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Relaxin induces matrix-metalloproteinases-9 and -13 via RXFP1: Induction of MMP-9 involves the PI3K, ERK, Akt and PKC-ζ pathways ☆

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ABSTRACT

We determined the precise role of relaxin family peptide (RXFP) receptors-1 and -2 in the regulation of MMP-9 and -13 by relaxin, and delineated the signaling cascade that contributes to relaxin's modulation of MMP-9 in fibrocartilaginous cells. Relaxin treatment of cells in which RXFP1 was silenced resulted in diminished induction of MMP-9 and -13 by relaxin, whereas overexpression of RXFP1 potentiated the relaxin-induced expression of these proteinases. Suppression or overexpression of RXFP2 resulted in no changes in the relaxin-induced MMP-9 and -13. Studies using chemical inhibitors and siRNAs to signaling molecules showed that PI3K, Akt, ERK and PKC-ζ and the transcription factors Elk-1, c-fos and, to a lesser extent, NF-κB are involved in relaxin's induction of MMP-9. Our findings provide the first characterization of signaling cascade involved in the regulation of any MMP by relaxin and offer mechanistic insights on how relaxin likely mediates extracellular matrix turnover.

1. Introduction

Relaxin H2, the major stored and circulating form of relaxin in humans, is a 6-kDa polypeptide belonging to the insulin family of structurally related hormones, whose activities are distinct from other members of this family (James et al., 1977; Schwabe and McDonald, 1977). It is primarily synthesized in the corpus luteum and placenta, and is involved in multiple and diverse physiologic functions including extracellular matrix (ECM) remodeling, neoangiogenesis, and vasodilation (Bani, 1997; Bathgate et al., 2003; Ivell and Bathgate, 2006; Schwabe and Bullesbach, 2007; Sherwood, 2004). Of these, the most important physiologic function of relaxin appears to be the remodeling of ECM in reproductive and non-reproductive tissues. In these target tissues, relaxin is known to decrease collagen and glycosaminoglycan content including that of small proteoglycans, decorin and biglycan that contribute to collagen fibril organization and formation (Downing and Sherwood, 2011; Unemori et al., 1992). In most reproductive tissues, including the cervix, uterus, ovary, breast, deciduas and the fibrocartilaginous pubic symphysis, relaxin mediates changes in matrix composition and organization by modulating the synthesis of matrix macromolecules (Hwang et al., 1996) or altering the expression of matrix-degrading enzymes (Mushayandebvu and Rajabi, 1995) or both (Hwang et al., 1996; Mushayandebvu and Rajabi, 1995). Further evidence of the role of relaxin in ECM turnover is provided by observations in relaxin knockout mice that demonstrate accumulation of collagen in the nipple and diminished relaxation of the pubic symphysis during parturition (Zhao et al., 1999), as well as progressive fibrosis of the lungs (Samuel et al., 2005a,b; Zhao et al., 1999).

Besides its modulation of matrix turnover in reproductive tissues, relaxin regulates tissue turnover in lung and alveolar fibroblasts, renal and vascular tissues (Lekgabe et al., 2005; McGuane and Parry, 2005; Unemori and Amento, 1990; Unemori et al., 1996), and in synovial joint fibrocartilage (Kapila, 2003; Naqvi et al., 2005). In both the synovial joint and pubic symphysis fibrocartilaginous tissues, the relaxin-mediated alterations in matrix composition appear to result largely due to increased degradative responses rather than due to changes in matrix synthesis (Naqvi et al., 2005; Samuel et al., 1996, 1998). More specifically, relaxin enhances degradation of these tissues by upregulating specific members of the matrix metalloproteinase (MMP) family of enzymes, namely MMP-1 (collagenase-1), -3 (stromelysin-1), -9 (92 kDa gelatinase) and -13 (collagenase-3) (Kapila and Xie, 1998; Kapila et al., 2009a; Naqvi et al., 2005). MMPs are a family of up to 25 enzymes that are characterized by their ECM substrate specificity, zinc-dependent activity, inhibition by tissue inhibitors of metalloproteinase, secretion as a zymogen and sequence similarities (Kessenbrock et al., 2010; Morrison et al., 2009). Between them, MMPs can degrade all the major matrix macromolecules of connective tissues including collagen, fibronectin and proteoglycans as well as many minor proteins.

The net matrix remodeling activities of relaxin within target tissues are likely determined by the levels of its receptors and the subsequent signaling initiated by activation of the receptors by

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the binding with the hormone ligand. Relaxin is known to bind to and activate the leucine-rich guanine nucleotide-binding (G protein)-coupled receptors (LGR) previously known as LGR7 and LGR8 (Hsu et al., 2002), which have since been renamed as relaxin family peptide (RXFP) RXFP1 and RXFP2, respectively. The human relaxin H2 activates both RXFP1 and RXFP2 resulting in an increase in intracellular cAMP concentrations (Dessauer and Nguyen, 2005; Halls et al., 2006; Nguyen and Dessauer 2005a,b; Nguyen et al., 2003) in THP-1, MCF-7 and HEK cells. Although the signaling by relaxin in fibroblastic cells has not yet been well characterized, in rat renal and cardiac fibroblasts, nitric oxide instead of cAMP appears to be the major relaxin H2 signaling molecule (Samuel et al., 2004).

Both relaxin receptors and their transcripts have been identified in reproductive and non-reproductive tissues such as the brain, kidney, lung, anterior cruciate ligament of the knee joint and in synovial joint fibrocartilaginous cells (Faryniarz et al., 2006; Hsu et al., 2002; Wang et al., 2006, 2009). Although the precise contributions of RXFP1 and RXFP2 to modulation of MMPs upon activation by relaxin are not known, indirect evidence for the role of RXFP1 to *in vivo* remodeling of matrices is provided by the phenotypic characteristics of the female RXFP1 null mice that are similar to those described for relaxin-deficient mice (Kamat et al., 2004; Krajnc-Franken et al., 2004; Zhao et al., 1999). Although relaxin binds to both RXFP1 and 2, further indirect evidence that RXFP1 rather than RXFP2 is the likely candidate receptor for MMP regulation by relaxin are suggested by findings showing that the latter is a known cognate receptor for Insulin3 (INSL3) peptide (Bogatcheva et al., 2003; Del Borgo et al., 2006; Kumagai et al., 2002), and that the phenotypes of mice with INSL3 or RXFP2 mutations have little in common with those with relaxin-1 or RXFP1 deficiency (Ivell et al., 2011; Kamat et al., 2004; Krajnc-Franken et al., 2004; Samuel et al., 2004; Samuel et al., 2005a,b). Finally, recent studies have demonstrated that relaxin-3 also modulates tissue remodeling in a manner similar to that by relaxin H2 through RXFP1 and that human relaxin-3 does not activate RXFP2 (Hossain et al., 2011; Samuel et al., 2007a,b). These findings taken together indirectly, but not conclusively, demonstrate that the tissue remodeling by relaxin likely occurs through RXFP1 rather than RXFP2. While these studies suggest that RXFP1 is a likely candidate receptor in the modulation of tissue remodeling, its role and that of RXFP2 in the induction of MMPs by relaxin has not been determined. Furthermore, although relaxin is known to modulate several signaling pathways on activating RXFP1 or RXFP2 (Halls et al., 2005, 2006, 2007; Halls et al., 2009a,b), the cascade of signals that lead to relaxin's induction of MMPs by one or both of these receptors have not been determined.

In this investigation we sought to determine the precise contributions of RXFP1 and RXFP2 to the regulation of MMP-9, and -13, and to elucidate the downstream signaling pathways from the receptors in the induction of MMP-9 in fibrochondrocytes from a mouse synovial joint. We chose to investigate the mechanisms of relaxin's regulation of MMPs in synovial joint fibrochondrocytes since the induction of MMP-9 and -13 by relaxin as well as relaxin receptor expression has been well characterized in this cell system (Hashem et al., 2006; Kapila, 2003; Kapila et al., 2009a, 2009b; Kapila and Xie, 1998; Wang et al., 2007). We also studied the regulation of MMP-14 by relaxin since this proteinase, like MMP-13 is a collagenase, but is regulated substantially differently than the other collagenases (Chakraborti et al., 2003; Yan and Boyd, 2007) thus serving as a suitable control. Our results show that relaxin H2 induces MMP-9 and -13 in fibrochondrocytes through the RXFP1 receptor, and that relaxin's modulation of MMP-9 occurs via PI3K–AKT–PKCζ–ERK1/2 signaling pathway and involves Elk-1 and c-fos transcription factors. These findings provide the first characterization of signaling cascade involved in the regulation of any MMP by relaxin and offer critical mechanistic information on the relaxin-mediated turnover of the ECM in fibrocartilaginous cells.

2. Material and methods

2.1. Reagents and animals

All cell culture reagents and media were purchased from Invitrogen Corp. (Carlsbad, CA) and chemicals were from Sigma–Aldrich Corp. (St. Louis, MO) unless otherwise mentioned. Recombinant human relaxin-2 was a gift from BAS Medical (San Mateo, CA). C57BL/6J female mice were obtained from Charles River Laboratories (Wilmington, MA).

2.2. Fibrochondrocyte isolation and culture

Temporomandibular joint (TMJ) disc fibrochondrocytes were isolated from 12-week-old female C57BL/6J mice as described previously (Kapila et al., 1995) and cultured in α-MEM supplemented with 10% fetal bovine serum (FBS). The doses of siRNA, cDNA, signaling inhibitors and optimal timeframe for each experiment were determined by preliminary dose–response and time course studies. A minimum of three early passage (P2–P4) fibrochondrocyte preparations were used for each experiment.

2.3. Overexpression of relaxin receptors

The fibrochondrocytes were seeded at 1.0×10^6 cells/6 cm dish and transfected after 16 h with 2 μg of RXFP1 cDNA, or RXFP2 cDNA (Hsu et al., 2000, 2002) (both kindly provided by Dr. Teddy Hsu) or control pcDNA vector (Qiagen, Valencia, CA) using Effectene transfection reagent according to the manufacturer's instructions (Qiagen) in serum-free Opti-MEM media, with about 40–60% transfection efficiency. After 6 h of incubation, the Opti-MEM was replaced with α-MEM containing 20% FBS plus antibiotics (1% penicillin/streptomycin) and maintained for a period of 12 h. The cells were then washed and maintained in serum-free medium (α-MEM with 0.2% lactalbumin hydrolysate; LAH) for 4 h, before being incubated in fresh serum-free medium with or without 0.1 ng/ml relaxin. We used 0.1 ng/ml of relaxin because it induces maximum levels of MMPs in fibrochondrocytes (Kapila and Xie, 1998), and because this is within the range of physiologic concentrations of relaxin in serum of cycling women (Petersen et al., 1995; Stewart et al., 1990). After 48 h of incubation cell conditioned media, cell lysates, or mRNA were collected and stored at −80 °C until assayed.

2.4. Suppression of relaxin receptors

The fibrochondrocytes were seeded at 1.0×10^6 cells per 6 cm dish and transfected after 16 h with 250 pm RXFP1 or RXFP2 or control siRNA (sc-40178, sc-40180 and sc-37007, respectively; Santa Cruz Biotechnology, Santa Cruz, CA) using Lipofectamine 2000 (Invitrogen Corp.) in serum-free Opti-MEM media according to the manufacturer's instructions. The siRNA combinations used are products of three target specific 20–25 nuleotide siRNAs. Lipofectamine was used to transfect siRNA since it provided adequate knockdown of the receptors of 50% or more, while being compatible with cell survival and health throughout the duration of the experiment. The remaining procedures were as described for the relaxin receptor overexpression experiments.

2.5. Analyses of signaling responses

Since the PI3K, PKC-ζ, and ERK signaling pathways that are modulated by relaxin converge on regulatory promoter elements, ETS/Elk-1, NF-κB, AP-1 that are common to several MMPs including MMP-1, -9 and -13, these candidate molecules were the focus of our studies.