

## Expression of leptin receptors in hepatic sinusoidal cells

Kenichi Ikejima\*, Tie Lang, Yan-Jun Zhang, Shunhei Yamashina, Hajime Honda, Mutsuko Yoshikawa, Miyoko Hirose, Nobuyuki Enomoto, Tsuneo Kitamura, Yoshiyuki Takei and Nobuhiro Sato

Address: Department of Gastroenterology, Juntendo University School of Medicine, 2-1-1, Hongo, Bunkyo-ku, Tokyo 113-8421, Japan

Email: Kenichi Ikejima\* - ikejima@med.juntendo.ac.jp; Tie Lang - ikejima@med.juntendo.ac.jp; Yan-Jun Zhang - ikejima@med.juntendo.ac.jp; Shunhei Yamashina - ikejima@med.juntendo.ac.jp; Hajime Honda - ikejima@med.juntendo.ac.jp; Mutsuko Yoshikawa - ikejima@med.juntendo.ac.jp; Miyoko Hirose - ikejima@med.juntendo.ac.jp; Nobuyuki Enomoto - nobu@med.juntendo.ac.jp; Tsuneo Kitamura - ikejima@med.juntendo.ac.jp; Yoshiyuki Takei - ytakei@med.juntendo.ac.jp; Nobuhiro Sato - nsato@med.juntendo.ac.jp

\* Corresponding author

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### Abstract

Emerging evidence has suggested a critical role of leptin in hepatic inflammation and fibrogenesis, however, the precise mechanisms underlying the profibrogenic action of leptin in the liver has not been well elucidated. Therefore, the present study was designed to investigate the expression and functions of leptin receptors (Ob-R) in hepatic sinusoidal cells. Hepatic stellate cells (HSCs), Kupffer cells and sinusoidal endothelial cells (SECs) were isolated from rat livers by *in situ* collagenase perfusion followed by differential centrifugation technique, and expression of Ob-Ra and Ob-Rb, short and long Ob-R isoforms, respectively, were analyzed by RT-PCR. Ob-Ra mRNA was detected ubiquitously in HSCs and SECs. In contrast, Ob-Rb was detected clearly only in SECs and Kupffer cells, but not in 7-day cultured HSCs. Indeed, tyrosine-phosphorylation of STAT-3, a downstream event of Ob-Rb signaling, was observed in SECs, but not in HSCs, 1 hr after incubation with leptin. Further, leptin increased AP-1 DNA binding activity and TGF-beta 1 mRNA levels in Kupffer cells and SECs, whereas leptin failed to increase TGF-beta 1 mRNA in HSCs. These findings indicated that SECs and Kupffer cells, but not HSCs, express functional leptin receptors, through which leptin elicits production of TGF-beta 1. It is hypothesized therefore that leptin, produced systemically from adipocytes and locally from HSCs, up-regulates TGF-beta 1 thereby facilitate tissue repairing and fibrogenesis in the sinusoidal microenvironment.

### Introduction

Increasing lines of evidence indicate that obesity is an important risk factor for exacerbation of alcoholic and non-alcoholic fatty liver diseases. Leptin, an obese gene product mainly produced from adipocytes, is a cytokine-type hormone that regulates food intake and fat metabolism through actions on the central nervous system [1]. Recently, McCullough et al. reported that serum leptin

levels are increased in patients of alcoholic cirrhosis [2]. Further, hepatic stellate cells (HSCs) have been shown to produce leptin when they get activated [3]. Furthermore, co-administration of recombinant leptin augments inflammation and fibrogenesis in the liver caused by hepatotoxic xenobiotics [4]. Moreover, the ob/ob mouse, a natural occurring leptin-deficient animal, shows extremely poor progression of hepatic fibrosis induced by

chronic thioacetamide (TAA) treatment [5]. These findings lead to the hypothesis that leptin plays a pivotal role in profibrogenic responses in the liver.

Leptin receptors (Ob-R) have originally been shown in hypothalamic neurons, through which leptin regulates food intake and body weight [6]. In fact, homozygous mutations of leptin receptor gene have been identified in rodents (i.e., db/db mice and Zucker rats), which are also associated with obesity [7,8]. There are several isoforms of leptin receptors (Ob-R), which are splice variants with the same extracellular domain. The most ubiquitous form of Ob-R is a short-form receptor (Ob-Ra); however, the function of this receptor isoform remains unclear. In contrast, a long-form leptin receptor (Ob-Rb), which contains longer intracellular domain, is known to activate the Janus kinase (JAK)-STAT-3 pathway, leading to transcriptional regulation of target genes. In the present study, we investigated the expression and functions of leptin receptors in hepatic sinusoidal cells in order to elucidate the precise mechanisms underlying the profibrogenic action of leptin in the liver.

## Methods

### Isolation and primary culture of rat sinusoidal cells

Hepatic stellate cells (HSCs) were isolated from livers of Wistar rats and Zucker rats by *in situ* collagenase perfusion and differential centrifugation using Metrizamide density gradients. To isolate Kupffer cells and sinusoidal endothelial cells (SECs), two-step Percoll density gradients were used for differential centrifugation. HSCs were cultured on polystyrene culture dishes using D'MEM with 10% fetal bovine serum (FBS) for up to seven days. Kupffer cells were cultured in RPMI1640 medium supplemented with 10% FBS for 24 hr prior to experiments. Isolated SECs were cultured in EBM-2 medium (Bio Whittaker Co., Walkersville, MD) supplemented with 10% FBS, the EGM-2 bullet kit (Bio Whittaker Co.) and 20 ng/ml human recombinant VEGF (Wako Pure Chemical Co., Osaka, Japan) on type I collagen-coated plastic dishes for three days prior to experiments.

### RNA isolation, RT-PCR and ribonuclease protection assay

Total RNA from cultured cells was prepared by a phenol-chloroform extraction method using Trizol reagent (Gibco/Life Technologies, Grand Island, NY).

For RT-PCR, 1 microgram of total RNA was reverse transcribed using Moloney murine leukemia virus reverse transcriptase (Gibco/Life Technologies) and an oligo dT<sub>12-18</sub> primer (Invitrogen Corp., Carlsbad, CA). The cDNA (1 microgram l) obtained was amplified using Taq DNA polymerase (AmpliTaQ Gold™, PE Applied Biosystems, Foster City, CA). Primer sets used for amplifying specific cDNA were as follows; for rat Ob-Ra forward (5'-

AAG TGG CTT AGA ATC CCT TCG-3') and reverse (5'-GAG TGT CCG CTC TCT TTT GG-3'), yielding a 337-bp product size; for rat Ob-Rb forward (5'-TGA CCA CTC CAG ATT CCA CA-3') and reverse (5'-AAG CTC ATC CAA CCC TGA GA-3'), yielding a product size of 350-bp. After a 10-min denaturation period at 95°C, 35 cycles of 95°C for 45 sec, 58°C for 45 sec and 72°C for 60 sec followed by final extension at 72°C for 7 min were performed using GeneAmp PCR System 9700 (PE Applied Biosystems). The size and amount of PCR products were verified by electrophoresis in 1.5% agarose gels.

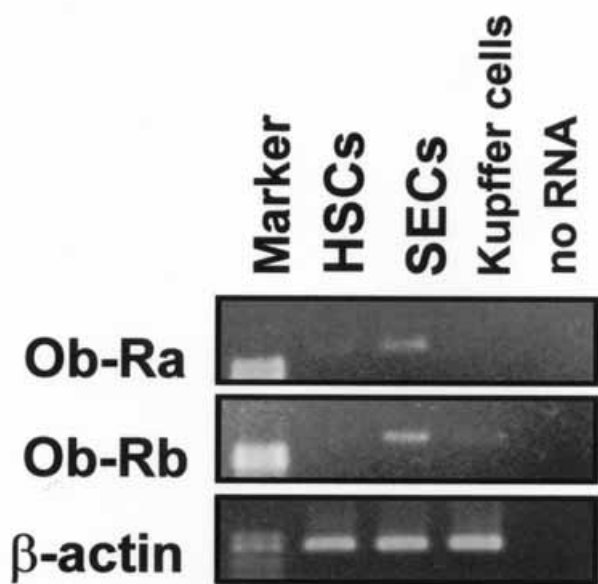
For ribonuclease protection assay, the template plasmid for rat TGF-beta 1 (subcloned into pCRII-TOPO vector) were linearized by appropriate restriction endonuclease digestion, and the radiolabeled riboprobes were generated using MAXIscript™ *in vitro* transcription kit (Ambion Inc., Austin, TX) in the presence of alpha <sup>32</sup>P UTP (Amersham Pharmacia Biotech). ribonuclease protection assay was performed using RPA III™ kit (Ambion Inc.). Briefly, twenty microgram of total RNA was hybridized with the radiolabeled probes (1 × 10<sup>5</sup> cpm each) in a hybridization solution at 42°C overnight. The reaction was then incubated with RNase A/T1 at 37°C for 30 min, and precipitated with RNase inactivation/precipitation solution. The reaction was separated on a denaturing 5% polyacrylamide/urea gel and exposed to X-ray films (X-Omat AR, Kodak) at -80°C.

### Western blotting

Whole cell protein extracts were obtained by homogenizing cells in a buffer containing 66 mM Tris pH 8.0, 5 mM ethylenediaminetetraacetic acid (EDTA), 1% Triton X-100 and protease/phosphatase inhibitors [9]. Twenty micrograms of protein was separated in 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred onto polyvinylidene fluoride (PVDF) membranes. Membrane were soaked with 5% nonfat dry milk in Tris-buffered saline (TBS), and then incubated with a polyclonal rabbit anti-phospho(Tyr705)-STAT3 (Cell Signaling Technology, Beverly, MA, 1:1000 dilution in TBS). After washes with TBS containing 0.05% TritonX-100 (TBS-T), membranes were incubated with a secondary antibody (horse radish peroxidase-conjugated anti-rabbit IgG, Santa Cruz Biotechnology Inc., Santa Cruz, CA). The ECL detection kit (Amersham Pharmacia Biotech Inc.) was applied according to the manufacturer's indication for the detection of specific bands.

### Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared according to the method of Dignam with slight modifications, and equal amounts of protein (1–10 micrograms) were analyzed. Binding reactions were performed for 20 min on ice in a buffer



**Figure 1**  
**Expression of Ob-R mRNA in sinusoidal cells.** Hepatic stellate cells (HSCs), sinusoidal endothelial cells (SECs), and Kupffer cells were isolated from Wistar rats, and cultured for 7 days, 3 days and 1 day, respectively. Total RNA (1 microgram) from each cell type was analyzed by RT-PCR for Ob-Ra, Ob-Rb, and beta-actin as described in Methods.

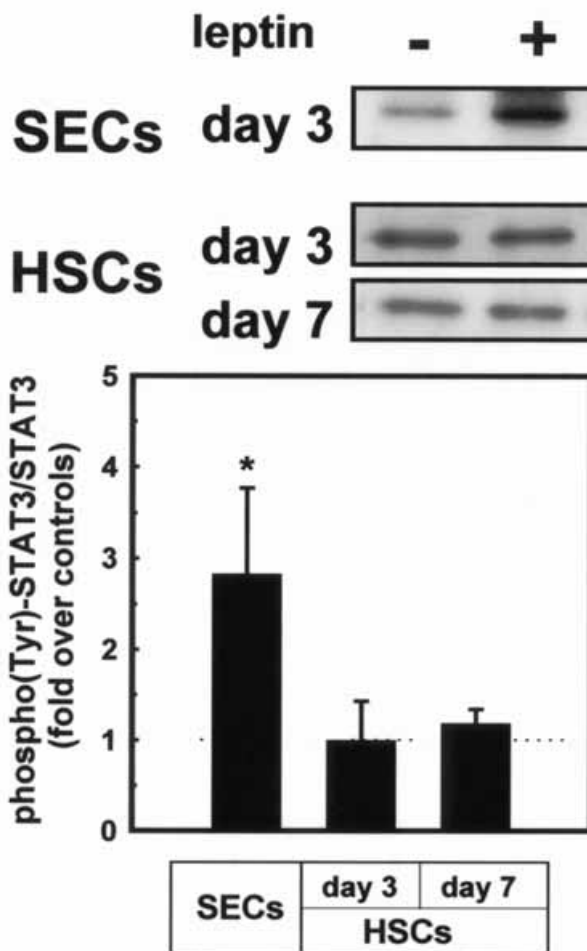
containing 5 micrograms poly [d(I-C)], 10 mM HEPES, pH 7.6, 50 mM KCl, 0.1 mM EDTA, 1 mM DTT, 10% glycerol, 5 mM MgCl<sub>2</sub> and 20,000 cpm of <sup>32</sup>P-labeled oligonucleotides containing consensus sequence of AP-1 (Santa Cruz Biotechnology Inc.). For competition experiments, 100–250-fold excess amount of unlabeled consensus oligo was added in the binding reactions and co-incubated. DNA-protein complexes were separated by native polyacrylamide gel electrophoresis (5%, 0.4X TBE). Gels were dried and exposed to X-OMAT films (Kodak).

**Results**

**Kupffer cells and SECs express a functional leptin receptor**

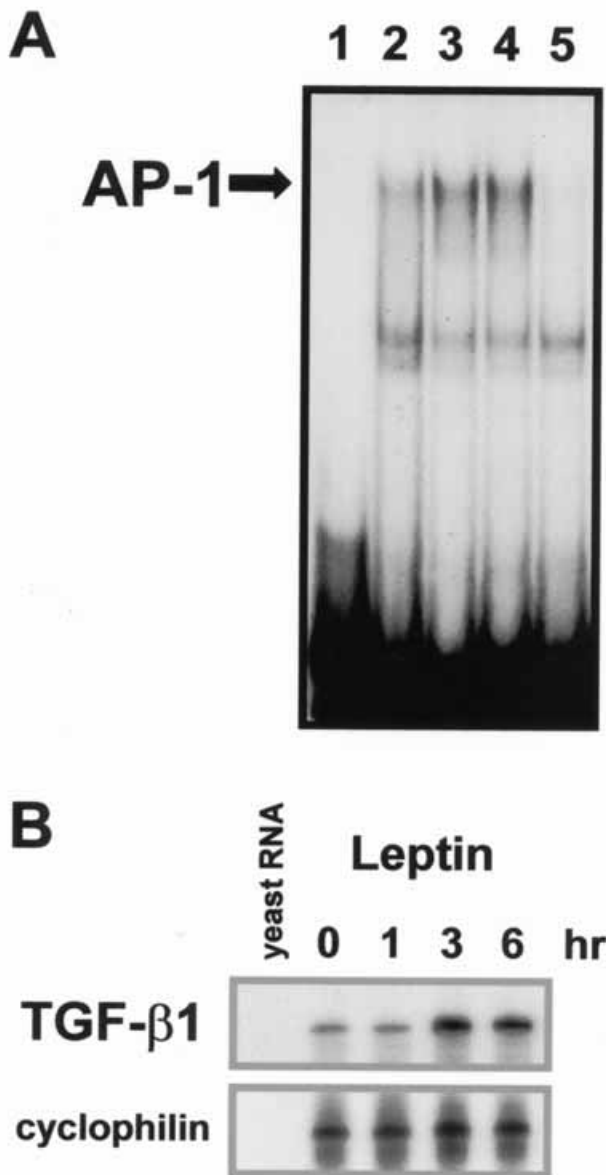
To determine whether sinusoidal cells express Ob-R isoforms, mRNA for Ob-Ra and Ob-Rb were detected by RT-PCR (Fig. 1). As expected, Ob-Ra mRNA was detected ubiquitously in HSCs and SECs. In contrast, Ob-Rb was detected clearly only in SECs and Kupffer cells, but not in seven-day cultured HSCs.

Next, to confirm the expression of functional receptors, phosphorylation of STAT-3 was measured by Western blotting. As shown in Fig. 2, tyrosine phosphorylation of STAT-3 was clearly detected in SECs when they were



**Figure 2**  
**Effect of leptin on phosphorylation of STAT3 in SECs and HSCs.** Leptin was added to cultured SECs (day 3) and HSCs (day 3 and 7) for 1 hr, and tyrosine phosphorylation of STAT3 was detected by Western blotting. Representative photographs of specific bands and densitometrical data were shown.

treated with leptin (100 nM) for 1 hr. In sharp contrast, STAT-3 phosphorylation was barely detectable in rat HSCs even they were incubated with the same dose of leptin. These findings indicated that primary cultured rat SECs express both Ob-Ra and functional Ob-Rb constitutively. Kupffer cells are also positive for constitutive expression of Ob-Rb. Isolated HSCs, however, appear to express only Ob-Ra, but not Ob-Rb.



**Figure 3**  
**Effect of leptin on AP-1 DNA binding activity and TGF-beta 1 mRNA levels in isolated Kupffer cells.** (A) Nuclear extracts were prepared from isolated Kupffer cells 1 hr after addition of leptin (100 nM), and AP-1 DNA binding activity was analyzed by EMSA. lane 1; probe alone, lane 2; control, lane 3; leptin, lane 4; IL-6 (positive control), lane 5; X250 competitor. (B) Kupffer cells were incubated with leptin for 3–6 hr, and TGF-beta 1 mRNA was detected by ribonuclease protection assay.

#### **Leptin activates AP-1 and increases TGF-beta 1 mRNA in Kupffer cells and SECs**

Next, we examine whether leptin activates AP-1 DNA bindings in isolated Kupffer cells. Addition of leptin to the medium for 1 hr increased both AP-1 DNA binding activities in a dose dependent manner (Fig. 3A). Further, the effect of leptin on steady state mRNA levels for TGF-beta 1 mRNA in Kupffer cells were measured by ribonuclease protection assay (Fig. 3B). TGF-beta 1 mRNA levels increased nearly 2-fold over controls 3–6 hr after leptin treatment (100 nM). Similarly, increases in AP-1 DNA binding activity and steady state mRNA levels of TGF-beta 1 were observed obviously in LSE cells, a human sinusoidal endothelial cell-derived cell-line [9]. In contrast, leptin does not increase steady state mRNA levels of TGF-beta 1 in isolated HSCs [5]. Collectively, these observations indicated that leptin most likely up-regulates transcriptional activities of TGF-beta 1 gene in Kupffer cells and SECs through activation of AP-1.

#### **Discussion**

Recently, we have evaluated the role of Ob-R in hepatic fibrogenesis using Zucker rats, which lack functional Ob-R due to a missense mutation in the common, extracellular domain [9]. Zucker rats presented extremely poor profibrogenic responses in the liver caused by chronic TAA treatment as compared to their lean littermates, indicating that Ob-R is involved in the profibrogenic response in the liver. Overt expression of alpha SMA in the liver was observed in lean littermates given TAA, whereas alpha SMA staining was almost negative in Zucker rats even they were treated with the equivalent amount of TAA. In clear contrast, HSCs isolated from Zucker rats transactivated *in vitro* almost as same as the cells isolated from lean littermates in terms of induction of alpha SMA and steady state mRNA levels of alpha 1(I)procollagen. This discrepancy in HSC transactivation *in vivo* and *in vitro* led us to investigate the expression of Ob-R isoforms in sinusoidal cells.

RT-PCR analysis for the expression of Ob-R isoforms demonstrated that Ob-Rb mRNA is expressed constitutively in SECs and Kupffer cells, but it was barely detectable in activated HSCs (Fig. 1). On the other hand, Ob-Ra mRNA was detectable both in SECs and HSCs. Further, leptin obviously increased phosphorylation of STAT3 in isolated SECs but not in HSCs (Fig. 2). Moreover, leptin increased DNA binding activity of AP-1 in Kupffer cells and LSE cells, thereby increasing steady state mRNA levels of TGF-beta 1 in these cells (Fig. 3). In contrast, the induction of TGF-beta 1 by leptin was not detected in isolated HSCs, although the autocrine regulation of HSCs by TGF-beta 1 is well characterized. Collectively, these data suggested that HSCs lack functional Ob-Rb, while the direct effect of leptin on HSCs has not been ruled out completely. It is hypothesized that Ob-Rb-mediated signaling is

predominant in Kupffer cells and SECs, which upregulates production/activation of TGF-beta thereby facilitates tissue repairing and profibrogenic responses in the sinusoidal microenvironment.

In conclusion, leptin and its functional receptors play a crucial role in hepatic fibrogenesis, most likely through up-regulation of TGF-beta expression in the liver. It is postulated therefore that leptin, produced systemically from adipose tissue and locally from HSCs, contributes the progression of hepatic fibrosis in a variety of obesity-related chronic liver diseases including non-alcoholic fatty liver diseases.

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