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Short communication

The tissue factor pathway inhibitor 1 of *Sciaenops ocellatus* possesses antimicrobial activity and is involved in the immune response against bacterial infection

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ABSTRACT

Tissue factor pathway inhibitor 1 (TFPI-1) is a Kunitz-type serine protease inhibitor that regulates the activation of tissue factor-induced coagulation. In teleosts, TFPI-1-like sequences have been found to exist in two species (*Danio rerio* and *Cyprinus carpio*); however, the potential function of fish TFPI-1 has not been investigated. In this study, we identified and analyzed a TFPI-1 homologue, SoTFPI-1, from red drum (*Sciaenops ocellatus*). The deduced amino acid sequence of SoTFPI-1 is 284 residues in length and contains three Kunitz domains, an acidic N-terminus, and a basic C-terminus. SoTFPI-1 shares 49.5% and 46.9% overall sequence identities with the TFPI-1 of *D. rerio* and *C. carpio*, respectively. Quantitative real time RT-PCR analysis showed that constitutive SoTFPI-1 expression occurred, in increasing order, in kidney, brain, liver, gill, blood, spleen, muscle, and heart. Bacterial infection and lipopolysaccharide exposure upregulated SoTFPI-1 expression in kidney in time-dependent manners. Recombinant SoTFPI-1 (rSoTFPI-1) purified from *Escherichia coli* exhibits not only serine protease inhibitor activity but also bactericidal activity in a manner that is independent of any host factors. A synthetic peptide, TO17, corresponding to the C-terminal basic region of SoTFPI-1 also possesses antibacterial effect that is more potent than that of the full-length rSoTFPI-1. Taken together, these results demonstrate that (i) SoTFPI-1 is a biologically active serine protease inhibitor endowed with bactericidal property; (ii) provide the first indication that teleost TFPI-1 is likely to be involved in anti-microbial infection and thus is linked to innate immune defense.

1. Introduction

Tissue factor pathway inhibitor 1 (TFPI-1, commonly referred to as TFPI) is an anticoagulant protein that inhibits tissue factor (TF)-mediated coagulation activation. TF is a member of the class II cytokine receptor superfamily and a high-affinity receptor for the coagulation factor VII (FXII). It initiates the extrinsic pathway of blood coagulation by binding in the presence of calcium to FXII, resulting in the formation of the TF–FVII/FVIIa complex. TF–FVII/FVIIa activates factor X (FX) and factor IX (FIX), leading to the generation of thrombin and the conversion of blood fibrinogen to fibrin (Lwaleed and Bass, 2006; Neuenschwander et al., 1993; Osterud and Rapaport, 1977). TFPI-1 blocks the activation of the coagulation cascade by forming a stable complex with Xa, whereby inactivating FXa. In addition, the TFPI–Xa complex also interacts with and inhibits TF–FVIIa, thus preventing FXa production by the latter (Broze, 1995).

Structurally, TFPI-1 contains three bovine pancreatic trypsin inhibitor (BPTI)/Kunitz domains arranged in tandem (Bajaj et al., 2001). The first and second BPTI/Kunitz domains bind FVIIa and FXa, respectively, while the third Kunitz domain is involved in cell surface and lipoprotein association (Girard et al., 1989; Piro and Broze, 2004). The C-terminus of TFPI-1 is positively charged and required for interactions with FXa, heparin, and certain types of cells (Cunningham et al., 2002; Ettelaie et al., 1999; Wesselschmidt et al., 1992). The C-terminal region of TFPI-1 can be cleaved off at different positions, resulting in TFPI-1 truncates with various sizes (Holst et al., 1993; Warshawsky et al., 1995). In humans, most plasma TFPI-1 are 34–41 kDa in molecular mass and complexed with different forms of lipoproteins (Girard et al., 1989; Lwaleed and Bass, 2006). A small population of plasma TFPI-1, which includes full-length and truncated TFPI-1, exists in free and uncomplexed forms. Compared to truncated TFPI-1, full-length TFPI-1 binds more effectively to FXa and thus is a more potent anticoagulant (Lindhout et al., 1995).

Studies have indicated that, in addition to functioning as a major regulator of blood coagulation, mammalian TFPI-1 is associated with innate immunity and involved in inflammation response. It

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is known that human TFPI-1 is able to reduce mortality in rabbit models of sepsis induced by lethal bacterial challenge (Camerota et al., 1998; Creasey et al., 1993; Matyal et al., 2001), and that TFPI-2, a structural homologue of TFPI-1, can regulate extracellular matrix remodeling through inhibiting plasmin and matrix metalloproteinase activity, whereby playing an important role in tumor growth and invasion (Chand et al., 2004; Kondraganti et al., 2006; Sierko et al., 2007). In addition, the C-terminal fragment of recombinant human TFPI-1 possesses complement-dependent antibacterial activity (Schirm et al., 2009). In contrast to mammalian TFPI-1, which has been studied extensively in recent years, very little study has been carried out with fish TFPI-1. To date, TFPI-2 has been discovered in several fish species, whereas TFPI-1-like sequences have only been identified in zebrafish (*Danio rerio*) (Hanumanthaiah et al., 2002) and common carp (*Cyprinus carpio*). The expression and potential function of fish TFPI-1 have not been investigated.

Red drum (*Sciaenops ocellatus*) was discovered originally in the Atlantic Ocean and the Gulf of Mexico; it was introduced into China in 1991 and has since been cultured extensively in China in a number of provinces. In spite of its economic importance, red drum is essentially unexplored with respect to its immunological mechanisms. In this study, we identified from this fish species a TFPI-1 homologue, SoTFPI-1, and analyzed its expression and functional property. We found that SoTFPI-1 exhibits serine protease inhibitor activity and bactericidal activity, the latter being likely mediated by a C-terminal segment of SoTFPI-1.

2. Materials and methods

2.1. Fish

Red drum (*S. ocellatus*) were purchased from a commercial fish farm in Fujian Province, China and maintained at 22 °C in aerated seawater. Fish were acclimatized in the laboratory for two weeks before experimental manipulation. Fish were anaesthetized with tricaine methanesulfonate (Sigma, St. Louis, MO, USA) prior to experiments involving tissue collection.

2.2. Bacterial strains and culture condition

Edwardsiella tarda TX1 is a fish pathogen that has been reported previously (Zheng et al., 2010). *E. coli* BL21(DE3) was purchased from Tiangen (Beijing, China). All strains were cultured in Luria–Bertani broth (Sambrook et al., 1989) medium at 28 °C (for *E. tarda*) or 37 °C (for *E. coli*).

2.3. Cloning of SoTFPI-1

Plasmid was isolated from ~1200 clones of a cDNA library constructed from the total RNA of *E. tarda*-challenged red drum head kidney, spleen, and liver (Dang et al., 2010). Sequence analysis revealed that one of the plasmids contains the open reading frame of a TFPI-1 homologue (named SoTFPI-1 for "*S. Ocellatus* TFPI-1") with a 5′ – untranslated region (UTR). The 3′-UTR of SoTFPI-1 were determined by rapid amplification of cDNA ends (RACE) using the SMART RACE cDNA Amplification Kit (Clontech, Mountain View, CA, USA) according to manufacturer's instructions and as described previously (Hu et al., 2010). The nucleotide sequence of SoTFPI-1 has been deposited in GenBank database under the accession number HM581689.

2.4. Sequence analysis

The cDNA and amino acid sequences of SoTFPI-1 were analyzed using the BLAST program at the National Center for Biotechnology Information (NCBI) and the Expert Protein Analysis System. Domain search was performed with the simple modular architecture research tool (SMART) version 4.0 and the conserved domain search program of NCBI. The calculated molecular mass and theoretical isoelectric point were predicated by EditSeq in DNASTAR software package (DNASTAR Inc. Madison, WI, USA). Multiple sequence alignment was created with the ClustalX program. Signal peptide search and subcellular localization prediction were performed with SignalP 3.0 and WoLF PSORT, respectively.

2.5. Quantitative real time reverse transcriptase-PCR (qRT-PCR) analysis of SoTFPI-1 expression in fish tissues

Brain, heart, gill, kidney, spleen, liver, muscle, and blood were taken aseptically from five fish and used for total RNA extraction with the RNAprep Tissue Kit (Tiangen, Beijing, China). One microgram of total RNA was used for cDNA synthesis with the Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA). qRT-PCR was carried out in an ABI 7300 Real-time Detection System (Applied Biosystems, Foster City, CA, USA) by using the SYBR ExScript qRT-PCR Kit (Takara, Dalian, China) as described previously (Hu et al., 2010). Each assay was performed in triplicate with β-actin mRNA as the control. All data are given in terms of relative mRNA, expressed as means plus or minus standard errors of the means (SE).

2.6. Expression of SoTFPI-1 in kidney in response to bacterial and lipopolysaccharide (LPS) challenge

E. tarda TX1 was cultured in LB medium to mid-logarithmic phase. The cells were washed with phosphate-buffered saline (PBS) and resuspended in PBS to 5×10^6 CFU/ml. LPS of a *Listonella anguillarum* was prepared as described previously (Liu et al., 2010) and suspended in PBS to 500 μg/ml. Red drum (~8.1 g) were divided randomly into groups of five fish and injected i.p. with 100 μl of TX1, LPS, or PBS. Fish were sacrificed at 4 h, 8 h, 12 h, 24 h, and 48 h post-challenge, and tissues were collected under aseptic conditions. Total RNA extraction, cDNA synthesis, and qRT-PCR were performed as described above.

2.7. Plasmid construction

To construct pEtTFPI-1, the coding sequence of SoTFPI-1 without signal sequence was amplified by PCR with primers F1 (5′-GATATCAGAAGAGACAGACAAGCA-3′; underlined sequence, EcoRV site) and R1 (5′-GATATCGATGGAGCGATGAAGAAT -3′; underlined sequence, EcoRV site); the PCR products were ligated with the *T–A* cloning vector pBS-T (Tiangen, Beijing, China), and the recombinant plasmid was digested with EcoRV to retrieve the 0.78 kb fragment, which was inserted into pET259 (Zheng et al., 2010) at the SwaI site.

2.8. Purification of recombinant SoTFPI-1 (rSoTFPI-1)

E. coli BL21(DE3) was transformed with pEtTFPI-1. The transformant was cultured in LB medium at 37 °C to mid-log phase, and expression of SoTFPI-1 was induced by adding isopropyl-β-D-thiogalactopyranoside to a final concentration of 1 mM. After growth at 37 °C for an additional 5 h, recombinant proteins were purified under denaturing conditions by using nickel–nitrilotriacetic acid (Ni–NTA) columns (GE Healthcare, USA) as recommended by the manufacturer. Briefly, the cells were harvested by centrifugation, and the cell pellet was resuspended in Buffer B (100 mM NaH_2PO_4, 10 mM Tris–Cl, and 8 M urea) at 5 ml/g wet weight. After incubation at room temperature for 1 h with stirring, the cells were centrifuged at 10,000× *g* for 30 min at room temperature. The supernatant was collected and applied to