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The antimicrobial activity of the appetite peptide hormone ghrelin

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Abstract

The present study examined the antimicrobial activity of the peptide ghrelin. Both major forms of ghrelin, acylated ghrelin (AG) and desacylated ghrelin (DAG), demonstrated the same degree of bactericidal activity against Gram-negative *Escherichia coli* (*E. coli*) and *Pseudomonas aeruginosa* (*P. aeruginosa*), while bactericidal effects against Gram-positive *Staphylococcus aureus* (*S. aureus*) and *Enterococcus faecalis* (*E. faecalis*) were minimal or absent, respectively. To elucidate the bactericidal mechanism of AG and DAG against bacteria, we monitored the effect of the cationic peptides on the zeta potential of *E. coli*. Our results show that AG and DAG similarly quenched the negative surface charge of *E. coli*, suggesting that ghrelin-mediated bactericidal effects are influenced by charge-dependent binding and not by acyl modification. Like most cationic antimicrobial peptides (CAMPs), we also found that the antibacterial activity of AG was attenuated in xphysiological NaCl concentration (150 mM). Nonetheless, these findings indicate that both AG and DAG can act as CAMPs against Gram-negative bacteria.

Keywords

ghrelin; peptide; *Escherichia coli*; *Pseudomonas aeruginosa*; LL37; antimicrobial activity

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Conflict of interest

The authors declare that they have no conflict of interest to disclose.

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1. Introduction

Ghrelin is a 28-amino acid peptide that exists in two forms: desacylated ghrelin (DAG) and acylated ghrelin (AG). While ghrelin is expressed mainly in the stomach and pancreas, it is also present in plasma and saliva in concentrations of approximately 100–200 pg/ml [1]. With its unique hydrophobic octanoyl moiety, AG binds as an endogenous ligand of the growth hormone secretagogue receptor (GHS-R1a) in pituitary cells, thereby activating the secretion of growth hormone and inducing orexigenic signaling in the brain, while DAG lacks ability to bind and activate GHS-R1a [3,11,14]. Its inherent association with GHS-R1a, AG has also been demonstrated to play multiple physiological roles in memory and learning, glucose homeostasis, thymopoiesis, and prevention of neuronal cell death [14,15]. Moreover, our group recently reported that AG is secreted in the oral cavity and may have anti-inflammatory effects on gingival epithelial cells [18]. However, while these reported AG- and DAG-mediated effects are all related to host biological events, the specific effects of AG and DAG on microorganisms remain unexplored.

In a previous study, we found that two cationic neuropeptides, vasoactive intestinal peptide (VIP) and orexin B (ORXB), demonstrate antimicrobial effects in a similar manner to the cationic antimicrobial peptide LL-37[17]. The chemical properties of ghrelin, including net charge and isoelectric point, are, interestingly, very similar to those of VIP, orexin B, and LL-37 (Table 1). Furthermore, although the complete secondary structure of ghrelin has yet to be elucidated, a study suggests that ghrelin possesses an amphipathic helix structure in neutral aqueous solution [4] similar to that of LL-37, the first amphipathic alpha-helical peptide isolated from human[22]. Thus, we hypothesized that ghrelin, with its positive net charge and putative amphipathic helix structure, could also exert antimicrobial activity. To prove our hypothesis, we tested the bactericidal effect of AG and DAG against the Gram-negative bacteria *Escherichia coli* (*E. coli*) and *Pseudomonas aeruginosa* (*P. aeruginosa*), as well as the Gram-positive bacteria *Staphylococcus aureus* (*S. aureus*) and *Enterococcus faecalis* (*E. faecalis*), and we further evaluated the effect of cationic AG and DAG on the negative cell surface charge of *E. coli*.

2. Materials and Methods

2.1. Bacterial strains and growth conditions

E. coli (ATCC 27325), *P. aeruginosa* (ATCC 15692), *S. aureus* (ATCC 6538P), and *E. faecalis* (ATCC 29212) were aerobically cultured in brain heart infusion (BHI) broth (BD BBL, Franklin Lakes, NJ, USA) overnight at 37° C

2.2. Peptides

AG and DAG were obtained from a commercial vendor (Genscript, Piscataway, NJ, USA), and LL37 was custom-synthesized using a commercial service (NeoBio, Cambridge, MA, USA).

2.3. Agar plate-based bactericidal assay

Following a previously published protocol, the overnight bacterial cultures were washed twice with phosphate-buffered saline (PBS) and suspended in 10 mM sodium phosphate buffer (NaPi; pH 6.8) [16,17]. Bacterial suspensions in NaPi (10^5 bacterial cells/mL, in a total sample volume of 76 μ l) were incubated aerobically in the absence or presence of various concentrations of AG and DAG for 2 h at 37°C. The bacterial suspensions were then diluted in PBS, plated on BHI agar (BD BBL, Franklin Lakes, NJ, USA) plates, and incubated at 37°C overnight. Colony-forming units (CFU) were assessed by counting the number of bacterial colonies on each agar plate. The bactericidal effect was calculated as the

percentage of bacterial cell survival after peptide treatment[16,17]. The same bactericidal assay was performed in NaPi with various concentrations of NaCl to evaluate the effects of NaCl on the antimicrobial activity of the peptides.

2.4. Fluorescent-based bacterial viability assay

Bacterial suspensions of mid-log phase *E. coli* in NaPi (10^8 bacterial cells/mL, in a total sample volume of 100 μ l) were incubated aerobically in the absence or presence of AG in concentrations of 100 and 200 μ g/ml for 2 h at 37°C. The staining mixture was prepared using equal ratios of SYTO9 and propidium iodide (PI) from the LIVE/DEAD BacLight Bacterial Viability kit (Invitrogen, Grand Island, NY, USA) and added to the bacterial suspensions in a 1:200 volume ratio. After the suspensions were incubated in the dark at room temperature for 15 min, the samples were applied to glass slides and visualized using an Olympus FSX100 fluorescence microscope.

2.5. Measurement of zeta-potential

Overnight cultures of *E. coli* were harvested, washed with PBS, and resuspended in NaPi to give a final concentration of 10^8 cells/mL in 10mM NaPi. Ten μ l of bacterial suspension were inoculated into 100 μ l of NaPi with LL37, AG, or DAG to yield final concentrations of 5 μ g and 20 μ g peptide/ 10^5 bacterial cells, followed by incubation for 20 min at 37°C. The bacterial suspensions were then applied to a 90-Plus particle sizer/zetasizer (Brookhaven Instruments, NY, USA), and the mean zeta potentials were computed using the Smoluchowski equation [26].

2.6. Statistical analysis

The collected data were analyzed using the Student's *t*-test, and the results are presented as values of mean $\pm$ standard deviation.

3. Results

3.1. Antibacterial activity of AG and DAG

The bactericidal effects mediated by ghrelin were examined for Gram-negative *E. coli* and *P. aeruginosa*, as well as Gram-positive *S. aureus* and *E. faecalis*. In the agar plate-based assays, AG and DAG demonstrated similar bactericidal activity against the Gram-negative bacterial species in a dose-dependent manner (Fig. 1). In particular, ghrelin concentrations equal to, or greater than, 12.5 μ g/ml showed a significant level of bacterial killing effects toward these two Gram-negative bacteria. Compared to the Gram-negative bacteria tested, AG showed less bactericidal activity against Gram-positive *S. aureus* (survival rate: 81.1 $\pm$ 6.5% and 70.1 $\pm$ 8.4% [average $\pm$ SD], AG at 25 μ g/ml and 50 μ g/ml, respectively). Furthermore, in the range of ghrelin concentrations tested, neither AG nor DAG showed significant bactericidal effects against *E. faecalis* (data not shown).

Fluorescence microscopy was also used to observe bactericidal activity. We monitored AG-treated *E. coli* using fluorescent dyes SYTO9 and PI, which, respectively, stain live cells green and dead cells red. The image of *E. coli* incubated with AG shows a notably higher number of red cells compared to untreated control *E. coli* (Fig. 2A). In concentrations of 100 μ g and 200 μ g/ml, AG killed about 15% and 75% of the bacteria, respectively (Fig. 2B). While the amount of bacteria and AG used in this experiment is different from that used in the agar plate-based assays, these results still support our novel finding: that ghrelin possess antibacterial activity.

3.2. Effects of AG and DAG on the zeta potential of *E. coli*

As shown in Table 1, ghrelin is similar to other CAMPs, such as LL37 and hBD2, in net charge and isoelectric point. To determine whether AG and DAG can affect the negative bacterial cell charge with their cationic charge, the effects of the two peptides on the zeta-potential of *E. coli* was examined (Fig. 3). AG in concentrations of 5 and 20 $\mu\text{g}/10^5$ bacterial cells increased the zeta-potential of *E. coli* from its base value of -45 mV to -39 and -27 mV, respectively, in a dose-responsive manner (Fig. 3). Interestingly, the addition of DAG to *E. coli* demonstrated a similar change in zeta potential values and showed no statistical difference from the zeta potential readings of AG-treated *E. coli* tested at the same concentrations. It is noteworthy that LL37, which was used as a positive control, also increased the zeta-potential of *E. coli* (Fig. 3). Similar to the results from the agar plate-based assays (Fig. 1), these measurements showed no significant difference in antibacterial strength between AG and DAG.

3.3. Effect of NaCl on the antimicrobial activity of AG

It was reported that bactericidal activities of CAMPs, such as human beta defensins and LL37, are often weaker at physiological NaCl concentration (150 mM) [16]. Therefore, the effects of NaCl on the antimicrobial activity of AG against *E. coli* and *P. aeruginosa* were investigated and compared against the effects of NaCl on the antimicrobial activity of LL37 (Fig. 4). As anticipated, the bactericidal activities of LL37 against the two tested bacterial species were attenuated by the addition of various concentrations of NaCl in a dose-dependent manner (Fig. 4). Similarly, the bactericidal activity of AG decreased in the presence of physiological NaCl concentration (150 mM) (Fig. 4).

4. Discussion

The present study demonstrates that both AG and DAG can act as CAMPs. In particular, we found that they exhibit similar bactericidal strength against Gram-negative *E. coli* and *P. aeruginosa*, while showing little or no bactericidal activity against Gram-positive *S. aureus* and *E. faecalis*. It was previously suggested that the cationic charge of CAMPs facilitated their binding to the negatively charged bacterial cell surface in order to perform their bactericidal activities. Ghrelin's positive net charge and isoelectric point are similar to those of the CAMPs LL37 and hBD2, as well as the more recently recognized CAMPs, ORXB and VIP [17](Table 1), suggesting its potential for antibacterial activity. Our zeta-potential measurements with *E. coli* support ghrelin's bactericidal mechanism based, in part, on its charge-dependent binding, essentially because AG and DAG could both quench the negative charge of *E. coli* cells. Furthermore, like LL37, hBD1, hBD2 [16,23,28], and other CAMPs, AG and DAG also showed attenuated bactericidal activity against *E. coli* and *P. aeruginosa* in physiological NaCl concentration (150 mM).

Recent evidence shows the potential regulatory role of ghrelin in the immune system. In particular, findings indicate that AG is expressed and secreted by T cells and that the RNA interference (RNAi)-mediated reduction of AG in T cells results in an increase in proinflammatory cytokines [5,6]. Our group recently reported that AG is also secreted in the oral cavity, where it elicits anti-inflammatory effects on gingival epithelial cells [18]. Hence, all reported effects mediated by AG are, thus far, related to host biological events, as noted above, while the effects of ghrelin on microorganisms have never been investigated until the present study. Very interestingly, in the Ohta, et al. study, a remarkably high amount of AG (10–100 ng/ml) was detected in the gingival crevice fluid (GCF) [18] in concentrations about 100-fold higher than found in saliva. Furthermore, DAG was detected in GCF at similar levels (unpublished data). Since an increasing amount of bacteria in the gingival crevice is considered to cause periodontal disease [8,24], it is plausibly postulated that the

both AG and DAG may be engaged in host innate immune antibacterial defense. Furthermore, we also found that additive antimicrobial effects among different CAMPs [17], the amount of each of different CAMPs required to elicit sufficient antimicrobial effects in the physiological context is thought to be much lower than that observed in the in vitro assay using a single species of CAMP. Ghrelin has been mainly recognized in its acetylated form and as an endocrine hormone [12,14]. However, the results from this study, along with our unpublished reports indicating the presence of AG and DAG in GCF, suggest that these two major forms of ghrelin can be classified as exocrine innate immune antimicrobial factors.

In the present study, the antimicrobial activities of ghrelin (AG; 25 µg/ml) against *E. coli* and *P. aeruginosa* were diminished in the presence of physiological concentration of NaCl (150 mM) (Figure 3). In the presence of physiological concentration NaCl (150 mM), attenuation of antimicrobial activity has been reported in several types of CAMPs including LL-37 and hBDs [2,7]. Salt sensitive property of CAMPs appears to be resulted from the reduction of CAMP's positive charge by chloride ions, thus, preventing binding to the microbial membrane. However, other studies showed that bactericidal activities of LL37 and hBD3 are rather salt-insensitive [9,13,19]. According to the study by Ouhara et al. that examined the antimicrobial activity of human 3-defensins and LL-37 against a variety of periodontal bacteria, even among the different substrains of *A. Aggregatibacter actinomycetemcomitans* (*A. actinomycetemcomitans*), various levels of zeta-potential along with a wide range of minimum inhibitory concentration (MIC) of tested CAMPs was detected [19]. Interestingly, the association between anti-microbial effects of CAMPs on different substrains of *A. actinomycetemcomitans* and the levels of bacterial zeta-potential of these tested substrains is rather weak [19], suggesting bacterial cell surface charge may not be the sole factor that affects the CAMPs' antimicrobial activity. For example of possible factor that affects the CAMPs' antimicrobial activity, a recent report revealed that OmpT family molecules expressed on the bacterial surface can deactivate LL-37 by OmpT-mediated enzymatic cleavage, suggesting that some bacteria possess mechanism that can attenuate CAMP's anti-microbial activity [25]. Nonetheless, the results obtained in this study suggested that salt-sensitive antimicrobial activity of ghrelin may be associated with bacterial cell charge.

The ghrelin gene encodes a precursor protein, preproghrelin, which, through posttranslational modification, is broken down into two smaller peptides, DAG and obestatin [10]. The multifunctional hormone AG is formed from DAG in a catalytic reaction mediated by ghrelin-O-acyltransferase (GOAT), where octanoic acid is conjugated to DAG at its third amino acid residue, serine [10,27]. Among more than 90 known neuropeptides, only ghrelin requires posttranslational acylation for its functional maturation, making the acyl modification of ghrelin particularly unique. Based on the fact that octanoic acid is an 8-carbon saturated fatty acid with antibacterial effects [21], we hypothesized that acylated ghrelin might possess stronger bactericidal activities than desacylated ghrelin. Among a number of different lipophilic acids with various aliphatic chain lengths [$\text{CH}_3(\text{CH}_2)_x\text{COOH}$; $x=1-10$], octanoic acid [$\text{CH}_3(\text{CH}_2)_6\text{COOH}$] demonstrates the most efficient bacterial growth inhibition of *E. coli* [21]. However, contrary to our hypothesis, neither AG nor DAG showed significant difference in their antimicrobial strength against either *E. coli* or *P. aeruginosa* (Fig. 1) or in their binding ability to bacterial surfaces (Fig. 3). Nonetheless, this is the first study to demonstrate the biological significance of DAG, i.e., its antimicrobial property. In the host serum, only about 6% of ghrelin is acylated, while the rest is desacylated (about 40%) or degraded (50–60%) [20]. Therefore, the collective amount of AG and DAG in serum that can facilitate antimicrobial effects, as an innate immune defense, is almost 10-fold higher than the amount of ghrelin active for hormonal effects which can be only mediated by AG. Taken together, these results indicate that the bactericidal effects of ghrelin

are mediated by its charge-dependent binding to bacteria and, hence, independent of ghrelin's acyl modification, thus supporting the novelty of our findings.

5. Conclusion

In conclusion, we demonstrated that AG and DAG possess similar antimicrobial activity against Gram-negative *E. coli* and *P. aeruginosa*, and that the antimicrobial activity may be derived from the cationic property of such AMPs that facilitates their charge-dependent binding to bacteria. Furthermore, we found that the antimicrobial effect of AG and DAG, like other CAMPs, was attenuated in physiological NaCl concentration (150 mM). These novel findings introduce a new area of study for the neuropeptide hormone ghrelin as an antimicrobial peptide. In future investigations, we will aim to elucidate its biological role in the context of innate immune responses, as well as its translational value for the development of therapeutic antimicrobial regimens.

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Highlights

1. Ghrelin possesses antimicrobial activities against Gram(+) bacteria.
2. Ghrelin's antimicrobial activities against Gram(−) bacteria seems to be minimal.
3. Ghrelin's cationic property appears to be responsible for its antimicrobial action.
4. Ghrelin loses its bactericidal activity in the physiological NaCl concentration.

Fig. 1. Antibacterial activity of AG and DAG against Gram-negative bacteria

Bacterial cells were incubated with or without various concentrations of AG and DAG for 2 hours at 37°C in 10 mM NaPi (pH 6.8). Subsequently, serial dilutions of the bacteria in PBS solution were plated on agar plates and incubated overnight at 37°C. The total colonies on each plate were then counted, and bacterial survival was calculated as the number of cells that survived after treatment with AG and DAG against the number of cells that survived without treatment with AG and DAG, and this number was reported as a percentage. The data are shown as the mean $\pm$ standard deviation of three independent experiments. Significantly different from control, nontreated bacterial cells (Student's *t*-test: * $P < 0.05$).

Fig. 2. Fluorescent staining for the determination of bacteria killed by AG

In order to monitor the real-time actions of ghrelin's bactericidal effects against *E. coli*, AG in 10 mM NaPi (pH 6.8) solution was incubated (0, 100, and 200 µg/ml AG in 10^8 cells/ml bacteria suspensions) for 2 hours at 37°C. Immediately after incubation, live and dead bacteria were distinguished by a fluorescent-based staining system (LIVE/DEAD® BacLight™). More specifically, SYTO9 and propidium iodide (PI) were used to stain live cells green and dead cells red, respectively. (A) Representative fluorescent microscopic images of bacteria incubated with or without AG (0 or 200 µg/ml AG in 10^8 cells/ml bacteria suspensions) are shown. (B) Histogram showing the percentage of dead bacteria stained red with PI. The data (percent of dead bacteria after treatment with 0, 100, and 200 µg/ml AG in 10^8 cells/ml bacteria suspensions) are shown as the mean $\pm$ standard deviation of three independent experiments. Significantly different from control, nontreated bacterial cells (Student's *t*-test: * $P < 0.05$, ** $P < 0.01$).

Fig. 3. Influence of AG and DAG on bacterial cell charge

The zeta-potential of *E. coli* reacted with AG, DAG, or LL37 in NaPi solution was examined following the protocol described in Materials and Methods. The data are shown as the mean $\pm$ standard deviation of three independent experiments. Significantly different from control, nontreated bacterial cells (Student's *t*-test: ** $P < 0.01$).

Fig. 4. Salt sensitivity of antimicrobial peptides against *E. coli* and *P. aeruginosa*

Bacterial suspensions in 10 mM NaPi (pH 6.8) with various concentrations of NaCl (0 to 150 mM) were treated with AG (25 μ g/ml) or LL37 (1 μ g/ml). Bacterial survival is expressed as a percentage, as noted in Figure 1. The data are shown as the mean $\pm$ standard deviation of three independent experiments. Significantly different from control, nontreated bacterial cells (Student's *t*-test: * $P < 0.05$, ** $P < 0.01$).

Table 1

Antimicrobial chemical properties of peptides

	# AA	Charge	IP
LL37 [*]	37	6.0	11.1
hBD2 [*]	41	5.8	9.2
Histatin 5 [*]	24	5.6	10.7
Ghrelin	28	5.1	11.5
Orexin B ^{**}	28	3.1	12.1
VIP ^{**}	28	3.1	10.2
Neuropeptide Y ^{**}	36	0.1	7.6

^{*}, antimicrobial peptide;^{**}, neuropeptide ;

AA, number of amino acids;

IP, Isoelectric point