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Functional characterization of insulin-like growth factors in an ancestral fish species, the Shovelnose sturgeon *Scaphirhynchus platorhynchus*

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ABSTRACT

Observations from the present study provide the first characterization of the GH-IGF axis in Shovelnose sturgeon *Scaphirhynchus platorhynchus*, an ancestral fish species. An initial characterization of steady-state IGF-I and IGF-II gene expression in multiple tissues was conducted using real-time RT-qPCR. Overall, the tissues had significantly different profiles of IGF-I gene expression, with the highest IGF-I expression observed in the liver. The highest IGF-II gene expression was also observed in the liver, with minimal or no detection in muscle. A comparison between IGF-I and IGF-II expression within individual tissues revealed higher levels of IGF-II than IGF-I mRNA in the spleen, stomach and trunk kidney, and higher levels of relative IGF-I mRNA expression in the intestine and muscle. The GH-IGF axis was further elucidated by observing the effects of exogenous GH on IGF-I and IGF-II expression in liver and muscle tissue. The results revealed a significant dose-dependent response of both hepatic IGF-I and IGF-II, and muscle IGF-I mRNA expression following rbGH administration. At the highest rbGH concentration (240 μg/g BW), IGF-I mRNA levels in liver and muscle peaked significantly at 48 h, indicating both hepatic and muscle IGF-I expression to be stimulated by GH. Hepatic IGF-II expression was also stimulated 48 h following rbGH administration. Expression of IGF-II mRNA was not inducible in the muscle. Few studies have evaluated the effects of exogenous GH on IGF expression in ancestral vertebrate species, and as such, this research provides valuable insight into the evolution of the somatotropic axis in vertebrates.

1. Introduction

Sturgeons (family: Acipenseridae) are of the order Acipenseriformes, a group of Chondrichthyans with an evolutionary history of at least 200 million years (Peng et al., 2007), which also includes paddlefish (family: Polyodontidae) and some extinct families (Helfman et al., 1997). Currently, the Acipenseridae family encompasses 25 species split among 4 different genera: 17 *Acipenser*; 2 *Huso*; 3 *Pseudoscaphihryncus*; and 3 *Scaphirhyncus* (Froese and Pauly, 2015). As living specimens, sturgeon hold a unique phylogenetic position, not having undergone the generally accepted fish specific whole genome duplication, which has been associated with the radiation of teleost fish diversity (Meyer and Van de Peer, 2005). As a result, the sarcopterygian (lobe-finned fishes and tetrapods) genome possessed originally only half as many genes compared to the derived fishes. Thus as a group, the Acipenseridae family provides researchers with the unique opportunity to study the functional and molecular evolution of physiological systems, including the evolution of the mechanisms associated with growth in vertebrates.

Little is known regarding the mechanisms of growth regulation in ancestral fishes. Sequence, immunohistochemistry and phylogenetic analysis exist for growth hormone (GH) in a few ancestral fish species (Rubin and Dores, 1994; Kawauchi et al., 2002; Azizzadeh Pormehr et al., 2013), however, functional characterization of the somatotropic axis is lacking. Similar to mammals, the teleost growth hormone-insulin-like growth factor (IGF) axis is integral in the regulation of cell differentiation and proliferation, and other physiological processes, including growth promotion and reproduction (Duan, 1997). Strong cross-reactivity of sturgeon GH in tetrapod radioimmunoassays and a high degree of conservation in GH structure between sturgeon and tetrapods, and to a lesser extent teleost fishes, is suggestive of corresponding functional conservation (Farmer et al., 1981). Similar to tetrapods, GH works in concert with the IGFs in teleost fishes, and together act as the primary hormonal pathway regulating growth (Moriyama et al., 2000; McCormick, 2001; Fuentes et al., 2013), development (Reinecke et al., 2005), and cell differentiation (Funkenstein et al., 2006; Rius-Francino et al., 2011; Salmerón et al., 2013). Growth hormone can function independently and directly on tissues through stimulation of specific cell types or, most notably, through the anabolic actions of IGFs (Duan and Plisetskaya, 1993; Reinecke et al., 2005). In fish and mammals alike, IGF-I is primarily synthesized in the liver, where it then circulates and binds to receptors in distant target tissues. In addition, distinct localization of IGF-I and IGF-II production and paracrine/autocrine signaling of IGFs can occur in extrahepatic tissues,

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which has been demonstrated in previous studies using fish as models (Duan and Plisetskaya, 1993; Sakamoto and Hirano, 1993; Reinecke et al., 1997). As such, the delineation of steady-state IGF messenger RNA (mRNA) expression in extrahepatic sites can provide further insight into the roles of IGFs in tissues not primarily involved with growth promotion or regulation. Previous studies have detected steady-state levels of IGF-I in extrahepatic tissues, including the kidneys, gonads, gills, and spleen, further revealing the intricacies of their broad physiological functions in fish (Berishvili et al., 2006; Reindl and Sheridan, 2012). In addition, researchers have detected an abundance of IGF-I and IGF-II mRNA expression in multiple species of bony fish at varying life stages, which further stresses the regulatory importance IGFs may have in both embryonic and post-natal development (Greene and Chen, 1999; Pozios et al., 2001; Peterson et al., 2005). Literature exploring steady-state levels of IGFs in extrahepatic tissues isn't extensively available for fish species, and the vast majority of research pertaining to steady-state IGF expression has been explored in commercially important species. However, ancestral vertebrate species can provide a more comparative perspective, evolutionarily and functionally, between primitive and more modernly derived species. In addition, measuring the responses of IGFs to exogenous GH stimulation can aid in the delineation of growth mechanisms. In fish, administration of exogenous growth hormone has proved valuable in understanding the mechanisms that regulate somatic growth, environmental adaptations, cell differentiation, reproduction, and many other complex physiological processes (Peterson et al., 2004; Codina et al., 2008; Ponce et al., 2008; Rius-Francino et al., 2011).

As in mammals, the somatic growth mechanisms of GH in teleosts are primarily mediated via hepatic IGF-I, with the liver expressing the greatest amount of IGF-I mRNA (Pierce et al., 2004; Reinecke et al., 1997). Experiments investigating the acute effects of exogenous GH injections in fish have supported this, revealing GH is highly effective in increasing hepatic IGF expression (Sakamoto and Hirano, 1993; Duan et al., 1994; Kajimura et al., 2001; Cheng et al., 2002; Biga et al., 2004). Previous studies have demonstrated a significant acute response (<3 days) of hepatic and circulating IGF-I following exogenous GH administration (Sakamoto and Hirano, 1993; Duan et al., 1994; Shamblott et al., 1995; Biga et al., 2004, 2005). Kajimura et al. (2001) discovered that in Mozambique tilapia *Oreochromis mossambicus* exogenous recombinant bovine GH (rbGH) significantly increased IGF-I expression in skeletal muscle, providing further support for GH's roles in somatic growth and muscle accretion in fish.

Elucidating the somatic growth axis in an ancestral species, such as the Shovelnose sturgeon *Scaphirhynchus platorhynchus* can provide insight, not only, into sturgeon physiology, but into the evolution of vertebrate growth processes. To further examine the conservation of the vertebrate somatotropic axis and delineate the roles of IGFs in an ancestral species, multi-tissue IGF-I and -II mRNA expression profiles in Shovelnose sturgeon were initially characterized followed by the characterization of hepatic and muscle IGF-I and IGF-II expression subsequent to rbGH administration.

2. Material and methods

2.1. Animal husbandry

The following experimental protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of Southern Illinois University Carbondale (SIUC) under Protocol Number 12-008. Shovelnose sturgeon were obtained as fry from the Bozeman Fish Technology Center (Bozeman, Montana, USA) and cultured for 18 months at the Center for Fisheries, Aquaculture, and Aquatic Sciences at Southern Illinois University Carbondale. Two weeks prior to the experiment, fifteen fish weighing approximately 150 g and measuring approximately 48 mm were stocked into a 2039 L raceway. Fish were hand fed a 50% protein starter feed (Aquamax® 200, Purina Mills, LLC, Richmond, Indiana, USA) four times daily during the two-week acclimation period, and fish were reared under a 12:12 h light:dark photoperiod. Feed was withheld 24 h prior to the start of the experiment.

Culture water was maintained at 22.5 ± 0.4 °C, and temperature was recorded daily (YSI Model 550A Dissolved Oxygen Meter, Yellow Springs, Ohio, USA). Total unionized ammonia (NH_3), alkalinity, nitrite, nitrate, hardness, and pH levels were measured weekly using a LaMotte Smart3® Colorimeter (La Motte Company, Chestertown, MD, USA), a salinity refractometer, and a S20 SevenEasy® pH meter (Mettler Toledo, Columbus, OH, USA) (0.02 ppm Nitrogen; 0.01 ppm Ammonia; 1 ppt Salinity; 160 ppm Total Alkalinity; 7.86 pH). Dissolved oxygen was maintained at 7.0 ± 0.04 mg/L.

2.2. Tissue collection and gene expression analysis

Shovelnose sturgeon were rapidly caught and euthanized using a sodium bicarbonate-buffered, 250-mg/L solution of tricainemethanesulfonate (MS-222; Western Chemicals Inc., Ferndale, WA, USA). Fish remained in the solution for 10-min after the cessation of opercular movement (American Veterinary Medical Association (AVMA), 2007). Analysis of steady-state expression in liver and muscle was determined using ten fish, and in other tissues was determined using four fish. Immediately following euthanasia, the trunk kidney, head kidney, liver, spleen, gill, muscle, brain, pituitary, hypothalamus, heart, intestine, and stomach were rapidly excised. Samples were placed in RNAse-free microcentrifuge tubes containing 1 mL TRIzol® (Life Technologies, Grand Island, NY, USA), flash frozen in liquid nitrogen, and stored at −80 °C until further RNA isolation and real-time qPCR analysis of IGF expression. Total RNA was isolated according to the manufacturer's instructions and was used for analysis of IGF-I and IGF-II mRNA from each of the tissues. Total RNA concentration and sample purity were quantified by measuring the absorbance at 260 nm and 280 nm with a NanoDrop® 2000 spectrophotometer (ThermoScientific, Wilmington, DE, USA). Each RNA sample was measured in triplicate and all samples analyzed had a 260/280 ratio ≥ 1.80. Prior to reverse transcription, total RNA was treated with DNase I® (Life Technologies) to prevent genomic DNA contamination. One microgram of total RNA was used to produce cDNA, following the manufacturer's protocol (iScript cDNA Synthesis Kit, BioRad, Hercules, CA, USA).

2.3. Primer design and sequence verification

Specific oligonucleotide primers for real-time RT-qPCR were designed by the using Beacon Designer 7 program (Premier Biosoft, Palo Alto CA). Gene-specific primer pairs for IGF-I and GAPDH (glyceraldehyde 3-phosphate dehydrogenase) were designed from the conserved regions of cross-species comparative alignments of sturgeon species (Table 1). IGF-II primer pairs were derived from Pallid sturgeon *Scaphirhynchus albus* gene sequence (Table 1). The best respective primer pairs were selected from several pairs based on PCR product quality and lengths after electrophoresis on a 1.3% agarose gel. PCR products were then sequenced to verify gene specificity. ExoSAP PCR was used to clean successful amplifications, and a sequencing reaction was conducted using the Big Dye Terminator Kit (Applied Biosystems, Valencia, CA, USA), following standard manufacturer protocol. Sequencing reactions contained 1 μl BigDye v3.1, 3 μl 5× Buffer, 1.0 μl primer (3.2 μM), 9.5 μl H_20, and 0.5 μl product. Subsequent reactions products were cleaned using Sephadex columns (General Electric Healthcare, Buckinghamshire, UK), and sequenced using an ABI 3130 XL genetic analyzer (Applied Biosystems).

2.4. Real-time quantitative polymerase chain reaction

IGF-I and IGF-II mRNA were quantified by real-time RT-qPCR using the SsoFast EvaGreen Supermix protocol (BioRad). The relative expression abundances were normalized using GAPDH as a reference gene,