Carnosine activates the CREB pathway in Caco-2 cells

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Abstract Imidazole dipeptide, carnosine, is a versatile compound composed of β -Ala and L-His. A recent study showed that carnosine might benefit the treatment of Alzheimer's disease and the maintenance of cognitive function. Based on the observation that carnosine is immediately degraded by serum carnosinase, we hypothesized that carnosine improves brain function by promoting brain-gut interaction. This study sought to present possible mechanisms regulating carnosine-induced activation of brain-gut interaction. We had previously found that carnosine augmented the expression of BDNF in human colorectal cancer cells, thus we became interested in cAMP-responsive element binding protein (CREB), which is a dominant regulator of BDNF transcription. We found that carnosine activates CREB and CREB-related pathways by activating Ca^{2+} -related pathways. Our findings suggest that carnosine augments the expression of CREB-regulated genes in the intestine;

this augmentation contributes to the carnosine-induced activation of brain-gut interaction.

Keywords CREB pathway · Ca^{2+} regulation · BDNF · Brain-gut interaction · Carnosine

Introduction

Carnosine is an imidazole dipeptide composed of β -Ala and L-His, and has many functions including buffering (Davey 1960), free radical scavenging (Chasovnikova et al. 1990), enzyme regulation (Johnson and Aldstadt 1984), and sarcoplasmic reticulum Ca^{2+} regulation (Batrakova and Rubtsov 1997; Culbertson et al. 2010). Recent reports indicate that carnosine may benefit the treatment of Alzheimer's disease (Herculano et al. 2013; Hisatsune et al. 2015), suggesting that carnosine activates brain function. However, carnosine is immediately degraded into β -Ala and L-His by serum carnosinase (Boldyrev et al. 2013). Thus, activation of brain function by carnosine would be elicited not by direct delivery of carnosine into the brain, but rather by carnosine activation of a brain-gut interaction. This observation suggests that carnosine is worth studying in its role as a food ingredient that may activate brain-gut interaction pathways.

Motivated by a previous observation that carnosine augmented the expression of BDNF (Kadooka et al.

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2015), the present study focused on CREB, a dominant regulator of BDNF transcription, and evaluated whether carnosine activates CREB-related pathways.

Material and method

Cell line

The present study used the human colorectal cancer cell line Caco-2 (Riken Bioresource Center, Tsukuba, Japan). Caco-2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Nissui, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS; Life Technologies, Gaithersburg, MD, USA) at 37 °C in an atmosphere containing 5% CO₂.

Transfection

CREB-S133A (a dominant-negative CREB mutant gene) and pcDNA expression vector were transfected into Caco-2 cells respectively using the HilyMax reagent (Dojin, Kumamoto, Japan). After transfection, the cells were selected with 400 µg/mL G418 (Wako, Osaka, Japan).

Reagents

Carnosine was purchased from Wako (Osaka, Japan).

Quantitative reverse transcription polymerase chain reaction

Total RNA was isolated using TRIzol Reagent (Life Technologies), and cDNA was prepared using a ReverTra Ace kit (Toyobo, Osaka, Japan) according to the manufacturer's protocols. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed using a Thunderbird kit and a Thermal Cycler Dice Real Time System TP-800 (TaKaRa, Shiga, Japan). Gene expression levels were normalized to those of β-actin. The following PCR primers were employed: BDNF forward primer, 5'-GTCAAG TTGGGAGCCTGAAATAGTG-3', and reverse primer, 5'-AGGATGCTGGTCCAAGTGGTG-3'; human BDKRB1 forward primer, 5'-AGGAGGTCAGCAG GACAAGG-3', and reverse primer, 5'-CAGGAAGG CAAAGAAGTGGTAAG-3'; human SLN forward primer, 5'-TAAACACCCGGGAGCTGTTTCT-3', and

reverse primer, 5'-ATAGGACCTCACAAGGAGCC ACATA-3'; human ATF3 forward primer, 5'-GTG CCGAAACAAGAAGAAGG-3', and reverse primer, 5'-TATGCAGGTCTTCTGGACCC-3'; human CREB 3L3 forward primer, 5'-TGCGCCTGTACGAGTGTT CT-3', and reverse primer, 5'-GGGGCTTCCTGGA GACTCTT-3'; human GDNF forward primer, 5'-CC AACCCAGAGAATTCCAGA-3', and reverse primer, 5'-TTTCATAGCCCAGACCCAAG-3'; human IL-6 forward primer, 5'-AAGCCAGAGCTGTGCAG ATGAGTA-3', and reverse primer, 5'-TGTCCTGC AGCCACTGGTTC-3'; human β-actin forward primer, 5'-TGGCACCCAGCACAATGAA-3', and reverse primer, 5'-CTAAGTCATAGTCCGCCTAGAAGCA-3' (Kadooka et al. 2015).

Immunoblotting

Cell lysate was prepared using NP-40 lysis buffer (0.5% Nonidet P-40, 5 mM EDTA, 2 mM Na₃VO₄, 10 mM Tris-HCl at pH 7.6, 150 mM NaCl, 50 mM NaF, 5 µg/mL aprotinin, and 1 mM PMSF). Proteins (25 µg) were separated using 10% SDS-PAGE, and transferred to a PVDF membrane (GE Healthcare, Little Chalfont, UK). The membrane was probed with anti-Phospho-CREB antibody (#9198; Cell Signaling, Danvers, MA, USA) or anti-CREB antibody (#9192; Cell Signaling). Horseradish peroxidase-labeled anti-rabbit IgG antibody (GE Healthcare) was used as the secondary antibody. The proteins were detected using ImmunoStar LD (Wako), and visualized using an LAS-1000 Lumino Image analyzer (Fujifilm, Tokyo, Japan).

Results

Carnosine activates the CREB pathway by activating Ca²⁺-dependent pathways

As reported previously (Kadooka et al. 2015), carnosine augmented the expression of BDNF in Caco-2 cells (Fig. 1a). Transcription factor CREB regulates BDNF promoter activity via binding to the cAMP/Ca²⁺-response element (Pruunsild et al. 2011), suggesting that carnosine activates CREB in Caco-2 cells. Furthermore, previous results suggested that carnosine might increase Ca²⁺ sensitivity, and activate the Ca²⁺-dependent signaling pathways in intestinal epithelial cells, by augmenting the gene

Fig. 1 Carnosine activates the CREB pathway by activating Ca^{2+} -dependent pathways. The expression of endogenous BDNF (a), BDKRB1 (b), and SLN (c) were assessed using qRT-PCR. The results are expressed as means $\pm$ standard errors for 3 replicates. Statistical significance was defined at $P < 0.05$ (** $P < 0.01$; *** $P < 0.001$)

expression of calcium channel and calcium transport proteins (Lamont and Miller 1992; Perego et al. 2013; Kadooka et al. 2015). Here we used qRT-PCR to study expression of genes related to Ca^{2+} homeostasis such as bradykinin receptor B1 (BDKRB1), which is involved in the regulation of cytosolic Ca^{2+} concentration, and sarcolipin (SLN), which is a sarcoplasmic reticulum Ca^{2+} -ATPase, whose expression was revealed by microarray analysis to be changed upon carnosine treatment (Kadooka et al. 2015). Carnosine augmented the expression of both genes (Fig. 1b, c).

These results suggest that carnosine activates the CREB pathway by activating Ca^{2+} -dependent pathways.

Carnosine activates the CREB pathway

We tested whether carnosine activates the CREB pathway. As shown in Fig. 2a, b, carnosine activated CREB in Caco-2 cells. Next, we evaluated the effects of carnosine on the expression of CREB-related genes such as ATF3 and CREB3L3, whose expression was found by microarray analysis to be changed upon carnosine treatment (Kadooka et al. 2015). Carnosine significantly augmented the expression of both genes. These gene expression data indicate that carnosine activates the CREB pathway in Caco-2 cells.

Carnosine augmented the expression of CREB-regulated genes in Caco-2 cells

CREB regulates the transcription of many genes. Among them, expression of glial cell-derived neurotrophic factor (GDNF) and interleukin 6 (IL-6) were

Fig. 2 Carnosine activates the CREB pathway. Phosphorylated and total CREB were quantified by immunoblot analysis (a). Relative amount of phosphorylated CREB was determined by quantifying the band intensity using ImageJ (b). The expression levels of endogenous ATF3 (c) and CREB 3L3 (d) were assessed using qRT-PCR. The results are expressed as means $\pm$ standard errors for 3 replicates. Statistical significance was defined at $P < 0.05$ (*** $P < 0.001$)

Fig. 3 Carnosine augmented the expression of CREB-regulated genes in Caco-2 cells. The expression of endogenous GDNF (a) and IL-6 (b) were assessed using qRT-PCR. The effects of dominant-negative mutant of CREB (CREB-S133A) on the expression of carnosine-induced augmentation of GDNF (c) and IL-6 expression (d) were assessed. The expression of endogenous GDNF and IL-6 in Caco-2 cells transduced with mock (pcDNA) or CREB-S133A were assessed using qRT-PCR. The results are expressed as means $\pm$ standard errors for 3 replicates. Statistical significance was defined at $P < 0.05$ (***) $P < 0.001$

both augmented upon treatment with carnosine in Caco-2 cells (Fig. 3a, b). Thus, carnosine was found to activate the Ca^{2+} /CREB pathway and augment expression of their target genes in Caco-2 cells. We next used a dominant-negative CREB mutant (CREB-S133A) (Zhu et al. 2004) to test for CREB-dependency in the augmented expression of GDNF and IL-6 upon carnosine treatment. We found that CREB-S133A blocked carnosine-induced augmented expression of GDNF and IL-6 in Caco-2 cells, suggesting that carnosine-induced activation of CREB resulted in the augmentation of CREB-regulated genes in Caco-2 cells.

Discussion

A double-blind randomized controlled trial (Hisatsune et al. 2015) indicates that carnosine supplementation is

capable of preserving cognitive function in elderly people. However, carnosine is promptly degraded into β -Ala and L-His by serum carnosinase (Boldyrev et al. 2013), suggesting that activation of brain-gut interaction cannot be elicited by direct delivery of carnosine into the brain. We hypothesized that gut-derived secretory factors would constitute this carnosine-induced brain-gut interaction. We had previously found that carnosine augmented the expression of BDNF in Caco-2 cells (Kadooka et al. 2015).

Meta-analyses have found abnormally low serum BDNF levels in depressed patients; antidepressant treatment normalizes these levels (Molendijk et al. 2014). These observations suggest that serum BDNF level can proxy for brain function. Furthermore, BDNF crosses the blood–brain barrier (Ahlskog 2011), indicating that peripheral augmentation of BDNF may activate brain-gut interaction.

In the present study, we focused on the dominant BDNF transcriptional activator CREB (Pruunsild et al. 2011), and we found that carnosine activates CREB and CREB-related pathways. Carnosine-induced activation of CREB pathway triggered secretion of secretory factors functioning in the brain-gut interaction, which suggests a molecular basis for the carnosine-induced activation of brain-gut interaction. In light of microarray results (Kadooka et al. 2015) and results of the present study, we conclude that carnosine activates Ca^{2+} -related pathways and subsequent CREB-related pathways, although detailed molecular mechanisms await future study. Future study may also reveal molecular mediators between CREB-regulated secretory factors and the brain.

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