Molecular Immunology 57 (2014) 171–180

Contents lists available at ScienceDirect

Molecular Immunology

journal homepage: www.elsevier.com/locate/molimm

Cyclic AMP synergizes with butyrate in promoting β-defensin 9 expression in chickens

Lakshmi T. Sunkara [a], Xiangfang Zeng [a,1], Amanda R. Curtis [a], Guolong Zhang [a,b,c,*]

[a] Department of Animal Science, Oklahoma State University, Stillwater, OK 74078, USA
[b] Department of Biochemistry and Molecular Biology, Oklahoma State University, Stillwater, OK 74078, USA
[c] Department of Physiological Sciences, Center for Veterinary Health Sciences, Oklahoma State University, Stillwater, OK 74078, USA

ARTICLE INFO

Article history:
Received 14 August 2013
Received in revised form
19 September 2013
Accepted 21 September 2013
Available online 16 October 2013

Keywords:
Host defense peptide
cAMP signaling
Butyrate
Disease resistance
Antibiotic alternative
Chickens

ABSTRACT

Host defense peptides (HDP) have both microbicidal and immunomodulatory properties. Specific induction of endogenous HDP synthesis has emerged as a novel approach to antimicrobial therapy. Cyclic adenosine monophosphate (cAMP) and butyrate have been implicated in HDP induction in humans. However, the role of cAMP signaling and the possible interactions between cAMP and butyrate in regulating HDP expression in other species remain unknown. Here we report that activation of cAMP signaling induces HDP gene expression in chickens as exemplified by β-defensin 9 (AvBD9). We further showed that, albeit being weak inducers, cAMP agonists synergize strongly with butyrate or butyrate analogs in AvBD9 induction in macrophages and primary jejunal explants. Additionally, oral supplementation of forskolin, an adenylyl cyclase agonist in the form of a *Coleus forskohlii* extract, was found to induce AvBD9 expression in the crop of chickens. Furthermore, feeding with both forskolin and butyrate showed an obvious synergy in triggering AvBD9 expression in the crop and jejunum of chickens. Surprisingly, inhibition of the MEK-ERK mitogen-activated protein kinase (MAPK) pathway augmented the butyrate-FSK synergy, whereas blocking JNK or p38 MAPK pathway significantly diminished AvBD9 induction in chicken macrophages and jejunal explants in response to butyrate and FSK individually or in combination. Collectively, these results suggest the potential for concomitant use of butyrate and cAMP signaling activators in enhancing HDP expression, innate immunity, and disease resistance in both animals and humans.

1. Introduction

Host defense peptides (HDPs), also known as antimicrobial peptides, constitute an ancient yet effective mechanism of innate immunity (Brogden, 2005; Hancock and Sahl, 2006; Zasloff, 2002). These peptides are generally characterized by their small size (<10 kDa), net positive charge, and amphipathicity. HDPs protect the host from infections by directly killing microbes and by acting as immunomodulators (Fjell et al., 2012; Steinstraesser et al., 2011). Two major families of HDPs exist in vertebrate animals, namely cathelicidins and defensins (Lai and Gallo, 2009; Selsted and Ouellette, 2005; Zanetti, 2004). All cathelicidins share a highly conserved, amino-terminal segment known as the cathelin domain, followed by a highly variable, mature peptide sequence in the carboxyl-terminal region, which often adopts either an α-helical or extended structure (Lai and Gallo, 2009; Zanetti, 2004). On the other hand, most defensins consist of six conserved cysteine residues in the C-terminal region and are further classified into α-, β-, and θ-defensins based on the cysteine spacing pattern (Selsted and Ouellette, 2005).

A total of 14 β-defensins known as AvBD1-14 have been reported in chickens, but with no α- or θ-defensins (Lynn et al., 2004, 2007; Xiao et al., 2004). Four cathelicidins known as fowlicidins 1–3 (Lynn et al., 2004; van Dijk et al., 2005; Xiao et al., 2006) and cathelicidin-B1 (Goitsuka et al., 2007) have been found in chickens as well. Albeit with a restricted expression pattern, these chicken HDPs collectively are synthesized in a variety of tissues including the liver and bone marrow as well as in the digestive, respiratory, and urogenital tracts (Goitsuka et al., 2007; Lynn et al., 2004; van Dijk et al., 2005; Xiao et al., 2004). Chicken HDPs have been shown to be broadly active against a range of microbes

Abbreviations: HDP, host defense peptide; cAMP, cyclic adenosine monophosphate; AvBD9, avian β-defensin 9; HBD1, human β-defensin 1; FSK, forskolin; BZB, benzyl butyrate; GTB, glyceryl tributyrate; CT, cholera toxin; PT, pertussis toxin; CF, *Coleus forskohlii*; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; DB-cAMP, dibutyryl-cAMP; DDA, 2′,5′-dideoxyadenosine; MAPK, mitogen-activated protein kinase; HDACi, histone deacetylase inhibitor; AP-1, activator protein-1; CRE, cAMP response element; CREB, CRE-binding protein; VDR, vitamin D receptor; VRE, VDR response element; ICER, inducible cAMP early repressor.

* Corresponding author at: Department of Animal Science, Oklahoma State University, Stillwater, OK 74078, USA. Tel.: +1 405 744 6619; fax: +1 405 744 7390.
E-mail address: zguolon@okstate.edu (G. Zhang).
[1] Present address: State Key Laboratory of Animal Nutrition, China Agricultural University, Beijing 100193, China.

0161-5890/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.molimm.2013.09.003

including foodborne pathogens (Bommineni et al., 2007, 2010; Derache et al., 2009, 2012; Evans et al., 1995; Harwig et al., 1994; Higgs et al., 2005; Milona et al., 2007; van Dijk et al., 2007; Xiao et al., 2006, 2009).

Suppressing HDP expression has been revealed as a major immune evasion strategy by several pathogens like *Shigella*, *Vibrio cholerae*, enterotoxigenic *Escherichia coli*, and *Neisseria gonorrhea* (Bergman et al., 2005; Chakraborty et al., 2008; Islam et al., 2001; Sperandio et al., 2008). Conversely, inducing the synthesis of endogenous HDPs confers on rabbits and chickens an enhanced capacity to fight off infections (Raqib et al., 2006; Sadeyen et al., 2006; Sarker et al., 2011; Sunkara et al., 2011, 2012). Unlike infection or injury that triggers HDP expression with an unwanted and often exaggerated inflammatory response, butyrate and vitamin D_3 have been found to be highly potent in augmenting HDP synthesis without provoking inflammation (Schauber et al., 2003; Sunkara et al., 2011). Because of no direct interactions with microbes, such HDP-inducing compounds may be difficult to trigger resistance. Therefore, dietary modulation of HDP synthesis may have potential to be developed as a novel antibiotic-free approach to antimicrobial therapy (van der Does et al., 2012).

Butyrate is a major species of short-chain fatty acid produced from microbial fermentation of dietary fiber in the large intestine (Canani et al., 2011). It has been found that butyrate induces HDP expression mainly by acting as a histone deacetylase inhibitor (HDACi) to cause hyper-acetylation of core histones in the target gene promoter (Kida et al., 2006). Additionally, mitogen-activated protein kinases (MAPKs) and cyclic AMP (cAMP) are among the major players involved in butyrate-mediated human cathelicidin LL-37 induction in human epithelial cells (Chakraborty et al., 2009; Kida et al., 2006; Schauber et al., 2003; Schwab et al., 2007). Cylic AMP signaling also directly regulates LL-37 expression (Chakraborty et al., 2009). Forskolin (FSK), a natural adenylate cyclase agonist present in the roots of *Coleus forskohlii* (Alasbahi and Melzig, 2012), enhances cathelicidin expression in undifferentiated human HT-29 epithelial cells (Chakraborty et al., 2009). Paradoxically, activation of cAMP signaling by FSK, cholera toxin (CT), and heat-labile toxin (LT) was found to suppress LL-37 and β-defensin 1 expression in butyrate-differentiated HT-29 cells (Chakraborty et al., 2008).

To examine the role of cAMP signaling and possible interactions between cAMP and butyrate in HDP induction in chickens, we studied the expression of a representative chicken β-defensin (AvBD9) in macrophage cells and jejunal explants in response to various cAMP agonists in the presence or absence of butyrate. We also evaluated the synergy between cAMP and butyrate in AvBD9 induction in the intestinal tract of chickens following oral supplementation. The involvement of MAPKs in AvBD9 induction by butyrate and FSK individually or in combination was further investigated.

2. Materials and methods

2.1. Media and chemicals

Cell culture medium RPMI 1640, penicillin, and streptomycin were obtained from Hyclone (Logan, UT), while phosphate buffered Saline was from Amresco (Solon, OH). Gentamicin and fetal bovine serum (FBS) were purchased from Cellgro (Herndon, VA) and Atlanta Biologicals (Lawrenceville, GA), respectively. Sodium butyrate, benzyl butyrate (BZB), glyceryl tributyrate (GTB), pertussis toxin (PT), and cholera toxin (CT) were purchased from Sigma-Aldrich (St. Louis, MO). Forskolin (FSK), 8-bromo-cAMP, dibutyryl-cAMP (DB-cAMP), 2′,5′-dideoxyadenosine (DDA), SB203580, PD98059, and SP600125 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). A *C. forskohlii* (CF) extract standardized to contain 10% FSK was procured from Vitacost (Lexington, NC). Sodium butyrate, 8-bromo-cAMP, and DB-cAMP were dissolved in RPMI 1640, whereas BZB and GTB were prepared in ethanol. FSK, DDA, SB203580, PD98059, and SP600125 were dissolved in dimethyl sulfoxide (DMSO), while CT and PT were made in sterile water. All chemical stocks were stored at −20 °C.

2.2. Culture and stimulation of chicken macrophage cells

Chicken HD11 macrophage cell line (Beug et al., 1979) was a generous gift from Dr. Hyun S. Lillehoj at the USDA-Agricultural Research Services (ARS), and chicken HTC macrophage cell line (Rath et al., 2003) was kindly provided by Dr. Narayan C. Rath at the USDA-ARS. Both HD11 and HTC macrophage cells were propagated in RPMI 1640 containing 10% FBS, 100 μg/ml streptomycin, and 100 U/ml penicillin. After being seeded overnight at 2×10^6/well in 6-well tissue culture plates, cells were treated with different concentrations of chemicals. For synergistic or signaling studies, cells were pre-treated with different signaling agonists or inhibitors for 1 h, followed by stimulation with sodium butyrate, GTB or BZB for another 24 h. Cells were then lysed in RNAzol (Molecular Research Center, Cincinnati, OH) for RNA extraction.

2.3. Preparation, culture, and stimulation of chicken jejunal explants

A segment of chicken jejunum was harvested from 1- to 2-week-old broiler chickens, washed thoroughly in cold PBS containing 100 μg/ml of gentamicin, 100 U/ml penicillin, and 100 μg/ml streptomycin, and then cut into a series of 0.5-cm-long segments. Jejunal segments were placed individually in 6-well plates and cultured in 4 ml RPMI 1640 containing 10% FBS, 20 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin, and 100 μg/ml gentamicin. Each segment was treated in triplicate with 4 mM butyrate with or without different concentrations of FSK, and then incubated in Hypoxia Chamber (StemCell Technologies, Vancouver, BC, Canada) filled with 95% O_2 and 5% CO_2 at 37 °C for 24 h. Jejunal segments were homogenized in RNAzol RT for RNA extraction.

2.4. Feeding supplementation of butyrate and CF extract and in broiler chickens

Day-old male broiler chickens, generously provided by Cobb-Vantress Hatchery (Siloam Springs, AR), were randomly divided into groups of 10, provided *ad libitum* with a standard antibiotic-free ration and tap water and acclimatized for 4 days. Birds were then fed with the standard ration supplemented with a *C. forskohlii* extract (CF extract; Vitacost) containing 10% FSK at 5, 10, 20 or 40 mg/kg feed with or without sodium butyrate at 1 g/kg feed. After 2 days of feeding, 8 birds from each were euthanized with isoflurane and cervically dislocated. The crop and jejunum were collected in RNAzol RT and then stored at −80 °C until homogenization for RNA extraction. The animal trial was repeated once under identical protocols with 8 chickens per group, supplemented with 10 mg/kg of the CF extract with or without 1 g/kg of butyrate. All animal procedures were approved by the Institutional Animal Care and Use Committee of Oklahoma State University under protocol no. AG126.

2.5. Real-time RT-PCR

Total RNA was extracted from chicken cells and tissues using RNAzol RT (Molecular Research Center) by following the manufacturer's protocol and quantitated using Nanodrop 1000 (Nanodrop Products, Wilmington, DE). RNA (0.3 μg) was reverse transcribed in a total of 4 μl reaction using Maxima First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Pittsburgh, PA) according