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Egr-1 is involved in the inhibitory effect of leptin on PPARγ expression in hepatic stellate cell in vitro

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ABSTRACT

Aims: Hepatic stellate cell (HSC) activation is a key step in the hepatic fibrogenic process. Increasing evidence demonstrates the pro-fibrogenic action of leptin in rodent liver. Peroxisome proliferator-activated receptor-γ (PPARγ) is a potential molecular target for inhibition of HSC activation. Our previous study suggested that leptin markedly down-regulated PPARγ gene expression in HSCs. The aim of this study is to explore the molecular mechanisms underlying the inhibitory effect of leptin on PPARγ expression in rat HSCs in vitro.
Main methods: The effects of leptin on the expression and trans-activation activity of early growth response-1 (Egr-1) are examined by using real-time PCR, Western blotting analysis, transient transfection, and electrophoretic mobility shift assay. The role of Egr-1 in PPARγ gene expression is demonstrated by co-transfection approach, Western blotting analysis and real-time PCR.
Key findings: We document that leptin increases Egr-1 expression at protein and mRNA levels, and significantly stimulates Egr-1 trans-activation activity. Moreover, leptin induces the expression and activity of Egr-1 through activation of extracellular signal-regulated kinase (ERK) or phosphatidylinositol 3-kinase/AKT signaling (PI-3K/AKT) pathway. Further investigation reveals that Egr-1 exerts a clear inhibitory effect on the promoter activity and expression of PPARγ gene and demonstrates that Egr-1 increases the expression of HSC activation markers and promotes HSC growth. Taken together, these findings suggest that Egr-1 is involved in the inhibitory effect of leptin on PPARγ expression in rat HSCs in vitro.
Significance: Our results provide novel insights into the mechanisms of leptin-induced inhibition of PPARγ expression in HSCs in vitro.

Introduction

Circulating levels of free leptin (an adipokine) are significantly higher in patients with nonalcoholic steatohepatitis (NASH), type 2 diabetes mellitus, and alcoholic cirrhosis (McCullough et al. 1998; Cortez-Pinto et al. 1999; Nakamura et al. 2001). The role of leptin in liver disease may be distinctly important as human obesity and type II diabetes mellitus become more prevalent (Alberti and Zimmet 1998). Leptin has been shown to be critical in the development of hepatic fibrosis in rodents (Saxena et al. 2002, 2004; Honda et al. 2002; Leclercq et al. 2002; Ikejima et al. 2002; Aleffi et al. 2005; Cao et al. 2006; Marra 2007). Hepatic stellate cell (HSC) activation is a key step in the hepatic fibrogenic process. Peroxisome proliferator-activated receptor-γ (PPARγ), a nuclear transcription factor, controls growth and differentiation in different tissues. PPARγ level is high in quiescent HSCs and its expression and activity are dramatically diminished during HSC activation in vitro and in vivo (Galli et al. 2000; Marra et al. 2000; Miyahara et al. 2000). PPARγ has been proposed as a potential molecular target for inhibition of HSC activation (Marra et al. 2000; Miyahara et al. 2000; Hazra et al. 2004; Galli et al. 2002). Our recent study has documented that leptin markedly down-regulated PPARγ gene expression through extracellular signal-regulated kinase (ERK) and phosphatidylinositol 3-kinase/AKT (PI-3K/AKT) signaling pathways in HSCs in vitro (unpublished data). However, whether other transcription factors mediate leptin regulation of PPARγ gene expression in HSCs is not known yet.

Transcription factor early growth response-1 (Egr-1) is a critical upstream mediator of cell proliferation and differentiation and can be rapidly and transiently induced by many growth factors and cytokines (Sukhatme et al. 1988; Sukhatme 1990; Fu et al. 2002). ERK and PI-3K/AKT signaling pathways play important roles in the fundamental cellular processes (Seger and Krebs 1995; Yoon and Seger 2006; Engelman et al. 2006). These two pathways appear to affect the expression and activity of Egr-1 in some cell types (Hodge et al. 1998; Schwachtgen et al. 1998; Guillemot et al. 2001; Ingram et al. 2006). Leptin stimulation invokes distinct ERK and PI-3K/AKT signaling pathways in many cell types (Takahashi et al. 1997; Tang et al. 2007; Saxena et al. 2007) including activated HSCs (Saxena et al. 2002). It is not clear yet whether leptin affects the expression and/or activity of Egr-1 in HSCs.

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Egr-1 controls the expression of a wide variety of genes (Fu et al. 2003; Svaren et al. 2000). It has been shown that many growth factors and cytokines up-regulate PPARγ gene expression via induction of Egr-1 in human aortic smooth muscle cells (HASMC) (Fu et al. 2002). As far as we know, the relationship between Egr-1 and PPARγ gene expression in HSCs has not been revealed.

Therefore, the focus of the present study is to determine the effect of leptin on the expression and activity of Egr-1 and determine the role of Egr-1 in PPARγ gene expression, to elucidate whether transcription factor Egr-1 is involved in leptin-induced inhibition of PPARγ gene expression in HSCs.

Materials and methods

Materials

Leptin was purchased from ProSpec-Tany TechnoGene (Rehovot, Israel) and used at 0–100 ng/ml (Saxena et al., 2004). LY294002 (a specific PI-3K inhibitor) and PD98059 (a specific MEK inhibitor) were purchased from CalBiochem (La Jolla, CA, USA) and used at 5 μM (Zhou et al. 2007).

HSC isolation and culture

HSCs were isolated from Sprague–Dawley rats as described previously (Zhou et al. 2007). Briefly, the liver of male Sprague–Dawley rat (200–250 g) was perfused in situ with pronase and collagenase. HSCs were isolated by using density gradient centrifugation and cultured in Dullbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum (FBS) on uncoated plastic dishes. Culture medium was changed every 24 h. The purity of the isolated HSCs was assessed by autofluorescence of the stored retinoids in HSC lipid droplets as well as immunohistochemistry using antibody against α-smooth muscle actin (α-SMA) (Abcam, Cambridge, UK; diluted 1:100). Cell viability was examined by Trypan blue exclusion. Both cell purity and viability were in excess of 90%. All the experiments were conducted on HSCs with 3–6 passages (between 2 and 3 weeks). By these time points, all HSCs showed myofibroblast-like phenotype (activated HSCs) (Rockey et al. 1992). After cells were synchronized by serum starvation in DMEM containing 0.1% FBS for 24 h, all treatments were administered with serum-free media in which cells were kept in DMEM with 0.1% FBS. Chinese national guidelines for the care of animals were followed.

RNA isolation and real-time PCR

Total RNA was extracted by using TRI-Reagent (Sigma) according to the protocol provided by the manufacturer. Real-time PCR was carried out as described previously (Zhou et al. 2007). The endogenous glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as internal control. The primers used in real-time PCR were as follows: Egr-1: (forward) 5′-GCCT GCGACATCTGTGGAA-3′; (reverse) 5′-GCCGCAAGTGGATCTTGGTA-3′; PPARγ: (forward) 5′-ATTCTGGCCCACCAACTTCGG-3′; (reverse) 5′-TGGAAGCCTGATGCTTTATCCCCA-3′; GAPDH: (forward) 5′-GGCAAATT-CAACGGCACAGT-3′; (reverse) 5′-AGATGGTGATGGGCTTCCC-3′.

Western blotting analyses

Whole cell lysates were prepared as described earlier (Zhou et al. 2007) and separated by SDS–PAGE. Target proteins were respectively detected by using primary antibodies against Egr-1(1:1000), PPARγ(1:500), α-SMA(1:5000), αI(I)procollagen (1:500), β-actin (1:4000), or GAPDH (1:1000) and subsequently by horseradish peroxidase-conjugated secondary antibodies (1:5000). Anti-α-SMA antibody was purchased from ABcam (Cambridge, UK) and other antibodies were purchased from Santa Cruz (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Protein bands were visualized by using a chemiluminescence reagent (Amersham Bioscience). In Western blotting analyses, the densities of bands were normalized with the internal invariable control β-actin or GAPDH. The levels of target protein bands were densitometrically determined by using Quantity One 4.4.1 (Bio-Rad). The variation in the density was expressed as fold change relative to the control in the blot.

Plasmid and transient transfection assays

The Egr-1 luciferase reporter plasmid pEgr-1-Luc, containing four Egr-1 binding sites cloned into the reporter luciferase plasmid, was a gift from Dr. Gerald Thiel (Thiel et al. 2000). The cDNA expression plasmid pdn-Egr-1cDNA was kindly provided by Dr. Gerald Thiel, as well (Zhang et al. 2003). This plasmid generates a trans-dominant negative mutant Egr-1, which competitively binds to Egr-1 binding sites, but lacks the trans-activation activity (Zhang et al. 2003). The cDNA expression plasmid pEgr-1cDNA, containing Egr-1 cDNA in a CMV-driven expression vector, was kindly provided by Dr. Alfred C. Johnson (Nishi et al. 2002). The PPARγ promoter luciferase reporter plasmid pPPARγ-Luc contains the 5'-flanking region (−2776 bp) of the PPARγ gene promoter cloned into the reporter luciferase plasmid (Fajas et al. 1997), and was a gift from Dr. Johan Auwerx (Pasteur Institute, Lille, France) (Fajas et al. 1997). The plasmid pdn-PTEN or pwt-PTEN contains a full-length cDNA encodning the dominant-negative form of phosphatase and tensin homolog (PTEN) or wild-type PTEN, respectively. They were kindly provided by Dr. Christopher G Kevil (Louisiana State University Health Sciences Center, Shreveport, USA). The plasmid pdn-ERK or pa-ERK contains cDNA encoding the dominant-negative form of ERK or the constitutively active form of ERK, respectively, which were described previously (Zhou et al. 2007).

Transient transfection assays were performed using LipofectAMINE reagent (Life Technologies) following the protocol provided by the manufacturer. Briefly, semiconfluent HSCs in six-well plastic plates were transiently transfected with a reporter plasmid (3 μg DNA/well, if no specific indication) or an expression plasmid. Luciferase activity assays were performed as we described earlier (Zhou et al. 2007). Transfection efficiency was controlled by co-transfection of a β-galactosidase reporter plasmid pSV-β-gal (1 μg DNA/well, if no specific indication) (Promega). β-galactosidase activities were measured using an assay kit from Promega Corp. Luciferase activities were normalized with β-galactosidase activities and expressed as fold changes.

Electrophoretic mobility shift assay (EMSA)

Nuclear proteins were extracted as previously described (Chen and Davis 1999). Briefly, HSCs in cell culture flasks were washed and harvested in cold PBS. The cell pellet was resuspended in Solution A (10 mM Hepes, pH 7.9, 1.5 mM $MgCl_2$, and 10 mM KCl) and incubated for 10 min on ice. Cells were re-collected by centrifugation. The cell pellet was resuspended in Solution A with the following inhibitors: 0.5 mM dithiothreitol, 10 μg/μl leupeptin, 2 mM phenylmethylsulfonyl fluoride, 10 mM NaF, 10 μg/ml aprotonin, 1 mM $NaVO_3$, and 60 mM β-glycerophosphate. Cells were gently stroked on ice in a homogenizer. Nuclei were recovered by centrifugation at 2500 rpm for 10 min at 4 °C. The nuclei pellet was resuspended in Solution A with the above inhibitors and subsequently centrifuged at 15,000 rpm for 20 min at 4 °C. The pellet was resuspended in Solution C (20 mM Hepes, pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol, 10 μg/μl leupeptin, 2 mM phenylmethylsulfonyl fluoride, 10 mM NaF, 10 μg/ml aprotonin, 1 mM $NaVO_3$, and 60 mM β-glycerophosphate), incubated for 90 min at 4 °C and then centrifuged at 15,000 rpm for 30 min. The supernatant was stored at 70 °C until use.

Nucleotide sequence of the sense strand of the double-stranded oligonucleotides (Egr-1 probe) was as follows: 5'-GGATCCA*GCGGGG GCGAGCGGGGGCG*A-3' containing two consensus Egr-1 binding sites (underlined) (Fu et al. 2002). The double-stranded oligonucleotide probe was prepared by mixing two single-strand of complementary