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Molecular Mechanisms of Natural Honey Against *H. pylori* Infection Via Suppression of NF-κB and AP-1 Activation in Gastric Epithelial Cells

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Background and Aims. Natural honey has been used as a medicine since ancient times. Honey is widely known for its antibacterial properties against *H. pylori*; however, the mechanisms of its antibacterial activity are not fully known. The present study was performed to examine the molecular mechanisms by which natural honey can inhibit *H. pylori* infection in gastric epithelial cells.

Methods. Electrophoretic mobility shift assay was used to measure NF-κB- and AP-1-DNA binding activity. Western blotting was used to detect IκB-α and COX-2 expression.

Results. H. pylori induced NF-κB and AP-1 DNA-binding activity in gastric epithelial cells. Manuka honey inhibited *H. pylori*-induced NF-κB and AP-1 in a time- and dose-dependent manner. Maximum inhibition of *H. pylori*-induced NF-κB and AP-1 by manuka honey was observed at concentrations of 20% at 1–2 h. Pre-treatment of AGS cells with other commercial natural honeys also inhibited *H. pylori*-induced NF-κB and AP-1 DNA-binding activity. Honey prevented *H. pylori*-induced degradation of IκB-α protein and downregulated COX-2 protein levels.

Conclusions. Our findings suggest that natural honey exerts its inhibitory effects against *H. pylori* by inhibiting NF-κB and AP-1 activation and downregulation of COX-2 expression. These results provide new mechanistic insights into honey effects in the suppression of *H. pylori* infection. © 2016 IMSS. Published by Elsevier Inc.

Key Words: Natural honey, *H. pylori*, NF-κB, AP-1, Gastric epithelial cells.

Introduction

Helicobacter pylori infects over half of the population worldwide (1,2). This pathogen initiates an inflammatory and immune response within the gastric mucosa, which subsequently leads to the activation of cell signaling pathways causing mucosal inflammation and cancer development (3–5). The induction of the host immune response elicits the production of pro-inflammatory proteins such as transcription factors, cytokines and adhesion molecules (6,7). *H. pylori* was reported to induce transcription factors such as NF-κB and AP-1 that regulate inflammation and signalling cascades leading to carcinogenesis (8,9).

Treatment of *H. pylori* infection has become a challenge in recent years. Successful treatment with antimicrobial agents is successful in 80–90% of patients and can lead to regression of *H. pylori*-associated diseases. However, the high rate of *H. pylori* infection in developing countries, patient incompliance and antibiotic resistance against *H. pylori* merits extensive investigations (10,11). Antibiotic resistance in *H. pylori* is a growing global concern that necessitates the search for novel therapeutic agents. Several new treatment alternatives have been introduced to overcome treatment failure. Several studies around the world have shown promising activities of natural remedies against *H. pylori* in *in vitro* and *in vivo* studies (12–17).

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One of these dietary natural products is honey, a natural substance formed from nectar by honeybees. Natural honey is used extensively as a health drink worldwide since ancient times. The pharmacologically active molecules in honey are flavanoids, phenolic acids and their esters, vitamins, trace elements, amino acids and proteins as well as certain enzymes including glucose oxidase, invertase and catalase (18). The revelation in the Holy Koran and documentation in the Hadith clearly referred to the effectiveness of honey in the healing of diseases for mankind (19). Honey has been the focus of research in recent years to modulate inflammation, microbial infection and cancer (20−26).

Research studies have revealed the promising effects of honey as a non-antimicrobial approach for reducing *H. pylori* infection (15,17,27,28). There have been a number of reports suggesting that honey can inhibit *H. pylori* growth in *in vitro* studies (19,29−33). The exact mechanism of the inhibition of *H. pylori* by honey is not yet well defined. The aim of the present study was to examine the molecular mechanisms by which natural honey inhibits *H. pylori* infection in gastric epithelial cells.

Materials and Methods

Materials

NF-κB and AP-1 consensus oligonucleotides were obtained from Promega (Promega Corp., Madison, WI). Polyclonal antibody to IκB-α was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal COX-2 antibody was purchased from Cayman Chemical Company (Ann Arbor, MI). [γ^{32}P]ATP (35 pmol, 3000 Ci/mmol) was purchased from Amersham International (Aylesbury, UK). Poly(dI-dC) was obtained from Pharmacia (Biosystems, Milton Keynes, UK).

Manuka Honey and Commercial Honey Brands

Different commercial honey brands available in the Irish market were used in this study such as manuka honey (from New Zealand), pure acacia honey, Boyne valley honey and Healy's natural honey. Manuka honey comes from the flowers of New Zealand's manuka bush from bees visiting *Leptospermum* trees. Initial experiments were done to determine the concentrations of manuka honey or other commercial honey brands to be used in our studies. Appropriate dilutions of natural honeys were made in the cell culture medium in which AGS cells are cultured just prior to use. All subsequent *in vitro* studies using manuka or other natural honeys were carried out at a concentration of 20%.

H. pylori *Culture*

H. pylori reference strain NCTC 11638 obtained from the National Collection of Type Cultures (Colindale, UK) was used in this study. Bacteria were grown in a microaerobic humidified atmosphere on 7% lysed horse blood Columbia agar at 37°C. After 48−72 h, bacteria were harvested in PBS (pH 7.4) containing 8 mmol Na_2HPO_4, 1.5 mmol KH_2PO_4, 137 mmol NaCl and 2.7 mmol KCl or RPMI 1640 medium without antibiotics and resuspended to a concentration of 6×10^8 colony-forming units CFU/mL using the McFarland standard kit and used immediately.

Cell Culture and Treatments

The gastric epithelial cell line AGS was obtained from the European Collection of Animal Cell Cultures, ECACC (Porton Down, Salisbury, UK). AGS cells were grown in RPMI 1640 medium supplemented with 10% filtered fetal calf serum (FCS), 100 Units/mL penicillin, 100 μg/mL streptomycin and 2 mmol L-glutamine. AGS cells were removed from flasks by trypsin/EDTA treatment and seeded at a density 5×10^5 cells/mL. AGS cells were removed from flasks by trypsin/EDTA treatment and seeded at a density 5×10^5 cells/mL for experiments. Confluent AGS cells were pre-incubated with various concentrations of manuka honey, pure acacia honey, Boyne valley honey and Healy's natural honey ranging from 1−20% followed by stimulation with a freshly prepared suspension of *H. pylori* (6×10^8 CFU/mL) for 2 h. The ratio of *H. pylori* to AGS cells is 100:1 and uninfected cells were used as a control in each experiment.

Preparation of Total Cell Extracts

Cellular extract were collected by centrifugation at 1400 rpm for 5 min. The pellet of cells was resuspended in lysis buffer containing 20 mmol Tris-HCl (pH 7.5), 1% (w/v) sodium dodecyl sulfate (SDS), 150 mmol NaCl, 1 mmol EGTA, 1 mmol EDTA, 0.5 mmol phenylmethylsulfonylfluoride (PMSF) and leupeptin (10 μg/mL) and then the cells were solubilized by boiling for 5 min. The protein concentration was determined on the cell extract by the Bradford method (34).

Western Blot Analysis

Total cell extracts (50 μg protein/lane) were resolved by electrophoresis through polyacrylamide gels using 10% separating gels according to the method of Laemlli (35). Proteins were electrotransfered onto PVDF membrane using a semidry blotting apparatus (Atto). Blots were blocked with 5% (w/v) dried skim milk in PBS for 1 h at room temperature and then incubated for 1 h at room temperature with the appropriate primary antibody (anti-IκB-α or anti-COX-2 at a dilution of 1:1000). Blots were then incubated with anti-rabbit horseradish peroxidase conjugated secondary antibody (at a dilution of 1:1000) for 1 h at room

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