whereas the solubility in case of KRB buffer was 3 µg/mL. Phase solubility studies revealed that the carnosic acid solubility was enhanced significantly with increase in concentration of HP-β-CD. The solubility of carnosic acid was enhanced to 0.009 M at 0.143 M HP-β-CD. However, the stoichiometry does not appear to reflect a covalent or insertion complexation between HP-β-CD and carnosic acid. Loftsson et al.^{21,22} demonstrated that the drug/cyclodextrin complexes can self-associate to form water-soluble aggregates/microaggregates of several drug/cyclodextrin complex units that eventually solubilize water-insoluble lipophilic drugs through noninclusion complexation. Carnosic acid-to-HP-β-CD ratio was 1:18, suggesting the formation of aggregates or micellar structures leading to enhanced solubility of carnosic acid (Fig. 2). Nevertheless, the solubility of carnosic acid was enhanced by approximately 900-fold at 0.143 M HP-β-CD over its inherent solubility in KRB buffer. Hence, it was decided to use 1:18 ratio of carnosic acid/HP-β-CD in further studies.

In Vitro Permeation Studies of Carnosic Acid Across Bovine Olfactory Mucosa

Bovine olfactory mucosa has been reported to be a closer model to human olfactory mucosa due to similarity in drug transport and metabolic pathways in nasal epithelium.²³ Chitosan enhanced the permeation of carnosic acid across the bovine olfactory mucosa by approximately threefold, approximately eightfold, and approximately sevenfold at 0.1%, 0.25%, and 0.5% (w/v) concentrations, respectively, over control (17.20 ± 6.67 µg/cm²; Fig. 3). Recently, chitosan was reported to lead to a significant drop in the electrical resistivity of the mucosa, indicating the interaction of the polymer with the mucosal membrane. Moreover, the electrical resistivity was also found to recover with time, suggesting that the permeabilization of the membrane by the chitosan is reversible. It is likely that the drop in electrical resistivity is due to opening of the tight junctions present in the membrane.¹⁷ Such

similar observations were found using chitosan in other biological membranes.^{24,25} As the amount of carnosic acid permeated with the use of 0.25% (w/v) chitosan was not significant statistically ($p > 0.05$) compared with that of 0.5% (w/v) chitosan, 0.25% (w/v) chitosan was used to develop i.n. formulation for *in vivo* studies.

***In Vivo* Delivery of Carnosic Acid**

Carnosic acid was reported to upregulate the expression of NGF and BDNF in the human glioblastoma and dopaminergic neuronal cell lines, respectively.^{7,9} It has been reported that the catechol portion of the carnosic acid plays an important role in stimulating NGF synthesis.^{26,7} However, the exact mechanism of BDNF elevation by carnosic acid is still unclear.

In most of the neurodegenerative disorders, the degeneration is localized in the hippocampus. Therefore, it would be appropriate to monitor the levels of neurotrophins in this region. Microdialysis is a minimally invasive technique of sampling therapeutic agents from the tissue fluids. The method allows sampling of drug from specific regions of the brain.^{27–30} Hence, microdialysis technique was employed in this study to sample the free neurotrophin (NGF and BDNF) levels in the hippocampus region of rat brain.

The recovery of microdialysis probes in case of NGF studies was $4.2 \pm 0.3\%$, whereas the recovery was found to be $2.98 \pm 0.2\%$ in studies involving BDNF. Upon i.n. administration of 0.25% (w/v) chitosan in HP- β -CD solution (vehicle control group) to group 1 rats, the endogenous NGF and BDNF levels were found to be 124.01 ± 28.06 and 206.91 ± 50.94 ng/L, respectively. The enhancement in the BDNF levels was about 50% in case of rats administered intranasally with carnosic acid (4 mg/kg) in HP- β -CD solution (group 2), which was significantly higher ($p < 0.05$) than the control group. On the contrary, there was no significant enhancement in the NGF levels in group 2 over group 1. This suggests that the threshold or effective concentration of carnosic acid to upregulate expression of significantly high levels of NGF is relatively higher than that of BDNF, which was obviously not attained with i.n. administration of only carnosic acid. In case of i.n. administration of carnosic acid solution (2 mg/kg) with chitosan (group 3), the NGF and BDNF levels in the rat hippocampus region were enhanced by approximately threefold and approximately twofold, respectively, over control group (Fig. 4). It is most likely that the chitosan in the formulations enhanced the bioavailability of carnosic acid, thus leading to a better biological response.

In order to study the effect of escalation of dose of carnosic acid on the upregulation of BDNF and NGF, 4 mg/kg dose of carnosic acid solution with chitosan was administered via i.n. route to group 4. Upon i.n. administration of 4 mg/kg carnosic acid solution with chitosan (0.25%, w/v), the NGF levels were enhanced by approximately 3.2-fold and the BDNF levels by approximately 2.7-fold as compared with the control group (Fig. 4). These findings were not in accordance with that reported by Kosaka and Yokoi,⁷ wherein they have observed an increase in NGF synthesis (~25-fold) in the human glioblastoma cell lines by carnosic acid in concentration-dependent manner (5–100 μ M). Additional mechanistic studies need to be carried out to explain the difference in dose-dependent response between *in vitro* and *in vivo* models. Till date, there are no reports regarding the mechanism of

absorption of carnosic acid across mucosal membranes and the nature of dose-dependent pharmacokinetics of carnosic acid. Hence, further studies on these lines would explain the differences observed between *in vitro* and *in vivo* results.

The systemic bioavailability of drugs is generally high following s.c. administration as compared with nonparenteral routes. However, the bioavailability of drugs into the brain may vary depending on physicochemical properties of drugs and the absorption mechanism across the blood-brain barrier. In this study, the levels of carnosic acid in the brain could not be quantified due to poor recovery ($1.1 \pm 0.8\%$) of carnosic by microdialysis, owing to its poor water solubility. However, when carnosic acid solution (4 mg/kg) was administered via s.c. route (group 5), there was again only a 50% increase in the BDNF and NGF levels in the brain compared with control group (Fig. 4). This suggests that the parenteral administration would also deliver effective levels of carnosic acid into brain. However, frequent administration and invasiveness limits the parenteral administration in patients.

Histological analysis of hematoxylin-and-eosin-stained brain sections from treated and control animals was also performed. In all animals, the brain was architecturally normal. Additionally, no cytopathologic abnormalities, that is, apoptosis/necrosis of neurons or glial cells, inflammatory infiltrates, or glial scars were found. Figure 5 depicts representative areas of the dentate gyrus of the hippocampus at low and high magnification. The tissues are architecturally and histologically normal. These studies demonstrate that the i.n. administration of formulations containing carnosic acid and/or chitosan has no adverse effects following frequent administration.

CONCLUSIONS

The novel findings of this study indicate that the nose–brain delivery of small molecular size therapeutic agents such as carnosic acid along with barrier-modulating agents (chitosan) will have a significant positive impact on the treatment of broad-spectrum neurodegenerative and CNS disorders. The brain bioavailability following parenteral administration appears to be poor. Therefore, the i.n. delivery strategy would circumvent the limitations associated with parenteral delivery. However, further studies demonstrating the safety of carnosic acid on the olfactory mucosa need to be performed to provide validity to the clinical applicability of carnosic acid i.n. formulations.

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Figure 1.
Structure of carnosic acid.

Figure 2. Phase solubility diagram of carnosic acid at different concentrations of hydroxypropyl-β-cyclodextrin. ($R^2 = 0.984$, $y = 0.0574x$). The data points provided are average of four trials and error bars represent standard error of mean.

Figure 3. Effect of different concentrations of chitosan (CH) on *in vitro* permeation of carnosic acid across the bovine olfactory mucosa. [-◆- Control, -●- 0.1% (w/v) CH, -▲- 0.25% (w/v) CH, -■- 0.5% (w/v) CH]. The data points provided are average of four trials and error bars represent standard error of mean.

Figure 4. Concentration of (a) nerve growth factor and (b) brain-derived neurotrophic factor in rat hippocampus after 4 days of administration of carnosic acid formulations by intranasal/subcutaneous route. The data points represent average values of three animals with standard error of mean as error bars.

Figure 5. Histology of the dentate gyrus of the hippocampus (20 X magnifications, bar = 500 μ m) (a) untreated (control), (b) carnosic acid (4 mg/kg) treated, and (c) carnosic acid (4 mg/kg) and chitosan (0.25%, w/v) treated. Histologically normal neurons and glial cells of the dentate gyrus of the hippocampus (400 X magnification, bar = 20 μ m) (d) untreated (control), (e) carnosic acid (4 mg/kg) treated, and (f) carnosic acid (4 mg/kg) and chitosan (0.25%, w/v) treated.