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Biochemical and Biophysical Research Communications 345 (2006) 377–384

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Structural and functional analogs of the novel mammalian neuropeptide, neuromedin S (NmS), in the dermal venoms of Eurasian bombinid toads

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Received 13 April 2006
Available online 2 May 2006

Abstract

We report the isolation and structural characterization of two neuromedin S (NmS) analogs, (NmS-17 and NmS-33), from the dermal venoms of Eurasian bombinid toads. NmS is a novel neuromedin U (NmU)-related peptide with potent anorexigenic and circadian rhythm-modulating properties recently discovered in mammals. Cloning of NmS precursor-encoding cDNAs from skin venom-derived libraries revealed the presence of a high degree of transcript splice variation comparable to that found previously for NmU in both amphibian skin and mammalian brain. Synthetic replicates of both amphibian NmS peptides evoked robust and dose-dependent transient increases in intracellular calcium ion concentrations in CHO cells that had been stably transfected with either FM-3/GPR66 or FM-4/TGR-1 human NmU receptors. The potency and efficacy of these amphibian skin peptides at such receptors were comparable to those observed with human NmS and rat NmS. These data show that NmS and NmU genes had already diverged at the level of the Amphibia and that differential splicing of their transcribed mRNAs has been highly conserved throughout tetrapod vertebrate evolution indicative of fundamental biological function. NmS is additionally a novel neuropeptide homolog that can be added to the biologically active peptide arsenal of amphibian venom/defensive skin secretions.
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Keywords: Amphibian; Venom; Peptide; Neuromedin; Cloning

Neuromedin U (NmU) was isolated by one of the present co-authors (K.K.) in 1985 from an extract of hog spinal cord due to its hypertensive and uterine smooth muscle contracting properties [1]. Two molecular forms were isolated and were designated as NmU-25 and NmU-8, the latter representing the biologically active C-terminal core of the former. NmU was found to have a classical brain-gut distribution and to be of ubiquitous occurrence in a wide range of vertebrates including human, dog, rat, guinea pig, rabbit, chicken, and frog [2–9]. In a variety of bioassays, NmU was found to possess additional peripheral biological effects, including modification of intestinal ion transport, adrenocortical function, and splanchnic blood flow [10–12]. However, intracerebroventricular administration of the peptide into rats induced a significant reduction in food intake (anorexigenic effect), augmentation of stress responses, and elevations in body temperature and heart rate [13–15].

Amphibian venoms/defensive skin secretions are rich sources of biologically active peptides many of which are structural and functional homologs of endogenous vertebrate neuropeptides leading several authors to speculate that every vertebrate neuropeptide may have a frog skin secretion equivalent [16,17]. In 2000, we reported the presence of an NmU homolog in the defensive skin secretion of the Australasian tree frog, *Litoria caerulea* [18]. This

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0006-291X/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2006.04.103

peptide, designated NmU-23, was found to exhibit full-NmU agonist activity in preparations of rat uterine and human urinary bladder smooth muscle and to be equipotent with NmU-25 in displacing this radiolabeled ligand in a rat uterine smooth muscle membrane radioreceptor assay. Subsequently, the cDNA encoding this peptide was cloned from a library manufactured from lyophilized skin secretion of this tree frog using a novel technique developed in our laboratory [19]. In addition to the full-length prepronευromedin U transcript, deemed to be such due to its alignment with the homologous human transcript cloned from a pituitary gland library, we identified a number of splice variants largely involving splicing events within short exons immediately upstream of the NmU encoding sequence. This prompted a study of frog, rat, and human brain in which the same splice variation was observed. Thus within the tetrapod vertebrates, the NmU gene appears to possess a multiplicity of short exons within the open-reading frame that undergoes a highly conserved and discrete differential regional splicing indicative of an as yet unidentified but presumably fundamental function.

Recently, a second NmU-related peptide, designated neuromedin S (NmS), has been identified in and isolated from rat, mouse, and human brain tissues [20]. This was achieved by reverse deorphanization of the NmU receptors, FM-3/GPR66, and FM-4/TGR-1, stably transfected into CHO cells, and interrogated with brain peptide extracts using a calcium mobilization assay. Using radioimmunoassay systems that employ phylogenetically conserved, site-specific antisera to neuropeptides, we have screened reverse phase HPLC-fractionated venoms/defensive skin secretions from some 140 different species of amphibians for at least 20 neuropeptides. In this screen, we confirmed the presence of NmU immunoreactivity in *L. caerulea* skin secretion [18], although this was somewhat heterogenous indicating the presence of molecular forms in addition to the major NmU-23. In addition, we detected NmU immunoreactivity in a closely related species, the white-lipped tree frog, *Litoria infrafrenata*. What was most surprising was the presence of NmU immunoreactivity in skin secretion fractions of the three species of bombinid toad subjected to analysis. All other species were consistently negative. Structural and bioinformatic analyses of the bombinid toad immunoreactive peptides indicated that they were in fact NmS homologs. Molecular cloning of these toad skin NmS cDNAs from venom-derived libraries revealed a spectrum of splice variants that reflected our previous findings for NmU transcripts in *L. caerulea* skin secretion [19]. Synthetic replicates of frog NmS-17 and frog NmS-33 interacted with stably transfected GPR66 and TGR-1 NmU receptors in a manner similar to endogenous human and rat NmS homologs. These data demonstrate, for the first time, that NmU and NmS genes had already diverged within the amphibians and that the systematic study of amphibian defensive skin secretions can provide many valuable insights into the molecular evolution of vertebrate regulatory peptides.

Materials and methods

Acquisition of skin secretions. Young adult specimens of the Oriental fire-bellied toad (*Bombina orientalis*, $n = 5$), the Chinese giant fire-bellied toad (*Bombina maxima*, $n = 3$), and the European yellow-bellied toad (*Bombina variegata*, $n = 10$) were captive-bred and were maintained in separate species terraria at a temperature of 22 °C under a 12 h/12 h light/dark cycle with three feeds of multi-vitamin loaded crickets per week. Following a 12-week period of acclimatization, venom was obtained by gentle transdermal electrical stimulation (4-ms pulse width, 50 Hz, 5–7 V) of deionized water-moistened skin for three periods of 15 s duration [21]. The viscous white granular gland secretions were washed from the skins of the toads using a stream of deionized water into pre-chilled (2 °C) glass beakers, snap-frozen in liquid nitrogen, and lyophilized. The secretions from each of the three species of toads were separately pooled. Approximately 10–15 mg dried weight of skin secretion could be obtained from each specimen on a monthly basis.

Reverse phase HPLC/MS. Ten milligrams of lyophilized venom from each species of toad were separately subjected to reverse phase HPLC/MS analysis using a gradient formed from 0.05/99.95 (v/v) trifluoroacetic acid (TFA)/water to 0.05/39.95/60.0 (v/v/v) TFA/water/acetonitrile in 60 min at a flow rate of 1 ml/min. A Thermoquest gradient reversed phase HPLC system, fitted with an analytical column (Phenomenex, C-18, 25 × 0.45 cm), and interfaced with a Thermoquest LCQ™ Deca electrospray ion-trap mass spectrometer was employed. The effluent from the chromatographic column was flow-split with approximately 10% entering the mass spectrometer source and 90% directed towards a fraction collector. Dead volume between column and fraction collector was minimal (20 μl). The molecular masses of polypeptides in each chromatographic fraction were further analyzed using matrix-assisted laser desorption/ionization, time-of-flight mass spectrometry (MALDI-TOF MS) on a linear time-of-flight Voyager DE PRO mass spectrometer (Perseptive Biosystems, MA, USA) in positive detection mode using α-cyano-4-hydroxycinnamic acid as the matrix. Internal mass calibration of the instrument with known standards established the accuracy of mass determination as ±0.1%. The peptides possessing masses coincident with those of NmS analogs deduced from cloned cDNAs were each subjected to primary structural analyses by automated Edman degradation using an Applied Biosystems 491 Procise sequencer in pulsed-liquid mode or by MS/MS fragmentation sequencing using the LCQ™ Deca electrospray ion-trap instrument.

Analysis of reverse phase HPLC fractions by NmU/NmS radioimmunoassay. Fifty microliter samples from each chromatographic fraction were removed and lyophilized prior to radioimmunometric analysis using a system described in detail previously [18]. Briefly, antiserum GP 9320 was raised in a guinea pig immunized with a (Lys^0)-NmU-8/glutaraldehyde/ovalbumin conjugate. The assay buffer, in which all reactants were diluted, consisted of 0.04 M sodium phosphate, pH 7.2, containing 0.14 M sodium chloride and 2% (v/v) horse serum. The assay volume was 400 μl consisting of 100 μl of diluted NmU-8 antiserum (1:38,000), 100 μl of monoradioiodinated NmU-8 tracer (100 Bq; 2 fmol), and 100 μl of NmU-8 standard (0–250 fmol/ml) or unknown sample. Addition of tracer was delayed for 24 h and bound/free tracer was separated after a further 24 h by addition of 1 ml of 0.05% dextran-coated microfine charcoal and centrifugation at 1100*g* for 30 min. All procedures were carried out at 4 °C and under the conditions described, the sensitivity of the assay, defined as the least amount that could be detected above zero with 95% confidence, was 1.2 fmol NmU-8/assay tube. The antiserum cross-reacted fully on a molar basis with NmU-8, NmU-25, frog NmU-23, human NmS, and rat NmS but exhibited no cross-reactivity with a wide range of vertebrate neuropeptides.

Cloning of NmS precursor cDNAs from venom-derived libraries. Five milligrams of lyophilized venom from each species were dissolved separately in 1 ml of cell lysis/mRNA stabilization buffer (Dynal Biotec, UK). Polyadenylated mRNA was isolated from the stabilization buffer/skin secretion mixtures using magnetic oligo(dT) beads as described by the

manufacturer (Dynal Biotec, UK) and reverse-transcribed. The cDNA was subjected to 3′- and 5′-RACE procedures to obtain full-length prepro-NmS nucleic acid sequence data using a SMART-RACE kit (Clontech UK) essentially as described by the manufacturer. Briefly, the 3′-RACE reactions employed an NUP primer (supplied with the kit) and a degenerate sense primer (NmS-S1; 5′-GGIATHGTIGGIMGICCITT-3′) that was complementary to the internal amphibian NmS amino acid sequence, -GIVGRPF-. 3′-RACE products were gel-purified and cloned using a pGEM-T vector system (Promega Corporation) and sequenced using an ABI 3100 automated DNA sequencer. The sequence data obtained from these 3′-RACE products were used to design a gene-specific primer, (NmS-AS1: 5′-TGTGTCCATAAGATCAGGCAGAAT-3′) to a sequence within the 3′-non-translated region. 5′-RACE reactions were performed using this primer in conjunction with the NUP and the resultant products were gel-purified, cloned, and sequenced as described above. Following acquisition of these data, a second gene-specific sense primer (NmS-S2: 5′-CCTCTACCACTGCTGCTTGCGATC-3′) was designed to a site within the putative signal peptide domain and was employed in 3′-RACE reactions. Products were likewise gel-purified, cloned, and sequenced as described previously. All identified sequences and splice variants were represented at least ten times in the several hundred clones that were sequenced.

Chemical synthesis of toad NmS-17 and NmS-33. Subsequent to unequivocal primary structural characterization of the toad defensive secretion peptides NmS-17 and NmS-33, each was separately synthesized using solid-phase Fmoc chemistry and an Applied Biosystems 433 peptide synthesizer. Following cleavage from the resin and deprotection, each respective peptide was purified by reverse phase HPLC, a process that was monitored and quality-controlled by means of electrospray mass spectrometry using an LCQ DECA instrument (Thermo-Electron Corporation, San Jose, CA, USA).

Production of human NmU-receptor transfected cell lines. CHO cells were stably transfected with either human FM-3/GPR66 (GenBank Accession No. BC036543) or with FM-4/TGR-1 (AF242874) NmU/NmS receptors by cloning into pcDNA3.1 vectors. The cell lines (CHO/FM-3-14 and CHO/FM-4-16), that exhibited the greatest elevations in free cytosolic Ca^{2+} ion concentrations when challenged with human NmU, were used in this study. The intracellular calcium mobilization assay was performed using a FLIPR system (Molecular Devices) that has been described in detail previously [20]. All peptide solutions contained 1% (w/v) bovine serum albumin and each synthetic peptide was subjected to amino acid analysis prior to construction of solutions for dose–response studies.

Results

Identification and structural characterization of toad venom NmS peptides

NmS/NmU immunoreactive peptides were identified in reverse phase HPLC fractions of venom from each of the three species of bombinid toad investigated. The profile obtained with *B. orientalis* is shown in Fig. 1. Two immunoreactive peptides were resolved in fractions of *B. maxima* and *B. orientalis* venoms in contrast to the single peptide in *B. variegata* that was coincident in retention time with the more abundant and hydrophilic peptide resolved in the other congeneric species. This more hydrophilic peptide was found to be of identical molecular mass (1964 Da—non-protonated) in all three species. A combination of automated Edman degradation and MS/MS fragmentation sequencing established the primary structure as: DSSGIVGRPFFLFR PRNamide. The minor, and more hydrophobic peptides from *B. maxima* and *B. orientalis* were found to be of similar (3800 and 3818 Da—non-protonated, respectively) but not identical molecular masses. The primary structure of the *B. maxima* peptide was established by automated Edman degradation as: FLFQFSRAKDPSLKIGDSSGIVGRPF FLFRP(RNamide). The last C-terminal amino acid residues were not detectable in the final cycles of Edman degradation but their inclusion matched the previously observed molecular mass of this peptide. The *B. orientalis* peptide was also subjected to automated Edman degradation and the two C-terminal residues were likewise not detected. However, as in the first instance, their inclusion provided an exact match to observed molecular mass. The *B. orientalis* peptide differed in primary structure from the *B. maxima* homolog at two sites—a Thr for Ala substitution at position 8 and a Thr for Ile substitution at position 15. This finding explained the discrepancy in molecular mass observed between the two

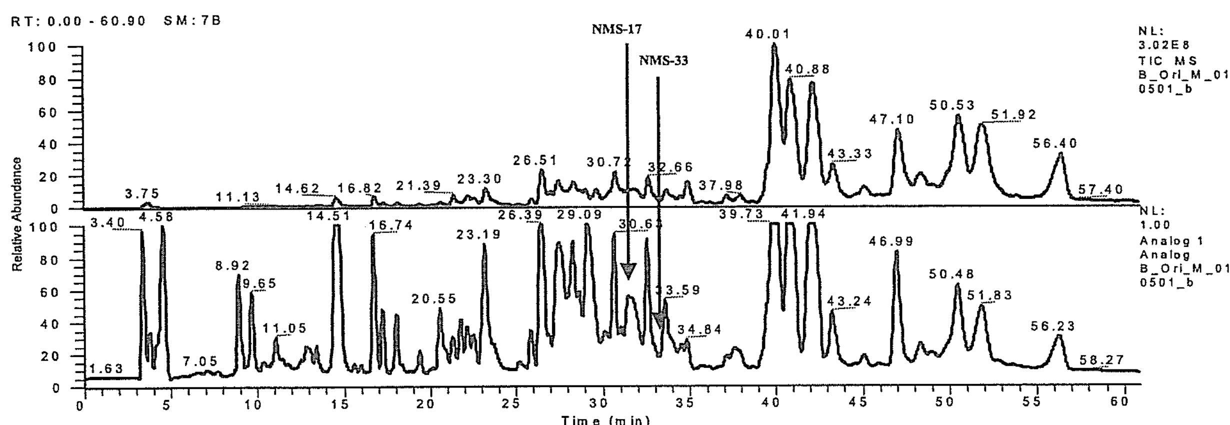

Fig. 1. A typical LC/MS spectrogram of skin venom from the Oriental fire-bellied toad, *B. orientalis*. The upper panel illustrates total ion count entering the mass spectrometer and the lower panel illustrates UV absorbance profile at λ214 nm. Both *y* axes are in arbitrary units where the highest peak is default expressed as 100% of signal to illustrate component relative abundance. The retention times of NmS-17 and NmS-33 are indicated by arrows.

```
Toad   NmS-17                     DSSGIVGRPFFLFRPRNa
Toad   NmS-33     FLFQFSRAKDPSLKIGDSSGIVGRPFFLFRPRNa
Human  NmS-33     ILQRGSGTAAVDFTKKDHTATWGRPFFLFRPRNa
Mouse  NmS-36  LPRLLRLDSRMATVDFPKKDPTTSLGRPFFLFRPRNa
Rat    NmS-36  LPRLLHTDSRMATIDFPKKDPTTSLGRPFFLFRPRNa
                                  *     ***********

Frog   NmU-25  LKPDEELQGPGGVLSRGYFVFRPRNa
Frog   NmU-23    SDEEVQVPGGVISNGYFLFRPRNa
Human  NmU-25  FRVDEEFQSPFGSRSRGYFLFRPRNa
Rat    NmU-23  YKVNE-YQGP-GAPSGGFFLFRPRNa
Pig    NmU-25  FKVDEEFQGPIVSQNRRYFLFRPRNa
Dog    NmU-25  FRLDEEFQGPIASQVRRQFLFRPRNa
                      * *        * *****
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Fig. 2. Comparative alignments of homologous amphibian and mammalian NmS and NmU sequences. * Denotes amino acid residue identities; *a* denotes C-terminal amide. Defining NmS motif is clearly a completely conserved C-terminal undecapeptide amide marked in bold italic contrasting with NmU whose defining motif is a completely conserved C-terminal pentapeptide amide marked in bold italic. Gaps have been inserted in the sequence of rat NmU-23 to maximize alignment.

peptides. The primary structures of toad NmS-17 and toad NmS-33 are compared with NmS peptides from human, mouse, and rat in Fig. 2. In addition, NmU peptides from amphibian and mammalian source tissues are included for comparison. The defining motif of NmS is evidently a fully conserved C-terminal undecapeptide amide that contrasts with the defining motif of NmU peptides, that is, a C-terminal pentapeptide amide. The C-terminal region of NmU has been established as the bioactive core of the molecule [1] and it is likely that this will also hold true for NmS peptides. However, NmS appears to have been more rigorously conserved in this region than its NmU relation during tetrapod evolution.

Cloning of NmS precursor cDNAs from venom-derived libraries

Using the strategy described in the Methods section, a single diffuse band was obtained following electrophoresis of 3′-RACE PCR products generated from each venom-derived library. Cloning of these PCR products revealed their heterogeneity as, following sequencing of over 200 clones, it was evident that six different transcript isoforms were present in *B. maxima* and in *B. orientalis* venom-derived libraries, but only a single transcript, corresponding to one of the *B. maxima/orientalis* isoforms, was present in the library from *B. variegata* venom. This may be of evolutionary significance as the first two species are of Asiatic origin whereas the latter is found in Central Europe. The longest open-reading frames encoded by the cloned cDNAs from each species are aligned in Fig. 3 and those of human, mouse, and rat are included as a sub-set for direct comparison. Figs. 4 and 5 show the aligned splice variant isoforms 1 through 6 obtained from *B. maxima* and *B. orientalis* venom-derived cDNA libraries, respectively. In common with the transcripts arising from the NmU gene in amphibians and mammals, the major region of splice variation occurs immediately upstream of the NmS-encoding domain. All

```
Human   MKHLRPQFPLILAIYCFCMLQIPSSGFPQPLADPSDGLDI
Mouse   MKHPLPHYSPILFIYCFCMLQIPSSGASPPLADSPDGLDI
Rat     MKHPFPQFPPILVIYCFCMLQIPSSGASPPLAGPPDGLDA
        ***  *    ** *************   ***   ****
BM      MRSEKHLLPLPLLLAICCLGTLHLSSGFPQSVPSYLEGLD
BO      MRSEKHLPPLPLLLAICCLGTLHPSSGFPQSVPSYMEALD
BV      MRSEKHLLPLPPLLAICCLGTLHLSSGFPQSVPSYLEGLD
        ******* *** *********** *********** * **

Human   VQLEQLAYCLSQWAPLSRQPKDNQDIYKRFLFHYSRTQEA
Mouse   VDPERLAYFLKQREIHSNQPKENQDVYKRFLFHYSRTRKP
Rat     VDPERLAHFLNQRETCSNQPKESRDVYKRFLFHYSRAWKS
        *  * **  * *    * ***   * **********
BM      IPESERHAFCFSQWTALQDQEQIPSFVMDLCSSIYNRMKV
BO      IPESEKLAFCFSQWTALPDQEQIPSFVMDLCSSIYNRMKV
BV      IPESE-----------------IPSFVMDLCSSITNRMKV
        *****                 ************ *****

Human   THPVKTGFPPVHPLMHLAAKLANRRMKRILQRGSGTAAVD
Mouse   THPVSAEFAPVHPLMRLAAKLASRRMKRLPRLLRLDSRMA
Rat     THPVNSEFAPVHPLMRLAAKLPSRRMKRLPRLLHTDSRMA
        ****   * ****** *****  *******
BM      NEENNHEIYKRFLFQFSRAKDPSLKIGESQIATAEYTKRD
BO      NEENNHEIYKRFLFQFSRTKDPSLKTGESQIATAEYTKRD
BV      NEENNHEIYKRFLFOFSRAKDPSLKIGESQIATAEYTKRD
        ****************** ****** **************

Human   ---FTKKDHTATWGRPFFLFRPRNGRNIEDEAQIQW
Mouse   TVDFPKKDPTTSLGRPFFLFRPRNGRYTDNNFQ---
Rat     TIDFPKKDPTTSLGRPFFLFRPRNGRYTDKV-Q---
           * *** *   *************      *
BM      SSGIVGRPFFLFRPRNGRKVSINEH
BO      SSGIVGRPFFLFRPRNGRKVSINEH
BV      SSGIVGRPFFLFRPRNGRKVSINEH
        *************************
```

Fig. 3. Alignment of full-length translated open-reading frames of NmS precursors from human, mouse, rat, and the toads, *B. maxima* (BM), *B. orientalis* (BO), and *B. variegata* (BV). * Denotes conserved residues in mammals; * denotes conserved residues in toads.

NmS cDNA isoforms from the bombinid toads studied here have been deposited in the EMBL Nucleotide Sequence Database under the Accession Nos. AM115659 through AM115671.

Pharmacological investigation of stable NmU receptor-transfected CHO cell lines

Synthetic replicates of toad NmS-17 and toad NmS-33 were investigated for activity using CHO cell lines (CHO/FM-3-14 and CHO/FM-4-16) that had been stably transfected with either human FM-3/GPR66 or FM-4/TGR-1 NmU/NmS receptors. Human and rat NmS were tested at the same time in all experiments as internal controls for comparative purposes. The dose–response curves for each peptide ligand and at each NmU receptor-transfected CHO cell line are shown in Fig. 6. All NmS peptides tested generated classical sigmoidal dose–response curves, as assessed by quantitative changes in fluorescence intensity within the concentration range (10^{-13} M through 10^{-7} M) employed, and were of a high and comparable order of potency effecting receptor activation in the sub-nanomolar range. The calculated EC_{50} values for each NmS peptide at each receptor are summarized in Table 1 for clarification of

```
BM1    MRSEKHLLPLPLLLAICCLGTLHLSSGFPQSVPSYLEGLD
BM2    MRSEKHLLPLPLLLAICCLGTLHLSSGFPQSVPSYLEGLD
BM3    MRSEKHLLPLPLLLAICCLGTLHLSSGFPQSVPSYLEGLD
BM4    MRSEKHLLPLPLLLAICCLGTLHLSSGFPQSVPSYLEGLD
BM5    MRSEKHLLPLPLLLAICCLGTLHLSSGFPQSVPSYLEGLD
BM6    MRSEKHLLPLPLLLAICCLGTLHLSSGFPQSVPSYLEGLD

BM1    IPESERHAFCFSQWTALQDQEQIPSFVMDLCSSIYNRMKV
BM2    IPESE-----------------IPSFVMDLCSSIYNRMKV
BM3    IPESERHAFCFSQWTALQDQEQIPSFVMDLCSSIYNRMKV
BM4    IPESERHAFCFSQWTALQDQEQIPSFVMDLCSSIYNRMKV
BM5    IPESERHAFCFSQWTALQDQEQIPSFVMDLCSSIYNRMKV
BM6    IPESERHAFCFSQWTALQDQEQIPSFVMDLCSSIYNRMKV

BM1    NEENNHEIYKRFLFQFSRAKDPSLKIGESQIATAEYTKRD
BM2    NEENNHEIYKRFLFQFSRAKDPSLKIGESQIATAEYTKRD
BM3    NEENNHEIYKRFLFQFSRAKDPSLKIG------------D
BM4    NEENNHEIYKR----------------ESQIATAEYTKRD
BM5    NE-------------------------ESQIATAEYTKRD
BM6    NE-------------FSRAKDPSLKIGESQIATAEYTKRD

BM1    SSGIVGRPFFLFRPRNGRKVSINEH
BM2    SSGIVGRPFFLFRPRNGRKVSINEH
BM3    SSGIVGRPFFLFRPRNGRKVSINEH
BM4    SSGIVGRPFFLFRPRNGRKVSINEH
BM5    SSGIVGRPFFLFRPRNGRKVSINEH
BM6    SSGIVGRPFFLFRPRNGRKVSINEH
```

Fig. 4. Alignment of full-length translated open-reading frames of NmS precursors generated by splice variation (BM1 through 6) and cloned from a skin secretion library of the toad, *B. maxima.* KR (-Lys-Arg-) indicates propeptide convertase processing sites generating the N-terminals of NmS peptides and GR (-Gly-Arg-) indicates propeptide convertase/amidation enzyme site that generates the C-terminal asparaginamide. Sequences encoded by "spliced out" exons are indicated by hatched lines.

```
BO1    MRSEKHLPPLPLLLAICCLGTLHPSSGFPQSVPSYMEALD
BO2    MRSEKHLPPLPLLLAICCLGTLHPSSGFPQSVPSYMEALD
BO3    MRSEKHLPPLPLLLAICCLGTLHPSSGFPQSVPSYMEALD
BO4    MRSEKHLPPLPLLLAICCLGTLHPSSGFPQSVPSYMEALD
BO5    MRSEKHLPPLPLLLAICCLGTLHPSSGFPQSVPSYMEALD
BO6    MRSEKHLPPLPLLLAICCLGTLHPSSGFPQSVPSYMEALD

BO1    IPESEKLAFCFSQWTALPDQEQIPSFVMDLCSSIYNRMKV
BO2    IPESEKLAFCFSQWTALPDQEQIPSFVMDLCSSIYNRMKV
BO3    IPESEKLAFCFSQWTALPDQEQIPSFVMDLCSSIYNRMKV
BO4    IPESEKLAFCFSQWTALPDQEQIPSFVMDLCSSIYNRMKV
BO5    IPESE-----------------IPSFVMDLCSSIYNRMKV
BO6    IPESEKLAFCFSQWTALPDQEQIPSFVMDLCSSIYNRMKV

BO1    NEENNHEIYKRFLFQFSRTKDPSLKTGESQIATAEYTKRD
BO2    NE---------FLFQFSRTKDPSLKTGESQIATAEYTKRD
BO3    NE-------------------------ESQIATAEYTKRD
BO4    NEENNHEIYKR----------------------------D
BO5    NE-------------------------------------D
BO6    NEENNHEIYKRFLFQFSRTKDPSLKTG------------D

BO1    SSGIVGRPFFLFRPRNGRKVSINEH
BO2    SSGIVGRPFFLFRPRNGRKVSINEH
BO3    SSGIVGRPFFLFRPRNGRKVSINEH
BO4    SSGIVGRPFFLFRPRNGRKVSINEH
BO5    SSGIVGRPFFLFRPRNGRKVSINEH
BO6    SSGIVGRPFFLFRPRNGRKVSINEH
```

Fig. 5. Alignment of full-length translated open-reading frames of NmS precursors generated by splice variation (BO1 through 6) and cloned from a skin secretion library of the toad, *B. orientalis* KR (-Lys-Arg-) indicates propeptide convertase processing sites generating the N-terminals of NmS peptides and GR (-Gly-Arg-) indicates propeptide convertase/amidation enzyme site that generates the C-terminal asparaginamide. Sequences encoded by "spliced out" exons are indicated by hatched lines.

this point. Toad NmS-17 and NmS-33 were obvious cognate ligands for both human receptors with similar orders of potency compared to human NmS, and rat NmS that in turn have been found previously to be comparable to human NmU [20]. Toad NmS-17 was virtually indistinguishable from human NmS in terms of potency at the expressed human FM-4/TGR-1 receptor (0.231 ± 0.015 nM vs. 0.237 ± 0.027 nM; $n = 5$, mean $\pm$ SEM) whereas it was more potent than human NmS as a ligand for the FM-3/GPR66 receptor (0.085 ± 0.005 nM vs. 0.139 ± 0.013 nM).

Discussion

Neuromedin S has been identified in this study as a new amphibian venom/defensive skin secretion peptide. Reverse phase HPLC fractions of venom from all three species of discoglossid toad from the genus, *Bombina*, which were included in a radioimmunometric screen for bioactive peptides in a sample of 140 species of amphibian, were found to contain peptides that were reactive with an antiserum raised to NmU. *B. maxima* and *B. orientalis* venom fractions contained two immunoreactive peptides, in contrast to that of *B. variegata*, in which a single immunoreactive peptide was detected. Isolation and primary structural analysis of all five NmU-immunoreactive peptides indicated that they exhibited greater structural similarity to the novel neuropeptide, neuromedin S (NmS) [20], than to NmU peptides from the same spectrum of tetrapod vertebrates (Fig. 2).

Neuromedin S (NmS) is a recently discovered neuropeptide in man and rodents which is an obvious structural homolog of NmU that cross-reacts fully with C-terminally directed NmU antisera, interacts with both nominate NmU receptors, and shares biological effects, such as hypertension induction, smooth muscle contraction, and induction of anorexia [20]. However, detailed RT-PCR analysis of NmS distribution revealed that it was mainly expressed in the central nervous system, spleen, and testis [20]. Specifically within the brain, NmS expression was localized predominantly to the core region of the suprachiasmatic nucleus within the hypothalamus—a regulatory center for circadian rhythm that intracerebroventricular administration of NmS was found to shift. This discrete nucleus also expresses mRNA encoding the TGR-1 NmU receptor that was found to be NmS-preferring and thus endogenous NmS may interact with in an autocrine or paracrine manner.

In amphibians, NmU was discovered in the skin secretion of the Australasian White's tree frog (*L. caerulea*) in our laboratory using an approach similar to that adopted in the present study [18]. This peptide, of 23 amino acid residues, was found to be of similar molar potency to porcine NmU-25 in contraction of rat uterine smooth muscle and

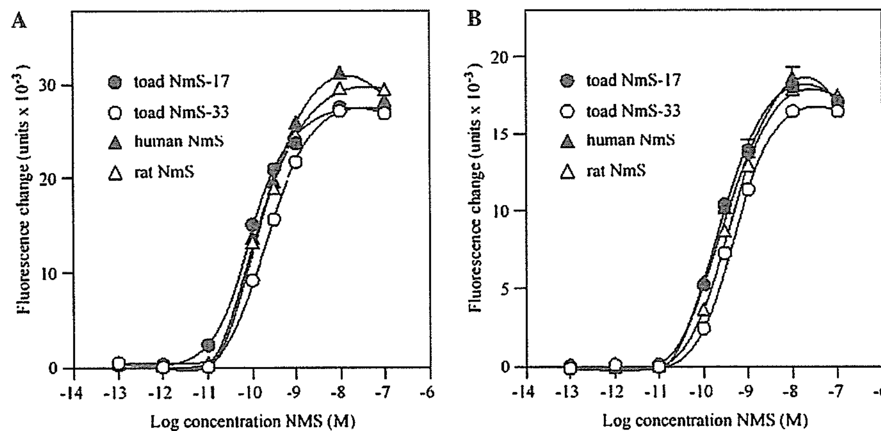

Fig. 6. Response curves for toad NmS-17 (filled circle), toad NmS-33 (open circle), human NmS (filled triangle), and rat NmS (open triangle) in the calcium mobilization assay using CHO cells stably expressing FM-3/GPR66 (A) or FM-4/TGR-1 (B) human NmU receptors. The calcium mobilization assay using the FLIPR system has previously been described in detail [20]. Data points represent the means ± SEM of six replicates.

Table 1
Half-maximal response concentrations (EC_{50}, in nM) of amphibian and mammalian NmS peptides for effecting calcium mobilization in FM-3/GPR66 or FM-4/TGR-1 stably transfected CHO cell lines

Peptide	FM-3/GPR66	FM-4/TGR-1
Toad NmS-17	0.085 ± 0.005	0.231 ± 0.015
Toad NmS-33	0.222 ± 0.018	0.439 ± 0.020
Human NmS	0.139 ± 0.013	0.237 ± 0.027
Rat NmS	0.148 ± 0.012	0.358 ± 0.021

EC_{50} was determined by the FLIPR assay [20], and data were analysed using GraphPad Prism ($n = 6$, mean ± SEM).

of similar affinity to rat NmU in displacing monoradioiodinated rat NmU ligand from rat uterine smooth muscle membranes. Molecular cloning of the frog NmU precursor cDNA from a frog skin library indicated that a series of splice variants were present in addition to the "full-length" open-reading frame transcript judged to be so by alignment with and structural similarities to the homologous human NmU precursor transcript [2]. Interrogation of a fetal human brain cDNA library indicated the same splice variants as discovered in the skin of the amphibian were also generated as a consequence of NmU gene expression in man and that the sites of splice variation occurred precisely at exon/intron boundaries within the human gene that consists of 10 exons [20]. Differential splicing of NmU mRNA is thus a highly conserved phenomenon within the tetrapod vertebrates—a finding that is certainly of biological relevance.

Molecular cloning of NmS cDNAs from libraries constructed using lyophilized venoms of *B. maxima* and *B. orientalis*, using a novel technique developed in our laboratory [19], revealed a series of six transcripts in each species that represented splice variants displaying a striking similarity to those obtained for NmU in amphibian skin and mammalian brain (Figs. 4 and 5). The venom library of *B. variegata* in contrast, apparently contained a single NmS-encoding transcript that represented one of the splice variants present in the other two congeneric species. The reason for this difference is unclear but of note is that *B. variegata* has a geographical distribution (Central Europe) that is different from the other Oriental species. The high degree of phylogenetic conservation in the splice variation observed following expression of the NmU gene in mammals and amphibians can be explained mechanistically by the structure of the human NmU gene that contains 10 exons. The coincident nature of amphibian and human transcripts would imply a very similar if not identical organisation of the amphibian NmU gene. The NmS gene in humans, although exhibiting relatively low nucleotide similarity (53%) with the NmU gene, is likewise composed of 10 exons with comparably conserved exon/intron boundaries. Though no evidence so far exists of differential splicing events occurring in the expression of the human NmS gene, the data presented in this study unequivocally demonstrate that such events occur following expression of the amphibian homolog. As in the case of the NmU gene, these differential splicing events occur predominantly within relatively short exons encoding peptide domains upstream of the NmS-encoding sequence within the precursor protein. Of particular note is the fact that several of these discrete exon-encoded peptide domains terminate in a typical –KR– propeptide convertase processing site such that differential splicing events within this region have the potential to alter the sites of cleavage and hence the nature of the peptide products so generated. This effect explains the presence of the two molecular variants of NmS detected in the venom of two species examined in the present study. NmS-17 was the predominant molecular form of NmS in two species of toad and the only molecular form in the third. This is considerably shorter in chain length than the 33-mers from human and the 36-mers from rat and mouse. Although a 33-mers NmS was isolated from the venom of two of the three species of toads studied, it arises in a different manner in the toads when compared with the human. Fig. 3 shows the alignments of mammalian and amphibian "full-length"

open-reading frames and the typical propeptide convertase processing sites (–KR–) that are indicated. As can be clearly seen, these differ in location between mammalian and amphibian precursors. The –KR– processing site in the amphibian precursors that resides immediately upstream of the NmS-17 encoding domain exhibits a site substitution in the mammalian homologs to –KK–. The specificity of the endogenous propeptide convertases in mammals obviously does not permit cleavage of this motif as no attenuated forms of mammalian NmS were reported in the original publication [20]. The generation of toad NmS-33 occurs as a result of a different process to the 33-mers human homolog. Alternative splicing of exons immediately upstream of the NmS encoding sequence in the amphibian gene (*B. maxima* and *B. orientalis*) results in the generation of a series of putative isomeric precursors. One of these isoforms within each of the two species (BM3 and BO6) encodes NmS-33 as a result of splicing out of an exon that encodes a peptide terminating in the –KR– processing site which generates NmS-17. The N-terminal domain of NmS-33 constitutes the N-terminal domain of the novel 33-mers peptide speculated to exist in the NmU precursor [2] and found to exist as a 34-mers in the human NmS precursor [20]. This novel NmU/NmS gene associated peptide was found to be a potent prolactin-releasing factor when administered intracerebroventricularly in rats [20]. However, intracerebroventricular administration of NmU in rats potently suppresses prolactin release [22]. Thus it would be intriguing to examine the effect of toad NmS-33, a peptide containing both contra-active domains, on prolactin release in this bioassay. While this was beyond the scope of the present study to assess, the ability of both toad NmS-17 and NmS-33 to activate stably transfected NmU receptors in CHO cell lines, as monitored by the generation of transient intracellular calcium fluxes, was studied. Both peptides were found to be equipotent with human NmS and rat NmS in activating both subtypes of NmU receptors, FM-3/GPR66 and FM-4/TGR-1, confirming the previous assertion that NmS peptides are cognate ligands for both of these NmU receptors [20]. Whether NmS or NmU peptides interact with these receptors appears to be determined by the differential spatial distribution of both components of this regulatory system. The fact that both NmU and NmS genes are obviously very closely related in many ways begs the question of their evolutionary origins. In other similar situations in peptide evolutionary biology, it is a general assumption that the degree of phylogenetic conservation of active site residues reflects two things: fundamental physiological importance and perhaps derivation. In Fig. 2, the primary structures of amphibian and mammalian NmS and NmU peptides are compared. Within this comparison that spans the tetrapod vertebrates, NmS displays a fully conserved C-terminal undecapeptide amide whereas a similar set of NmU peptides display a fully conserved pentapeptide amide. The C-terminal amidated region of NmU has been established as the active core with N-terminal extensions having subtle effects on bioactivity in a species-specific manner. This appears to be likewise true for NmS when comparing potencies of rat/mouse NmS-36 with toad NmS-17. In view of this evidence, we would contend that the original gene encoded NmS and that the gene duplication event that gave rise to NmU must have preceded tetrapod vertebrate evolution as both are separate entities in amphibians. Thus the results of this study have provided important insights into regulatory peptide biology and have posed several fundamental questions that will form the basis of further in-depth investigations to dissect the relative roles of NmS and NmU peptides in regulation of important physiological events such as control of feeding behavior and circadian rhythm modulation.

Acknowledgments

Mei Zhou is in receipt of an Overseas Postgraduate Studentship from Queen's University, Belfast. This work was supported in part by grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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Ghrelin stimulates phagocytosis and superoxide production in fish leukocytes

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Abstract

To clarify the role of ghrelin in the fish immune system, the *in vitro* effect of ghrelin was examined in phagocytic leukocytes of rainbow trout (*Oncorhynchus mykiss*). Administration of trout ghrelin and des-VRQ-trout ghrelin, in which three amino acids are deleted from trout ghrelin, increased superoxide production in zymosan-stimulated phagocytic leukocytes from the head kidney. Gene expression of growth hormone (GH) secretagogue-receptor (GHS-R) was detected by RT–PCR in leukocytes. Pretreatment of phagocytic leukocytes with a GHS-R antagonist, [D-Lys^3]-GHRP-6, abolished the stimulatory effects of trout ghrelin and des-VRQ-trout ghrelin on superoxide production. Ghrelin increased mRNA levels of superoxide dismutase and GH expressed in trout phagocytic leukocytes. Immunoneutralization of GH by addition of anti-salmon GH serum to the medium blocked the stimulatory effect of ghrelin on superoxide production. These results suggest that ghrelin stimulates phagocytosis in fish leukocytes through a GHS-R-dependent pathway, and also that the effect of ghrelin is mediated, at least in part, by GH secreted by leukocytes.
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Introduction

Ghrelin was originally discovered in rat stomach as an endogenous ligand for the growth hormone (GH) secretagogue-receptor (GHS-R) (Kojima *et al.* 1999). Ghrelin is recognized as an important regulator not only of GH secretion but also of feeding, glucose homeostasis, gastric motility, the cardiovascular system and cell proliferation (Muccioli *et al.* 2002, Yoshihara *et al.* 2002, Broglio *et al.* 2003). The effect of ghrelin on energy metabolism has suggested its potential use as a therapeutic target in disorders of GH secretion, feeding and nutritional condition (Muccioli *et al.* 2002, Yoshihara *et al.* 2002, Broglio *et al.* 2003). Signal transduction mediated by G protein has been detailed in fish GHS-R (Chan *et al.* 2004). However, expression of GHS-R gene was detected in human T cells, B cells and neutrophils, suggesting the action of ghrelin on the immune system (Hattori *et al.* 2001). Recently, Dixit *et al.* (2004) revealed that ghrelin inhibits expression of proinflammatory cytokine in human T cells and monocytes, suggesting a role of ghrelin in the immune system. However, evidence of the immunomodulatory effect of ghrelin is limited, and the effect of ghrelin on the defense mechanism is still unclear.

It is well known that GH stimulates immune functions in various vertebrates (Sakai *et al.* 1996*a*, 1996*b*, 1997, Balm 1997, Clark 1997, Cohen & Kinney 2001, Yada & Nakanishi 2002, Yada *et al.* 2004*a*, 2004*b*). In fish, *in vivo* and *in vitro* administration of GH enhances specific and nonspecific immune functions, such as phagocytosis by neutrophils and macrophages (Sakai *et al.* 1996*a*, 1997, Balm 1997, Yada & Nakanishi 2002, Yada *et al.* 2004*a*, 2004*b*). Extrapituitary expression of GH gene in fish leukocytes suggests autocrine or paracrine action of GH in the modulation of immune functions (Yada & Azuma 2002, Yada & Nakanishi 2002, Yada *et al.* 2005). Thus, ghrelin seems to be a likely candidate for modulator of the GH gene expression in the fish immune system.

We hypothesized that phagocytosis, one of the major defense mechanisms in primitive vertebrates, is activated by ghrelin in fish. Ghrelin has been isolated in rainbow trout, and its stimulatory action on GH secretion has been demonstrated in the same species (Kaiya *et al.* 2003*a*). This study examined the *in vitro* effects of ghrelin on the activity of phagocytic leukocytes isolated from the head kidney of trout, which is equivalent to the bone marrow in higher vertebrates. The effects of ghrelin on superoxide dismutase (SOD) and GH gene expression were also examined in trout phagocytic leukocytes by real-time PCR.

Journal of Endocrinology (2006) **189**, 57–65
0022–0795/06/0189–057 © 2006 Society for Endocrinology *Printed in Great Britain*

DOI: 10.1677/joe.1.06187
Online version via http://www.endocrinology-journals.org

Materials and Methods

Fish

Rainbow trout (*Oncorhynchus mykiss*), each weighing about 500 g, were reared at the National Research Institute of Fisheries Science at Nikko (Japan) for successive generations in outdoor concrete ponds supplied with a continuous flow of spring water at 10 °C under natural photoperiod. They were fed commercial dry diet (Oriental, Chiba, Japan).

Hormones and reagents

Rainbow trout ghrelin and des-VRQ-trout ghrelin were synthesized as described previously (Kaiya *et al.* 2003*a*). Salmon GH was isolated from chum salmon (*O. keta*) pituitaries, as described by Kawauchi *et al.* (1986). GHS-R-specific antagonist, [D-Lys^3]-GHRP-6, was purchased from Phoenix Pharmaceuticals (Belmont, CA, USA). The specificity of a polyclonal antisalmon GH antiserum (AS9–2) was validated by immunocytochemistry and RIA (Bolton *et al.* 1986). Nitroblue tetrazolium (NBT) and zymosan A were purchased from Sigma.

Isolation of leukocytes

Head-kidney leukocytes (HKL) were isolated as described by Sakai *et al.* (1996*a*) with slight modifications. Six fish were used for each experiment. Fish were anesthetized in 3-aminobenzoic acid ethyl ester (MS222; Sigma), killed by decapitation and the head kidney was placed in Eagle's minimum essential medium (MEM) with Earle's salt (Sigma), containing 0·2% heparin sodium and buffered with 7·5% $NaHCO_3$ (pH 7·6); minced with forceps; and filtered by nylon mesh (37 µm). The dissociated cells were placed on 34/51% Percoll (Pharmacia) cushions and centrifuged at 400 *g* for 25 min. The leukocyte band was harvested, washed with PBS (pH 7·6) and suspended in MEM containing 0·5% trout serum. Viable leukocytes were counted by trypan blue exclusion (viability of >90%), and were used for *in vitro* experiments, as described below. To isolate peripheral blood leukocytes (PBL) for RT–PCR, blood was collected from the caudal vessels with a heparinized syringe. Blood was diluted 1:2 in MEM containing 0·2% heparin sodium. The mixture was placed on a 54% Percoll cushion, and centrifuged at 400 *g* for 25 min, and the leukocyte band was harvested.

Superoxide production in phagocytic cells

Quantification of superoxide anion production by adherent and phagocytic leukocytes as a killing mechanism after phagocytosis was used to examine the effects of trout ghrelin, des-VRQ-trout ghrelin and salmon GH. The superoxide production in adherent cells isolated from HKL was determined as the reduction of NBT (Sakai *et al.* 1996*a*). In brief, isolated leukocytes from each fish were suspended in MEM containing 0·5% trout serum at a density of 10^7 cells/ml, and 100 µl were seeded onto 96-well microplates in duplicate. The cells were preincubated overnight in an atmosphere of 95% O_2/5% CO_2 at 15 °C. After removal of nonadherent cells, adherent cells on the bottom of the well (about 10^5 cells) were incubated in MEM with or without hormones for 4 h at 15 °C. The adherent leukocytes from trout head kidney contain about 90% macrophages and 10% granular neutrophils (Sakai *et al.* 1996*a*). After incubation, the medium was aspirated, and the cells were further incubated in 100 µl MEM containing NBT (1 mg/ml) with zymosan A as a stimulant for superoxide production. After incubation for 1 h at 15 °C, the medium was aspirated, and the cells were fixed with methanol for several minutes. They were air-dried, and dissolved in 120 µl of 2 M KOH and 140 µl dimethyl sulfoxide (DMSO; Sigma). Absorbance at 620 nm was measured with a microplate reader (SpectraMax 190; Nihon Molecular Devices, Tokyo, Japan).

RNA extraction and RT–PCR for GHS-R mRNA

Tissues (stomach, intestine, gills, skin, body and head kidney, spleen and pituitary) were frozen in liquid nitrogen immediately after isolation, and stored at −80 °C until use for RNA extraction. Skin was taken from the dorsal side of the body. Isolated PBL and HKL were seeded onto 24-well microplates, and incubated overnight in an atmosphere of 95% O_2/5% CO_2 at 15 °C. Nonadherent cells from PBL were harvested by centrifugation at 500 *g* for 5 min and immediately used for RNA extraction.

Total RNA was extracted from tissues and cells by the guanidium-isothiocyanate-phenol method (Chomczynski & Sacchi 1987), and treated with RNase-free DNase I (Takara, Shiga, Japan). After inactivation of DNase, reverse transcription was carried out with the SuperScript First-Strand Synthesis System (Invitrogen). PCR was performed with AmpliTaqGold DNA Polymerase (Applied Biosystems, Foster City, CA, USA) and the GeneAmp 9700 PCR System (Perkin Elmer, Norwalk, CT, USA). Nucleotide sequences of full-length rainbow trout GHS-R 1a and 3′-end truncated form (GHS-R 1b) have recently been determined (H Kaiya, unpublished data), and PCR primers were designed to amplify trout GHS-R 1a (5′-TGCCTTTCCACTTGCATCGT-3′, forward; 5′-TTCCCCCTCCAAATTGGCT-3′, reverse) and GHS-R 1b (5′-GCGGTTGAGTCTGGGCTT CTA-3′, forward; 5′-TTATGCAGTGGTTAAAAT AGT-3′, reverse). The amplification condition was 40 cycles of 94 °C for 1 min, 57 °C for 1 min, and 72 °C for 1 min. The amplified products were analyzed by a 1·5% agarose gel and stained with ethidium bromide.

Quantification of SOD and GH mRNA levels by real-time PCR

To determine absolute amounts of mRNA, standard partial cDNA of rainbow trout SOD were cloned and sequenced. The SOD 1 fragment was purified with rainbow trout HKL cDNA with AmpliTaq Gold DNA Polymerase (Applied Biosystems) and primers (5′-GGCTTCCACGTCCATGCTTA-3′, forward; 5′-CCCAGATCATCAGCCTTCTCAT-3′, reverse), as described by GeneBank accession no. AF469663. The amplification regime was 40 cycles consisting of 94 °C for 1 min, 54 °C for 30 s and 72 °C for 1 min. The products were purified and subcloned into pT7 Blue (Merck). Cloned cDNA fragments were digested by KpnI at the ends of the insert, separated by agarose gel electrophoresis, and purified with GENECLEAN (Qbiogene, Carlsbad, CA, USA).

The number of HKL from each fish was adjusted to 10^7 cells/ml in MEM containing 0·5% trout serum, and 300 µl were seeded onto 24-well microplates. The cells were cultured overnight in an atmosphere of 95% O_2/5% CO_2 at 15 °C. Nonadherent cells were removed, and adherent cells (about 3×10^5/well) were incubated in MEM with or without hormones for 4 h at 15 °C. Total RNA was extracted and treated with RNase-free DNase I, and reverse transcription of RNA was carried out as described above. Real-time PCR was performed with an ABI Prism 7900HT Sequence Detection System (Applied Biosystems), as described previously (Yada *et al.* 2005). The PCR mixture (20 µl) contained 1X TaqMan Universal PCR Master Mix (Applied Biosystems), 300 nM each forward and reverse primers, 100 nM fluorogenic probe, and standard (6×10^2–6×10^7 copies/reaction) or template cDNA (0·04–40 ng/reaction). After denaturation at 95 °C for 10 min, PCR amplification of 50 cycles of 95 °C for 15 s and 60 °C for 1 min was conducted. The sequences of the primers and probe were as follows: 5′-GACAAC ACCAACGGCTGTATGA-3′, forward; 5′-CTCCG TGGGTCTGGTTGTG-3′, reverse; 5′-TGCCGGACC CCACTTCAACCC-3′, probe for trout SOD 1, 5′-GGAGCGGAGCAGCAAGAG-3′, forward; 5′-TGC AGGGAAAGGTCTCATCTG-3′, reverse; 5′-CTGTC TCCAGGGTTCGGTTTCCCAG-3′, probe for trout GH 2, and 5′-TTCAACACCCCTGCCATGTA-3′, forward; 5′-ACGGCCAGAGGCGTATAGG-3′, reverse; 5′-TGGCCATCCAGGCCGTGTTGT-3′, probe for trout β-actin. SOD and GH mRNA levels were standardized with β-actin mRNA levels in each sample.

Statistical analysis

The significance of differences between control and experimental groups was evaluated by analysis of variance followed by paired Student's *t*-test for parametric groups or Wilcoxon's rank sum test for nonparametric groups. Calculations were performed with a computer program, STATISTICA (Statsoft, Tulsa, OK, USA).

Results

Rainbow trout ghrelin and des-VRQ-trout ghrelin (10 nM) produced significant increases in superoxide production in phagocytic HKL (Fig. 1). Although the stimulatory effects of ghrelin were significant without stimulation by zymosan A, the largest responses were observed in the presence of zymosan A at 2 mg/ml. Salmon GH used as a positive control of enhancement of superoxide production produced a tendency similar to that of ghrelin. Then, experiments of superoxide production were done with that condition of stimulation. Trout ghrelin and des-VRQ-trout ghrelin at concentrations from 1 pM to 10 nM increased superoxide production in phagocytic HKL in a dose-dependent manner (Fig. 2).

Figure 3 shows the expression of GHS-R genes in various tissues and lymphoid cells. Both forms of GHS-R mRNAs (GHS-R 1a and 1b) were expressed ubiquitously in lymphoid tissues and cells, such as head kidney, spleen, nonadherent and adherent PBL, and HKL. The stimulatory effects of trout ghrelin and des-VRQ-trout ghrelin on superoxide production in phagocytic HKL were abolished by pretreatment with a GHS-R antagonist, [D-Lys^3]-GHRP-6 (Fig. 4). That inhibitory effect of GHS-R antagonist was not significant in GH-enhanced superoxide production. Quantification of SOD mRNA levels revealed that both trout ghrelin and des-VRQ-trout ghrelin, and salmon GH stimulated gene expression of SOD in phagocytic HKL (Fig. 5).

Effects of trout ghrelin and des-VRQ-trout ghrelin on GH mRNA levels in HKL are shown in Fig. 6. Both ghrelins produced significant increases in GH gene expression. In Fig. 7, immunoneutralization of GH by addition of anti-salmon GH serum to the medium resulted in significant inhibition of superoxide production in HKL enhanced by two trout ghrelins.

Discussion

The present study demonstrated that ghrelin stimulates superoxide production associated with phagocytosis in trout leukocytes. The effect of ghrelin was abolished by a GHS-R antagonist, [D-Lys^3]-GHRP-6, which is known to inhibit signal transduction mechanism also in fish GHS-R (Chan *et al.* 2004). Those facts suggest that ghrelin activates trout phagocytes through a GHS-R-mediated mechanism. Administration of ghrelin increased mRNA levels of SOD, which catalyzes the dismutation of superoxide into oxygen and hydrogen peroxide. This result suggests that ghrelin and GH stimulate both

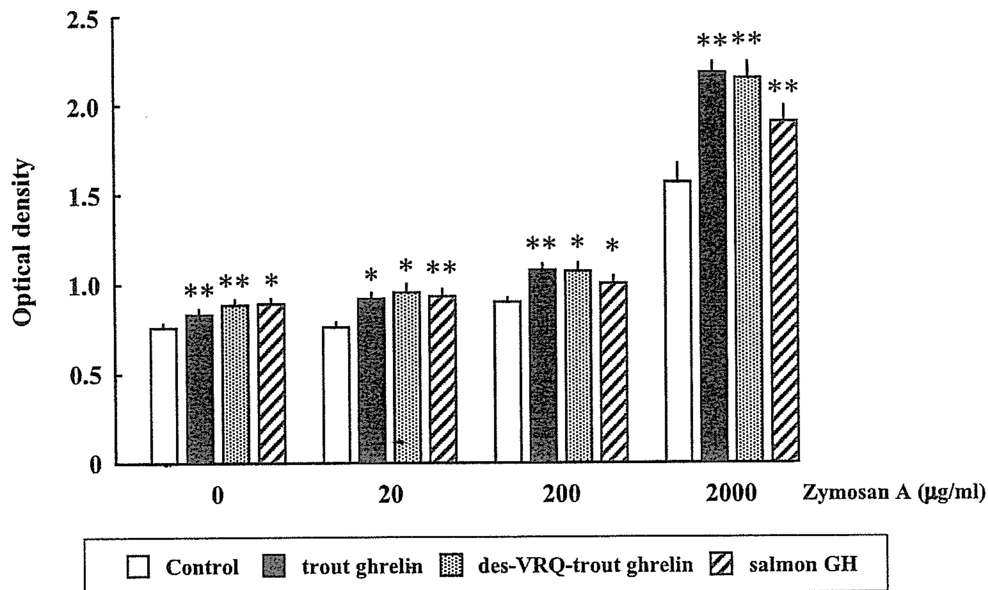

Figure 1 Dose-related influence of zymosan A on the effects of trout ghrelin, des-VRQ-trout ghrelin and salmon GH on superoxide production in phagocytic head-kidney leukocytes. Cells were incubated with each hormone (10 nM) for 4 h, and then treated with zymosan A for 1 h. Data are expressed as means ± S.E.M. (n=6). *, **Significantly different from the control at P<0·05 and P<0·01 respectively.

superoxide and hydrogen peroxide as reactive oxygen species during the process of phagocytosis in fish phagocytic leukocytes (Secombes 1996). To our knowledge, this is the first report to demonstrate the effect of ghrelin in enhancing the immune function of phagocytic leukocytes in vertebrates.

The present study revealed that trout ghrelin and des-VRQ-trout ghrelin are equipotent in stimulating

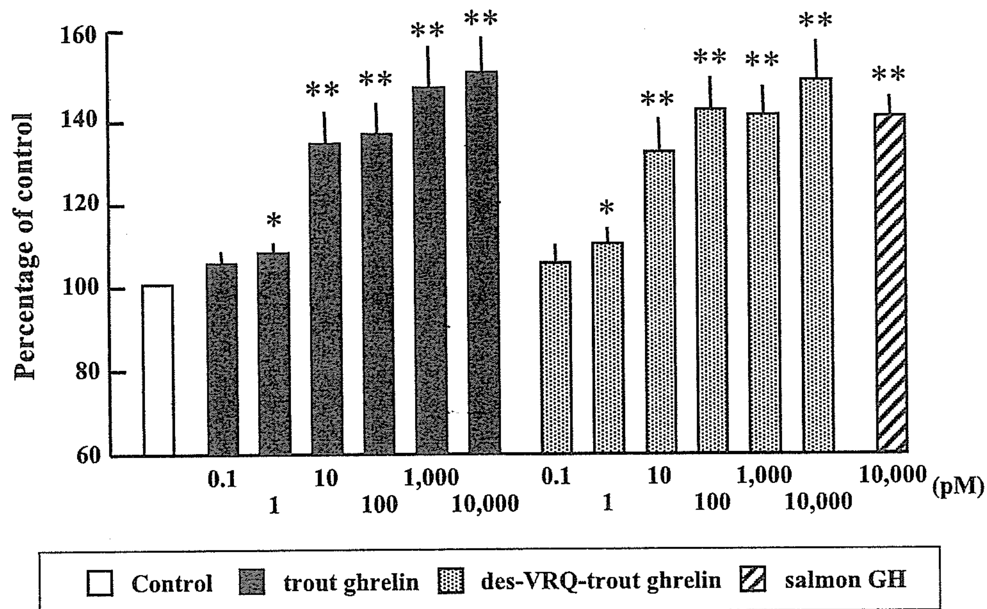

Figure 2 Dose-related effects of trout ghrelin and des-VRQ-trout ghrelin on zymosan A-stimulated superoxide production in phagocytic head-kidney leukocytes. Cells were incubated with each hormone (10 nM) for 4 h, and then treated with zymosan A (2 mg/ml) for 1 h. Data are expressed as the percentage of the control in each individual (mean ± S.E.M., n=6). *,**Significantly different from the control at P<0·05 and P<0·01 respectively.

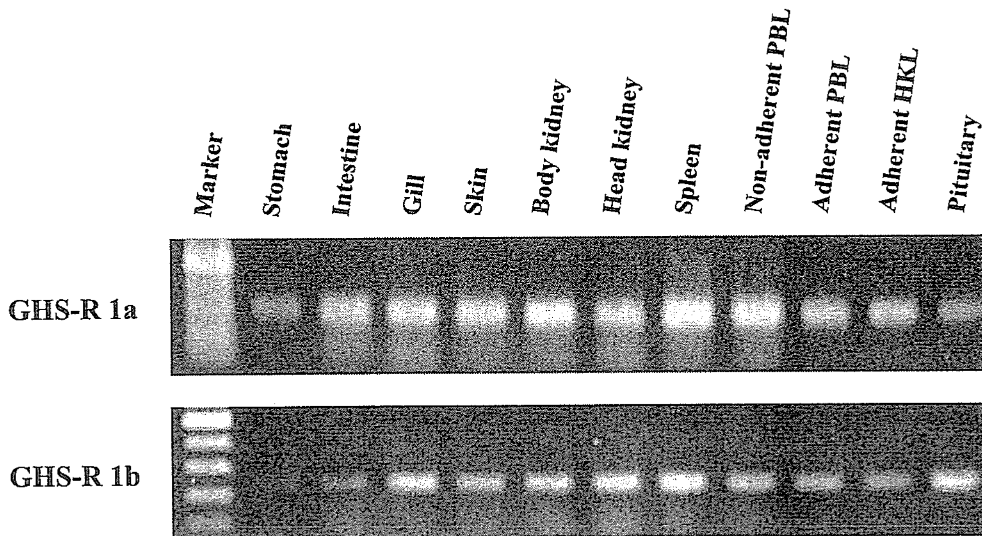

Figure 3 Expression of GHS-R mRNAs (GHS-R 1a and 1b) detected by RT–PCR in tissues and cells of trout.

superoxide production in phagocytic leukocytes from head kidney. The two trout ghrelin isoforms are derived from a single gene by alternative splicing, and show similar effects on GH secretion from trout pituitary (Kaiya *et al.* 2003*a*). A similar isoform of ghrelin is also present in rat and man as des-Gln^{14}-ghrelin, which is also a ligand for GHS-R (Hosoda *et al.* 2000, 2003). Signal transduction mediated by G protein has been detailed also in fish GHS-R (Chan *et al.* 2004). Carboxyl-terminus is amidated in teleost ghrelins, but not in tetrapod ghrelins (Kaiya *et al.* 2003*a*). It is known that the active core of ghrelin is the amino-terminal tetrapeptide including acylation (Bednarek *et al.* 2000, Matsumoto *et al.* 2001). Thus, results in this study indicate that the conserved structure at the N-terminus is important for immunomodulatory activity, the amide structure at the C-terminus being unrelated to its biologic activities.

The stimulatory effect of ghrelin on GH secretion from the pituitary is well established in mammals, birds, amphibians and several species of fish, including rainbow trout (Kojima *et al.* 1999, Kaiya *et al.* 2001, 2002, 2003*a*, 2003*b*, Muccioli *et al.* 2002, Riley *et al.* 2002, Yoshihara *et al.* 2002, Broglio *et al.* 2003, Unniappan & Peter 2004). Poppi *et al.* (2002) reported that hexarelin, a synthetic GHS-R ligand, stimulates GH release from bovine and

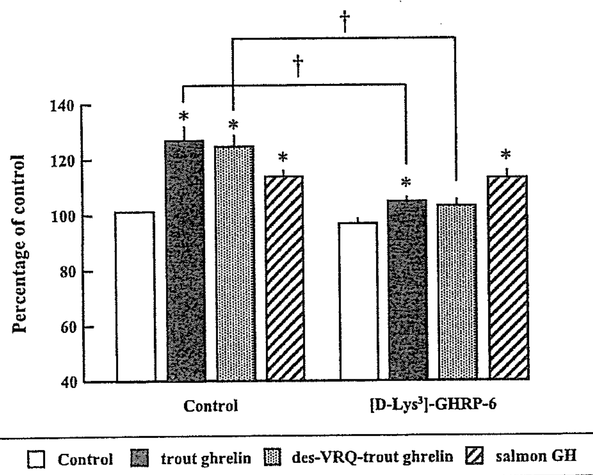

Figure 4 Effects of a GHS-R-specific antagonist, [D-Lys^3]-GHRP-6, on superoxide production in phagocytic head-kidney leukocytes. Cells were preincubated with [D-Lys^3]-GHRP-6 (10 μM) for 1 h, incubated with trout ghrelin (10 nM) or des-VRQ-trout ghrelin (10 nM) for 4 h, and then treated with zymosan A (2 mg/ml) for 1 h. Data are expressed as the percentage of the control in each individual (mean ± S.E.M., n=6). *Significantly different from control at P<0·05. †Significant difference between columns at P<0·05.

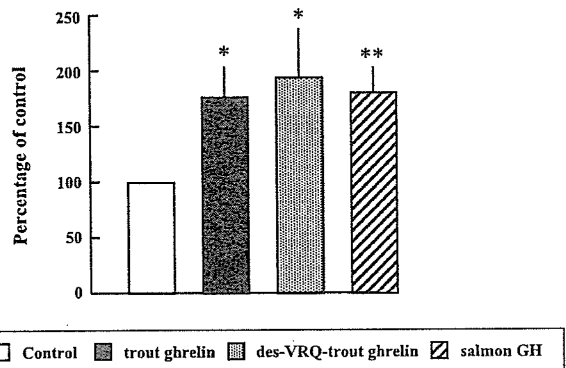

Figure 5 Effects of trout ghrelin, des-VRQ-trout ghrelin and salmon GH on SOD mRNA levels in phagocytic head-kidney leukocytes. Cells were incubated with each hormone (10 nM) for 4 h. Data are expressed as the percentage of the control in each individual (mean ± S.E.M., n=6). *, **Significantly different from control at P<0·05 and P<0·01 respectively.

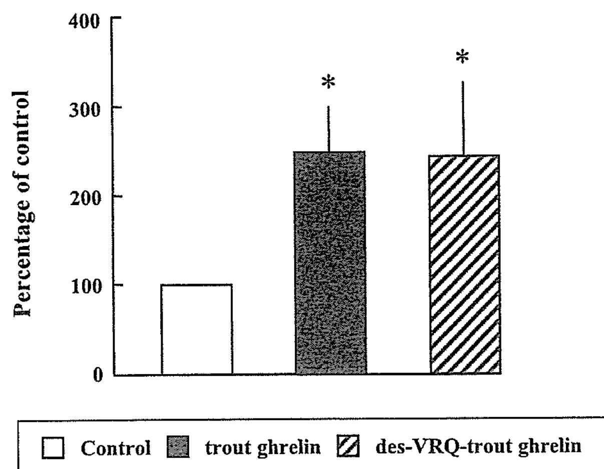

Figure 6 Effects of trout ghrelin and des-VRQ-trout ghrelin on GH mRNA levels in phagocytic head-kidney leukocytes. Cells were incubated with each hormone (10 nM) for 4 h. Data are expressed as the percentage of the control in each individual (mean ± S.E.M., n=6). *Significantly different from the control at P<0·05.

porcine lymphocytes. In this study, stimulation of GH gene expression by trout ghrelin and des-VRQ-trout ghrelin was observed in phagocytic leukocytes of trout. Furthermore, the stimulatory effect of ghrelin on superoxide production was abolished by immunoneutralization with anti-GH serum added to the medium, suggesting the

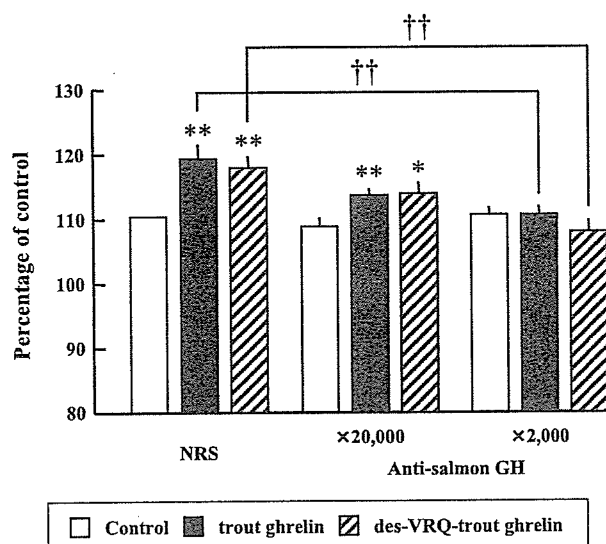

Figure 7 Effects of immunoneutralization against GH with anti-salmon GH serum on the superoxide production in phagocytic head-kidney leukocytes. Cells were incubated with trout ghrelin (10 nM) or des-VRQ-trout ghrelin (10 nM) in the presence of antisalmon GH for 4 h, and then treated with zymosan A (2 mg/ml) for 1 h. Data are expressed as the percentage of the control in each individual (mean ± S.E.M., n=6). *,**Significantly different from control at P<0·05 and P<0·01 respectively. ††Significant difference between columns at P<0·01.

importance of GH secreted by leukocytes. GH is now known to be produced in many immune tissues in tetrapods (Venters *et al.* 2001, Jeay *et al.* 2002). Distribution of GH mRNA in lymphoid tissues and leukocytes has also been demonstrated in fish, including rainbow trout (Calduch-Giner & Pérez-Sánchez 1999, Mori & Devlin 1999, Yada & Azuma 2002, Yada & Nakanishi 2002, Yada *et al.* 2005). GH is known to stimulate phagocytosis in both mammals and fish (Edwards *et al.* 1988, Fu *et al.* 1991, Sakai *et al.* 1996*a*, 1997, Yada & Nakanishi 2002). In rainbow trout, *in vivo* administration of GH enhances serum bactericidal activity and resistance to artificial infection of *Vibrio anguillarum* (Sakai *et al.* 1997). Ghrelin may enhance phagocytosis in part by stimulation of the autocrine pathway of GH. Hypothalamic and extrahypothalamic regulation of GH gene expression in the pituitary is well documented also in teleost fish (Melamed *et al.* 1998, Argenton *et al.* 2002). Our recent study revealed that GH mRNA levels in trout leukocytes are increased by *in vitro* administration of cortisol (Yada *et al.* 2005). However, regulation of GH gene expression in the fish immune system has not been fully elucidated. Further studies are needed on the immunomodulatory effects of other endocrine factors regulating GH secretion in the pituitary and lymphoid cells.

Apart from the stimulation of GH secretion, ghrelin has been shown to regulate prolactin and adrenocorticotropin secretion, feeding, glucose homeostasis, gastric motility, the cardiovascular system and cell proliferation in mammals (Muccioli *et al.* 2002, Yoshihara *et al.* 2002, Broglio *et al.* 2003). In fish, ghrelin is known to stimulate not only GH but also prolactin and luteinizing hormone secretion (Riley *et al.* 2002, Unniappan & Peter 2004). Expression of ghrelin in the stomach of female tilapia (*Oreochromis niloticus*) is higher than that in male fish, suggesting involvement of ghrelin in sexual dimorphism (Parhar *et al.* 2003). Ghrelin also stimulates food intake in goldfish (*Carassius auratus*) (Unniappan *et al.* 2004). The multifunction of ghrelin coincides well with distribution of GHS-R in various tissues (Muccioli *et al.* 2002). The present study revealed that two genes of GHS-R are ubiquitously expressed in trout tissues. Distribution of GHS-R mRNAs in the adherent leukocytes coincides with the *in vitro* stimulation of phagocytosis by ghrelin. The nonadherent leukocytes separated from peripheral blood also express GHS-R genes. The nonadherent leukocytes were microscopically identified as 95% lymphocytes (Yada *et al.* 2004*b*). Lymphocytes seem to be targets of ghrelin in teleost fish, as shown in human lymphocytes (Hattori *et al.* 2001, Poppi *et al.* 2002). In trout, GHS-R gene expression is also detected in the skin and osmoregulatory tissues, such as the gills and body kidney. Unlike mammals, fish show distinct endocrine regulation of pigmentation and body color change (Bentley 1998). Hormonal control of osmoregulation is also characteristic of euryhaline fish.

GH and insulin-like growth factor-I play important osmoregulatory roles in stimulating secretion of excess ions in several fish species, including rainbow trout (Sakamoto *et al.* 1993, McCormick 1995). Expression of GHS-R in the skin, gills and kidney of trout suggests that ghrelin is involved in the regulation of pigmentation and osmoregulation in teleost species.

It is becoming clear that ghrelin, like GH, is an important regulator of energy balance (Muccioli *et al.* 2002, Yoshihara *et al.* 2002, Broglio *et al.* 2003). GH is known to enhance proliferation of fish lymphocytes (Sakai *et al.* 1996*a*, 1996*b*, Yada *et al.* 2004*b*). However, ghrelin causes inhibition of cell proliferation in the human thyroid tumor cell line (Muccioli *et al.* 2002), although it stimulates the proliferation of prostate cancer cell lines (Jeffery *et al.* 2002). These contradictory results in the role of ghrelin in cell proliferation could be due to differences in the type of cell lines. Recently, De Vriese *et al.* (2005) revealed that administration of anti-ghrelin serum inhibits proliferation of erythroleukemic cells, suggesting that the autocrine pathway of ghrelin can stimulate the proliferation of immune cells. Studies are needed on the proliferative effect and autocrine pathway of ghrelin in the immune system in fish.

Interactions between the endocrine and immune systems via hormones and cytokines are important to adjust defense mechanisms in both mammals and fish (Weyts *et al.* 1999, Yada & Nakanishi 2002). Administration of homologous interleukin (IL)-1β activates the hypothalamo-pituitary-interrenal axis in rainbow trout (Holland *et al.* 2002). In man, ghrelin inhibits expression of IL-1β, IL-6, and tumor necrosis factor (TNF)-α in T cells and monocytes, suggesting that ghrelin modulates cell-to-cell interaction in leukocytes during inflammatory processes (Dixit *et al.* 2004). Although several cytokine genes, including IL-1β and TNF-α, are known to be expressed in PBL and/or HKL of rainbow trout, endocrine regulation of cytokine production in fish has yet to be determined (Yada & Nakanishi 2002). GH affects the production of several cytokines in human leukocytes (Derfalvi *et al.* 1998, Malarkey *et al.* 2002, Uronen-Hansson *et al.* 2003), and it seems to play a role in inhibiting the inflammatory response accompanied by increased levels of plasma ceruloplasmin, an acute-phase protein, in rainbow trout (Yada *et al.* 2004*a*). These results imply that ghrelin is involved in regulation of inflammation through cytokines and/or GH production in trout leukocytes. The role of ghrelin in the regulation of cytokine production in the fish immune system should be clarified in future studies.

In summary, the present study revealed that ghrelin stimulates phagocytosis, superoxide production and GH gene expression in rainbow trout leukocytes. Fish are considered to be the most primitive vertebrates possessing an immune system similar to that of mammals, characterized by lymphocytes, immunoglobulin, major histocompatibility complex (MHC) and T-cell receptor (TCR) (Yada & Nakanishi 2002). Even in fish, phagocytosis by macrophages, after antigen presentation through cell-to-cell interaction with the MHC/TCR system, is necessary for production of specific antibodies (Manning 1994). Besides the energy homeostasis shown in mammals, ghrelin may, like GH, possess multiple functions in the immune system (Clark 1997, Cohen & Kinney 2001, Yada & Nakanishi 2002, Yada *et al.* 2004*b*).

Acknowledgements

We thank Professor T Hirano, University of Hawaii, for valuable discussion and critical review of the manuscript, and Professors Y Takei, University of Tokyo, and M Sakai, Miyazaki University, for their encouragement during the course of this study. This study was supported by a grant-in-aid from the Ministry of Agriculture, Forestry and Fisheries, Japan. It is also funded by the Cooperative Research System of the Ocean Research Institute, University of Tokyo (#22) to TY. The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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Received in final form 25 December 2005
Accepted 6 January 2006

J Pharmacol Sci **100**, 398 – 410 (2006)

Journal of Pharmacological Sciences
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Review

Biological, Physiological, and Pharmacological Aspects of Ghrelin

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Received January 24, 2006

Abstract. Ghrelin, identified as an endogenous ligand for the growth hormone secretagogue receptor, functions as a somatotrophic and orexigenic signal from the stomach. Ghrelin has a unique post-translational modification: the hydroxyl group of the third amino acid, usually a serine but in some species a threonine, is esterified by octanoic acid and is essential for ghrelin's biological activities. The secretion of ghrelin increases under conditions of negative energy-balance, such as starvation, cachexia, and anorexia nervosa, whereas its expression decreases under conditions of positive energy-balance such as feeding, hyperglycemia, and obesity. In addition to having a powerful effect on the secretion of growth hormone, ghrelin stimulates food intake and transduces signals to hypothalamic regulatory nuclei that control energy homeostasis. Thus, it is interesting to note that the stomach may play an important role in not only digestion but also pituitary growth hormone release and central feeding regulation. We summarized recent findings on the integration of ghrelin into neuroendocrine networks that regulate food intake, energy balance, gastrointestinal function and growth.

Keywords: ghrelin, growth hormone (GH) secretagogue receptor, GH-releasing peptide, appetite regulation, stomach

I. Introduction

The pulsatile release of growth hormone (GH) from the pituitary somatotrophs is regulated by two hypothalamic peptides, growth-hormone-releasing hormone (GHRH) and somatostatin (1 – 3). GHRH is secreted by arcuate neurons into the hypothalamic portal vessels and stimulates GH release by activating GHRH receptor on pituitary somatotrophs. Somatostatin inhibits the activation of GHRH neurons through its receptor and hyperpolarizes somatotroph membranes to inhibit GH release. In addition, a third independent pathway regulating GH release has been identified from studies of GH secretagogues (GHSs) (4 – 8). GHSs are synthetic compounds that are potent stimulators of GH release, working through a new G-protein-coupled receptor (GPCR), the GHS receptor (GHS-R) (9 – 11). Because GHSs are a group of artificial compounds, it was postulated that there must exist an endogenous ligand that binds to GHS-R and carries out similar function to GHSs (12 – 14). Unexpectedly, we succeeded in the purification and identification of the endogenous ligand for the GHS-R from the stomach and named it "ghrelin" (15, 16). Ghrelin is a GH-releasing and appetite-stimulating peptide.

With the discovery of the orexigenic and adipogenic effects of ghrelin (17, 18), studies focused on defining neural circuits responsible for mediating ghrelin actions in the brain. The hypothalamus is the center for the integration of feeding and associated autonomic, neuroendocrine, and gastro-entero-pancreatic activities. Ghrelin regulates, in an antagonistic manner to leptin, synthesis and secretion of several neuropeptides in the hypothalamus that regulate feeding and energy balance. Here, we review the purification, structure, distribution, and physiological and pharmacological functions of ghrelin.

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Published online in J-STAGE: April 13, 2006
DOI: 10.1254/jphs.CRJ06002X

Invited article

II. Discovery of ghrelin

GHSs are a family of small synthetic peptides and non-peptide molecules that stimulate the secretion of GH in several species and in humans. In 1977, Bowers and colleagues developed the first peptides of GHS derived from methionine-enkephalin, which stimulated in vitro the release of GH from pituitary cells, although their potency was rather weak (19, 20). Further development produced several more potent peptides, including GH-releasing peptide-6 (GHRP-6) and hexarelin, which had improved GH-releasing potency and were active in vitro and in vivo (8, 21). Based on the structure of GHRP-6, nonpeptidyl compounds of GHS, such as MK-0677, were developed by Merck (8).

In 1996 the Merck group made another outstanding contribution by cloning the gene for the GHS receptor, a new GPCR with seven transmembrane domains (7-TM) (9). A receptor specifically different from GHRH was strongly supported by the differences in the GH releasing activity of GHS versus GHRH as well as the pituitary intracellular signal pathways of these compounds (Fig. 1). GHRH was known to act on the GHRH receptor to increase intracellular cyclic AMP through the protein kinase A pathway (22). This indicates that the GHRH receptor is coupled to a G_s subclass of G protein. On the other hand, GHS stimulates the phospholipase C pathway, resulting in an increase in the intracellular Ca^{2+} through inositol 1,4,5-triphosphate-mediated signal transduction, indicating that the ghrelin receptor is coupled to a G_q subclass of G protein (23, 24).

A cultured cell line expressing GHS-R was established and used to identify tissue extracts that could stimulate the GHS-R, as monitored by increases in intracellular Ca^{2+} levels. After screening several rat tissue extracts, very strong activity was unexpectedly found in stomach extracts (15). The purified ligand from the rat stomach through four steps of chromatography procedures was a 28-amino acid peptide, and it was named ghrelin ("ghre" is the Proto-Indo-European root of the word "grow"). Ghrelin has a unique post-translational modification: the hydroxyl group of the third serine residue (Ser3) is esterified by octanoic acid and is essential for ghrelin's biological activities (Fig. 2A). Human ghrelin is identical to rat ghrelin apart from two amino acids; however, amphibian (bullfrog) ghrelin undergo acylation at the third amino acid threonine (25). Thus, the acyl modification of hydroxyl group on the third residue represents an invariant and essential covalent change for the activation of ghrelins across multiple species (Fig. 2B). The amino acid sequences of mammalian ghrelins are well conserved; in particular, the 10 amino acids in their NH_2 termini are identical. In rat stomach, a second type of ghrelin peptide has been identified as des-Gln14-ghrelin (26). The ligand des-Gln14-ghrelin, a 27-amino acid peptide with an *n*-

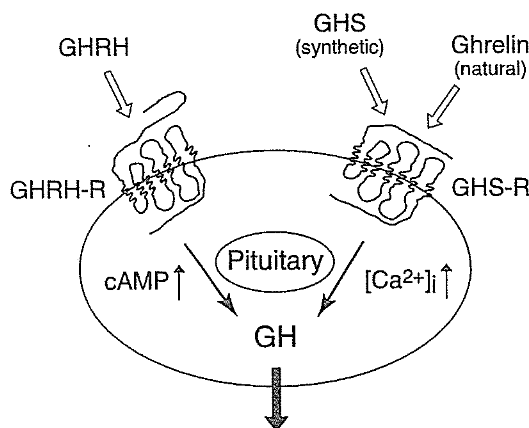

Fig. 1. Regulation of GH release from the pituitary. In the pituitary somatotroph cells, GHRH stimulates GH release through binding to the GHRH receptor and increasing intracellular cyclic AMP, while ghrelin and GHSs stimulate GH release through the GHS receptor to increase intracellular Ca^{2+} levels.

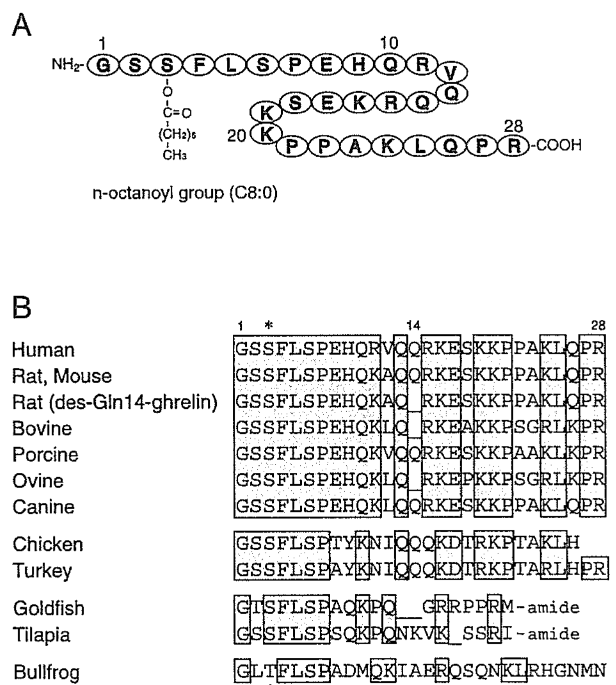

Fig. 2. The structure of ghrelin. A: structure of human ghrelin. The third amino acid, serine, is modified by an octanoic acid and this modification is essential for ghrelin's activity. B: sequence comparison of vertebrate ghrelins. Identical amino acids in each species are colored. The asterisks indicate the acyl-modified third amino acid. NH_2-terminal cores with acyl-modification sites are well conserved among vertebrate ghrelins.