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Urotensin II upregulates migration and cytokine gene expression in leukocytes of the African clawed frog, *Xenopus laevis*

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ABSTRACT

Urotensin II (UII) exhibits diverse physiological actions including vasoconstriction, locomotor activity, osmoregulation, and immune response via the UII receptor (UTR) in mammals. However, in amphibians the function of the UII–UTR system remains unknown. In the present study, we investigated the potential immune function of UII using leukocytes isolated from the African clawed frog, *Xenopus laevis*. Stimulation of male frogs with lipopolysaccharide increased mRNA expression of UII and UTR in leukocytes, suggesting that inflammatory stimuli induce activation of the UII–UTR system. Migration assays showed that both UII and UII-related peptide enhanced migration of leukocytes in a dose-dependent manner, and that UII effect was inhibited by the UTR antagonist urantide. Inhibition of Rho kinase with Y-27632 abolished UII-induced migration, suggesting that it depends on the activation of RhoA/Rho kinase. Treatment of isolated leukocytes with UII increased the expression of several cytokine genes including tumor necrosis factor-α, interleukin-1β, and macrophage migration inhibitory factor, and the effects were abolished by urantide. These results suggest that in amphibian leukocytes the UII–UTR system is involved in the activation of leukocyte migration and cytokine gene expression in response to inflammatory stimuli.

1. Introduction

Urotensin II (UII) is a cyclic peptide originally isolated from the urophyses of teleost fish based on its ability to contract smooth muscles (Pearson et al., 1980). Subsequently, isoforms of UII have been isolated in various vertebrate species including amphibians (Conlon et al., 1992; Konno et al., 2013), rodents (Coulouarn et al., 1999), and human (Coulouarn et al., 1998). Recently, a second gene encoding a precursor of a UII analog, termed UII-related peptide (URP), has been reported in the Japanese eel (Nobata et al., 2011), African clawed frog (Konno et al., 2013), birds (Tostivint et al., 2006), and rodents and humans (Sugo et al., 2003). The putative mature form of URP is an octapeptide (ACFWKYCV/I), and shares the same cyclic moiety with UII, although its precursor sequences differ among vertebrate species. UII and URP act through the G-protein-coupled receptor-14, recently renamed urotensin II receptor (UTR). Complementary DNAs that encode UTR have been cloned in teleost fish (Evans et al., 2011; Lu et al., 2006), rodents (Marchese et al., 1995), cats (Aiyar et al., 2005), and humans (Ames et al., 1999). However, there was no information on amphibian UTR until we recently cloned a functional UTR from the African clawed frog, *Xenopus laevis* (Konno et al., 2013).

It is well established that UII in mammals is a potent vasoconstrictor with a potency of greater than that of endothelin-1 (Ames et al., 1999; Douglas and Ohlstein, 2000). Furthermore, recent studies in fish and mammals have shown that UII regulates diverse physiological actions including locomotor activity (Do-Rego et al., 2005), osmoregulation (Balment et al., 2005; Evans et al., 2011; Lu et al., 2006; Song et al., 2006), and immune response (Segain et al., 2007; Singh and Rai, 2011; Watanabe et al., 2005) through UTR. However, few studies have investigated the physiological roles of UII/URP in amphibians with the exception of the effects of UII on smooth muscle contraction (Yano et al., 1994) and vasoconstriction (Yano et al., 1995). One of the reasons may be that the target sites of UII/URP have not been well defined in amphibians. Thus, findings from amphibians would provide valuable information for understanding the diverse roles and functional evolution of the UII–UTR system in vertebrates.

We previously cloned cDNAs encoding URP and UTR from *X. laevis* and characterized the properties of UTR in the presence of UII, URP, and a UTR antagonist (urantide) using a calcium mobilization assay in the Chinese hamster ovary cells transiently expressing *Xenopus* UTR (Konno et al., 2013). Furthermore,

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immunohistochemical localization of *Xenopus* UTR suggested that the UII–UTR system acts in the kidney and urinary bladder (osmoregulatory organs), splenocytes and leukocytes (immune cells), and hyaline chondrocytes (connective tissue) (Konno et al., 2013). However, direct physiological actions in the target tissues of UII/URP have not yet been demonstrated. Because UTR was expressed in splenocytes and leukocytes in our previous study, we focused in the present study on potential immune functions of UII/URP in amphibian leukocytes.

Recent studies have revealed that UII and UTR were expressed respectively in lymphocytes and in monocytes/macrophages isolated from human peripheral blood mononuclear cells (PBMCs) of healthy subjects (Bousette et al., 2004; Segain et al., 2007). UTR is likely to function as a chemoattractant receptor for UII in human PBMC and rat splenocytes (Segain et al., 2007). In addition, the interaction between the UII–UTR system and cytokines, which are released from innate immune cells and play key roles in the regulation of immune response, has been reported in pathologies such as fibrotic disorders (Dai et al., 2007, 2011; Tian et al., 2008). Liang and colleagues showed that the inhibition of the UII–UTR system with urantide reduced the serum levels of TNF-α, IL-1β, and IFN-γ in lipopolysaccharide (LPS)/D-galactosamine (GaIN)-challenged mice (Liang et al., 2013). However, the direct actions of UII on cytokine production in leukocytes remain to be elucidated even in mammals. Thus, in the present study, we investigated the potential immune functions of the UII–UTR system in *X. laevis*, which is used to study the immune system, because it possesses both innate and acquired immune systems, as observed in mammals. The present study may shed light on the mechanism of control of inflammatory response via the UII–UTR system in leukocytes.

2. Materials and methods

2.1. Reagents

LPS (*Escherichia coli* strain O111), which was used in previous immune studies with *X. laevis* (Cui et al., 2011; Nagata et al., 2013), was purchased from Sigma (St. Louis, MO, USA). Rho-kinase inhibitor (Y-27632) was purchased from Wako Pure Chemical Industries (Osaka, Japan). *Xenopus* UII peptide (GNLSECFWKYCV) was synthesized by GenScript (Piscataway, NJ, USA). Human URP (ACFWKYCV) and urantide (a potent UTR antagonist) were purchased from the Peptide Institute (Osaka, Japan).

2.2. Animals and isolation of leukocytes

Immature male *X. laevis* 1.5 years of age (30–50 g body weight) were purchased from a commercial supplier (*Xenopus* Inbreed Strain Resource Center, Hyogo, Japan) and maintained in plastic containers (40 × 30 × 20 cm) containing dechlorinated tap water at 18–22 °C under a 12-h light/12-h dark photoperiod until use. Frogs were anesthetized with 0.1% ethyl 3-aminobenzoate methanesulfonate (MS-222; Sigma). In an experiment testing the inflammatory response, frogs ($n = 8$) were intraperitoneally injected with 100 μg of LPS in 200 μl Dulbecco's phosphate-buffered saline (PBS, pH 7.4). The dose of LPS and schedule for blood sampling were determined as previously described in *X. laevis* (Nagata et al., 2013). In the study, the injection of 100 μg LPS to immature *X. laevis* (30–40 g body weight) resulted in marked increases in the serum X-lectin concentration, which plays a role in antibacterial innate immunity, to maximum levels on day 3 after injection (Nagata et al., 2013). Three days after injection, the frogs were anesthetized in 0.1% MS-222 and peripheral blood was collected by cardiac puncture in 1-mL syringes. Blood samples were overlaid onto discontinuous Percoll (GE Healthcare BioScience, Piscataway, NJ, USA) gradients [90, 60, 50, 40% (v/v)] buffered with 7/9 diluted Dulbecco's PBS and centrifuged at 500*g* for 15 min at room temperature to separate erythrocytes and leukocytes (Aizawa et al., 2005) and the leukocyte suspension was used for histological observation, migration assay, and molecular analysis. All the experiments were performed in accordance with the guide for the care and use of laboratory animals and approved by the ethics committees of the University of Toyama.

2.3. Cytology of leukocytes

To discriminate leukocyte cell type, smear preparations of leukocytes were stained with May–Grunwald Giemsa (MGG) (Nacalai Tesque, Kyoto, Japan) following the manufacturer's instructions. In myeloperoxidase (MPO) staining, which is important for the cytomorphological diagnosis and classification of leukocytes, smear preparations were fixed in fixative containing 54% acetone and 2.7% glutaraldehyde for 30 s and then stained for MPO with an ImmPACT DAB Peroxidase Substrate kit (Vector Laboratories, Burlingame, CA). The cells were observed with an inverted microscope (Nikon ECLIPSE C1; Nikon, Tokyo, Japan).

2.4. Reverse-transcription (RT)-PCR

The expression of UII, URP, and UTR in leukocytes isolated from peripheral blood of immature male frogs was confirmed by the detection of each mRNA expression by RT-PCR. Total RNA was isolated from leukocytes, erythrocytes, and brain as positive control using RNAiso Plus reagent (Takara Bio, Otsu, Japan). Complementary DNA was synthesized from 1 μg of total RNA using a PrimeScript RT reagent Kit with gDNA Eraser (Takara Bio). Specific primers (Table 1) used in PCR were designed for UII (GenBank accession NM_001280580), URP (NM_001280582), UTR (AB727592), and elongation factor 1 alpha (EF1α; NM001101761) as reference. The PCR conditions comprised 32 cycles (UII, URP, and UTR) and 25 cycles (EF1α) of 30 s at 94 °C, 30 s at 58 °C, and 30 s at 72 °C, using 25-μl reaction mixtures. Sequences of PCR products amplified using each of these primer sets were confirmed by sequencing.

2.5. Migration assay of leukocytes

The migration ability of the isolated leukocytes was assessed using a transwell migration assay (a modified Boyden chamber assay) with polycarbonate membranes (5 μm pore size; Fig. S1) (Millicells; Millipore Corp., Billerica, MA, USA) as described previously (Mori et al., 2003). Leukocytes (2×10^5 cells) isolated from peripheral blood were suspended in Leibovitz's L-15 medium (Life Technologies Corp.) adjusted to amphibian osmolality (220 mOsm) with Dulbecco's PBS and were added to the upper chamber. After preincubation for 30 min, each UII, URP (10^{-9}–10^{-8} M), and LPS (2 μg/ml medium) was added to the lower chamber. The dose of LPS and incubation time with ligands and LPS in the migration assay were determined according to previous studies with human PBMCs and *Xenopus* leukocytes (Chadzinska and Plytycz, 2004; Cui et al., 2011; Segain et al., 2007). When preincubated, the leukocytes were pretreated with urantide (10^{-6} M) or Y-27632 (10^{-7} M) for 30 min at 22 °C. The leukocytes were allowed to migrate for 12 h at 22 °C. After this period, leukocytes migrating to the lower chamber were collected by centrifugation at 300*g* for 3 min and then counted with an automatic cell counter (Countess, Life Technologies Corp.). Cell migration was expressed as the chemotactic index calculated by the following ratio: cells migrating to reagents (LPS, UII, and URP)/cells migrating to control (medium). Furthermore, to determine the cell types of migrating