論文の内容の要旨

応用生命化学専攻
平成 17 年度博士課程　進学
氏　　名　孫　動玉
指導教員名　清水　誠

論文題目

Regulatory effect of carnosine on interleukin-8 production in intestinal epithelial cells
（腸管上皮細胞におけるカルノシンのインターロイキン-8 産生調節作用に関する研究）

Enhanced intestinal production of pro-inflammatory cytokines has been considered to contribute to the pathogenesis of inflammatory bowel disease (IBD) such as Crohn's disease and ulcerative colitis. In the course of inflammation, interleukin-8 (IL-8), a CXC chemokine, attracts and activates neutrophils at the site of infection, which may subsequently culminate into epithelial cell damage. Because the migration of neutrophils and their activation by IL-8 has the potential to perpetuate inflammation by producing reactive oxygen species (ROS), which are not only directly injurious but further increase IL-8 production, the down-regulation of IL-8 is vital in the prevention of inflammation.

Carnosine is a dipeptide composed of β-alanine and L-histidine, its physiological concentration in human skeletal muscle ranges from 2 to 25 mM. Carnosine has been reported to serve as a physiological buffer, divalent metal ion chelator, antioxidant and free radical scavenger. Several studies have demonstrated that carnosine inhibited the angiotensin-converting enzyme, delayed aging, and stimulated wound-healing. Since carnosine is supplied from normal meals like meat and also from dietary supplements through the intestines, the intestinal tissues are thought to be exposed to high concentration of carnosine. However, the functions of carnosine in the intestines have not previously been reported. In the present study, cell culture experiments with Caco-2 cells were therefore conducted to examine the hypothesis that carnosine regulates the secretion of inflammatory cytokines under

inflammatory conditions

The regulatory effects of carnosine on IL-8 secretion

The secretion of IL-8 was increased by H_2O_2, whereas the secretion of IL-1β, TNF-α, or IFN-γ was not significantly affected in Caco-2 cells. We examined whether or not carnosine could suppress the H_2O_2-induced IL-8 secretion in Caco-2 cells. A co-treatment with carnosine and H_2O_2 did not affect the level of H_2O_2-induced IL-8 secretion. The inhibitory effect of carnosine on the H_2O_2-induced IL-8 secretion was marked after a pretreatment with carnosine than by a simultaneous treatment with carnosine and H_2O_2[1]. However, cycloheximide, a protein synthesis inhibitor, had no effect on the inhibitory activity of carnosine, suggesting that *de novo* protein synthesis during the pretreatment was not required for the carnosine-mediated inhibition of IL-8 secretion. On the other hand, the increased uptake of carnosine that occurred during the preincubation did seem to be important. The competition experiment using Gly-Sar, a PepT1 substrate, indicated that a sufficient uptake and high intracellular content of carnosine were required to significantly inhibit IL-8 secretion under inflammatory conditions. In other words, the uptake of carnosine seems to have been the rate-limiting step in the inhibition of IL-8 secretion.

The inhibition of IL-8 secretion may have occurred at a various stages of protein synthesis such as transcription, translation and secretion. To test whether or not carnosine regulated the mRNA level, we determined the relative levels of IL-8 mRNA in the presence of carnosine. Interestingly, carnosine did not reduce the level of H_2O_2-induced IL-8 mRNA after a co-treatment or pretreatment with carnosine. We also examined whether carnosine inhibited another stimulus-induced IL-8 secretion through the same mechanism. Significant inhibition of IL-8 secretion by carnosine was observed in TNF-α-, cadmium- and humulene-treated Caco-2 cells without reducing the IL-8 mRNA level. These results suggest that carnosine did not inhibit the transcription of IL-8 gene.

Requirement of carnsoine structure for inhibition of IL-8 secretion

Using the component amino acids of carnosine and various histidine-containing dipeptides, we found that there was certain structural requirement for carnosine to show its unique inhibitory characteristic. The H_2O_2-induced IL-8 secretion in intestinal epithelial cell lines was markedly inhibited by a treatment with histidine while there was no inhibitory effect by β-alanine, suggesting that histidine is responsible for the carnosine action. The suppressive effect of histidine on IL-8 secretion was, however, mediated by inhibition of the transcriptional level, especially the inhibition of NF-κB activation[2]. These results indicate that the inhibitory mechanism of carnosine on IL-8 secretion differed from that of histidine, a component amino

acid. To examine whether other histidine-containing dipeptides would also show an inhibition pattern similar to carnosine, we investigated the effects of Gly-His, Ala-His, and anserine (β-Ala-1-methyl-His) on the H_2O_2-induced IL-8 secretion. These three peptides inhibited the H_2O_2-induced IL-8 secretion in a dose-dependent manner. They also reduced the H_2O_2-induced IL-8 mRNA expression, indicating that the anti-inflammatory mechanism for these histidine-containing dipeptides differed from that for carnosine. By the analysis of intracellular amino acids, we observed that Gly-His and Ala-His had been degraded to the component amino acids. It is therefore reasonable to think that the inhibitory effect of Gly-His and Ala-His on IL-8 secretion and mRNA expression could be attributed to histidine. Although anserine, a methylated derivative of carnosine, remained intact in the cells, the inhibitory mechanism for anserine also differed from that of carnosine. This result suggests that the structure of intact carnosine was required to inhibit IL-8 secretion without affecting the level of mRNA. Although we do not yet know how the carnosine structure is recognized and which molecule in the cells is involved in this recognition, the inhibitory effect of carnosine on IL-8 secretion was uniquely related to its structure.

Mechanism for the inhibitory effect of carnosine on IL-8 production

Several potential mechanisms could be proposed to explain this unique mechanism of carnosine. (1) carnosine may overwhelm the H_2O_2-induced IL-8 mRNA stabilization, (2) carnosine may down-regulate the translation of IL-8, (3) carnosine may promote IL-8 degradation, or (4) carnosine may inhibit IL-8 secretion through the Golgi complex. IL-8 is secreted after the cleavage of a signal peptide *via* common secretory apparatus involving the Golgi complex and small secretory vesicles. In this respect, the fourth proposed mechanism can be excluded, because the intracellular IL-8 concentration was significantly decreased in the presence of carnosine. The data for laser scanning confocal microscopy also supports carnosine inhibiting stimulus-induced IL-8 synthesis rather than the secretion of IL-8 through the Golgi complex. The data with the treatment of actinomycin D, a transcriptional inhibitor, show that there was no difference in the degradation rate of IL-8 mRNA in the presence or absence of carnosine, demonstrating that carnosine did not inhibit the stability of IL-8 mRNA in the Caco-2 cells. The inhibitory effect of carnosine on the IL-8 secretion without altering the expression and degradation of IL-8 mRNA suggests that carnosine exerts a direct effect at the translation or post-translational level. Translation is divided into three distinct phases: initiation, elongation, and termination. Among these, the most important step in the translational process is the binding of mRNA to ribosome (i.e. initiation). eIF4E is one of the main regulatory initiation factors and the activation of eIF4E is regulated by its abundance, by its phosphorylation state, and by its binding proteins (4E-BP). We found that carnosine

strongly inhibited H_2O_2-induced eIF4E phosphorylation. Carnosine also significantly inhibited the phosphorylation of Akt, which is the key event regulating the 4E-BP phosphorylation in the mTOR/FRAP pathway. The suppression of Akt phosphorylation by carnosine could inhibit a 4E-BP phophorylation and lead to the suppression of eIF4F complex formation, including eIF4E, eIF4A and eIF4G. These results suggest that the second proposed mechanism, in which carnosine is presumed to down-regulate the translation of IL-8, would be the most plausible.

Carnosine, a bioactive peptide found in most types of dietary meat, was tested for its ability to modulate immune reactions, specifically with respect to the IL-8 secretion in intestinal epithelial cells. Our results demonstrate that carnosine inhibited the stimulus-induced IL-8 production and secretion in the intestinal cells. This inhibition was not likely to have occurred at a transcriptional level, but down-regulation of the translation process *via* the decreased phosphorylation of eIF4E would have been involved in this inhibition[3].

Such a unique inhibitory mechanism of carnosine toward IL-8 secretion would effectively serve to suppress inflammation in the intestinal epithelium, providing a better effect by collaborating with other anti-inflammatory substances such as anserine and histidine which would inhibit IL-8 production at the transcriptional level. Although a more detailed investigation into the translational or protranslational level is required to elucidate the mechanism involved in this phenomenon, the results of this present study provide the first evidence for carnosine acting as an effective suppressor of the inflammatory response of intestinal epithelial cells to various stimuli. These results will help to provide practical intervention for inhibiting intestinal inflammation.

1. Son DO, Satsu H, Kiso Y, Shimizu M. (2004) Characterization of carnosine uptake and its physiological function in human intestinal epithelial Caco-2 cells. *Biofactors* 21; 395-398

2. Son DO, Satsu H, Shimizu M. (2005) Histidine inhibits oxidative stress- and TNF-alpha-induced interleukin-8 secretion in intestinal epithelial cells. *FEBS Letters* 579; 4671-4677

3. Son DO, Satsu H, Kiso Y, Totsuka M, Shimizu M. Inhibitory effect of carnosine on interleukin-8 production in intestinal epithelial cells through posttranscriptional regulation. *Cytokine* submitted