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Papiliocin, an antimicrobial peptide, rescues hyperoxia-induced intestinal injury

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Abstract

Reactive oxygen species (ROS) induce a variety of cellular responses, such as proliferation, differentiation, senescence, and apoptosis. Intestinal epithelial cells are continuously exposed to ROS, and excessive generation of ROS severely damages cells via oxidative stress. Pro-inflammatory cytokines may lead to intestinal inflammation and damage by inducing excessive ROS generation. In this study, we showed that papiliocin, an antimicrobial peptide, significantly inhibited ROS production, without affecting cell viability. Moreover, TNF-α and IL-6 expression was decreased in the intestinal epithelial cells. The activity of papiliocin may significantly contribute to preserving the integrity of the intestinal mucosa against oxidative damage and inflammation-related disorders.

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Introduction

Antimicrobial peptides (AMPs), first discovered in insects, have been extensively studied for their functions in the systemic immune response as a major component of natural host defense molecules (Hwang *et al*., 2011; Zasloff *et al*., 2002). These peptides have inhibitory effects against invading pathogens, such as bacteria, yeasts, fungi, and viruses (Kim *et al*., 2010). To date, many AMPs have been identified and characterized in insects.

AMPs are produced in organisms ranging from prokaryotes to humans (Zhang and Gallo, 2016). Microbes also produce AMPs to defend their environmental niche (Kim *et al*., 2010). Penicillin was first discovered in 1928 as a natural AMP and has further inspired interest in the therapeutic potential of natural AMPs as host defense molecules (Zhang and Gallo, 2016). Lysozyme was first identified as an AMP in humans. In higher eukaryotic organisms, AMPs can also act as immunomodulators (Zhang and Gallo, 2016). These activities are diverse, however, specific to the type of AMP, and include a variety of cytokines and growth factor-like effects that are relevant to maintaining immune homeostasis (Zhang and Gallo, 2016).

Recently, several reports have suggested that AMPs exert potent anti-inflammatory and antibacterial responses. Papiliocin, a 37-residue cecropin-like AMP derived from the swallowtail

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butterfly *Papilio xuthus* larvae, has been reported to exhibit significant antimicrobial activity against several human pathogenic bacterial and fungal strains (Hwang *et al*., 2011; Kim *et al*., 2010; Kim *et al*., 2020(a); Zhang and Gallo, 2016). This peptide showed anti-inflammatory response in LPS-stimulated RAW264.7 cells by inhibiting nitrite production and inducible nitric-oxide synthase (iNOS) mRNA expression (Kim *et al*., 2011).

ROS can be generated by oxidative stress and cause oxidation of proteins, RNAs, and DNAs and peroxidation of membrane lipids, resulting in aging as well as chronic diseases and cancer (Balakrishnan *et al.,* 2014; Baynes, 1991; Felton and Summers, 1995; Kim *et al*., 2020(b); Oghenesuvwe and Paul, 2019; Pardini, 1995; Sosa *et al.,* 2013; Zhang *et al.,* 2016; Zielińska *et al.,* 2017). As a defense mechanism, several endogenous antioxidants can be produced that are known to prevent an imbalance between pro- and anti-inflammatory status of the cell (Dhinaut *et al.,* 2017; Kim *et al*., 2020(b)). However, the function of these antioxidants under high oxidative stress is reduced, and therefore, an excess supply of antioxidants is required (Chew and Park, 2004; Kim *et al*., 2020(b)).

In our previous studies, we compared the antioxidant activity of hemolymph extracts from immune-challenged and unchallenged larvae. Human epithelial Caco-2 cells were first exposed to oxidative stress, treated with various concentrations of either immune-challenged or unchallenged *Bombyx mori* hemolymph extracts in vitro, and incubated for varying durations (Kim *et al*., 2020(b)). In particular, immune-challenged *B. mori* hemolymph extracts showed higher antioxidant activities than the unchallenged counterparts. These results indicate that the immune-challenged silkworm hemolymph extracts have antioxidant properties.

To confirm the rescue of hyperoxia-induced intestinal injury by papiliocin, we measured its ability to inhibit pro-inflammatory cytokine production and intracellular ROS levels in H_2O_2-stressed Caco-2 cells. This study investigated the role of papiliocin in rescuing hyperoxia-induced intestinal injury by significantly neutralizing ROS generation.

Materials and Methods

Peptide Synthesis and Cell Culture

Papiliocin peptide was synthesized in an automated solid-phase peptide synthesizer at a peptide synthesis facility, ANYGEN Co. (Korea). The human epithelial cell line Caco-2 was supplied by the Korean Cell Line Bank (Seoul, Korea). Cells were cultured in DMEM medium containing 10% fetal bovine serum and antibiotics (all from Welgene, South Korea) and incubated at 37°C in a humidified 5% CO_2 atmosphere.

Cell viability assay

Cells were seeded at a density of $1x10^4$ cells/well in 96-well plates and incubated with PBS, 50 mM Hydrogen peroxide (H_2O_2), 50 uM Melittin (Control), and 50 uM papiliocin at 37°Cfor 24 hr. Cell numbers were measured with the Cell Titer 96 Aqueous One solution which contained phenazine ethosulfate (PES) and 3-(4,5-dimethyl-2-yl)-5-(3-carboxymethoxyphenyl) -2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS; Promega Corporation, Madison, WI, USA). Absorbance was determined at 490 nm, with background subtraction at 650 nm using an Emax microplate reader (Molecular Devices, LLC, Sunnyvale, CA, USA).

Quantification of Pro-inflammatory Cytokine (TNF-α and IL-6) Production by RT-qPCR in Caco-2 Cells

Total RNA was isolated from Caco-2 cells treatment with papiliocin peptide using the TRIZOL reagent (Life Technologies, Frederick, MD, USA), following the manufacturer's instructions. The isolated RNA was quantified spectrophotometrically by measuring the absorbance at 260 nm. Total RNA was treated with *DNase* I (Life Technologies) for 15 min at 37°C to remove the genomic DNA. After purification, oligo dT-primed cDNA was prepared from the total RNA isolated using the High-Capacity cDNA Archive kit (Applied Biosystems, Foster city, CA, USA), following the manufacturer's instructions. The reaction was allowed to proceed for 2 hr at 37°C. To detect the expression of antioxidant genes, RT-qPCR was performed using Top-Taq PreMix polymerase (CoreBioSystems, South Korea). RT-qPCR was performed on a StepOnePlus Real-Time PCR system with Power SYBR Green PCR Master Mix (Takara, Japan). RT-qPCR was carried out using the following protocol: 40 cycles of denaturation at 95°C for 5 sec and annealing and elongation at 60°C for 35 sec. Fluorescence was detected at the end of every 60°C extension phase. Quantification of gene expression data was performed using the $2^{-\Delta\Delta Ct}$ method, and the crossing point of the target genes with *β-actin* was calculated using the formula $2^{-(\text{target gene-β-actin})}$ for quantification of relative

expression. The sequences of the gene-specific primers used (Bioneer Corporation, South Korea) are using the following specific primer pairs: IL-6, 5`-GTGTTGCCTGCTGCCTTC-3` (sense) and 5`-AGTGCCTCTTTGCTGCTTTC-3` (antisense); TNF-α, 5`-ATCTTCTCGAACCCCGAGTG-3` (sense) and 5`-GGGTTTGCTACAACATGGGC-3` (antisense).

ROS measurements

Intracellular ROS (H_2O_2, HO, and ONOO−) levels were measured using the oxidation-sensitive fluorescent probe carboxy-H2DCFDA (Thermo Fisher Scientific, Waltham, MA, USA). H_2O_2 was used as an oxidant. To induce oxidative stress, H_2O_2 was prepared from a 30% stock solution. At 24 hr after seeding, Caco-2 cells were subjected to oxidative stress for 2 hr. H_2O_2 was diluted to 2.5 mM in DMEM with 10% FBS. After treatment with H_2O_2, the cultures were rinsed twice with PBS and incubated in DMEM containing 10% FBS. The oxidative stress-induced Caco-2 cells were incubated with 50 μM melittin and papiliocin. The harvested cells were loaded with 5 μM carboxy-H2DCFDA dye in PBS and incubated at 37°C for 1 hr in the dark. The DCF fluorescence signal was measured immediately using a microplate reader at excitation and emission wavelengths of 485 and 528 nm, respectively.

Results and Discussions

The cytotoxic effect on Caco-2 cells was examined by exposing the cells to effective concentrations of H_2O_2 and AMPs for 24 hr, and cell proliferation was examined using a PES/MTS-based assay. As shown in Fig. 1, treatment of Caco-2 cells for 24 h with H_2O_2, melittin, and papiliocin did not affect cell viability, indicating that the concentrations selected for the study did not damage cell integrity during the incubation period.

To assess the anti-inflammatory activities of the peptide, we measured their ability to inhibit pro-inflammatory cytokine production in Caco-2 cells using RT-qPCR. Cells were treated with the peptide at 50 μM, and TNF-α and IL-6 levels were measured and compared with those in the controls. As shown in Fig. 2, papiliocin efficiently inhibited pro-inflammatory cytokine production in Caco-2 cells. Among the pro-inflammatory cytokines, TNF-α and IL-6 production was significantly inhibited by 50 μM papiliocin, with reductions of 30% and 32%, respectively; this finding suggests that papiliocin acts a potent anti-inflammatory agent in the human intestine.

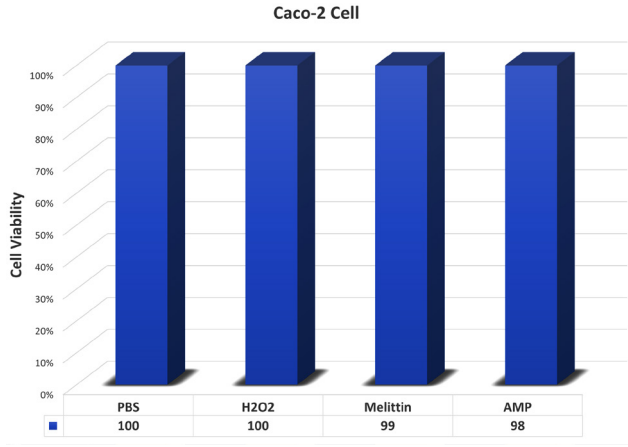

Fig. 1. Effects of papiliocin on the proliferation of human intestinal epithelial cells (Caco-2). Caco-2 cells were treated with papiliocin for 24 hr. Cell viability was then determined with a PES/MTS based assay. The data are representative of three independent experiments performed in triplicate.

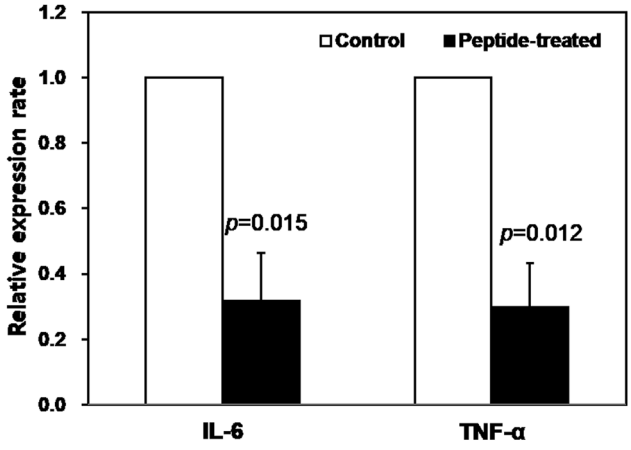

Fig. 2. Inhibition of proinflammatory cytokines production by papiliocin in Caco-2 cells.

To measure intracellular ROS levels, assays were conducted using two fluorescent probes, DCFH and DHR. As shown in Fig. 3, melittin increased ROS production in H_2O_2-stressed Caco-2 cells. However, papiliocin significantly decreased ROS levels. Moreover, treatment with papiliocin significantly neutralized the ROS-generating ability of Caco-2 cells. The highest protection was observed in the papiliocin-treated group, in which ROS levels were reduced by 88.6% as compared to the melittin-treated group.

ROS play an important role as signaling intermediates that induce a variety of cellular responses, such as proliferation,

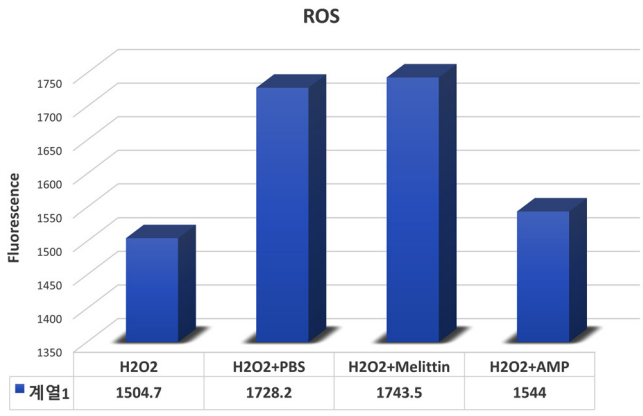

Fig. 3. Protective effects of papiliocin against intracellular ROS generation. H2O2-stressed Caco-2 cells were incubated with medium treated with papiliocin or medium supplemented with PBS and melittin, and the intracellular ROS levels were measured using DCFH as fluorescent probe.

differentiation, senescence, and apoptosis (Fujisawa *et al*., 2007; Zhao *et al*., 2017). Cells are continuously exposed to ROS, and excessive generation of ROS causes severe damage to cells in the form of oxidative stress (Fujisawa *et al*., 2007; Zhao *et al*., 2017). Caco-2 cells, originally derived from human colon carcinoma, can spontaneously differentiate into the intestinal epithelium under standard culture conditions, and the differentiated cells express enterocyte-like features (García-Nebot *et al*., 2014). ROS induce cytokine production during inflammation (Kina *et al*., 2009; Zhao *et al*., 2017). Subsequently, TNF-α may lead to cell damage by inducing ROS production, thereby increasing intestinal inflammation and damage (Baregamian *et al*., 2009; Zhao *et al*., 2017). The high expression of TNF-α in intestinal epithelial cells may be indicative of intestinal damage (Baregamian *et al*., 2009; Zhao *et al*., 2017). Interestingly, in this study, we showed that TNF-α and IL-6 expression was decreased in the intestinal epithelial cells upon treatment with the AMP papiliocin. To date, few peptides have demonstrated protective effects against oxidative damage in the intestine. Previously, Kim *et al*. (2011) showed the inhibitory effect of papiliocin on the production of the inflammatory cytokines TNF-α and MIP-2 in LPS-stimulated RAW264.7 cells (Kim *et al*., 2011). These data corroborate with the observed inhibition of NO and cytokine production by papiliocin, validating that the cecropin-like peptide papiliocin possesses potent anti-inflammatory activity (Kim *et al*., 2011). Recently, some studies have revealed similar ability of caseinophosphopeptides obtained after digestion of casein under simulated gastrointestinal digestion (García-Nebot *et al*., 2014).

In conclusion, intestinal epithelial cells were damaged and ROS levels increased under hyperoxia. Treatment with papiliocin inhibited the expression of pro-inflammatory cytokines. Thus, papiliocin may play an important role in rescuing hyperoxia-induced intestinal injury caused by ROS.

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