Effect of casein glycomacropeptide on subunit p65 of nuclear transcription factor-κB in lipopolysaccharide-stimulated human colorectal tumor HT-29 cells

Jianmiao Gong, Qingsen Chen *, Yali Yan, Guangchang Pang

Tianjin Key Laboratory of Food Biotechnology, College of Biotechnology and Food Science, Tianjin University of Commerce, Tianjin 300134, China

Abstract

Objective: The effect of casein glycomacropeptide (CGMP) on the expression of NF-κB subunit p65 in human colorectal cancer HT-29 cells induced by lipopolysaccharide (LPS) was investigated to explore the therapeutic efficacy of CGMP for human colorectal cancer.

Methods: HT-29 cells cultured in 96-well plates were stimulated with LPS for 30 min at the concentrations of 0, 0.001, 0.01, 0.1, 1 and 10 μg/mL. After stimulation, the expression of p65 in HT-29 cells was evaluated by immunofluorescence method. Similarly, HT-29 cells were incubated with 0, 0.001, 0.01, 0.1, 1, 10 and 100 μg/mL CGMP for 24 h at the optimal LPS concentration. After treatment with CGMP under the incubation with optimal LPS concentration for 30 min, the protein expression of p65 was analyzed by Western blotting. The optimum culture time was evaluated by incubating HT-29 cells with the optimal CGMP concentration for 6, 12, 24, 48 and 72 h.

Results: NF-κB-p65 revealed that the highest protein expression was achieved with 1 μg/mL LPS treatment. Meanwhile, CGMP could inhibit the protein expression of NF-κB-p65 in LPS-stimulated HT-29 cells, and the optimal inhibitory effect was observed at a CGMP concentration of 0.01 μg/mL with 48 h incubation.

Conclusion: CGMP can regulate NF-κB signaling pathway through inhibiting the expression of its subunit p65, which is beneficial for the further improvement of human colorectal cancer treatment.

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Keywords: Casein glycomacropeptide (CGMP); Human colorectal tumor cell HT-29; Lipopolysaccharide; p65

CGMP is a glycopeptide containing sialic acid discovered by Delfour in 1965 [1]. It is a polypeptide fragment of κ-casein (κ-CN, a unique sugar composition in CN) in milk. During the production process of cheese, rennet casein can hydrolyze the Phe-Met peptide bond of κ-CN in milk to generate insoluble sub-κ-CN (the 1–105 amino acid residual part of the peptide chain) and soluble polypeptide (106–169 amino acid residual part of the peptide chain). Such polypeptide containing a large number of carbohydrates is called glycomacropeptide. Correspondingly,

the glycomacropeptide from casein is named as casein glycomacropeptide (CGMP). During the past decade, CGMP, as a bioactive component, has attracted extensive attention due to its unique chemical and functional properties. Its most promising applications are to inhibit hemagglutinin of influenza virus, inhibit the secretion of gastric juice, promote the proliferation of *Bifidobacteria*, and modulate the response of immune systems [2–5]. In recent years, under the continuous support by the National Natural Science Foundation of China, our research group has systematically investigated the effect of CGMP on the regulation of the intestinal immune system, and the change of intestinal flora and intestinal inflammatory reactions. Our investigations have confirmed the anti-informatory activity of CGMP, which can improve, alleviate and cure inflammatory bowel diseases (IBD) to some extents [6]. However, the specific anti-inflammatory activity and corresponding clear mechanisms of CGMP still need further investigation to explore its effect on relevant immune molecules and immune signaling pathways, which will be beneficial for further elucidating the mechanism of IBD treatment.

NF-κB is discovered by Baltimore from Cancer Research Center of Massachusetts Institute and Rwiansen from

* Corresponding author at: Tianjin Key Laboratory of Food Biotechnology, College of Biotechnology and Food Science, Tianjin University of Commerce, Tianjin 300134, China. Tel.: +86 22 26667631; fax: +86 22 27725382.

E-mail address: chqsen@tjcu.edu.cn (Q. Chen).

Biomedical Research of Whitehead Institute at MIT [7] in 1986, as a transcription factor widely present in mammalian cells. It can bind to the specific site of a promoter or an enhancer to promote the transcription and expression of a variety of genes, which can regulate apoptosis, cell adhesion, cell proliferation, natural and adaptive immune response, inflammation, stress response and intracellular tissue remodeling processes [8–10]. The ubiquitous distribution of NF-κB in the body has gained more and more attention, and NF-κB has become an important treatment target of many diseases [11]. The nuclear translocation of p65 is the signal for NF-κB activation, and the initiation of physiological and pathological changes of NF-κB. The content of p65 in cells is extremely low, but it can contact with DNA extensively due to its high affinity to DNA. Therefore, p65 is highly efficient and accurate in recognizing target sites. In addition, nuclear transfer can be achieved through the dissociation of p65 and IκB without the requirement of new protein synthesis process. Thus it may induce fast gene transcription, and is involved in the transcriptional regulation of instant genes associated with stress defense responses [12]. Furthermore, CGMP plays an important role in the nuclear translocation of p65 during NF-κB activation or inhibition process. If CGMP inhibits the starting point of a series of cascade reactions, CGMP can subsequently terminate the pathophysiological processes.

1. Materials and methods

1.1. Chemicals and reagents

HT-29 cells were purchased from Qishi Biotechnology Co., Ltd., China. Fetal bovine serum (FBS) was ordered from Gibco, USA. DMEM medium with high glucose was purchased from HyClone Laboratories, Inc., USA. CGMP was ordered from Tatua Company, New Zealand. LPS was purchased from Sigma–Aldrich, USA. Nuclear and plasma protein extraction kits, BCA protein assay kit, $5\times$ protein sample buffer, horseradish peroxidase (HRP)-labeled goat anti-rabbit IgG, and fluorescein isothiocyanate (FITC)-labeled goat anti-rabbit IgG were purchased from Beijing Kangwei Century Biotechnology Co., Ltd., China. Rabbit anti-human polyclonal antibody p65 and rabbit anti-human LaminA polyclonal antibody were purchased from Abcam, USA. ECL chemiluminescent substrate hypersensitivity test kit was purchased from Millipore, USA. Other reagents were of analytical grade.

1.2. Human colon cancer HT-29 cells culture

HT-29 cells were cultured in DMEM medium with high glucose supplemented with 10% penicillin (100 U/mL) and streptomycin (100 U/mL). The cells were cultured at CO_2 incubators (HERAcell 240i, Thermo Fisher Scientific, USA,) with 37 °C and 5% CO_2. The cell culture medium was changed every two days. After cells were seeded in 96-well plates or cell culture dishes, the cell density was adjusted to 10^6 cells/mL for future experiments.

1.3. Study design

After the cells grown to sub-confluence stage, serum-free synchronization was conducted for 6 h. The experiment was conducted into two parts.

Part A: eight groups include a control group (N group), a LPS group (L group), and six CGMP groups with different CGMP concentration (C1: 0.001 μg/mL, C2: 0.01 μg/mL, C3: 0.1 μg/mL, C4: 1 μg/mL, C5: 10 μg/mL, and C6: 100 μg/mL). The cells were cultured at 37 °C with 5% CO_2 for 24 h. Except for the control group, the cells were incubated with LPS at 37 °C with 5% CO_2 for 30 min. Cells were then rinsed with PBS buffer 2 times (5 min each time) prior to digestion with 0.25% trypsin for 2 min. After the termination of digestion, the cells were collected and counted after centrifugation at 4 °C and 1000 rpm/min.

Part B (five groups): the cells were treated with the optimum CGMP dose at 37 °C and 5% CO_2 for 6, 12, 24, 48 and 72 h and processed similarly as described above.

1.4. Protein expression of p65 evaluated by immunofluorescence

Cells were seeded in 96-well microplates containing DMEM culture medium with high glucose supplemented with 10% FBS. After the cell population reached 60%–70% of the well (in logarithmic phase), the serum-free medium was used for synchronized culture for 6 h. The experiment was divided into six groups including control group (Group B) and five LPS concentration groups (L1: 0.001 μg/mL; L2: 0.01 μg/mL; L3: 0.1 μg/mL; L4: 1 μg/mL; and L5: 10 μg/mL). After the LPS treatment for 30 min, the cells were washed with PBS buffer 3 times for 5 min each time. Then, the cells were fixed with chilled 4% formaldehyde for 10 min, and washed with PBS buffer 3 times for 5 min each time. The cells were blocked with PBS buffer containing 0.3% Triton X-100, 2% BSA and 2% goat serum for 1 h at room temperature. The treated cells were incubated with primary antibody of p65 at 37 °C for 1 h, followed by PBS buffer washing for 3 times (5 min each time). Sequentially, the p65 was probed with Cy3-labeled secondary antibody at 37 °C for 1 h, and then the probed cells were washed with PBS buffer 3 times (5 min each time). The fluorescence intensity of the mixture was determined by a fluorescence microplate reader (SpectraMax M5; Molecular Devices, USA) with the excitation wavelength of 488 nm and the emission wavelength of 525 nm.

1.5. Expression of p65 protein in cells evaluated by Western blotting

1 mL protein extraction reagent containing phosphatase inhibitor was added to 10^7 cells, and the mixture was incubated on ice for 15 min. The supernatant was collected after centrifugation at 4 °C and 12,000 r/min for 20 min. The protein concentration was first determined by BCA protein assay before sample loading. 40 μL nuclear protein solution was mixed with $5\times$ sample buffer at a volume ratio of 1:4. The protein was denatured by boiling for 5 min, and cooled down to room

temperature. The electrophoresis was conducted at a voltage of 80 V using 5% polyacrylamide stacking gel and at 100 V using 12% separating gel. Upon the completion of electrophoresis, the protein was transferred to PVDF membrane (Millipore, USA). The PVDF membrane was blocked in 5% skim milk at room temperature for 1 h. Sequentially, the protein on the PVDF membrane was probed with rabbit anti-human p65 antibody at a dilution ratio of 1:1000 at room temperature for 2 h. The PVDF membrane was washed with PBS-T buffer 4 times for 5 min each time. After incubating with primary antibody, the PVDF membrane was targeted with peroxidase-labeled goat anti-rabbit IgG antibody at a dilution ratio of 1:5000 at room temperature for 1 h. Finally, ECL color reaction was conducted for gel imaging analysis. The target protein bands and reference protein bands were analyzed by Quantity One gel imaging software.

1.6. Statistical analysis

The experimental data were analyzed using SPSS 17.0 statistical software through variance analysis. The results were expressed as mean ± standard deviation ($\bar{x} \pm s$). The significant difference was considered at $P < 0.05$.

2. Results

2.1. Fluorescence intensity changes of p65 in HT-29 cells under the stimulation of LPS

The green fluorescence of FITC was excited at the wavelength of 488 nm, and relative fluorescence units (RFUs) were detected using a multifunctional plate reader with dual-grating full wavelength scanning. The fluorescence intensity of p65 in HT-29 cells from each group is shown in Fig. 1. Compared with the control group, the fluorescence intensity of p65 revealed an initial increase and a final decrease with the increase of LPS dosage. Although the fluorescence intensity of p65 exhibited a final reduction, the fluorescence intensity of p65 in LPS groups was higher than that in the control group. Among the LPS groups, the fluorescence intensity of p65 from L2 group (0.01 μg/mL), L3 group (0.1 μg/mL) and L4 group (1 μg/mL) revealed a very significant increase ($P < 0.01$), as well as a significant increase in L5 group (10 μg/mL) ($P < 0.05$).

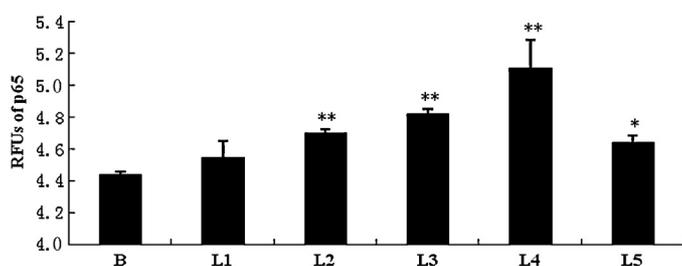Fig. 1. Effect of LPS on RFUs of p65. *Note*: B: blank control group; L1: 0.001 μg/mL LPS group; L2: 0.01 μg/mL LPS group; L3: 0.1 μg/mL LPS group; L4: 1 μg/mL LPS group; and L5: 10 μg/mL LPS group; *difference from the blank control group ($P < 0.05$); **significant difference ($P < 0.01$).
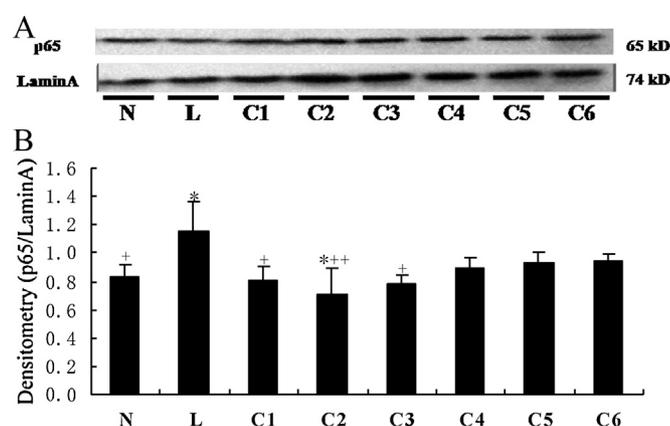

Fig. 2. Effect of CGMP on the protein expression of p65. (A) Western blot of p65 and LaminA in the control group; (B) densitometry of p65/LaminA. *Note*: N: control group; L: LPS group; C1: 0.001 μg/mL CGMP group; C2: 0.01 μg/mL CGMP group; C3: 0.1 μg/mL CGMP group; C4: 1 μg/mL CGMP group; C5: 10 μg/mL CGMP group; C6: 100 μg/mL CGMP group; *difference from blank control group ($P < 0.05$); **significant difference ($P < 0.01$); +difference from LPS group ($P < 0.05$); ++significant difference ($P < 0.01$).

2.2. Effect of CGMP dosages in protein expression of p65

Effect of CGMP on the protein expression of p65 is shown in Fig. 2. Compared with the control group, the content of p65 protein in HT-29 cells from LPS groups revealed a significant enhancement ($P < 0.05$). On the other hand, the protein expression of p65 in CGMP groups exhibited an initial decrease and a final increase. Among these CGMP groups, the protein expression of p65 in HT-29 cells from C2 revealed the most obvious inhibition ($P < 0.05$). Compared with the LPS group, C1, C2 and C3 revealed a significant reduction in expression of p65 protein under the stimulation of LPS ($P < 0.05$), which was obviously lower than that in the control group. Although the protein expression of p65 in C4, C5 and C6 revealed a reduction, it was still higher than that in the control group without significant difference.

2.3. Effect of treatment duration on the protein expression of p65

The effect of treatment duration on the expression of p65 with the optimal CGMP dose is shown in Fig. 3. The expression of p65 revealed an increase as the extension of treatment duration and reached the maximum after 12 h treatment; however, the expression of p65 exhibited a decreasing trend after 12 h, and reached the lowest level after the treatment of 48 h. Cells treated with CGMP for 12 h showed the highest expression of p65, which was very significantly different compared with other four groups ($P < 0.01$). In contrast, cells treated with CGMP for 48 h showed the lowest expression level of p65, which was very significantly different compared with the that in the cells treated for 6, 12 and 72 h ($P < 0.01$), but it showed no significant difference compared with that in the cells treated for 24 h. As the extension of CGMP treatment time, 72 h treatment revealed the increase in the expression of p65 again, which exhibited an obvious enhancement when compared with the treatment for 24

A

p65   65 kD
LaminA   74 kD

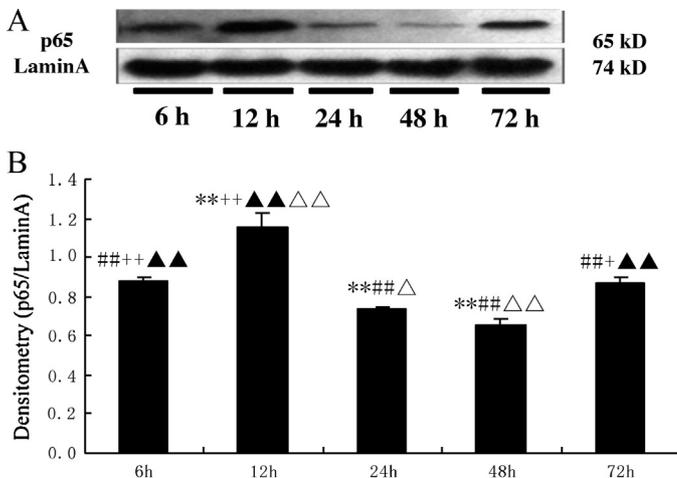**6 h   12 h   24 h   48 h   72 h**

B

Fig. 3. Effect of treatment duration on p65 expression in the presence of CGMP. (A) Western blot of p65 and LaminA in control group; (B) densitometry of p65/LaminA. *Note*: *Difference from 6 h group ($P < 0.05$); **significant difference ($P < 0.01$); #difference from 12 h group ($P < 0.05$); ##significant difference ($P < 0.01$); +difference from 24 h group ($P < 0.05$); ++significant difference ($P < 0.01$); ▲difference from 48 h group ($P < 0.05$); ▲▲significant difference ($P < 0.01$); △difference from 72 h group ($P < 0.05$); △△significant difference ($P < 0.01$).

and 48 h ($P < 0.05$), but was still significantly lower than the expression of p65 in cells with 12 h CGMP treatment ($P < 0.01$).

3. Discussion

CGMP, as a novel biologically active functional factor, has been confirmed to exert many biological functions which make it particularly suitable for functional health products. The in-depth exploration of the functional activity of CGMP will not only improve the utilization of milk resources, but also further explore its more potential value. Currently, health-promoting roles of CGMP have been discussed from different respects [13,14]; however, its mechanisms of the action are not discussed and explored. Foods can not only provide the nutrition and energy for life events, but also can adjust cytokine network through regulating immune network, message-passing network and metabolic network, thus regulating the health of the body and life events correspondingly [15,16]. The activity of NF-κB is strictly regulated by multiple factors due to its role as a transcription factor. The cells can only survive and play their physiological functions by maintaining the homeostasis of NF-κB activity. Previous studies have demonstrated that persistent activation of NF-κB exists in a number of disease statuses, while the reduced activity of NF-κB is observed in some other autoimmune diseases. The disturbed homeostasis, i.e., the excessive activation or inhibition of NF-κB, will cause physiological disorders or lead to diseases such as rheumatoid arthritis, atherosclerosis, multiple sclerosis and asthma inflammation, as well as cancers including prostate cancer, glioma and lymphoma [8–10]. Thus it is important to maintain the balance of NF-κB activity. In most of the cells at the resting state, the dimer subunit of NF-κB in cell cytoplasm can bind with its inhibitor (inhibitor of NF-κB, IκB) to form a trimeric complex of p65–p50–IκB. Upon the stimulation

by pathogens, stress, cytokines, oxygen radicals, oncoproteins and ultraviolet light, IκBs were degraded by phosphorylation or ubiquitination. The inhibition of the p65–p50 dimer by IκBs will be released quickly and nuclear translocation will occur, leading to initiate or promote the transcription of certain genes [17]. Requena et al. [18] have demonstrated that CGMP at high concentrations may activate the NF-κB signaling pathway, and that bovine casein glycomacropeptide (BGMP) in the gut may exert an anti-inflammatory effect. Therefore, to explore the mechanisms of physiological functions, we have investigated the protein expression levels of p65 in LPS-stimulated HT-29 cells through CGMP intervention at low concentrations.

In the present study, the activation of NF-κB signaling pathway in the presence of LPS was determined by immunofluorescence, which revealed that the expression of p65 was increased with LPS in a dose-dependent manner. However, the fluorescence intensity of p65 revealed an initial increase and a final decrease with the increase of LPS doses, with the most effective dose at 1 μg/mL. LPS may enter the intestinal tract during infection, thereby activating monocytes, macrophages and endothelial cells to synthesize and release various cytokines and inflammatory mediators. This thus leads to increased vascular permeability, fluid exudation and lymphocyte migration to inflammation sites, a defensive reaction of the body to remove pathogens and recover the body. Excessive defensive reactions can cause hardly controlled inflammatory cascades or serious disorders in immune functions [19]. Therefore, LPS was applied to activate NF-κB signaling pathway in our study. It can be seen from the Western blotting results that CGMP at the six concentrations could inhibit the expression of p65 in HT-29 cells after LPS stimulation, especially the low concentrations (0.001, 0.01 and 0.1 μg/mL). Although high concentrations (1, 10 and 100 μg/mL) of CGMP had inhibitory effect on the expression of p65, it was still higher than that in the control group. Requena et al. [18] have employed monocytic leukemia cells (THP-1) as a model. The cells are cultured in complete medium containing BGMP at a gradient concentration of 0.01–10 mg/mL for 24 h. WB detection has confirmed the phosphorylation of IκBα32 at the serine sites and protein expression of p50 and p65 at different time points. Results have revealed that BGMP can activate NF-κB. One hour after addition of BGMP, the highest expression of p-IκBα, p50 and p65 was observed, which is contrary to our experimental results. Therefore, the correlation between CGMP intake and its immunomodulatory effects needs to be further explored. During the investigation of optimum CGMP treatment duration, the expression of p65 revealed an initial enhancement and a final reduction with the extension of treatment duration, with the highest expression level occurring after treatment for 12 h and the lowest expression level after treatment for 48 h. Therefore, longer treatment duration does not mean higher suppression efficiency on p65. Various cytokines are released with the growth of cells, which may interact with CGMP to reduce its suppression effect on p65.

In summary, CGMP at low dose levels can significantly inhibit the expression of p65 in the NF-κB signaling pathway, which may serve as a target for treatments of colorectal cancer and IBD. This study also provides a theoretical support for

the application of CGMP in the field of functional health products as well as a scientific reference for nutrition therapies of colorectal cancer. However, the interactions of CGMP and other proteins such as 26S, ubiquitin proteasome and NEMO in the NF-κB signaling pathway need to be further explored to provide a more complete theoretical basis to illustrate the mechanisms of its anti-inflammatory and anti-cancer activity.

4. Conclusion

Based on the fluorescence intensity changes of p65 in this study, LPS at concentration of 0.01, 0.1 and 10 μg/mL can significantly activate NF-κB signaling pathway ($P < 0.05$) and stimulate the expression of p65. The maximum express was achieved at a LPS concentration of 1 μg/mL and treatment duration of 30 min ($P < 0.01$). CGMP with the tested doses can all inhibit the expression of p65 in LPS-stimulated HT-29 cells, with the optimum concentration of 0.01 μg/mL and treatment duration of 48 h ($P < 0.05$). Therefore, CGMP can inhibit the activation of NF-κB signaling pathway by inhibiting the expression of p65, which provides a theoretical basis for the improvement and treatment of colorectal cancer triggered by IBD through the regulation of NF-κB signaling pathway.

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