Antibacterial and antiviral potential of neuropeptides

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Abstract

The emergence of multidrug resistant bacteria is a global health threat and the discovery of new antimicrobial agents is an absolute priority. In this context endogenous peptides are emerging as novel potential candidates. In this work, we assessed the antimicrobial effects of orexins and ghrelin neuropeptides against gram-negative (Escherichia coli, Salmonella typhimurium, Klebsiella pneumoniae) and gram-positive (Staphylococcus aureus) bacteria. Orexin-B and ghrelin showed a potent bactericidal effect at concentration equal to or greater than 25 µg/ml. No antimicrobial activity has been observed for orexin-A. Furthermore, we investigated the antiviral proprieties of the three peptides against herpes simplex virus 1 (HSV-1). We found that orexin-B, but not orexin-A is effective for HSV-1 infectivity inhibition.

Introduction

Nowadays, antibiotics resistance is a major public health concern. The identification of new antimicrobial compounds is a top priority to face this situation. In this scenario neuropeptides are emerging as novel promising antimicrobial agents. Synthesized in neurons, neuropeptides act as neurotransmitters in the central and peripheral nervous system. These peptides have a key role in the control of several biological processes included immune and inflammatory responses. In the recent past, some neuropeptides, such as the vasoactive intestinal polypeptide and neuropeptide Y, have been reported having antimicrobial activities.

In this work we explored both the antimicrobial and antiviral proprieties of orexins and ghrelin. Orexin-A and orexin-B are neuropeptides produced in the lateral hypothalamus. The two peptides are synthesized from a precursor, prepro-orexin, that is then cleaved to generate the active molecules. Orexin-A is a 33 amino acids peptide including 4 cysteines linked by two intra-chain disulfide bonds. Post-translational modifications of this peptide include a pyrogulanyl cyclization at the N-terminal and a C-terminal amidation. Orexin-B is a 28 residue neuropeptide and like orexin-A is characterized by a C-terminal amida- tion. The two neuropeptides share 46% of sequence homology. Two-dimensional NMR spectroscopy analysis of soluble orexin-B revealed that its structure consists of two α-helices linked via a flexible loop. The lateral hypothalamus, where the neuropeptides are generated, is responsible for eating behavior and energy homeostasis. Indeed, orexins play a key role in feeding behavior and body weight regulation, sympathetic activation, control of metabolic status and energy expenditure, sleep-wake cycles, and regulation of glucose level in the blood. These neuropeptides fulfill their functions interacting with two G-protein-coupled receptors (orexin receptor 1 and 2). Recently, orexin-B has been reported having bactericidal effects.

Another neuropeptide suggested to posses some antimicrobial activity is the appetite peptide ghrelin. Ghrelin, also known as lenomorelin, is 28-amino-acid (3,3kDa) peptide hormone that was first isolated and identified from rat stomach endocrine cells. It is produced mainly from the enteroendocrine cells in the mucosal layer of the stomach, but it was found also in the pancreas, kidney, small and large intestines and in salivary glands.

The human Ghrelin gene (GHRL) encodes a 117 residue precursor (preproghrelin), that is cleaved by endoproteases to produce the mature ghrelin hormone. The ghrelin peptide exists in two forms acylated ghrelin and desacylated ghrelin, the functions of the latter are poorly understood. Ghrelin is characterized by the presence of an n-octanoyl group on the serine in third position (in some species on the threonine) that increases the hydrophobicity of the ghrelin molecule and it is important for the activity of the peptide. Ghrelin has been discovered as a natural ligand of the growth hormone secretagogue receptor. Increasing evidence supports a role for ghrelin in the regulation of hunger and metabolism. Ghrelin induces orexigenic signaling in the brain and regulates food intake, body weight and glucose metabolism. Moreover, this peptide boosts the immune response and inhibits the production of proinflammatory cytokines.

We investigated the antimicrobial activity of orexin-A, orexin-B and ghrelin against Escherichia coli, Salmonella typhimurium, Klebsiella pneumoniae and Staphylococcus aureus. Moreover, we evaluated the antiviral proprieties of the same peptides against herpes simplex virus 1 (HSV-1).

Materials and Methods

Peptide synthesis

Peptides were prepared by standard 9-fluorenlymethoxycarbonyl polymeine solid-phase synthesis, using a PSSM8 multispecific peptide synthesiser (Shimadzu Corporation Biotechnology Instruments Department, Kyoto, Japan). The TGA resin (substitution 0.3 mmol g⁻¹) was used as the solid-phase support, and syntheses were performed on a scale of 100 mmol. All amino acids, 4 equiv. relative to resin loading, were coupled according to the TBTU/HOBT/DIEA method: 1 equiv. of Fmoc-amino acid, 1 equiv. of TBTU, 1 equiv. of HOBT (1 M HOBT in DMF) and 2 equiv. of DIEA (2 M DIEA in DMF). The Fmoc protecting group was removed with 30% piperidine in DMF (ν/v). Peptides were fully deprotected and cleaved from the resin by hydrofluoric acid treatment: 89%

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trifluoroacetic acid (TFA) solution containing 5.5% tioanisole, 3.3% ethanediol and 2.2% anisole as scavengers; the crude peptides were precipitated with ice-cold ethyl ether, filtered, re-dissolved in water and lyophilised. The crude peptides were purified to homogeneity by preparative reverse-phase high-pressure liquid chromatography (HPLC) on a Waters Delta Prep 3000 chromatographic system, equipped with an UV Lambda Max Mod. 481 detector. The samples were injected on a Jupiter (Phenomenex) C18 column (21.20 mm×25 cm, 15 μm) eluted with a H2O/0.1% TFA (A) and CH3CN/0.1% TFA (B) solvent mixture. A linear gradient from 20 to 75% of B over 55 min at a flow rate of 20 mL min⁻¹ was employed. The collected fractions were lyophilised to dryness and analysed by analytical reverse-phase HPLC on a Shimadzu class-LC10 equipped with a diode array detector SPD-M10AV using a Phenomenex C18 analytical column (10×250 mm, 10 μm); a linear gradient from 20 to 75% of B over 55 min at a flow rate of 1 mL min⁻¹ was employed. The identity of purified peptides was confirmed by Maldi spectrometry. Their sequences, molecular weight and net charges are indicated in Table 1. All purified sequences, molecular weight and net charges were confirmed by Maldi spectrometry. Their sequences, molecular weight and net charges are indicated in Table 1. All purified peptides were obtained with high yields (50-60%).

Cells and viruses

African green monkey kidney cells (Vero) (ATCC CCL-81) were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum. HSV-1 (strain SC16), carrying a lacZ gene driven by the CMV IE-1 promoter to express β-galactosidase, was propagated on Vero cells monolayers. The following bacterial strains were included in our study: the Gram-negative bacteria E. coli ATCC 11219, Pseudomonas aeruginosa ATCC 13388, Salmonella enterica serovar typhimurium ATCC 14028, and K. pneumoniae ATCC 10031 and the Gram-positive bacterium S. aureus ATCC 6538. To standardize the bacterial cell suspension for assay of antibacterial activity, some colonies of each strain grown overnight on MHA plates were resuspended in MHB and incubated at 37°C until visibly turbid. This log-phase inoculum was resuspended in 0.9% sterile saline to reach an appropriate optical density at 600 nm (OD600) (with a Bio-Rad microplate reader; Bio-Rad Laboratories, Hercules, CA) corresponding to a concentration of about 1×10⁸ CFU/ml. This standardized inoculum was diluted 1:10 in MHB, and the inoculum size was confirmed by colony counting.

Antimicrobial-activity assay

Susceptibility testing was performed using the broth microdilution method outlined by the Clinical and Laboratory Standards Institute using sterile 96-well microtitrator plates (Falcon, NJ). Serial 2-fold dilutions (from 100 to 0.78 μg/ml) of each peptide were prepared in cation-adjusted MHB at a volume of 100 μl/well. Each well was inoculated with 5 μl of the standardized bacterial inoculum, corresponding to a final test concentration of about 5×10⁵ CFU/ml. Antimicrobial activities were expressed as the % reduction of microbial growth observed after 24 h of incubation at 37°C. Each assay was performed in triplicate, and the mean antimicrobial activity is reported.

Table 1. Sequences, molecular weights and net charges of the peptides.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
<th>MW (as TFA salt)</th>
<th>Net charge (pH 7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ghrelin</td>
<td>Gly-Ser-Ser(n-octanoyl)-Phe-Leu-Ser-Pro-Glu-His-Gln-Val-Gln-Gln-Arg-Lys-Glu-Lys-Lys-Pro-Pro-Pro-Pro-Ala-Lys-Leu-Gln-Pro-Pro-OH</td>
<td>3370.9</td>
<td>5.1</td>
</tr>
<tr>
<td>Orexin-B</td>
<td>Arg-Ser-Gly-Pro-Pro-Pro-Glu-Leu-Glu-Gln-Arg-Leu-Glu-Leu-Leu-Gln-Ala-Ser-Gly-Asn-His-Asn-Ala-Gly-Ile-Leu-Thr-Met-NH₂</td>
<td>2899.36</td>
<td>4.1</td>
</tr>
</tbody>
</table>

MW, molecular weights; TFA, trifluoroacetic acid.
Results

Antimicrobial-activity of orexins and ghrelin

We assessed the antimicrobial activity of the synthesized peptides orexin-A, orexin-B and ghrelin on the bacterial strains E. coli, S. enterica serovar typhimurium, K. pneumoniae and S. aureus, following the broth microdilution method outlined by the Clinical and Laboratory Standards Institute. As shown in the Figure 1, a dose-dependent effect has been evidenced for orexin-B and ghrelin from concentrations equal to 12.5 μg/mL, or greater. In particular, at 25 μg/ml or higher concentrations of orexin-B or ghrelin the relative % of viable bacteria was minimal, revealing a potent antimicrobial effect. Moreover, the results obtained for orexin-B and ghrelin are in accordance with what has been reported previously reported for E. coli, whereas S. aureus, tested with the broth microdilution method, resulted sensitive. In contrast, no bacterial killing effect has been observed for orexin-A, tested on the four bacterial strains.

Anti-viral proprieties of orexin-B

The ability of the peptides to reduce HSV-1 infectivity has been evaluated by a co-treatment assay. As negative control we included the medium in which the peptides have been dissolved (vehicle) and as positive control the cytolytic peptide mellitin. As shown in Figure 2, only for orexin-B a significative, dose-dependent antiviral effect has been found. The effect started at 25 μg/mL, exceeding the IC 50 value at 100 μg/mL. No antiviral effect has been detected for orexin-A and ghrelin at the tested concentrations.

Cytotoxicity of orexins and ghrelin

As shown in Table 2, orexin-A, orexin-B and ghrelin are not cytotoxic. However,

<table>
<thead>
<tr>
<th>Peptide concentrations (µg/ml)</th>
<th>Cell viability (%)</th>
<th>Hemolysis (%)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Orexin-A</td>
<td>Orexin-B</td>
</tr>
<tr>
<td>100</td>
<td>92</td>
<td>89</td>
</tr>
<tr>
<td>50</td>
<td>94</td>
<td>96</td>
</tr>
<tr>
<td>25</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>12,5</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>6,25</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>3,125</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>1,56</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>0,78</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>
we observed a slight reduction of cell viability in Vero cells at the highest concentrations tested (50-100 μM). The difference between the cytotoxic effects of the three peptides was mild, and slightly more marked only at 100 μM, where orexin-B was found to be the most endowed with a mild cytotoxic effect.

The results of the hemolytic assays are reported in Table 2. The peptides do not induce hemolysis at concentrations less than 25μM. A modest hemolytic effect appears at 50 and 100μM concentrations for all three peptides.

Discussion and Conclusions

In the last years several endogenous neuropeptides have been reported having antimicrobial activity, and among these some neuropeptides. It has been suggested that these cationic peptides bind to the negatively charged bacterial cell wall exerting their antimicrobial effect. In line with this, orexins sequence is rich in positive charged residues.

In this study we assessed the antimicrobial activity of orexin-A and orexin-B against the gram-negative E. coli, S. pyogenes and K. pneumoniae and the gram-positive S. aureus. Orexins share 46% of sequence homology, despite this only orexin-B showed antimicrobial proprieties.

We found that orexin-B was effective against all the strains tested. Our results are consistent with the results of Ohta et al. reporting bactericidal activity of orexin-B against E. coli and P. aeruginosa. Furthermore, we assessed the antiviral activity of these neuropeptides against HSV-1. Orexin-B showed a dose-response antiviral effect, exceeding the IC 50 value at 100 μg/mL. On the other hand, ghrelin did not show antiviral activity at the assessed concentrations. However, it was effective against all the gram positive and gram negative tested bacteria. Ghrelin, like orexin-B, is a cationic peptide and shows a similar isoelectric point and it has been suggested that it could share the same bactericidal mechanism. Further studies are required to investigate the mechanism underlying the anti-viral activity of orexin-B. However, taken together our results indicate that neuropeptides represent promising candidates as both antiviral and anti-bacterial agents.

References

