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Activation of AMPK by chitosan oligosaccharide in intestinal epithelial cells: Mechanism of action and potential applications in intestinal disorders

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ABSTRACT

Chitosan oligosaccharide (COS), a biomaterial derived from chitin, is absorbed by intestinal epithelia without degradation and has diverse biological activities including intestinal epithelial function. However, the mode of action is still unclear. This study aimed to investigate the effect of COS on AMP-activated protein kinase (AMPK) in intestinal epithelial cells (IEC) and its potential applications in the intestinal diseases benefited from AMPK activation. COS with molecular weights (MW) from 5,000 Da to 14,000 Da induced AMPK activation in T84 cells. That with MW of 5,000-Da was the most potent polymer and was used in the subsequent experiments. COS also activated AMPK in other IEC including HT-29 and Caco-2 cells. Mechanism of COS-induced AMPK activation in T84 cells involves calcium-sensing receptor (CaSR)-phospholipase C (PLC)-IP_3 receptor channel-mediated calcium release from endoplasmic reticulum (ER). In addition, COS promoted tight junction assembly in T84 cells in an AMPK-dependent manner. COS also inhibited NF-κB transcriptional activity and NF-κB-mediated inflammatory response and barrier disruption via AMPK-independent mechanisms. Interestingly, luminal exposure to COS suppressed cholera toxin-induced intestinal fluid secretion by ~30% concurrent with AMPK activation in a mouse closed loop model. Importantly, oral administration of COS prevented the development of aberrant crypt foci in a mouse model of colitis-associated colorectal cancer (CRC) via a mechanism involving AMPK activation-induced β-catenin suppression and caspase-3 activation. Collectively, this study reveals a novel action of COS in activating AMPK via CaSR-PLC-IP_3 receptor channel-mediated calcium release from ER. COS may be beneficial in the treatment of secretory diarrheas and CRC chemoprevention.

1. Introduction

AMP-activated protein kinase (AMPK), a heterotrimeric protein composed of α, β and γ subunits, is an energy sensor that controls energy homeostasis at both cellular and whole-body levels [1]. In response to increased ADP/ATP ratio or increased intracellular calcium concentration ($[Ca^{2+}]_i$), AMPK activity is enhanced through phosphorylation at threonine-172 (Thr-172) of AMPK-α subunit by liver kinase B1 (LKB1) or calcium/calmodulin-dependent protein kinase kinase β (CaMKKβ), respectively [1]. Upon its activation, AMPK phosphorylates target proteins and modulates their functional activities, leading to stimulation of energy-production processes and inhibition of energy-utilizing processes [1].

Apart from its role in controlling energy balance, AMPK regulates epithelial functions including tight junction assembly and ion transport [2–4]. For instance, AMPK promotes an assembly of tight junction proteins to apical junctional complexes in epithelial cells [5,6]. In addition, AMPK phosphorylates CFTR Cl^- channel, leading to suppression of CFTR channel activity [7]. Located in the apical membrane of intestinal epithelial cells

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(IEC), CFTR mediates cAMP-induced Cl^- secretion. Hence, overstimulation of the CFTR-mediated Cl^- secretion by enterotoxins (e.g. cholera toxin or CT) plays important roles in providing a driving force for intestinal fluid secretion in secretory diarrheas [8]. AMPK activators including AICAR and metformin abrogated CT-induced fluid secretion in excised mouse intestinal loops [9]. Interestingly, AMPK activators retarded growth of colorectal cancer (CRC) cell lines and prevented colitis-associated carcinogenesis in mice by inducing caspase-3 cleavage-mediated apoptosis of cancer cells [10–12]. Of particular interest, AMPK activation in human intestinal tissues is associated with superior prognosis in CRC patients [13]. Therefore, AMPK has been proposed as a drug target for CRC chemoprevention [14].

Chitosan oligosaccharide (COS) is a degradation product of chitosan, a polymer of β-(1-4)-linked D-glucosamine, derived from deacetylation of chitin found in exoskeletons of shrimps, crabs and insects. Due to its water solubility, biocompatibility, intestinal absorbability, and bioactivity, COS has received considerable interest for potential applications as dietary supplements or nutraceuticals [15]. To date, COS possesses a variety of biological activities such as anti-oxidative, anti-inflammatory and anti-bacterial activities [15]. After ingestion, COS is not digested by either gastrointestinal enzymes or gut floras, but is readily absorbed through intestinal epithelium [16,17]. Therefore, it is crucial to investigate the effects of COS on IEC, which is directly exposed to ingested COS and plays pivotal roles in health and diseases. Indeed, COS has recently been shown by our group to alleviate inflammation and its associated damages in mouse models of inflammatory bowel disease through suppression of nuclear factor kappa B (NF-κB)-mediated inflammatory responses in IEC [18]. However, the effect of COS on AMPK, which is an important regulator of intestinal epithelial functions, has not yet been reported. Therefore, this study aimed to investigate the effects of COS on AMPK activity and the underlying mechanisms in IEC. Furthermore, COS was evaluated for its effect on barrier function and potential application in the treatment of secretory diarrheas and in the chemoprevention of CRC.

2. Materials and methods

2.1. Materials

T84, HT-29 and Caco-2 cells were obtained from American Type Culture Collection (Manassas, VA, USA). Cholera toxin (CT) was from List Biological Laboratories, Inc. (Campbell, CA, USA). Fetal bovine serum (FBS) and culture media were from Thermo Fisher Scientific Inc. (Waltham, MA, USA). Other chemicals were from Sigma–Aldrich Co. (Saint Louis, MO, USA).

2.2. Preparation of chitosan oligosaccharides

Chitosan oligosaccharides (COS) with molecular weight (MW) of 5000 Da, 8000 Da, and 14,000 Da, and chitosan with MW of ~100,000 Da (all at the degrees of deacetylation >90%) were prepared according to the protocol used in the previous study [18]. Molecular weight of COS was defined by gel permeation chromatography. Degree of deacetylation of COS was defined by UV spectroscopic method. COS was dissolved in 1% acetic acid.

2.3. Cell culture

T84 cells were cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's F-12 medium included with 5% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin (Life Technologies, Carlsbad, CA, USA). Caco-2 and HT-29 cells were cultured in DMEM supplemented with 10% FBS, 1% non-essential amino acids, 100 U/mL penicillin and 100 μg/mL streptomycin. Cells were maintained in a humidified 95% O_2/5% CO_2 atmosphere at 37 °C.

2.4. Western blot analysis

Cells were seeded on 6-well plates at a density of 1×10^6 cells/well (Corning Life Sciences, Tewksbury, MA, USA). After treatments, cell lysates or mouse tissue lysates were harvested using lysis buffer. Protein concentrations in cell lysates were measured by Lowry method. Thirty-microgram proteins were separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) before transferring to nitrocellulose membrane. The membrane was blocked for 1 h with 5% non-fat dried milk (Bio-Rad, Hercules, CA, USA), and incubated overnight with rabbit antibodies to phospho-AMPK (Thr-172), AMPKα, phospho-acetyl Co-A carboxylase (Ser-79) (p-ACC), acetyl Co-A carboxylase (ACC), inducible nitric oxide synthase (iNOS), cyclooxygenase 2 (COX-2), β-catenin, cleaved caspase-3, or β-actin (Cell Signaling Technology, Boston, MA, USA). The membrane was then washed for 4 times with Tris-Buffered Saline Tween-20 (TBST) and incubated for 1 hour at room temperature with horseradish peroxidase-conjugated goat antibody to rabbit immunoglobulin G (Cell Signaling Technology, Boston, MA, USA). The signals were detected using Luminata Crescendo Western HRP Substrate (Merck Millipore, Billerica, MA, USA). Densitometry analysis was performed using Image J software (version 1.46r, National Institute of Health, Bethesda, MD, USA).

2.5. Determination of ADP/ATP ratio

ADP/ATP ratio was determined using the ADP/ATP Ratio Assay Kit (bioluminescent, ab65313, Abcam, Cambridge, MA, USA). Briefly, T84 cells (5×10^3 cells/well) were cultured for 24 h in 96-well plates before 24-h treatment with COS (100 μg/mL), metformin (5 mM) or vehicle. Cell lysates were determined for ADP/ATP ratio according to the manufacturer's instructions. Luminescence was detected using a Wallac Victor^2 microplate reader (PerkinElmer, Waltham, MA, USA).

2.6. Intracellular calcium measurement

T84 cells were harvested and incubated for 1 h at 37 °C with 1 mM indo-1 (Life Technologies, Carlsbad, CA, USA). Cells were then washed with fresh buffer containing 0.441 mM KH_2PO_4, 5.33 mM KCl, 4.17 mM $NaHCO_3$, 5.56 mM D-glucose, 137.93 mM NaCl, 0.338 mM Na_2HPO_4, 1 mM $CaCl_2$ and 1% (w/v) BSA, or the buffer without $CaCl_2$ for 3 times. The mean fluorescence intensity ratio between Ca^{2+}-bound indo-1 (excitation wavelength of 338 nm, emission wavelength of 405 nm) and Ca^{2+}-free indo-1 (excitation wavelength of 338 nm, emission wavelength of 490 nm) was detected by an FP-6200 spectrofluorometer (JASCO, Essex, UK).

2.7. Assay of NF-κB transcriptional activity

T84 cells (2×10^5 cells/well) were seeded and grown overnight on a 24-well plate before 24-h incubation in Opti-MEM (Life Technologies, Carlsbad, CA, USA) containing NF-κB GFP reporter (Qiagen Inc., Valencia, CA, USA) and Lipofectin Transfection Reagent (Life Technologies, Carlsbad, CA, USA) (at the ratio of 50:1:3.33). Cells were then incubated for 6 h in DMEM/F-12 media containing vehicle, TNFα (10 ηg/mL), TNFα (10 ηg/mL) plus COS (100 μg/mL) with or without compound C (80 μM), or compound C (80 μM) alone. Cells were trypsinized and determined for GFP