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The role of neuromedin U during inflammatory response in the common carp

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ABSTRACT

In the current study, we cloned and characterized the neuromedin U (NMU) gene from the common carp *Cyprinus carpio* L., and identified its participation in immune responses in the teleost. Five isoforms of the preproNMU genes were generated by alternative splicing and isolated from carp. The longest form of the carp preproNMU1 (isoform 1) cDNA was composed of 803 bp, and contained an 18 bp 5′-UTR, a 212 bp 3′-UTR and a 573 bp open reading frame, which translates into a peptide comprising 190 amino acid (aa) residues. The remaining carp preproNMU isoforms were composed of 175 (preproNMU2), 158 (preproNMU3), 150 (preproNMU4) and 133 (preproNMU5) aa residues. Isoforms 1–3 contained four processing signals (KR or RR), while isoforms 4 and 5 contained only two processing signals. High homology was demonstrated among fish and other vertebral NMU at the biologically active C-terminal region (aa position 175–182). Carp preproNMU transcript variants were identified in various tissues, and the expression pattern has been shown to change depending on feeding status. Moreover, it was shown that the expression of preproNMU3 and preproNMU5 was increased following treatment with bacterial or viral mimics. Finally, we investigated the functional aspect of carp NMU using a synthetic NMU peptide. The peptide was found to increase the expression of inflammation-related cytokine genes in intestinal cells within 1 h of treatment. In addition, the activation of phagocytic cells was also stimulated by the NMU peptide. The discovery of NMU in carp allows for a further understanding of immune regulation by biologically active substances.

1. Introduction

Neuromedin U (NMU) is a neuropeptide that was first purified from porcine spinal cord in 1985 [1]. The first identified biological function of NMU was the control of smooth muscle contraction of the uterus and regulation of arterial blood pressure [1]. Following this initial identification and characterization of NMU, it was revealed that the peptide had various biological activities including the regulation of the stress response [2], alteration of ion transport in the jejunum [3], reduction of food intake and body weight [4] and immune regulation [5]. Recent studies regarding the role of NMU in the regulation of inflammation during immune response have shown that its regulation is under the control of cytokine release [6–8].

Four molecular forms of NMU have been identified in mammals and are known as NMU-8, NMU-9, NMU-23 and NMU-25. These forms have been purified and sequenced from porcine (8 and 25) [1], dog (8 and 25) [9], pig (9) [10], rat (23) [11], rabbit (25) [12] and human (25) [13]. Among these peptides, octapeptides from porcine and dog have been shown to be generated by cleavage at a di-basic Arg–Arg motif present in the longer NMU-25 form [9]. However, the source of the nonapeptides identified in the pig remains unclear, as larger NMU molecules have not been isolated. In non-mammalian species, nonapeptides have been identified in the chicken [14]. This peptide is thought to be produced independently of NMU-25, as the NMU-25 form lacks a di-basic Arg–Arg cleavage motif. Among amphibian species, NMU-25 has been purified and sequenced from the frog [15]. It has also been shown that the C-terminus of the peptides from all species contains a conserved Arg–Pro–Arg–Asn–NH_2 sequence that may be involved in the enhancement of NMU bioactivity [16].

The NMU gene has been recently isolated from goldfish *Carassius auratus* [17]. The goldfish NMU cDNA was found to encode four precursor/transcript variants that generate three NMU peptides

Abbreviations: NMU, neuromedin U; LPS, lipopolysaccharide; polyI:C, polyinosinic-polycytidylic acid; RT, reverse transcription; PCR, polymerase chain reaction; qPCR, quantitative real-time PCR.

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termed NMU-21, NMU-25 and NMU-38. Among these peptides, the henicosa peptide NMU-21 was able to potently inhibit food intake and locomotor activity. It was also shown that NMU-21 induced anorexigenic action that was mediated by the corticotrophin-releasing hormone 1/2 receptor-signalling pathway [18]. In addition, two NMU receptor genes have also been recently isolated and characterized from the goldfish [19]. Goldfish NMU receptors expressed on human embryonic kidney 293 cells responded to rat NMU-23 and goldfish NMU-21, -25, -28 but not to goldfish ghrelin. To date, studies on NMU and its receptor in teleost fish are limited to the above reports, and all other functional roles of fish NMU remain unclear.

In this report, we have identified five transcriptional variants of the NMU gene expressed in the common carp, and investigated the tissue distribution of the transcriptional variants. Simultaneously, we analysed the expression of the carp NMU receptor genes to further our understanding of the ligand–receptor interaction. Next, we examined the expression of the transcriptional variants of the NMU gene by using quantitative real-time PCR (Q-PCR) to observe the involvement of NMU in intestinal cells stimulated with lipopolysaccharides (LPS), one of the most powerful bacterial virulence factors in terms of pro-inflammatory properties, or polyinosinic-polycytidylic acid (polyI:C), a substance that mimics innate immune responses elicited by viral infections. In addition, the gene expression levels of inflammatory cytokines including interleukin (IL)-1β [20], tumour necrosis factor (TNF)-α [21,22] and anti-inflammatory cytokine IL-10 [23] in intestinal cells treated with a synthetic 40-residue peptide derived from the NMU protein were analysed by Q-PCR. Finally, the activation of phagocytic cells treated with the synthetic 40-residue peptide was analysed using measurements of respiratory burst. To the best of our knowledge this is the first report to demonstrate the participation of NMU during inflammatory response in teleost fish.

2. Materials and methods

2.1. Fish maintenance and handling

The common carp *Cyprinus carpio* L. (mean weight 100 g) was obtained from Mera Fisheries Farm (Miyazaki, Japan). The fish were firstly acclimatized in an aerated fresh water tank at 20 °C and fed a commercial diet comprising min. 35% crude protein, min. 3% crude fat, max. 5% crude fibre, max. 10% moisture, max. 12% crude ash and 35% additional components (Hikari Staple, KYORIN Co. Ltd., Hyogo, Japan) at 1% body weight per day for two weeks under a natural photoperiod prior to their use in the study. For expression analysis of the NMU gene, two feeding status groups were defined in order to investigate the involvement of NMU in appetite regulation. The first group was termed hunger status, and comprised fish that were not fed for one week. The second group was termed repletion status, and comprised fish that were fed at 5% body weight per day for one week by force feeding using a disposable feeding needle (1.2φ × 75 mm; Fuchigami, Kyoto, Japan). All experiments were conducted in accordance with the guidelines for the care and use of laboratory animals at the University of Miyazaki.

2.2. cDNA synthesis

Fish were anaesthetized with 0.05% 2-phenoxyethanol (Sigma, MO, USA) and tissues, including the brain, gills, fore-gut, mid-gut, hind-gut, spleen, head kidney (HK), liver, skin and muscle, were dissected 12 h after feeding under sterile conditions. Tissues were collected from three individual fish for each feeding status group and each tissue type combined.

Total RNA was then extracted using ISOGEN (Nippon Gene, Tokyo, Japan) in accordance to the manufacturer's instructions. Poly(A) mRNA was purified using the quick prep micro mRNA kit (Amersham Pharmacia Biotech, Uppsala, Sweden) and was treated with RNase-free DNase (Takara Bio, Shiga, Japan). cDNA was synthesized via reverse transcription from 2 μg mRNA using ReverTra Dash (Toyobo, Osaka, Japan).

2.3. Cloning and sequencing

In order to isolate the carp NMU gene, we used PCR on brain cDNA prepared according to the method described above with the gzNMU Fw and Rv primers (Table 1). These primers were designed from a completely conserved region of the NMU genes identified in goldfish (*Carassius auratus*; AB499530) and zebrafish (*Danio rerio*; XM_002665712). Having isolated a partial carp NMU sequence, the 5′ and 3′ ends were then obtained by RACE-PCR using gene-specific primers (Table 1). For 5′-RACE, cDNA was transcribed from poly(A) mRNA using an oligo-dT primer (Invitrogen, CA, USA), treated with *Escherichia coli* RNase H (Promega, WI, USA), purified using a PCR purification kit (Qiagen, Netherlands) and tailed with poly(C) at the 3′ end of the single strand cDNA using terminal deoxynucleotidyl transferase (TdT, Promega). PCR was then performed using Cyca NMU Rv, a carp NMU-specific reverse primer and the oligo(dG) primer (Table 1). For 3′-RACE, cDNA was transcribed from poly(A) mRNA using an oligo(dT) adapter primer (Table 1). PCR was then performed using Cyca NMU Fw (Table 1), a carp NMU-specific forward primer, and the adapter primer.

PCR amplification was performed in a 50 μl reaction volume containing 5.0 μl dNTP mixture and 10× Gene *Taq* Universal buffer, 0.5 μl *Taq* polymerase (5 units/ml, Nippon Gene), 5.0 μl each primer set (F and R; 2.5 μM), 28.5 μl distilled water and 1.0 μl carp genomic DNA (300 ng). The amplification regime was 3 min at 94 °C, followed by 35 cycles consisting of 94 °C for 30 s, 53 °C for 30 s and 72 °C for 45 s. The products were cloned into the pGEM-T Easy vector (Promega) and transformed into DH5α (Promega). Recombinants were identified using red-white colour selection when grown on MacConkey agar (Sigma). Plasmid DNA from at least three independent clones was recovered using a QIAprep Spin Miniprep Kit (Qiagen) and sequenced using a CEQ 8000 Automated Sequencer (Beckman Coulter, Inc., CA, USA). Sequences generated were analysed for similarity with other known sequences using the BLAST and FASTA programs.

2.4. Structural analysis

Multiple sequence alignments were generated using ClustalX version 1.81 [24] and homology analysis was performed using MatGat software version 2.02 [25]. Phylogenetic analysis was performed on the full-length amino acid sequences of the known NMU using the neighbour-joining method (NJ) [26]. MEGA4 was used to construct the tree with high confidence limits [27].

2.5. Expression analysis in tissues (hunger and repletion)

Initially, primers were designed to individually amplify the five transcript variants of the carp NMU gene analysed for the cloning step. RT-PCR with the primer combinations NMU1 (NMUex1345F-NMUex1R), NMU2 (NMUex2F-NMUex2R), NMU3 (NMUex1345F-NMUex3R), NMU4 (NMUex1345F-NMUex4R) and NMU5 (NMUex1345F-NMUex5R) (see Table 1) was performed using brain, gill, fore-gut, mid-gut, hind-gut, spleen, HK, liver, skin and muscle cDNA as prepared above (see Section 2.2). Expression in the gut and brain was confirmed in both cDNAs synthesized from mixed tissue and three individuals. Primers for the carp β-actin gene (Table 1) were used as an internal control for RT-PCR. PCR conditions were: 1 cycle at 94 °C for 3 min, 40