Expression In Fish Of Hepcidin, A Putative Antimicrobial Peptide And Iron Regulatory Hormone

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Abstract

Hepcidin is a small, cationic peptide that was originally identified in mammals as an antimicrobial peptide and later shown to be an important iron regulatory hormone. Highly conserved hepcidin sequences have been identified in numerous fish species and there is evidence that the antimicrobial and iron-regulatory functions are conserved in fish as well. This review describes the expression of hepcidin genes in fish. In fish, hepcidin is primarily expressed in the liver. Expression is up-regulated by exposure to Gram-positive bacteria, Gram-negative bacteria, bacterial components such as lipopolysaccharides (LPS), vaccination with attenuated bacteria, and double-stranded RNA (a viral mimic). Hepcidin expression is also induced in response to excess iron and reduced by hypoxia and anemia. The expression patterns of hepcidin genes in some fish species suggest that the possible iron regulatory and antimicrobial activities may be separated into distinct hepcidin peptides in some fish species.

Key words: hepcidin, fish, expression

Introduction

Hepcidin is a small, cysteine-rich protein that is required for the maintenance of normal homeostatic levels of iron in humans and other mammals (Nicolas et al. 2001, Nicolas et al. 2003, Roetto et al. 2003). Iron is a required component of proteins that transport and store oxygen (hemoglobin and myoglobin) and a necessary cofactor of many enzymes involved in redox reactions; however, excess iron causes destructive organ damage, apparently through the production of free radicals (Andrews 1999). Thus maintaining normal iron homeostasis is critical. Iron levels are conserved through the recycling of senescent erythrocytes; cellular iron is bound by ferritin and stored in macrophages and hepatocytes. In addition, dietary iron is absorbed from the small intestine. Release of iron from hepatocytes and macrophages and uptake of iron from the small intestine is facilitated by the iron transporter ferroportin (Donovan et al. 2005, Fraenkel et al. 2005). Ferroportin is the only known iron exporter in vertebrate animals; ferroportin is an integral membrane protein found in enterocytes, hepatocytes, and macrophages (Donovan et al. 2005). When iron levels are too high, hepcidin, which is primarily expressed in liver (Krause et al. 2000, Park et al. 2001), circulates in plasma and binds to the iron transporter ferroportin. The resulting complex is then internalized and degraded, thus blocking release of stored iron (Nemeth et al. 2004b, Knutson et al. 2005). Disruption of hepcidin results in hemochromatosis, where excess iron is found in the liver and pancreas (Nicolas et al. 2001) due to unregulated uptake of iron from the small intestine. Overproduction of hepcidin results in severe iron deficiencies (Nicolas et al. 2002a). Mutations in the hepcidin gene in humans are associated with juvenile hemochromatosis, where patients exhibit a severe overload of iron in the heart and liver (Roetto et al. 2003). Hepcidin expression is stimulated by excess iron (Pigeon

et al. 2001) and decreased by anemia (Nicolas et al. 2002b, Weinstein et al. 2002) and hypoxia (Nicolas et al. 2002b). When oxygen is limited, the physiological response is to produce more erythrocytes, which requires iron for oxygen-transporting hemoglobin.

Hepcidin may also act as an antimicrobial peptide; however, the *in vivo* antimicrobial activity of hepcidin is unclear. Hepcidin was originally identified as a cysteine-rich protein similar to β-defensins and isolated from human urine (Park et al. 2001) and human plasma ultrafiltrate (Liver-Expressed Antimicrobial Protein; LEAP-1) (Krause et al. 2000). The synthetic peptide has *in vitro* antimicrobial activity against Gram-negative bacteria, Gram-positive bacteria, and several fungal species at concentrations similar to those seen with other antimicrobial peptides (Krause et al. 2000, Park et al. 2001). However, the concentration of hepcidin in urine is not high enough for antimicrobial activity, even during infection (Ganz 2006). Hepcidin does localize to the phagosomes of mouse macrophages upon infection with mycobacteria and synthetic hepcidin exhibits antimycobacterial activity *in vitro* (Sow et al. 2007). Furthermore, production of hepcidin in mice macrophages and neutronphils, but not in liver hepatocytes, upon exposure to bacteria requires the toll-like receptor TLR4, which recognizes bacterial lipopolysaccharides (Peyssonnaux et al. 2006).

The role of hepcidin in infection may be through the stimulation of 'anemia of inflammation'. Hepcidin expression is induced by infection (Nemeth et al. 2003) and by inflammation (Nicolas et al. 2002b). Hypoferremia, the reduction of iron available in plasma, occurs shortly after an inflammatory stimulus and is mediated by the IL-6 induction of hepcidin (Nemeth et al. 2004a). Hepcidin then blocks the release of iron from macrophages and the uptake of iron the small intestine. This iron sequestration may increase resistance to microbial infection (Jurado 1997).

Fish hepcidins

Hepcidins have been identified in many fishes from multiple orders including Perciformes, Cypriniformes, Siluriformes, Gadiformes, and Salmoniformes; from freshwater, saltwater, and diadromous fish; and from fish living in extreme habitats. These include hepcidins from hybrid striped bass (*Morone saxatilis x M. chrysops*; Shike et al. 2002), winter flounder (*Pseudopleuronectes americanus*; Douglas et al. 2003), Atlantic salmon (*Salmo salar*; Douglas et al. 2003), rainbow trout (*Oncorhynchus mykiss*; Douglas et al. 2003), medaka (*Oryzias latipes*; Douglas et al. 2003), zebrafish (*Danio rerio*; Shike et al. 2004), olive flounder (*Paralichthys olivaceus*; Hirono et al. 2005, Kim et al. 2005), red sea bream (*Chrysophrys major*; Chen et al. 2005), channel catfish (*Ictalurus punctatus*; Bao et al. 2005), black porgy (*Acanthopagrus schlegelii*; Wang et al. 2006), turbot (*Scophthalmus maximus*; Chen et al. 2007), Atlantic cod (*Gadus morhua*; Solstad et al. 2008), sea bass (*Dicentrarchus labrax*; Rodrigues et al. 2006), and several Antarctic notothenoid fish species (Xu et al. 2008).

Gene structure and gene sequence are remarkably conserved, both within fish and between fish and mammals. Hepcidin genes in fish and mammals contain three exons separated by two introns and encode a prepropeptide that consists of a highly conserved signal peptide sequence (24 residues), an acidic propiece (approximately 38-40 residues), and a mature peptide (19-27 residues) (Douglas et al. 2003). The prepropeptides contain a consensus furin cleavage

site and the mature peptide contains eight conserved cysteines and a conserved glycine residue.

Fish hepcidin proteins are divided into two main clusters by phylogenetic analysis; the mature peptide of the hepcidins in one group contain an amino-terminal sequence similar to the Q-S-H-L-S-L sequence in largemouth bass (*Micropterus salmoides*) hepcidin-1, while the mature peptides in the other group lack this amino-terminal sequence (Robertson et al. 2009). It is possible that this Q-S-H-L-S-L sequence may be a metal binding site. Small amino-terminal Cu(II)- and Ni(II)-binding motifs (ATCUN motifs) contain a histidine in the third position and are involved in binding metal ions. Human Hep25 contains an amino-terminal ATCUN motif that is required for Hep25 activity as an iron-regulatory hormone: aggregation (Hunter et al. 2002), binding to ferroportin (Nemeth et al. 2006), and binding to metal (Melino et al. 2005). Many fish encode multiple hepcidin genes and some of these fish (*e.g.*, largemouth bass, smallmouth bass (*Microp-terus dolomieu*), tilapia (*Oreochromis mossambicus*), and yellow croaker (*Pseudosciaena crocea*) produce both hepcidin(s) with a sequence similar to Q-S-H-L-S-L and hepcidin(s) that lack this sequence (Huang et al. 2007, Martin-Antonio et al. 2009, Robertson et al. 2009). It is possible that in fish, an iron regulatory function and an antimicrobial activity are performed by separate proteins (Ganz 2006, Robertson et al. 2009).

Tissue expression of fish hepcidins

Fish hepcidins, like mammalian hepcidins, are predominantly expressed in the liver. However, fish hepcidin expression is also detected in spleen, anterior kidney, kidney, blood, esophagus, stomach, intestine, heart, muscle, brain, gonad, gill, and skin. Hepcidin is expressed in the liver, esophagus, and cardiac stomach of winter flounder and in the liver, blood, muscle, gill, and skin of Atlantic salmon (Douglas et al. 2003). In Atlantic cod, hepcidin expression is detected in spleen and anterior kidney (Feng et al. 2009). In olive flounder, one hepcidin is expressed in liver, while another hepcidin is expressed in liver, anterior kidney, spleen, peripheral blood leukocytes, and stomach, but not brain, eye, gill, heart, intestine, muscle, ovary, skin, nor posterior kidney (Hirono et al. 2005). Hepcidin is expressed in liver, kidney, spleen, heart, and stomach in yellow croaker (Wang et al. 2009b). Redbanded seabream (*Pagrus auriga*) encodes four hepcidin genes, each with different expression patterns: *HAMP1* is expressed predominantly in kidney and spleen, but also in stomach, gill, heart, intestine, skin, liver, and brain; *HAMP2* is predominately expressed in kidney, spleen, and intestine; and *HAMP3* and *HAMP4* are mainly expressed in liver (Martin-Antonio et al. 2009). Hepcidin expression has also been detected in the gonad, heart, brain, anterior kidney, intestine, spleen, liver, gill, stomach, kidney, muscle, and skin of red sea bream (Chen et al. 2005).

Hepcidin induction by bacterial exposure

In fish, expression of hepcidin is induced by exposure to bacteria, bacterial components (*e.g.*, LPS), infection, inflammation, vaccination, and polyI:C (a double-stranded RNA molecule). This induction is seen not only in liver, but also in kidney, spleen, gill, intestine, and other tissues. The diverse expression of hepcidin in multiple tissues and the differential tissue induction of distinct hepcidins by infection suggest that some fish hepcidins may have antimicrobial activities other than regulation of iron homeostasis.

In Atlantic salmon infected with *Aeromonas salmonicida*, hepcidin expression of one Atlantic salmon hepcidin is upregulated mainly in esophagus, stomach, pyloric caecae, liver, spleen, intestine, posterior kidney, rectum, and muscle, while a second Atlantic salmon hepcidin is upregulated mainly in stomach pyloric caecae, liver, spleen, intestine, brain, heart, and muscle (Douglas et al. 2003). During an *in vitro* infection, rainbow trout macrophages exposed to *E. coli* exhibit increased hepcidin expression (Chiou et al. 2007). In largemouth bass, *hep-1* expression is induced in spleen and *hep-2* expression is induced in liver after exposure to *Edwardsiella ictaluri* (Robertson et al. 2009). Hepcidin expression in hybrid striped bass and white bass (*Morone chrysops*) is induced in the liver by the Gram-positive bacterium *Streptococcus iniae* and the Gram-negative bacterium *Aeromonas salmonicida* (Shike et al. 2002, Lauth et al. 2005,). Catfish hepcidin is induced in the spleen and in head kidney, but not in liver, by Gram-negative *E. ictaluri* (Bao et al. 2005). Red sea bream hepcidin is induced in the spleen, gill, liver, and intestine by exposure to *E. coli* DH5α (Chen et al. 2005). Hepcidin expression in zebrafish (Shike et al. 2004), black porgy (Yang et al. 2007), and sea bass (Rodrigues et al. 2006) is induced by bacterial infection.

In Atlantic cod, hepcidin expression is upregulated in spleen and anterior kidney by intracoelomic injection of formalin-killed *Aeromonas salmonicida* (Feng et al. 2009). Hepcidin is upregulated in Atlantic salmon after vaccination with an attenuated *Aeromonas salmonicida* strain (Martin et al. 2006). Rainbow trout hepcidin induced in liver by intracoelomic injection of killed Gram-negative *Vibrio anguillarum* (Bayne et al. 2001). Hepcidin expression is induced in the liver of rainbow trout in response to an inflammatory stimulus (Bayne et al. 2001).

Both olive flounder hepcidins (Hep-JF1 and Hep-JF2) are induced by LPS in the liver and one hepcidin (Hep-JF2) is induced by LPS in the kidney (Hirono et al. 2005, Kim et al. 2005). Hepcidin expression is induced in the spleen, heart, and stomach, but not in liver, of yellow croaker exposed to LPS (Wang et al. 2009b). Expression of three hepcidin genes (*HAMP1*, *HAMP3*, and *HAMP4*, but not *HAMP2*) is induced in the liver, kidney, and spleen of yellow croaker injected intracoelomically with LPS (Martin-Antonio et al. 2009). In tilapia, expression of TH1-5 and TH2-3, both of which display antimicrobial activity *in vitro*, is stimulated by LPS; expression of TH2-2, which does not display antimicrobial activity *in vitro*, is not stimulated by LPS (Huang et al. 2007).

In vitro antimicrobial activity of hepcidin

In vitro antimicrobial activity against Gram-negative bacteria, Gram-positive bacteria, and some fungi has been demonstrated with several synthetic fish hepcidin peptides. Synthetic bass hepcidin peptide is active *in vitro* against Gram-negative bacteria and fungi, but not Gram-positive bacteria (Lauth et al. 2005). Synthetic yellow croaker hepcidin displays *in vitro* antimicrobial activity against fungi and the Gram-negative bacterial fish pathogens *Aeromonas hydrophila*, *Vibrio parahaemolyticus*, *V. alginolyticus*, and *V. harveyi* (Wang et al. 2009b). Olive flounder expresses two different hepcidins (Hirono et al. 2005), one with an H-I-S-H-I-S-M at the amino terminus of the predicted mature peptide (Hep-JF1) and one without (Hep-JF2). The Hep-JF1 peptide, which is expressed only in liver and contains the amino-terminal H-I-S-H-I-S sequence, displays no antimicrobial activity *in vitro*;

while Hep-JF2, which lacks this amino terminal sequence and is expressed in many tissues, displays *in vitro* antimicrobial activity against both Gram-positive and Gram-negative bacteria (Hirono et al. 2005). Tilapia expresses three different hepcidins: one (TH2-3) with an amino-terminal Q-S-H-L-S-L sequence and two (TH1-5 and TH2-2) without; TH1-5 is active against Gram-positive bacteria, TH2-3 is active against Gram-negative bacteria, and TH2-2 is inactive *in vitro* (Huang et al. 2007).

Induction of hepcidin by double-stranded RNA and viral infection

Hepcidin expression can also be induced by double-stranded RNA and viral infection. Hepcidin expression is upregulated by the double-stranded viral mimic poly I:C in rainbow trout macrophages in an *in vitro* model and in spleen, but not the liver, of whole fish (Chiou et al. 2007). Hepcidin expression is also upregulated in rainbow trout macrophages *in vitro* by infection with the rhabdovirus that causes infectious hematopoietic necrosis (Chiou et al. 2007). In tilapia, expression of TH1-5 and TH2-3, both of which are stimulated by LPS, are also stimulated by poly I:C; expression of TH2-2 is not stimulated by LPS or poly I:C (Huang et al. 2007). Hepcidin expression is upregulated in the spleen of large yellow croaker by poly I:C (Zheng et al. 2006).

Regulation of fish hepcidin by iron, anemia, and hypoxia

Hepcidin expression is stimulated by excess iron and reduced by anemia and hypoxia in fish, similar to in mammals. Hepcidin expression is induced by high levels of iron in zebrafish (Fraenkel et al. 2005) and sea bass (Rodrigues et al. 2006). In olive flounder, expression of Hep-JF1 is inhibited in the liver, while expression of Hep-JF2 is stimulated in kidney in response to iron overloading (Hirono et al. 2005). Hepcidin expression is reduced by anemia in zebrafish (Fraenkel et al. 2005) and in catfish (Shi and Camus 2006). Hepcidin expression is also reduced by hypoxia in the goby *Gillichthys mirabilis* (Gracey et al. 2001).

Hepcidin expression by estrogenic compounds and contaminants

Our laboratory showed that constitutive expression of *hep-1* and bacterial-induction of *hep-2* was reduced by estradiol in the liver of largemouth bass (Robertson et al. 2009); regulation of hepcidin expression by estrogen-related chemicals (*e.g.*, estrogenic endocrine-disrupting compounds) is currently being investigated. Hepcidin expression is induced in red sea bream and black porgy by the contaminant benzo[a]pyrene (Wang et al. 2009a). Benzo(a)pyrene is a polycyclic aromatic hydrocarbon (PAH) found in coal tar and cigarette smoke that has been shown to impair the immune system in fish and decrease resistance to bacteria (Carlson et al. 2002). Hepcidin is upregulated in liver of medaka exposed to the contaminant 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD or dioxin; (Volz et al. 2005). TCDD is a ubiquitous and persistent contaminant that causes defects in reproduction, development, and the immune response. In a comparison of winter flounder from highly polluted estuaries in New Jersey and winter flounder from less polluted, less industrialized New Jersey estuaries, hepcidin was found to be induced in the livers of fish from the more industrialized estuaries, while vitellogenin is downregulated (Straub et al. 2004).

Conclusions

Sequence and expression analyses suggest that antimicrobial activity and iron regulatory function may reside in distinct hep-

cidin peptides in some fish species. Phylogenetic analysis divides fish hepcidin peptide sequences into two groups: one group contains a putative metal-binding motif at the amino terminus and the other group lacks this amino-terminal sequence. Some fish species express multiple hepcidin genes that encode predicted peptides from both groups. Most of these hepcidin genes are predominately expressed in the liver, similar to mammalian hepcidin; however, fish hepcidins are expressed in many tissues, including kidney and spleen. Expression of many of these hepcidin genes is induced by exposure to bacteria or bacterial components. Interestingly, in tilapia, the expression of two hepcidin genes that encode peptides with *in vitro* antimicrobial activity is increased by LPS, while the expression of another hepcidin gene that encodes a peptide that lacks *in vitro* antimicrobial activity is not increased by LPS. Several studies have found that the expression of hepcidin genes is affected in fish that have been exposed to contaminants. If hepcidin functions as an antimicrobial peptide in fish, this disruption of normal hepcidin expression by contaminants might affect the host defenses of fish.

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