Table 2 continued

		Tacrolimus group ($n = 32$)	Placebo group ($n = 31$)	p value
Steroids				
Dose (mg/day)	Patients (%)	15 (51.7)	11 (35.5)	0.297[a]
	Mean ± SD	5.0 ± 2.2	4.8 ± 1.7	0.802[b]

CRP C-reactive protein, *ESR* erythrocyte sedimentation rate, *DAS28* Disease Activity Score 28

[a] Fisher's exact test

[b] t test

[c] Wilcoxon rank sum test

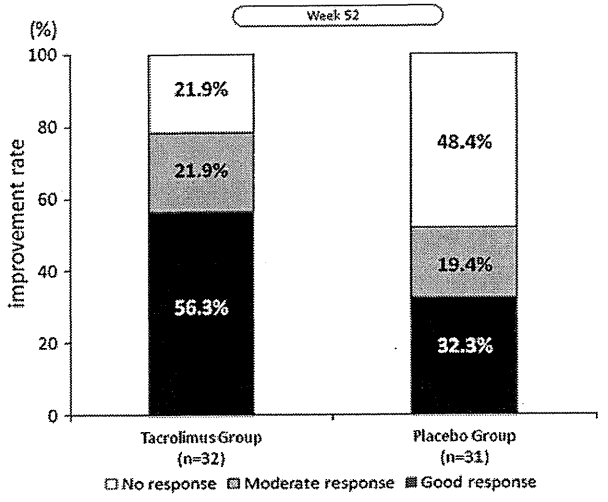


Fig. 2 Improvement rates [according to the EULAR (DAS28-CRP) criteria] in the tacrolimus group and the placebo group. *$p < 0.05$ by the Wald test for estimated parameter values

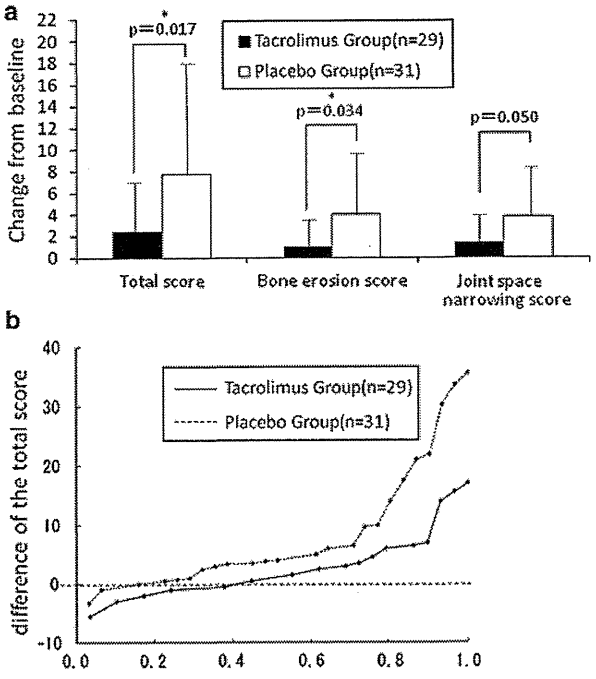


Fig. 3 **a** Evaluation of joint destruction. Joint destruction was evaluated by monitoring ΔTSS, the change in the ES, and the change in the JSN score by week 52 in the tacrolimus group and the placebo group, respectively. *TSS* total Sharp score, *ES* erosion score, *JSN* joint space narrowing; *$p < 0.05$ by analysis of covariance versus before administration in patients with or without MTX. **b** Cumulative probability of ΔTSS ≤0 up to week 52 in the tacrolimus group and the placebo group

three months until remission or low disease activity is achieved. However, there is insufficient evidence regarding the prevention of joint destruction by DMARDs apart from MTX. Tacrolimus is approved for the treatment of RA in Japan and shows good efficacy, suggesting that it could be useful for controlling joint destruction.

We previously investigated patients with a disease duration of RA of less than three years who showed an inadequate response to DMARDs. A double-blinded, placebo-controlled study of tacrolimus treatment was carried out for 12 months, with suppression of joint destruction as the primary outcome measure. Although baseline TSS showed no significant differences between the tacrolimus group and the placebo group, ΔTSS ≤0 was achieved in 24.1 % of the tacrolimus group versus 14.0 % of the placebo group [7]. Accordingly, the present subgroup analysis was performed, and CRP <1.5 mg/dL was identified as a factor that influenced the suppression of joint destruction by tacrolimus therapy according to univariate analysis. There were significantly smaller changes in TSS and ES in the patients with CRP <1.5 mg/dL from the tacrolimus group compared with those from the placebo group, as well as a smaller change of the JSN score, indicating that tacrolimus suppressed the progression of joint destruction in patients with early RA who had low disease activity and comparatively low CRP levels.

There have already been several reports about the prevention of joint destruction by biological agents [15–20],

Table 3 Adverse events (patients targeted for safety analysis and patients with CRP < 1.5 mg/dL)

	Tacrolimus group ($n = 32$)	Placebo group ($n = 31$)	Test[a]
Adverse event rate	81.3 % (26 patients, 102 events)	90.3 % (28 patients, 85 events)	$p = 0.474$
Severe adverse event rate	3.1 % (1 patient)	22.6 % (7 patients)	$p = 0.026$
Discontinuation rate due to adverse events	6.3 % (2 patients)	16.1 % (5 patients)	$p = 0.257$
Infections and infestations	40.6 % (13 patients, 25 events)	38.7 % (12 patients, 24 events)	$p = 1.000$
Benign, malignant and unspecified neoplasms (incl. cysts and polyps)	3.1 % (1 patient, 1 event)	3.2 % (1 patient, 1 event)	$p = 1.000$
Blood and lymphatic system disorders	6.3 % (2 patients, 2 events)	3.2 % (1 patient, 1 event)	$p = 1.000$
Psychiatric disorders		3.2 % (1 patient, 1 event)	$p = 0.492$
Nervous system disorders	9.4 % (3 patients, 4 events)	9.7 % (3 patients, 6 events)	$p = 1.000$
Eye disorders	9.4 % (3 patients, 3 events)	3.2 % (1 patient, 1 event)	$p = 0.613$
Vascular disorders	3.1 % (1 patient, 1 event)	6.5 % (2 patients, 2 events)	$p = 0.613$
Respiratory, thoracic, and mediastinal disorders	15.6 % (5 patients, 7 events)	16.1 % (5 patients, 6 events)	$p = 1.000$
Gastrointestinal disorders	31.3 % (10 patients, 15 events)	19.4 % (6 patients, 11 events)	$p = 0.387$
Skin and subcutaneous tissue disorders	21.9 % (7 patients, 7 events)	22.6 % (7 patients, 7 events)	$p = 1.000$
Musculoskeletal and connective tissue disorders	9.4 % (3 patients, 4 events)	9.7 % (3 patients, 3 events)	$p = 1.000$
Reproductive system and breast disorders	3.1 % (1 patient, 2 events)		$p = 1.000$
Congenital, familial and genetic disorders	3.1 % (1 patient, 1 event)		$p = 1.000$
General disorders and administration site conditions	6.3 % (2 patients, 3 events)	6.5 % (2 patients, 2 events)	$p = 1.000$
Investigations	40.6 % (13 patients, 26 events)	35.5 % (11 patients, 17 events)	$p = 0.797$
Injury, poisoning and procedural complications	3.1 % (1 patient, 1 event)	9.7 % (3 patients, 3 events)	$p = 0.355$

[a] Fisher's exact test

and the 2012 ACR Recommendations [14] suggest the use of biological agents combined with MTX for patients with early RA whose disease activity is high. However, it was reported that patients with early RA show no difference in their response to biological agents plus MTX versus DMARDs with regard to improvement of symptoms and suppression of bone erosion [21]. Thus, biological agents prevent further joint damage in patients with early RA who have higher disease activity and significant joint destruction, while the present study suggested that tacrolimus can suppress joint destruction in patients with early RA and CRP <1.5 mg/dL.

Tacrolimus has been reported to suppress the production of inflammatory cytokines, such as tumor necrosis factor-α, interleukin-1, and interleukin-6 [2, 22, 23], and it also delays the maturation of osteoclasts by inhibiting calcineurin and prevents the activation of T cells. In fact, animal studies have revealed the dose-dependent suppression of collagen-induced arthritis in rats by tacrolimus [24, 25], as well as the concentration-dependent induction of chondrocyte differentiation of progenitor cells in mouse [26]. In addition to an indirect action via the suppression of inflammatory cells, tacrolimus inhibits the maturation of osteoclasts by reducing the activation of NFATc1, a key regulator of osteoclast differentiation. Thus, tissue repair due to the promotion of bone/cartilage differentiation through direct action on osteoclasts helps tacrolimus to lessen joint destruction, and such a mechanism seems to support the results of the present subgroup analysis.

Our analysis revealed that joint destruction was prevented by adding treatment with tacrolimus at 3 mg daily in patients with early RA and CRP < 1.5 mg/dL who showed resistance to DMARDs. These results suggest that the combination of DMARDs and tacrolimus safely achieves clinical improvement in patients with early RA and CRP <1.5 mg/dL by preventing the progression of joint destruction. However, further studies will be required to confirm the suppression of joint destruction by tacrolimus in other patient populations.

Acknowledgments The authors thank Astellas Pharma Inc., which sponsored the study and provided editorial support. Statistical analysis was also performed by Astellas Pharma Inc. under the supervision of the authors.

Conflict of interest Y. Tanaka, S. Kawai, T. Takeuchi, K. Yamamoto, and N. Miyasaka have received consulting fees from Astellas Pharma Inc.

References

1. Sakuma S, Kato Y, Nishigaki F, Sasakawa T, Magari K, Miyata S, et al. FK506 potently inhibits T cell activation induced TNF-alpha and IL-1beta production in vitro by human peripheral blood mononuclear cells. Br J Pharmacol. 2000;130:1655–63.

2. Sakuma S, Kato Y, Nishigaki F, Magari K, Miyata S, Ohkubo Y, et al. Effects of FK506 and other immunosuppressive anti-rheumatic agents on T cell activation mediated IL-6 and IgM production in vitro. Int Immunopharmacol. 2001;1:749–57.
3. Kawai S, Hashimoto H, Kondo H, Murayama T, Kiuchi T, Abe T. Comparison of tacrolimus and mizoribine in a randomized, double-blind controlled study in patients with rheumatoid arthritis. J Rheumatol. 2006;33:2153–61.
4. Kawai S, Yamamoto K. Safety of tacrolimus, an immunosuppressive agent, in the treatment of rheumatoid arthritis in elderly patients. Rheumatology (Oxford). 2006;45:441–4.
5. Morita Y, Sasae Y, Sakuta T, Satoh M, Sasaki T, Kashihara N. Efficacy of low-dose tacrolimus added to methotrexate in patients with rheumatoid arthritis in Japan: a retrospective study. Mod Rheumatol. 2008;18:379–84.
6. Ogasawara M, Tamura N, Kageyama M, Onuma S, Kusaoi M, Toyama S, et al. Single-center, retrospective analysis of efficacy and safety of tacrolimus as a second-line DMARD in combination therapy and the risk factors contributing to adverse events in 115 patients with rheumatoid arthritis. Clin Rheumatol. 2011; 31:251–7.
7. Kawai S, Takeuchi T, Yamamoto K, Tanaka Y, Miyasaka N. Efficacy and safety of additional use of tacrolimus in patients with early rheumatoid arthritis with inadequate response to DMARDs—a multicenter, double-blind, parallel-group trial. Mod Rheumatol. 2011;21:458–68.
8. Arnett FC, Edworthy SM, Bloch DA, McShane DJ, Fries JF, Cooper NS, et al. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. Arthritis Rheum. 1988;31:315–24.
9. Felson DT, Anderson JJ, Boers M, Bombardier C, Furst D, Goldsmith C, et al. American College of Rheumatology. Preliminary definition of improvement in rheumatoid arthritis. Arthritis Rheum. 1995;38:727–35.
10. van Gestel AM, Prevoo ML, van't Hof MA, van Rijswijk MH, van de Putte LB, van Riel PL. Development and validation of the European League Against Rheumatism response criteria for rheumatoid arthritis. Comparison with the preliminary American College of Rheumatology and the World Health Organization/ International League Against Rheumatism Criteria. Arthritis Rheum. 1996;39:34–40.
11. van Gestel AM, Haagsma CJ, van Riel PL. Validation of rheumatoid arthritis improvement criteria that include simplified joint counts. Arthritis Rheum. 1998;41:1845–50.
12. Prevoo ML, van't Hof MA, Kuper HH, van Leeuwen MA, van de Putte LB, van Riel PL. Modified disease activity scores that include twenty-eight-joint counts. Development and validation in a prospective longitudinal study of patients with rheumatoid arthritis. Arthritis Rheum. 1995;38:44–8.
13. van der Heijde DM. Plain X-rays in rheumatoid arthritis: overview of scoring methods, their reliability and applicability. Baillieres Clin Rheumatol. 1996;10:435–53.
14. Singh JA, Furst DE, Bharat A, Curtis JR, Kavanaugh AF, Kremer JM, et al. Update of the 2008 American College of Rheumatology Recommendations for the use of disease-modifying antirheumatic drugs and biologic agents in the treatment of rheumatoid arthritis. Arthritis Care Res. 2012;2012(64):625–39.
15. Lipsky PE, van der Heijde DM, St Clair EW, Furst DE, Breedveld FC, Kalden JR, et al. Anti-Tumor Necrosis Factor Trial in Rheumatoid Arthritis with Concomitant Therapy Study Group. Infliximab and methotrexate in the treatment of rheumatoid arthritis. N Engl J Med. 2000;343:1594–602.
16. Bathon JM, Martin RW, Fleischmann RM, Tesser JR, Schiff MH, Keystone EC, et al. A comparison of etanercept and methotrexate in patients with early rheumatoid arthritis. N Engl J Med. 2000;343:1586–93.
17. Klareskog L, van der Heijde DM, de Jager JP, Gough A, Kalden J, Malaise M, et al. TEMPO (Trial of Etanercept and Methotrexate with Radiographic Patient Outcomes) study investigators. Therapeutic effect of the combination of etanercept and methotrexate compared with each treatment alone in patients with rheumatoid arthritis: double-blind randomised controlled trial. Lancet. 2004;363:675–81.
18. St Clair EW, van der Heijde DM, Smolen JS, Maini RN, Bathon JM, Emery P, et al. Active-Controlled Study of Patients Receiving Infliximab for the Treatment of Rheumatoid Arthritis of Early Onset Study Group. Combination of infliximab and methotrexate therapy for early rheumatoid arthritis: a randomized, controlled trial. Arthritis Rheum. 2004;50:3432–43.
19. Nishimoto N, Hashimoto J, Miyasaka N, Yamamoto K, Kawai S, Takeuchi T, et al. Study of active controlled monotherapy used for rheumatoid arthritis, an IL-6 inhibitor (SAMURAI): evidence of clinical and radiographic benefit from an X-ray reader-blinded randomised controlled trial of tocilizumab. Ann Rheum Dis. 2007;66:1162–7.
20. Emery P, Breedveld FC, Hall S, Durez P, Chang DJ, Robertson D, et al. Comparison of methotrexate monotherapy with a combination of methotrexate and etanercept in active, early, moderate to severe rheumatoid arthritis (COMET): a randomised, double-blind, parallel treatment trial. Lancet. 2008;372:375–82.
21. Ma MH, Kingsley GH, Scott DL. A systematic comparison of combination DMARD therapy and tumour necrosis inhibitor therapy with methotrexate in patients with early rheumatoid arthritis. Rheumatology (Oxford). 2010;49:91–8.
22. Sakuma S, Kato Y, Nishigaki F, Sasakawa T, Magari K, Miyata S, et al. FK506 potently inhibits T cell activation induced TNF-alpha and IL-1beta production in vitro by human peripheral blood mononuclear cells. Br J Pharmacol. 2000;130:1655–63.
23. Magari K, Miyata S, Nishigaki F, Ohkubo Y, Mutoh S. Comparison of anti-arthritic properties of leflunomide with methotrexate and FK506: effect on T cell activation-induced inflammatory cytokine production in vitro and rat adjuvant-induced arthritis. Inflamm Res. 2004;53:544–50.
24. Magari K, Nishigaki F, Sasakawa T, Ogawa T, Miyata S, Ohkubo Y, et al. Anti-arthritic properties of FK506 on collagen-induced arthritis in rats. Inflamm Res. 2003;52:524–9.
25. Magari K, Miyata S, Ohkubo Y, Mutoh S. Inflammatory cytokine levels in paw tissues during development of rat collagen-induced arthritis: effect of FK506, an inhibitor of T cell activation. Inflamm Res. 2004;53:469–74.
26. Nishigaki F, Sakuma S, Ogawa T, Miyata S, Ohkubo T, Goto T. FK506 induces chondrogenic differentiation of clonal mouse embryonic carcinoma cells, ATDC5. Eur J Pharmacol. 2002;437: 123–8.

Leptin stimulates interleukin-6 production *via* janus kinase 2/signal transducer and activator of transcription 3 in rheumatoid synovial fibroblasts

S. Muraoka[1], N. Kusunoki[1], H. Takahashi[2], K. Tsuchiya[2], S. Kawai[1]

[1]Division of Rheumatology, Department of Internal Medicine; [2]Department of Orthopaedic Surgery Toho University School of Medicine, Tokyo, Japan.

Abstract

Objectives

The aim of this study was to determine the influence of leptin on the production of proinflammatory cytokines by rheumatoid synovial fibroblasts (RSFs).

Methods

Synovial tissue was obtained from patients with rheumatoid arthritis (RA). Leptin receptor mRNAs were detected by reverse transcription-polymerase chain reaction (RT-PCR). Productions of mRNA and protein of interleukin (IL)-1β, tumour necrosis factor-α (TNF-α), and IL-6 in the culture medium were detected by real-time PCR and ELISA kit, respectively. Small interfering RNA (siRNA) was transfected into RSF to down-regulate the expression of leptin receptor. Effects of inhibitors of janus kinase 2 (JAK2), phosphatidylinositol 3-kinase (PI3K), and mitogen-activated protein kinase (MAPK) on IL-6 production were evaluated. Phosphorylation of signal transducer and activator of transcription 3 (STAT3) in RSF were determined by Western blot analysis.

Results

We detected leptin receptor mRNAs in RSFs. Expression of IL-1β and IL-6 mRNA was enhanced in a concentration-dependent manner by addition of leptin to RSFs. IL-6 secretion by RSFs showed an increase after leptin stimulation. Leptin-induced production of IL-6 by RSFs was decreased after exposure to siRNA targeting leptin receptor (Ob-Rb). A JAK2 inhibitor, but not PI3K and MAPK inhibitors, decreased leptin-induced IL-6 production. Enhanced phosphorylation of STAT3 was observed in RSFs after stimulation by leptin.

Conclusion

Leptin may be one of the proinflammatory cytokines that up-regulates IL-6 production in RSFs via activation of JAK2/STAT3. Leptin and JAK/STAT pathway may represent a new alternative therapeutic target in the treatment of RA.

Key words

leptin, rheumatoid arthritis, IL-6, JAK2, STAT3

Sei Muraoka, MD
Natsuko Kusunoki, PhD
Hiroshi Takahashi, MD, PhD
Kazuaki Tsuchiya, MD, PhD
Shinichi Kawai, MD, PhD

Please address correspondence and reprint requests to:
Natsuko Kusunoki, PhD,
Division of Rheumatology,
Department of Internal Medicine,
Toho University School of Medicine,
6-11-1 Omori-Nishi, Ota-ku,
Tokyo 143-8541, Japan.
E-mail: kusunoki@med.toho-u.ac.jp

Received on November 22, 2012; accepted in revised form on February 5, 2013.

Funding: This work was supported in part by Heisei 20 and 23 Strategic Research Foundation Grants from the Japanese Ministry of Education, Culture, Sports, Science & Technology to Toho University; grants from the Japan Society for the Promotion of Science to S.K. (23591449) and N.K. (24791008); and grants from the Nakatomi Foundation and Takeda Science Foundation to N.K.

Competing interests: none declared.

Introduction

Adipose tissue is a structural component of many organs and a site for energy storage. In addition, recent studies have demonstrated that the major cellular component of adipose tissue, the adipocyte, has the ability to synthesise and release physiologically active molecules such as leptin, adiponectin, and resistin, as well as cytokines like interleukin (IL)-6 and tumour necrosis factor-α (TNF-α) (1). These molecules are called adipokines or adipocytokines. Some adipokines may have a central role in the regulation of insulin resistance (2), as well as being involved in many aspects of inflammation and immunity (3). Leptin is the product of the *ob* gene, and is a 16-kDa nonglycosylated peptide hormone synthesised almost exclusively by adipocytes that regulates appetite and energy expenditure centrally at the hypothalamic level (4). It is also suggested that leptin may contribute to inflammation and autoimmunity (5). This is not explained by gene background such as polymorphism of LEP rs2167270 (19 G>A) (6).

Rheumatoid arthritis (RA) is characterised by extensive inflammation and proliferation of the synovium that affects multiple joints. Since proinflammatory cytokines, including TNF-α, IL-1β, and IL-6, play a central role in the pathophysiologic mechanisms of RA, novel methods of neutralising these cytokines with monoclonal antibodies or soluble receptors have recently been developed as new treatments for this disease (7). Although blockade of the above-mentioned cytokines is beneficial, it is not curative and the effect is only partial, with many patients failing to respond. Therefore, it seems possible that other proinflammatory cytokines may also contribute to inflammation in RA. We previously reported that adiponectin (one of the adipokines) stimulates the production of IL-8 (8) and prostaglandin E_2 (9) by rheumatoid synovial fibroblasts (RSFs). We also reported that the serum levels of leptin and adiponectin were elevated in patients with RA (10). Moreover, leptin levels are increased in synovial fluid of RA patients (11). These findings suggest that some adipokines may contribute to synovial inflammation in RA. Accordingly, we examined the direct effects of leptin on cultured RSFs in the present study.

Materials and methods

Materials

Recombinant human leptin was purchased from Sigma-Aldrich (St. Louis, MO, USA) and was dissolved in 15 mM HCl and 7.5 mM NaOH at a pH of approximately 5.2, in accordance with the manufacturer's instructions. Recombinant IL-6 was purchased from R&D Systems, Inc. (Minneapolis, MN, USA) and was dissolved in sterile phosphate-buffered saline (PBS) containing 0.1% (volume/volume) bovine serum albumin to prepare stock solutions. Mouse anti-human IL-6 antibody was obtained from R&D Systems, Inc. Rabbit anti-human signal transducer and activator of transcription (STAT) 3 polyclonal antibody and rabbit anti-human phosphorylated STAT (phospho-STAT) 3 (Tyr705) polyclonal antibody were sourced from Cell Signalling Technology (Beverly, MA, USA). Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG was obtained from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA, USA), ECL Western blotting detection reagent was purchased from GE Healthcare UK Ltd. (Buckinghamshire, UK), and polyvinylidene difluoride membranes (Immobilon-P) were obtained from Millipore Corp. (Billerica, MA, USA). AG490 (2-cyano-3-[3.4-dihydroxyphenyl]-*N*-[phenylmethyl]-2-propenamide), a janus kinase (JAK) 2 inhibitor came from Merck KGaA (Darmstadt, Germany), while LY294002 (2-[4-morpholinyl]-8-phenyl-4H-1-benzopyran-4-one), a phosphatidylinositol 3-kinase (PI3K) inhibitor and PD98059 (2-[2-amino-3-methoxyphenyl]-4H-1-benzopyran-4-one), a mitogen-activated protein kinase (MAPK) inhibitor for extracellular signal-regulated kinase (ERK) were from Sigma-Aldrich. RPMI 1640 medium, penicillin/streptomycin solution, fetal bovine serum (FBS), and 0.25% trypsin/EDTA were sourced from Invitrogen Corp. (Carlsbad, CA, USA). PBS was purchased from

Takara Shuzo Co., Ltd. (Shiga, Japan), and all other chemicals were obtained from Wako Pure Chemical Industries (Osaka, Japan).

Cell culture

RSFs were prepared from synovial tissue as described previously (12). RA tissue specimens were obtained from patients undergoing total knee replacement who fulfilled the revised criteria (13) for the classification of RA. The protocol for this study was approved by the Toho University Ethics Committee (approval number: 19021), and all patients gave written consent to the use of their tissue for the research. Synovial tissue was digested for 2 hours with 0.25% (weight/volume) bacterial collagenase (ImmunoBiological Laboratories, Gunma, Japan) and then was suspended in RPMI 1640 medium with 10% (v/v) FBS, 100 units/ml of penicillin, and 100 μg/ml of streptomycin. The cells were incubated at 37°C under 5% CO_2 for several days, after which nonadherent cells were removed. Fibroblast-like adherent cells from the third or fourth passages were used as RSFs at a concentration of 2.5×10^6 cells/75 cm^2 flask.

Reverse transcription – polymerase chain reaction (RT-PCR)

Cells were seeded in culture medium containing 10% (v/v) FBS, and total RNA was extracted with an RNeasy mini kit (Qiagen GmbH., Hilden, Germany), in accordance with the manufacturer's instructions. Reverse transcription was performed with a SuperScript first-strand synthesis system for RT-PCR (Invitrogen Corp.) was employed according to the manufacturer's instructions, using 2 μg of total RNA from the cells as a template. Equal amounts of each reverse-transcribed product were amplified by PCR with HotStar Taq polymerase (Qiagen GmbH). The primer sequences and number of cycles were 5'-TTCAGGTGCGCTGTAAGAGGCT (sense) and 5'-AGGCTCCAAAAGAAGAGGACCACA (antisense) with 38 cycles for Ob-Rb (920 bp), 5'-TCCCATATCTGAGCCCAAAG and 5'-CATCAGGGGCTTCCAAAGTA with 32 cycles for Ob-Re (565 bp), and 5'-CCTCGCCTTTGCCGATCC and 5'-GGATCTTCATGAGGTAGTCAGTC with 28 cycles for β-actin (626 bp). After initial denaturation for 15 minutes at 95°C, PCR involved amplification for a variable number of cycles of 30 seconds at 95°C (β-actin and Ob-Re) or 94°C (Ob-Rb), 30 seconds at 56°C (β-actin) or 59°C (Ob-Re) or 55°C (Ob-Rb), and 45 seconds (β-actin and Ob-Re) or 30 seconds (Ob-Rb) at 72°C, followed by elongation for 5 minutes at 72°C. The amplified complementary DNA (cDNA) fragments were resolved by electrophoresis on 2% (w/v) agarose gel, and were detected under ultraviolet light using an LAS-3000 (Fujifilm Corp. Tokyo, Japan) after the gel was stained with ethidium bromide.

Real-time PCR

To semi-quantitatively evaluate the expression of messenger RNA (mRNA) for IL-6, IL-1β, and TNF-α, real-time PCR was performed using real-time TaqMan technology with a Sequence Detection System model 7000 according to the manufacturer's recommendations (Applied Biosystems, Foster City, CA, USA). Cells were cultured under various conditions in medium containing 1% (v/v) FBS, after which extraction of total RNA and synthesis of cDNA were performed as described above. Specific probes for IL-6, IL-1β, and TNF-α were obtained from TaqMan Gene Expression Assay (Applied Biosystems), with the ID numbers of the products being Hs99999032_m1 for IL-6, Hs99999029_m1 for IL-1β and Hs00174128_m1 for TNF-α. The threshold cycle was calculated from a standard curve and expression of the target mRNA was normalised for the expression of β-actin mRNA.

Western blot analysis

Cells were cultured under various conditions at a density of $5\times10^4/cm^2$ in medium containing 1% (v/v) FBS. Subsequently, the cells were lysed in mammalian protein extraction reagent containing Halt™ phosphatase inhibitor cocktail (Pierce Biotechnology, Rockford, IL, USA). The protein content of the lysates was determined with bicinchoninic acid protein assay reagent (Pierce Biotechnology), using bovine serum albumin as the standard. Then cell lysates were adjusted to 10 μg of protein and were subjected to sodium dodecyl sulfate (SDS) polyacrylamide gel (10–15% [w/v]) electrophoresis. Next, the proteins were electroblotted onto Immobilon-P poly (vinylidene difluoride) membranes with a semidry blotter (Atto Corp., Tokyo, Japan). After the membranes had been blocked in 10 mM Tris-buffered saline (TBS) containing 0.1% (v/v) Tween 20 (TBST) and 5% (w/v) skim milk, the primary antibody (anti-human STAT3 antibody or anti-human phospho-STAT3 antibody) was added at a dilution of 1:1000 in TBST, and incubation was done for 18 hours at 4°C. After the membranes had been washed with TBST, the secondary antibody (HRP-conjugated goat anti-rabbit antibody) was added at a dilution of 1:10,000 in TBST and incubation was performed for 1 hour. After further washing with TBST, protein bands were detected with an enhanced ECL Western blotting detection reagent (GE Healthcare UK Ltd.) using LAS-3000 (Fujifilm Corp.).

Measurement of cytokines in the culture medium

Cells were seeded in 24-well plastic plates (1×10^5/well) and cultured for 24 hours under various conditions in medium containing 1% (v/v) FBS under an atmosphere of 5% CO_2. Then the concentrations of IL-6, IL-1β, and TNF-α in the medium were measured with an enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's recommendations (Invitrogen Corp.). Experiments using RSFs were done in triplicate wells, and the concentrations of IL-6, IL-1β, and TNF-α was measured in triplicate.

RNA interference assay with Ob-Rb

An RNA interference assay was performed to assess the effect on RSFs of down-regulating Ob-Rb expression. Small interfering RNA (siRNA) for Ob-Rb (Stealth™ RNAi) and negative control siRNA were purchased from Invitrogen Corp. For gene knockdown

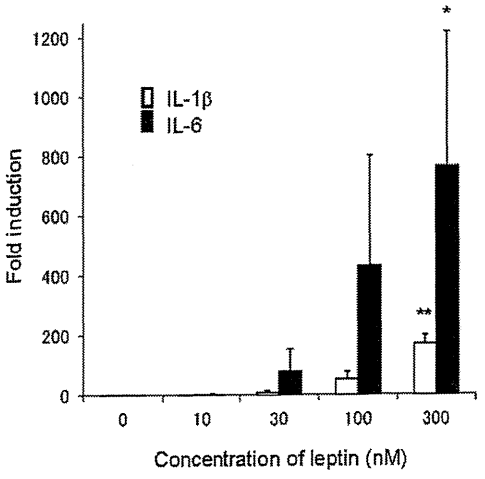


Fig. 1. Effect of leptin on mRNA expressions of inflammatory cytokines by RSFs. Rheumatoid synovial fibroblasts (RSFs) were incubated with leptin for 6 hours and the expression of interleukin (IL)-1β and IL-6 mRNA by RSFs was assessed by real-time polymerase chain reaction. The results showed that leptin significantly increased the expression of IL-1β and IL-6 mRNA by RSFs in a concentration-dependent manner. Expression of the target mRNA was normalised for the expression of β-actin mRNA, and fold induction was determined relative to expression by cells incubated without leptin. Bars show the mean and SEM (n=3). $^{*}p<0.05$; $^{**}p<0.01$ *vs.* no treatment. Significance was evaluated by one-way analysis of variance with Dunnett's *post hoc* test.

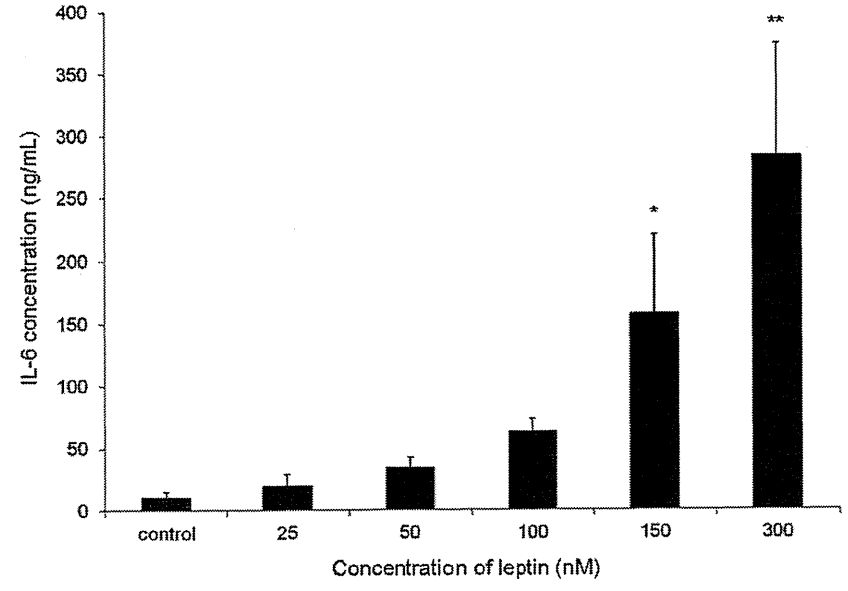


Fig. 2. Effect of leptin on the production of IL-6 by RSFs. Rheumatoid synovial fibroblasts (RSFs) were incubated with leptin at the indicated concentrations for 24 hours and the interleukin (IL)-6 concentration in the culture medium was measured by enzyme-linked immunosorbent assay. Leptin significantly increased IL-6 production by RSFs in a concentration-dependent manner. Bars show the mean and SEM (n=3). $^{*}p<0.05$; $^{**}p<0.01$ *vs.* no treatment. Significance was evaluated by one-way analysis of variance with Dunnett's *post hoc* test.

experiments, RSFs were plated in 10 cm plastic dishes (3×10^5/dish) in RPMI 1640 medium with 10% (v/v) FBS and cultured for 18 hours. Then the medium was changed to serum-free RPMI 1640 medium, and the cells were transfected with siRNA (10 pmol/ml) for Ob-Rb or with control siRNA (10 pmol/ml) using Lipofectamine™ RNAiMAX (Invitrogen Corp.) according to the manufacturer's recommendations. After 72 hours, the cells were replated into 35-mm plastic dishes for PCR or into 96-well plastic plates for measurement of IL-6 by ELISA.

Statistical analysis

Data are expressed as the mean ± SEM. Statistical analysis was performed with Prism ver. 5.0 software (Graphpad Software, San Diego, CA, USA). To compare two groups, Student's *t*-test was employed. Groups (≥3) were compared by using one-way analysis of variance (ANOVA). One-way ANOVA with Bonferroni's *post hoc* test was used to determine differences among all the groups. One-way ANOVA with Dunnett's *post hoc* test was used for comparison to control (no treatment). In all analyses, $p<0.05$ was considered significant.

Results

Effect of leptin on production of inflammatory cytokines by RSFs

To determine whether leptin increased the production of IL-1β, IL-6, and TNF-α by RSFs, real-time PCR was performed. The results showed that leptin significantly increased the expression of IL-1β and IL-6 mRNA by RSFs in a concentration-dependent manner (Fig. 1). In contrast, expression of TNF-α mRNA was not increased by leptin (data not shown). To confirm the production of IL-1β and IL-6 proteins, we measured the concentrations of these cytokines in the culture medium of RSFs incubated with leptin (Fig. 2). We found that leptin significantly increased IL-6 production by RSFs in a concentration-dependent manner. In contrast, concentrations of IL-1β and TNF-α were not detectable ever after stimulation with leptin.

Effect of siRNA for leptin receptor on IL-6 production by RSFs

mRNAs for both leptin receptors (Ob-Rb and Ob-Re) were expressed by cells from 3 patients with RA (data not shown). RSFs were transfected with siRNA targeting Ob-Rb (the leptin receptor) or with negative control siRNA, and then expression of Ob-Rb and Ob-Re mRNA was detected by RT-PCR. This showed that Ob-Rb mRNA expression by RSFs was decreased after exposure to the siRNA for Ob-Rb (Fig. 3A). When cells were seeded in 96-well plates and incubated with leptin for 18 hours, IL-6 production by RSFs transfected with the siRNA targeting Ob-Rb was significantly lower than that by RSFs transfected with negative control siRNA (Fig. 3B).

Effect of leptin on STAT3 phosphorylation in RSFs

We then examined more details of the signal transduction involved in these effects of leptin. To determine whether leptin induced STAT3 phosphorylation in RSFs, Western blotting was performed. This revealed that leptin increased STAT3 phosphorylation in a concentration-dependent manner (Fig. 4A). To investigate whether phosphorylation of STAT3 was related to the induction of IL-6 production by leptin,

RSFs were incubated with an anti-IL-6 antibody (Fig. 4B). Phosphorylation of STAT3 in response to leptin was not inhibited by addition of the anti-IL-6 antibody, but STAT3 phosphorylation in response to IL-6 was inhibited by the antibody.

Effects of signalling pathway inhibitors on leptin-induced IL-6 production by RSFs

We examined the effects of inhibitors of major signalling pathways on leptin-induced IL-6 upregulation in RSFs. As a result, leptin-induced IL-6 production was significantly inhibited by addition of AG490, a JAK2 inhibitor (Fig. 5), but not by LY294002, a PI3K inhibitor (Fig. 6A) or PD98059, a MAPK inhibitor for ERK (Fig. 6B). These findings suggested that leptin induces IL-6 production in RSFs *via* the JAK2/STAT3 pathway.

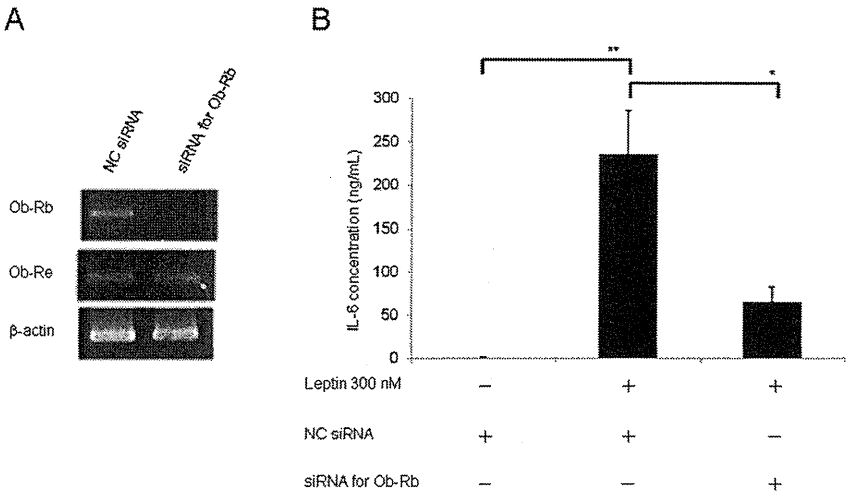


Fig. 3. Effect of siRNA targeting the leptin receptor on IL-6 production by RSFs. (**A**) Rheumatoid synovial fibroblasts (RSFs) were transfected with small interfering RNA (siRNA) for Ob-Rb or negative control (NC) siRNA, and Ob-Rb and Ob-Re mRNA levels were analysed by reverse transcription – polymerase chain reaction. Representative results obtained with fibroblasts from 3 patients are shown. Ob-Rb mRNA expression by RSFs was decreased after exposure to the siRNA for Ob-Rb when compared with exposure for NC siRNA. (**B**) After transfection with siRNA, RSFs were treated with 300 nM leptin or phosphate-buffered saline for 18 hours, and the interleukin (IL)-6 concentration in the culture medium were measured by enzyme-linked immunosorbent assay. IL-6 production by RSFs transfected with the siRNA targeting Ob-Rb was significantly lower than that by RSFs transfected with NC siRNA. Bars show the mean and SEM (n=3). $^{*}p<0.05$; $^{**}p<0.01$. Significance was evaluated by one-way analysis of variance with Bonferroni's *post hoc* test.

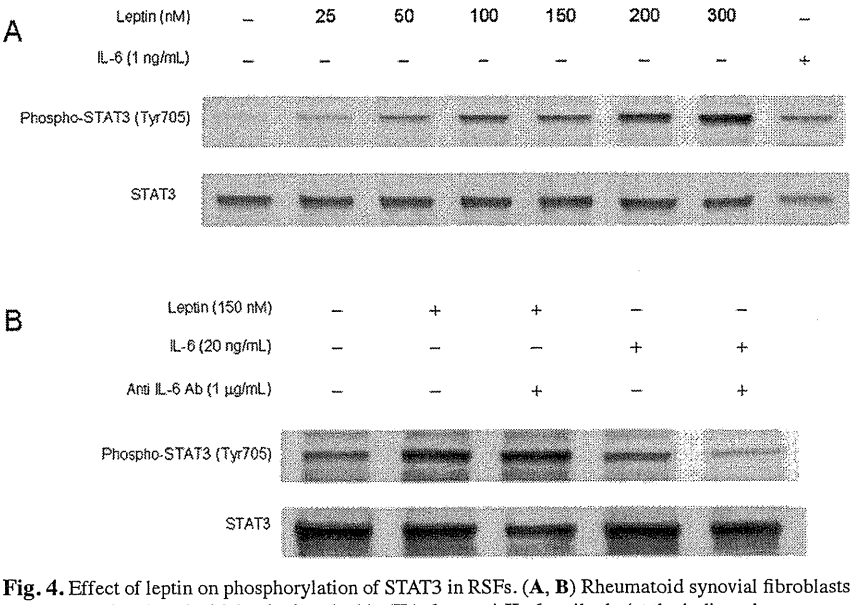


Fig. 4. Effect of leptin on phosphorylation of STAT3 in RSFs. (**A**, **B**) Rheumatoid synovial fibroblasts (RSFs) were incubated with leptin, interleukin (IL)-6, or anti-IL-6 antibody (at the indicated concentrations), and then Western blot analysis was performed. Leptin increased signal transducer and activator of transcription (STAT)3 phosphorylation in a concentration-dependent manner (**A**). Phosphorylation of STAT3 in response to leptin was not inhibited by addition of the anti-IL-6 antibody, but STAT3 phosphorylation in response to IL-6 was inhibited by the antibody (**B**). Representative results obtained with fibroblasts from 3 patients are shown.

Discussion

In the present study, we demonstrated that leptin induced the expression of IL-6 mRNA and protein in RSFs *via* the JAK2/STAT3 pathway. This finding is supported by data obtained in leptin-deficient *ob/ob* mice by Busso *et al.* (14), who reported that leptin-deficient mice were partly protected against antigen-induced arthritis, showing less synovial tissue proliferation and a weaker humoral response to the injected antigen. Moreover, Sugioka *et al.* (15) reported that acquired leptin resistance by high-fat feeding reduces inflammation from collagen antibody-induced arthritis in mice.

Harigai *et al.* (16) reported that TNF-α induced IL-6 production by synovial fibroblasts in a dose dependent manner. On the other hand, the present study showed that leptin stimulated IL-6 production. Gonzalez-Gay *et al.* (17) reported that leptin concentration was not changed by administration of anti-TNF-α-blocker infliximab. Therefore, TNF-α-induced IL-6 production might not be mediated by leptin.

IL-6 is a pleiotropic cytokine that is overexpressed in the synovial tissue of RA patients, who have elevated concentrations of IL-6 in both serum and synovial fluid (18). IL-6 influences the function of neutrophils, T cells, B cells, monocytes, and osteoclasts. It is a major inducer of the hepatic acute phase response, which is also a key feature of RA that is correlated with disease activity and joint destruction. Thus, IL-6 is thought to play a pivotal role in RA. Tocilizumab is a humanised anti-IL-6 receptor monoclonal antibody that has shown efficacy for treating RA in clinical trials (19). The average levels

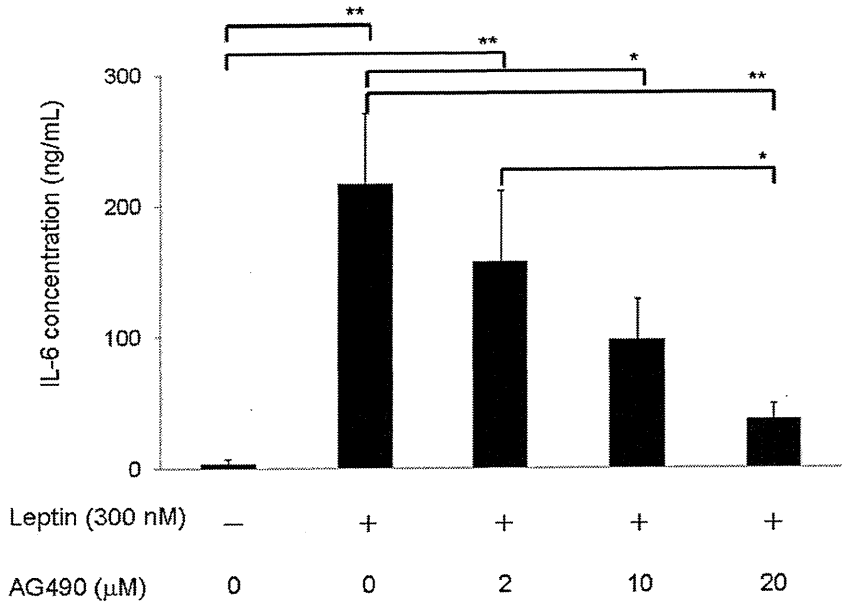


Fig. 5. Effect of a JAK2 inhibitor on IL-6 production by RSFs. Interleukin (IL)-6 level in culture medium of rheumatoid synovial fibroblasts incubated for 18 hours with/without leptin and AG490. The IL-6 concentration in the culture medium was measured by enzyme-linked immunosorbent assay. Leptin-induced IL-6 production was significantly inhibited by addition of AG490, a janus kinase 2 inhibitor. Bars show the mean and SEM (n=3). *$p<0.05$; **$p<0.01$. Significance was evaluated by one-way analysis of variance with Bonferroni's *post hoc* test.

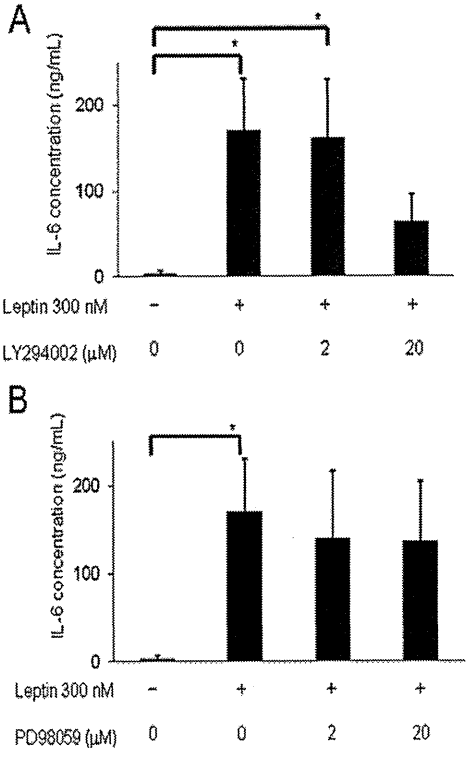


Fig. 6. Effect of signalling pathway inhibitors on IL-6 production by RSFs. (**A, B**) Interleukin (IL)-6 level in culture medium of rheumatoid synovial fibroblasts incubated for 18 hours with/without leptin, LY294002, and PD98059. The IL-6 concentration in the culture medium was measured by enzyme-linked immunosorbent assay. Leptin-induced IL-6 production was not inhibited by LY294002, a phosphatidylinositol 3-kinase inhibitor (**A**) or PD98059, a mitogen-activated protein kinase inhibitor for extracellular signal-regulated kinase (**B**). Bars show the mean and SEM (n=3). *$p<0.05$; **$p<0.01$. Significance was evaluated by one-way analysis of variance with Bonferroni's *post hoc* test.

of IL-6 in serum and synovial fluid of RA patients were 14 pg/mL and 4 ng/mL, respectively (20). In our study, 20–283 ng/mL of IL-6 were produced by 25–300 nM of leptin (Fig. 2). Thus, our present results suggest a contribution of leptin to the pathogenesis of RA *via* its influence on IL-6.

Although IL-1β mRNA in RSFs was increased by leptin, IL-1β protein was not detectable in culture medium of RSFs. In general, synovial fibroblasts are not the principal sources of inflammasome-mediated IL-1β production in the synovium (21). This might be one of the reasons of the discrepancy between changes in mRNA and protein of IL-1β in our study.

Six isoforms of the leptin receptor have been identified (22). The Ob-Re isoform is a soluble receptor that lacks the transmembrane and cytoplasmic domains, while Ob-Rb is a long form that has an intracellular signalling domain and is thought to be involved in intracellular signalling. In the present study, we found that both Ob-Rb and Ob-Re mRNAs were expressed by RSFs. In addition, the response of IL-6 to leptin was reduced when RSFs were transfected with siRNA targeting Ob-Rb. Therefore, the induction of IL-6 production by leptin was mediated by Ob-Rb.

It is known that JAK/STAT pathway is activated by leptin in several kinds of human cells, that is hepatocellular carcinoma (23), peripheral blood mononuclear cells (24), colorectal adenoma (25). However, these report have not shown upregulation of IL-6 by leptin. In addition, this is the first report that leptin stimulates IL-6 production *via* JAK2/STAT3 in RSFs. Although leptin has been shown to stimulate IL-6 production in human osteoarthritic cartilage (26), this was mediated by the nuclear factor κB and MAPK pathway rather than the JAK2/STAT3 pathway. Since Migita *et al.* (27) reported that IL-6 induced acute-phase serum amyloid A genes *via* JAK2/STAT3 activation in RSFs, we determined whether STAT3 phosphorylation was affected by the leptin-induced upregulation of IL-6. Phosphorylation of STAT3 in response to leptin was not inhibited by the anti-IL-6 antibody, suggesting that STAT3 phosphorylation might be due to a direct effect of leptin on RSFs.

A previous study demonstrated that leptin activated two signalling pathways (PI3K and MAPK) in RSFs and human peripheral blood mononuclear cells (28, 29). Therefore, we investigated the effect of LY294002 (a PI3K inhibitor) and PD98059 (a MAPK inhibitor for ERK) on RSFs incubated

with leptin. As a result, leptin-induced IL-6 production was not mediated by signalling of PI3K and/or MAPK.

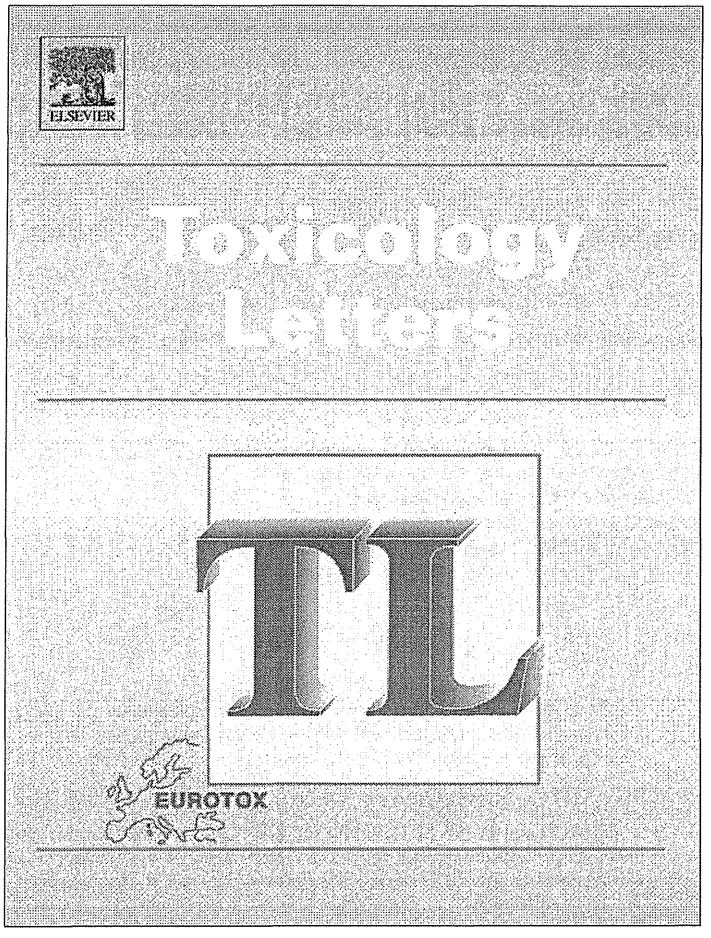
The serum leptin level in RA patients was reported to be in the 1–30 nM range (10), so the concentration of leptin used in this study was higher, but it might be possible that leptin stimulates a vicious cycle of inflammation by a paracrine effect in the articular cavity (30). Further studies will be necessary to confirm the mechanism by which leptin influences RA.

Acknowledgements

We wish to thank Prof. Chiaki Nishimura for advice regarding the statistical analysis, Dr Rika Yarita for support conducting *in vitro* experiments, and Ms Sonoko Sakurai for secretarial assistance.

References

1. AHIMA RS, FLIER JS: Adipose tissue as an endocrine organ. *Trends Endocrinol Metab* 2000; 11: 327-32.
2. MEIER U, GRESSNER AM: Endocrine regulation of energy metabolism: review of pathobiochemical and clinical chemical aspects of leptin, ghrelin, adiponectin, and resistin. *Clin Chem* 2004; 50: 1511-25.
3. TILG H, MOSCHEN AR: Adipocytokines: mediators linking adipose tissue, inflammation and immunity. *Nat Rev Immunol* 2006; 6: 772-83.
4. HALAAS JL, GAJIWALA KS, MAFFEI M *et al.*: Weight-reducing effects of the plasma protein encoded by the obese gene. *Science* 1995; 269: 543-6.
5. STOFKOVA A: Leptin and adiponectin: from energy and metabolic dysbalance to inflammation and autoimmunity. *Endocr Regul* 2009; 43: 157-68.
6. GARCÍA-BERMÚDEZ M, GONZÁLEZ-JUANATEY C, RODRÍGUEZ-RODRÍGUEZ L *et al.*: Lack of association between LEP rs2167270 (19 G>A)polymorphism and disease susceptibility and cardiovascular disease in patientswith rheumatoid arthritis. *Clin Exp Rheumatol* 2011; 29: 293-8.
7. BRENNAN FM, MCINNES IB: Evidence that cytokines play a role in rheumatoid arthritis. *J Clin Invest* 2008; 118: 3537-45.
8. KITAHARA K, KUSUNOKI N, KAKIUCHI T, SUGURO T, KAWAI S: Adiponectin stimulates IL-8 production by rheumatoid synovial fibroblasts. *Biochem Biophys Res Commun* 2009; 378: 218-23.
9. KUSUNOKI N, KITAHARA K, KOJIMA F *et al.*: Adiponectin stimulates prostaglandin E(2) production in rheumatoid arthritis synovial fibroblasts. *Arthritis Rheum* 2010; 62: 1641-9.
10. YOSHINO T, KUSUNOKI N, TANAKA N *et al.*: Elevated serum levels of resistin, leptin, and adiponectin are associated with C-reactive protein and also other clinical conditions in rheumatoid arthritis. *Intern Med* 2011; 50: 269-75.
11. BOKAREWA M, BOKAREW D, HULTGREN O, TARKOWSKI A: Leptin consumption in the inflamed joints of patients with rheumatoid arthritis. *Ann Rheum Dis* 2003; 62: 952-6.
12. KUSUNOKI N, YAMAZAKI R, KAWAI S: Induction of apoptosis in rheumatoid synovial fibroblasts by celecoxib, but not by other selective cyclooxygenase 2 inhibitors. *Arthritis Rheum* 2002; 46: 3159-67.
13. ARNETT FC, EDWORTHY SM, BLOCH DA *et al.*: The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. *Arthritis Rheum* 1988; 31: 315-24.
14. BUSSO N, SO A, CHOBAZ-PÉCLAT V *et al.*: Leptin signaling deficiency impairs humoral and cellular immune responses and attenuates experimental arthritis. *J Immunol* 2002; 168: 875-82.
15. SUGIOKA Y, TADA M, OKANO T, NAKAMURA H, KOIKE T: Acquired leptin resistance by high-fat feeding reduces inflammation from collagen antibody-induced arthritis in mice. *Clin Exp Rheumatol* 2012; 30: 707-13.
16. HARIGAI M, HARA M, KITANI A *et al.*: Interleukin 1 and tumor necrosis factor-alpha synergistically increase the production of interleukin 6 in human synovial fibroblast. *J Clin Lab Immunol* 1991; 34: 107-13.
17. GONZALEZ-GAY MA, GARCIA-UNZUETA MT, BERJA A *et al.*: Anti-TNF-alpha therapy does not modulate leptin in patients with severe rheumatoid arthritis. *Clin Exp Rheumatol* 2009; 27: 222-8.
18. NISHIMOTO N, KISHIMOTO T, YOSHIZAKI K: Anti-interleukin 6 receptor antibody treatment in rheumatic disease. *Ann Rheum Dis* 2000; 59 (Suppl. 1): i21-7.
19. NISHIMOTO N, HASHIMOTO J, MIYASAKA N *et al.*: Study of active controlled monotherapy used for rheumatoid arthritis, an IL-6 inhibitor (SAMURAI): evidence of clinical and radiographic benefit from an x ray reader-blinded randomised controlled trial of tocilizumab. *Ann Rheum Dis* 2007; 66: 1162-7.
20. USÓN J, BALSA A, PASCUAL-SALCEDO D *et al.*: Soluble interleukin 6 (IL-6) receptor and IL-6 levels in serum and synovial fluid of patients with different arthropathies. *J Rheumatol* 1997; 24: 2069-75.
21. KOLLY L, BUSSO N, PALMER G, TALABOT-AYER D, CHOBAZ V, SO A: Expression and function of the NALP3 inflammasome in rheumatoid synovium. *Immunology* 2010; 129: 178-85.
22. BERNOTIENE E, PALMER G, GABAY C: The role of leptin in innate and adaptive immune responses. *Arthritis Res Ther* 2006; 8: 217.
23. SAXENA NK, SHARMA D, DING X *et al.*: Concomitant activation of the JAK/STAT, PI3K/AKT, and ERK signaling is involved in leptin-mediated promotion of invasion and migration of hepatocellular carcinoma cells. *Cancer Res* 2007; 67: 2497-507.
24. SANCHEZ-MARGALET V, MARTIN-ROMERO C: Human leptin signaling in human peripheral blood mononuclear cells: activation of the JAK-STAT pathway. *Cell Immunol* 2001; 211: 30-6.
25. UCHIYAMA T, TAKAHASHI H, SUGIYAMA M *et al.*: Leptin receptor is involved in STAT3 activation in human colorectal adenoma. *Cancer Sci* 2011; 102: 367-72.
26. VUOLTEENAHO K, KOSKINEN A, KUKKONEN M *et al.*: Leptin enhances synthesis of proinflammatory mediators in human osteoarthritic cartilage-mediator role of NO in leptin-induced PGE2, IL-6, and IL-8 production. *Mediators Inflamm* 2009; 2009: 345838.
27. MIGITA K, KOGA T, KOMORI A *et al.*: Influence of Janus kinase inhibition on interleukin 6-mediated induction of acute-phase serum amyloid A in rheumatoid synovium. *J Rheumatol* 2011; 38: 2309-17.
28. MARTÍN-ROMERO C, SÁNCHEZ-MARGALET V: Human leptin activates PI3K and MAPK pathways in human peripheral blood mononuclear cells: possible role of Sam68. *Cell Immunol* 2001; 212: 83-91.
29. TONG KM, SHIEH DC, CHEN CP *et al.*: Leptin induces IL-8 expression via leptin receptor, IRS-1, PI3K, Akt cascade and promotion of NF-kappaB/p300 binding in human synovial fibroblasts. *Cell Signal* 2008; 20: 1478-88.
30. KONTNY E, PLEBANCZYK M, LISOWSKA B, OLSZEWSKA M, MALDYK P, MASLINSKI W: Comparison of rheumatoid articular adipose and synovial tissue reactivity to proinflammatory stimuli: contribution to adipocytokine network. *Ann Rheum Dis* 2012; 71: 262-7.



(This is a sample cover image for this issue. The actual cover is not yet available at this time.)

Toxicology Letters 215 (2012) 16–24

Contents lists available at SciVerse ScienceDirect

Toxicology Letters

journal homepage: www.elsevier.com/locate/toxlet

Liver X receptor α bidirectionally transactivates human *CYP1A1* and *CYP1A2* through two cis-elements common to both genes☆

Kikuko Araki, Keisuke Watanabe, Yasushi Yamazoe, Kouichi Yoshinari*

Division of Drug Metabolism and Molecular Toxicology, Graduate School of Pharmaceutical Sciences, Tohoku University, 6-3 Aramaki-aoba, Aoba-ku, Sendai, Miyagi 980-8578, Japan

HIGHLIGHTS

- LXRα activation induced transactivation of both h*CYP1A1* and h*CYP1A2* simultaneously.
- LXRα-mediated transactivation of h*CYP1A1* and h*CYP1A2* is through two regions.
- LXRα/RXRα heterodimer binds to two ER8 motifs in the proximal promoter of h*CYP1A1*.
- LXRα synergistically transactivated h*CYP1A1* and h*CYP1A2* with AhR but not CAR.
- LXRα ligand increased h*CYP1A1* and h*CYP1A2* mRNA levels in a cell-specific manner.

ARTICLE INFO

Article history:
Received 31 July 2012
Received in revised form
24 September 2012
Accepted 26 September 2012
Available online xxx

Keywords:
Nuclear receptor
Drug metabolism
Bidirectional transcription
Reporter assay
Hepatocytes
LXRα

ABSTRACT

CYP1A1 and CYP1A2 are involved in both detoxification and metabolic activation of xenobiotics. Human *CYP1A1* (h*CYP1A1*) and h*CYP1A2* exist in a head-to-head orientation in chromosome 15 with the overlapping 5′-flanking region. We have recently reported that nuclear receptor constitutive androstane receptor (CAR), in addition to aryl hydrocarbon receptor, bidirectionally transactivates these genes through common motifs. In this study, we have investigated a role of liver X receptor α (LXRα), another liver-enriched nuclear receptor, in the expression h*CYP1A1* and h*CYP1A2*. In reporter assays with dual-reporter constructs containing their promoter region between two different reporter genes, LXRα simultaneously transactivated h*CYP1A1* and h*CYP1A2* through two regions, independent of aryl hydrocarbon receptor. In electrophoretic mobility shift assays, LXRα/retinoid X receptor α heterodimer bound to two ER8-type motifs found at around −520 and −460 of h*CYP1A1*. The former corresponds to the CAR-binding motif previously identified. Reporter assays using mutated constructs confirmed the critical roles of these motifs in the LXRα-mediated simultaneous transcription of h*CYP1A1* and h*CYP1A2*. h*CYP1A1* and h*CYP1A2* mRNA levels were increased in human hepatoma HuH-7 cells and human primary hepatocytes, respectively, after treatment with the LXRα ligand GW3965. Our results suggest that LXRα transactivates the expression of h*CYP1A1* and h*CYP1A2* through common two cis-elements.

1. Introduction

CYP1A1 and CYP1A2 are involved in both detoxification and metabolic activation of xenobiotics including procarcinogens, and also important for the metabolism of endogenous compounds such as estrogens (Badawi et al., 2001; Lee et al., 2003). These enzymes are highly inducible after exposure to polycyclic aromatic hydrocarbons such as 3-methylcholanthrene (3MC) and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. This induction results from the enhanced transcription of both genes through the ligand-activated aryl hydrocarbon receptor (AhR) (Denison and Nagy, 2003). In response to ligands, AhR binds to the cis-element termed xenobiotics-responsive element (XRE) in the 5′-flanking region of target genes to activate their transcription. Human *CYP1A1* (h*CYP1A1*) and h*CYP1A2* are located in a head-to-head orientation in chromosome 15 and share an about 23-kb 5′-flanking region. We have recently demonstrated that both genes share a common regulatory region and that the region from −460 to −1826 of h*CYP1A1* containing five XREs works bidirectionally for the AhR-mediated transcription of h*CYP1A1* and h*CYP1A2* (Ueda et al., 2006;

Abbreviations: LXR, liver X receptor; CAR, constitutive androstane receptor; RXR, retinoid X receptor; AhR, aryl hydrocarbon receptor; 24HC, 24(S)-hydroxycholesterol; 3MC, 3-methylcholanthrene; CITCO, 6-(4-chlorophenyl)imidazo[2,1-*b*][1,3]thiazole-5-carbaldehyde-O-(3,4-dichlorobenzyl)oxime; DMSO, dimethyl sulfoxide; SEAP, secreted alkaline phosphatase; EMSA, electrophoretic mobility shift assay; h, human; ER, everted repeat; DR, direct repeat; XRE, xenobiotic-responsive element.

☆ A part of the data in this manuscript was presented at the 17th North American Regional ISSX Meeting, October 16–20, 2011, Atlanta, GA, USA.

* Corresponding author. Tel.: +81 22 795 6828; fax: +81 22 795 6826.
E-mail address: yoshinari@m.tohoku.ac.jp (K. Yoshinari).

0378-4274/$ – see front matter © 2012 Elsevier Ireland Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.toxlet.2012.09.021

Yoshinari et al., 2008). In addition, we have also found that constitutive androstane receptor (CAR), a xenobiotic-responsive nuclear receptor, transactivates h*CYP1A1* and h*CYP1A2* independent of AhR (Yoshinari et al., 2010). CAR heterodimerizes with retinoid X receptor α (RXRα), binds to an ER8 (everted repeat separated by 8 nucleotides)-type motif, located at around −520 of h*CYP1A1*, and transactivates both genes bidirectionally (Yoshinari et al., 2010). Thus, the mechanisms for the xenobiotic-induced transcription of h*CYP1A1* and h*CYP1A2* have been increasingly understood.

Liver X receptors α (LXRα) and LXRβ belong to the nuclear receptor superfamily. LXRα is highly expressed in the liver, intestine, brain and macrophages, while LXRβ is expressed ubiquitously (Lu et al., 2001). These receptors are activated by oxysterols including 4β-hydroxycholesterol and 24(S)-hydroxycholesterol (24HC) (Janowski et al., 1996) as well as high-affinity synthetic agonists such as GW3965 (Collins et al., 2002) and T0901317 (Repa et al., 2000; Schultz et al., 2000). Upon ligand binding, LXRs, as a heterodimer with RXR, bind to DR4 (direct repeat separated by 4 nucleotides)-type motifs found in the promoter regions of target genes to transactivate them (Willy et al., 1995). The target genes include those associated with lipid homeostasis, such as *CYP7A1*, *SREBP1*, *ABCA1 ABCG5* and *ABCG8* (Janowski et al., 1999; Lu et al., 2001). Recent reports suggest that LXRα is also involved in the expression of genes encoding drug-metabolizing enzymes such as mouse CYP3A11 (Gnerre et al., 2005) and human and mouse UGT1A3 (Verreault et al., 2006), and drug-transporters such as human OATP1B1 (Meyer Zu Schwabedissen et al., 2010) and MRP2 (Chisaki et al., 2009).

In the present study, we have investigated a possible involvement of LXRα in the transactivation of h*CYP1A1* and h*CYP1A2*. We have raised this possibility based on the following reasons: (1) LXRα, like CAR, is highly expressed in the liver and prefers DR4-type motifs as its binding sequences (Lu et al., 2001; Swales and Negishi, 2004; Willy et al., 1995); (2) LXRα is suggested to regulate the expression of genes associated with drug metabolism and disposition as mentioned above (Chisaki et al., 2009; Gnerre et al., 2005; Meyer Zu Schwabedissen et al., 2010; Verreault et al., 2006); (3) Treatment of mice with LXRα agonists (22(R)-hydroxycholesterol or GW3965), slightly but significantly increased hepatic *Cyp1a2* mRNA levels, although the authors concluded that LXRα effects on *Cyp1a2* expression was negligible (Gong et al., 2007); (4) Finally, recombinant CYP1A2 has been reported to catalyze the conversion of cholesterol to 4β-hydroxycholesterol, which is an LXRα agonist (Honda et al., 2011; Janowski et al., 1996). Using the dual-reporter system recently developed in our laboratory (Ueda et al., 2006), we here demonstrate that human LXRα is able to bidirectionally transactivate h*CYP1A1* and h*CYP1A2* through two cis-elements common to both genes.

2. Materials and methods

2.1. Materials

T4 polynucleotide kinase and restriction enzymes were purchased from New England BioLabs (Ipswich, MA). Poly(dI–dC) and [γ-^{32}P]ATP were purchased from GE Healthcare (Piscataway, NJ) and PerkinElmer (Waltham, MA), respectively. All other chemicals were of highest grade available purchased from Wako Pure Chemical Industries (Osaka, Japan) or Sigma–Aldrich (St. Louis, MO) unless otherwise mentioned. Oligonucleotide DNAs were commercially synthesized by Fasmac (Atsugi, Japan).

2.2. Plasmid DNA

Dual-reporter constructs used in Figs. 1 and 2, 6, 7 were prepared previously (Ueda et al., 2006; Yoshinari et al., 2010). A mutated construct, pd1AΔ-1827/-21975m was prepared previously (Yoshinari et al., 2008). pd1AΔ-1827/-21975dm was made by using QuikChange Lightning Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) with the primers, 5′-GCGGGGGCGGGGCTGCCCCGTGGTTACATCCTTCCCGGGGTTACTGAG-3′ and 5′-CTCAGTAACCCCGGGAAGGATGTAACCACGGGGCAGCCCCGCCCCCGC-3′.

The hLXRα cDNA was amplified by PCR using KOD-FX (TOYOBO, Tokyo, Japan) and the primers, 5′-CAGAAAGAGATGTCCTTGTG-3′ and 5′-AACAGTCATTCGTGCACATC-3′, and inserted into pTargeT (Promega, Madison, WI) to obtain mammalian expression plasmid pT-hLXRα. The cDNA fragment obtained by digesting pT-hLXRα with MluI and NotI was inserted into the same restriction sites of pTNT (Promega) for in vitro synthesis. pTarget-hCAR, pTNT-hRXRα and pTNT-hCAR were reported previously (Yoshinari et al., 2010).

2.3. Cell culture and reporter assay

HuH-7 and HepG2 cells (RIKEN BioResource Center, Tsukuba, Japan) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum (Nichirei Biosciences, Tokyo, Japan), minimum essential medium nonessential amino acids (Invitrogen, Carlsbad, CA) and antibiotic–antimycotic (Invitrogen). Cells were seeded in 48-well plates (BD Biosciences, San Jose, CA) 24 h before transfection. Reporter construct and pTarget expression plasmid (empty, pTarget-hLXRα, pTarget-hCAR) were transfected using jetPEI (PolyPlus Transfection, Illkirch, France). phRL-tk Control Vector was cotransfected to normalize transfection efficiency. Eight hours after transfection, cells were treated with 10 μM 24HC (Biomol, Plymouth Meeting, PA), 0.3 μM 6-(4-chlorophenyl)imidazo[2,1-*b*][1,3]thiazole-5-carbaldehyde-O-(3,4-dichlorobenzyl)oxime (CITCO; MERCK, Darmstadt, Germany), 1 μM 3MC (Toronto Research Chemicals, North York, Ontario, Canada) and vehicle (0.1% dimethyl sulfoxide; DMSO) for 40 h in medium without fetal bovine serum. Subsequently, the cells were harvested, and luciferase and secreted alkaline phosphatase (SEAP) activities were determined as described previously (Yoshinari et al., 2010). Representative results out of 2 or 3 independent assays are presented.

2.4. Electrophoretic mobility shift assay (EMSA)

hLXRα and hRXRα were synthesized in vitro with the corresponding pTNT plasmid, using TNT SP6 Quick Coupled Transcription/Translation System (Promega). Control lysate was prepared using empty pTNT. The sequences of double-stranded oligonucleotides used are shown in 3A, 4A and B. EMSAs were performed as described previously (Yoshinari et al., 2010). Briefly, nuclear receptor-containing or control lysates were incubated with each ^{32}P-labeled probe in reaction mixture containing 10 mM Tris–HCl, pH 8.0, 5% glycerol, 100 mM KCl, 1 mM dithiothreitol and 1 mg of poly(dI–dC) for 30 min at room temperature. For competition assays, unlabeled probes were added to the reaction mixture. Protein–DNA complexes were separated on 4% non-denaturing polyacrylamide gel in 0.25× TBE buffer (22.5 mM Tris–HCl, 22.5 mM boric acid, 0.5 mM EDTA, pH 8.0) and detected with FLA-3000 image analyzer (FujiFilm, Tokyo, Japan).

2.5. Quantitative reverse transcription-PCR (qRT-PCR)

HuH-7 cells seeded in 24-well plate (2×10^5 cells/well) in Dulbecco's modified Eagle's medium/10% fetal bovine serum were treated with vehicle (0.1% DMSO), 10 μM 24HC, 1 μM T0901317 (Sigma–Aldrich), 2 μM GW3965 (Sigma–Aldrich) or 1 μM 3MC for 48 h. Human hepatocytes (Biopredic International, Rennes, France; lot HEP187190 and HEP187111), seeded and cultured as described previously (Yoshinari et al., 2010), were treated with vehicle (0.2% DMSO), 10 μM 24HC, 1 μM T0901317, 2 μM GW3965 or 0.3 μM CITCO for 48 h, refreshing the inducer-containing medium after the first 24 h. Total RNA was prepared from the cells using Sepasol reagent (Nacalai Tesque, Kyoto, Japan) for HuH-7 and HepG2 cells and SV Total RNA Isolation System (Promega) for human hepatocytes according to the manufacturers' manuals. Determination of h*CYP1A1* and h*CYP1A2* mRNA levels were carried out as described previously (Yoshinari et al., 2010) except that GoTaq qPCR Master Mix (Promega) was used for real-time PCR. Primer sequences for *GAPDH* were 5′-ACATCAAGAAGGTGGTGAAG-3′ and 5′-CCACCACCCTGTTGCTGTAG-3′.

2.6. Statistical analysis

Student's *t*-test (2 group comparison) and one-way ANOVA followed by Dunnett's test or Bonferroni's multiple comparison test as post hoc test (multiple group comparison) were performed with Prism software (ver. 4.0, GraphPad Software, San Diego, CA). A *P*-value less than 0.05 ($P < 0.05$) was considered as statistically significant.

3. Results

3.1. Transcriptional activation of both hCYP1A1 and hCYP1A2 by hLXRα

We first performed reporter assays using the dual-reporter system, which had been developed in our laboratory (Ueda et al., 2006). With this system, transcriptional activities of h*CYP1A1* and h*CYP1A2* are simultaneously determined as luciferase and SEAP activities, respectively. In a system using pd-1A1/1A2, a

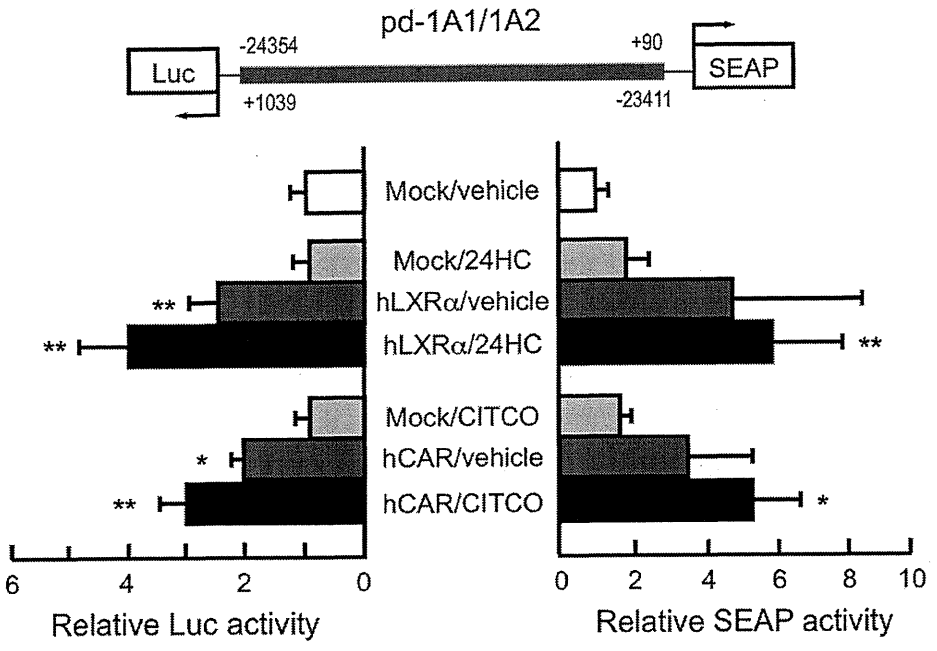


Fig. 1. Influence of hLXRα activation on the transcription of h*CYP1A1* and h*CYP1A2*. (A) Schematic structure of the dual reporter plasmid (pd-1A1/1A2) is shown on the top. The numbers above and below the plasmid represent the positions from the transcriptional starting points of h*CYP1A2* and h*CYP1A1*, respectively. (B) HuH-7 cells (3×10^4 cells in 48-well plate) were transfected with pd-1A1/1A2 (300 ng), phRL-tk (10 ng), and either control (mock) or nuclear receptor (hLXRα and hCAR) expression plasmid (20 ng). Eight hours after transfection, cells were treated with 10 μM 24HC, 0.3 μM CITCO or vehicle (0.1% DMSO) for 40 h, and reporter activities were measured. Firefly luciferase and SEAP activities normalized with *Renilla* luciferase activities are shown as ratios to those in control plasmid-transfected and vehicle-treated cells (Mock/vehicle). Data are shown as the mean ± S.D. ($n = 3$–4). Luc: luciferase. *$P < 0.05$, **$P < 0.01$ versus Mock/vehicle group (one-way ANOVA followed by Dunnett's post hoc test).

dual-reporter construct containing the whole 5′-flanking region (Fig. 1), co-transfection of hLXRα-expressing plasmid slightly increased both luciferase and SEAP activities in HuH-7 cells (Fig. 1). Treatment with the LXRα ligand, 24HC, further increased both activities of pd-1A1/1A2 to a similar extent with hCAR activation (Fig. 1). hLXRα activation also increased both reporter activities in HepG2 cells (data not shown). These results suggest that hLXRα simultaneously transactivates h*CYP1A1* and h*CYP1A2*.

3.2. Identification of hLXRα-responsive regions

To identify an hLXRα-responsive region(s), reporter assays were performed with various deletion constructs derived from pd-1A1/1A2 (Fig. 2A). Activation of hLXRα increased both luciferase and SEAP activities of pd-1A1/1A2, pd1AΔ-4618/-21975 and pd1AΔ-1827/-21975. But the deletion of nucleotides from −461 to −1826 of h*CYP1A1* resulted in the complete loss of the hLXRα-mediated increase in both reporter activities. These results suggested that the region from −461 to −1826 of h*CYP1A1* was responsible for the hLXRα-mediated transactivation of both h*CYP1A1* and h*CYP1A2*. Thus, we further investigated the hLXRα-responsive region using additional deletion constructs. As depicted in Fig. 2B, the deletion of nucleotides from −511 to −554 of h*CYP1A1*, which contains the CAR-responsive ER8 motif (termed $ER8_1$), reduced the hLXRα-mediated expression of both genes. Further deletion of nucleotides from −510 to −461 of h*CYP1A1* completely abolished the hLXRα-mediated expression of both genes. These results suggest that hLXRα transactivates both h*CYP1A1* and h*CYP1A2* simultaneously through two promoter regions, one from −511 to −554 of h*CYP1A1* and the other from −461 to −510 of h*CYP1A1*.

3.3. Identification of hLXRα/hRXRα heterodimer binding motifs

One of the LXRα-responsive regions that from −511 to −554 of h*CYP1A1*, contains the CAR-binding motif $ER8_1$ (Yoshinari et al., 2010). We thus investigated whether hLXRα/hRXRα heterodimer could bind to $ER8_1$ in EMSAs using various lengths of radiolabeled probes containing the AGGTCA half-site, one of the $ER8_1$ half-sites (Fig. 3A). As shown in Fig. 3B, hLXRα/hRXRα heterodimer strongly bound to P6 and weakly to P5 and P4, all of which contained $ER8_1$, while the binding of the heterodimer to P3, P2 and P1 was trivial. hLXRα or hRXRα alone did not bind to P6 (Fig. 3B). These results suggested that hLXRα binds to $ER8_1$ as a heterodimer with hRXRα like hCAR. Consistently, hLXRα/hRXRα heterodimer did not bind to probes P6m1, P6m2 and P6dm, which contained a mutation(s) in either half-site or both (Fig. 3C). In addition, the binding of the heterodimer to P6 was strongly competed by unlabeled P6 itself and weakly by shorter probes P5, P4, P3, P2 and P1 (data not shown).

The results of reporter assays suggested the presence of another hLXRα-responsive element at around −460 of h*CYP1A1*. To identify the responsive element, we carried out EMSAs with oligonucleotides probes shown in Fig. 4A. As shown in Fig. 4C, hLXRα/hRXRα heterodimer bound to P70 containing the oligonucleotides from −451 to −520 of h*CYP1A1*. Neither hLXRα nor hRXRα alone bound to P70 (Supplementary Fig. s1A). Fifty-fold excess of unlabeled P70 and P70A, but not P70B and P70C, abolished the binding of the hLXRα/hRXRα heterodimer to P70 (Fig. 4C). These results suggested that the heterodimer binds to the region from −451 to −487 of h*CYP1A1*. Scrutinizing the sequence of the region, we found an ER8-type motif (termed $ER8_2$) at around −460 of h*CYP1A1* (Fig. 4B). We thus investigated whether hLXRα/hRXRα heterodimer could bind to this motif using EMSAs with PAm1, PAm2 and PAdm, containing a mutation(s) in either half-site or both of $ER8_2$ (Fig. 4B). As results, hLXRα/hRXRα heterodimer did not bind to PAm1 and PAdm, while faint binding to PAm2 was observed

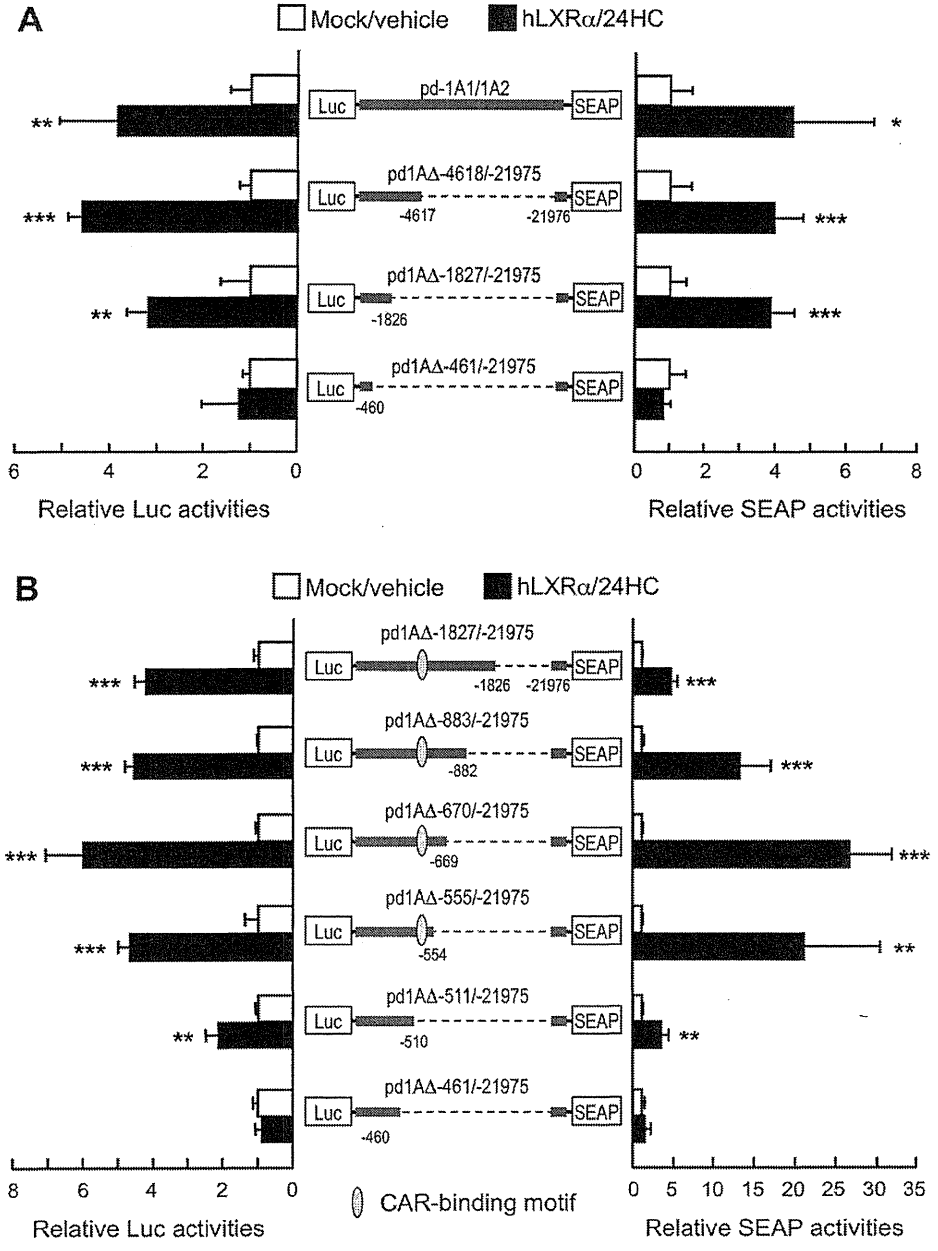


Fig. 2. Identification of hLXRα-responsive region(s) of h*CYP1A1* and h*CYP1A2*. (A and B) Schematic structures of the reporter plasmids used are shown in the middle. Reporter assays were performed as in Fig. 1. Normalized reporter activities in control plasmid-transfected and vehicle-treated cells are set at one for each reporter construct. Data are shown as the mean ± S.D. ($n = 3$–4). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus corresponding Mock/vehicle groups (Student's *t*-test).

(Fig. 4D). Consistently, the shifted complex of hLXRα/hRXRα heterodimer with P70A was competed by P70A itself and very weakly by PAm2, but not by PAm1 and PAdm (Supplementary Fig. s1B).

3.4. Role of two ER8-type motifs in the hLXRα-mediated bidirectional transactivation of hCYP1A1 and hCYP1A2

To examine whether $ER8_1$ and $ER8_2$ were essential for the hLXRα-mediated transactivation of both h*CYP1A1* and h*CYP1A2*, we carried out reporter assays with wild-type and mutated constructs (Fig. 5). When a mutated construct lacking the functional $ER8_1$ motif was used, hLXRα-responsiveness of both genes was much weaker than that with a wild-type construct. The introduction of mutations into both $ER8_1$ and $ER8_2$ completely abolished the hLXRα-mediated transcription of these genes. These results suggest that two ER8-type motifs, $ER8_1$ and $ER8_2$, cooperate on the hLXRα-mediated bidirectional transactivation of h*CYP1A1* and h*CYP1A2*.

3.5. Changes in mRNA levels of hCYP1A1 and hCYP1A2 after treatment with LXRα ligands

To investigate whether LXRα activation increases h*CYP1A1* and h*CYP1A2* mRNA levels in hepatocytes, HuH-7 cells and primary human hepatocytes were treated with LXRα ligands and qRT-PCR was performed. As shown in Fig. 6A, treatment with GW3965 or T0901317 increased h*CYP1A1* but not h*CYP1A2* mRNA levels in HuH-7 cells. Similar results were obtained with HepG2 cells (data not shown). On the other hand, LXRα ligand GW3965 increased

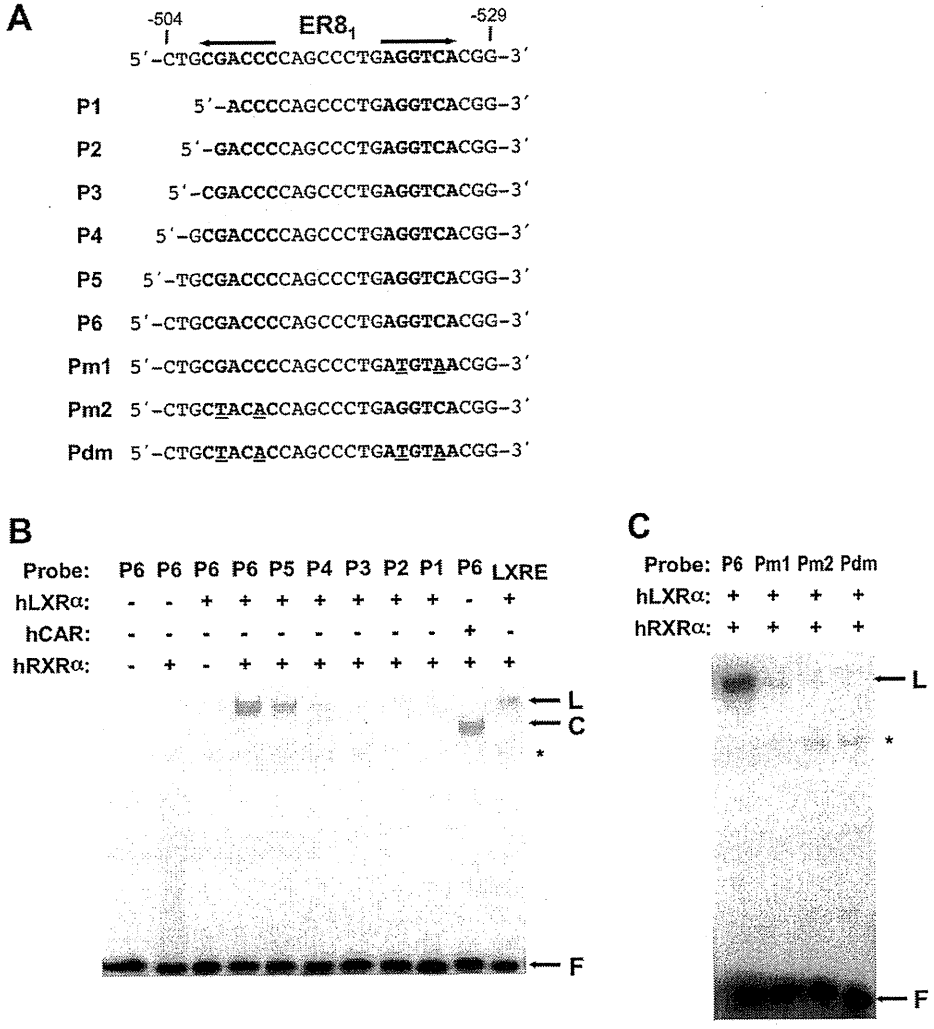


Fig. 3. Identification of a hLXRα/hRXRα heterodimer binding motif at around −520 of h*CYP1A1*. (A) Sequences of the oligonucleotide probes used are shown. Underlined nucleotides represent mutated ones. ER8$_1$ motif is indicated with bold letters and arrows. (B and C) EMSAs were carried out with radiolabeled probes and in vitro synthesized proteins as indicated. Probe LXRE contains the DR4 motif from rat *Cyp7a1* (Chiang et al., 2001). Asterisk indicates non-specific bindings. L; hLXRα–hRXRα-probe complex, C; hCAR–hRXRα-probe complex, F; free probe.

h*CYP1A2* but not h*CYP1A1* mRNA levels in primary human hepatocytes (Fig. 6B).

3.6. Co-regulation of hCYP1A1 and hCYP1A2 by LXRα, AhR and CAR

Recently, we have shown that the XRE cluster, a region from −461 to −1826 of h*CYP1A1*, is commonly essential for the AhR-mediated transcription of both h*CYP1A1* and h*CYP1A2*. The fact that ER8$_1$ and ER8$_2$ are located within the XRE cluster has raised a possibility of functional cooperation between LXRα and AhR in the expression of both h*CYP1A1* and h*CYP1A2*. This possibility was investigated in reporter assays. Both luciferase and SEAP activities of pd-1A1/1A2 were increased to a similar extent (25- to 30-fold) after treatment with the AhR ligand 3MC (Fig. 7). In the presence of 3MC at the concentration (1 μM) where maximum 3MC-dependent increases in reporter activities were observed (Supplementary Fig. s2), hLXRα activation further increased both reporter activities (Fig. 7A). These results suggest that hLXRα transactivates h*CYP1A1* and h*CYP1A2* independent of AhR. In contrast, no such synergistic transactivation of h*CYP1A1* and h*CYP1A2* was observed with LXRα and CAR, since both reporter activities after simultaneous activation of hLXRα and CAR were similar to those after hLXRα activation alone (Fig. 7B).

4. Discussion

In this study, we have investigated a possible involvement of hLXRα in the gene expression of h*CYP1A1* and h*CYP1A2*. With dual-reporter constructs, containing the whole or partially deleted h*CYP1A* promoter sequences between two different reporter genes, we have found that hLXRα transactivates the expression of both genes through two regions (from −554 to −511 and from −510 to −461 of h*CYP1A1*). EMSAs demonstrated that hLXRα binds to two ER8-type motifs (ER8$_1$ and ER8$_2$) found at around −520 and −460 of h*CYP1A1* as a heterodimer with RXRα. ER8$_1$ corresponds to the CAR-binding motif that we found previously (Yoshinari et al., 2010). The critical roles of these two ER8 motifs in the hLXRα-mediated transcription of h*CYP1A1* and h*CYP1A2* were confirmed by reporter assays using reporter constructs in which these motifs were individually or simultaneously mutated. These results suggest that the two ER8 motifs function as hLXRα-responsive elements for the bidirectional transactivation of both h*CYP1A1* and h*CYP1A2* and that

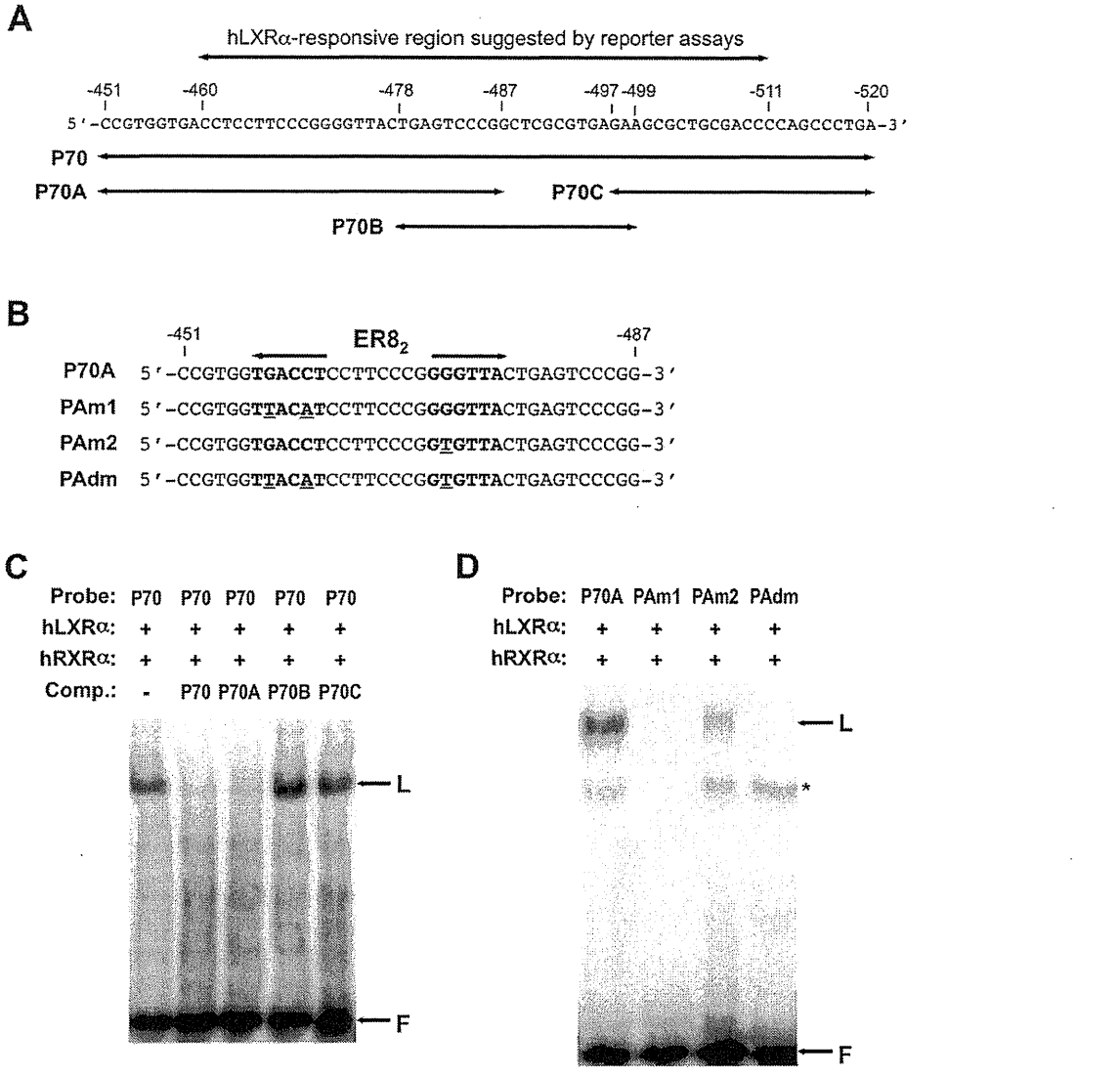


Fig. 4. Identification of a hLXRα/hRXRα heterodimer binding motif at around −460 of h*CYP1A1*. (A and B) Sequences of the oligonucleotide probes used are shown. Underlined nucleotides represent mutated ones. (C and D) EMSAs were carried out as in Fig. 3. Asterisk indicates non-specific bindings. L, hLXRα–hRXRα-probe complex; F, free probe; comp.; 50-fold excess non-labeled probe.

LXRα is a new member of transcription factors that control the expression levels of both h*CYP1A1* and h*CYP1A2*.

In contrast to the reporter assays, the influences of LXRα ligand treatment on h*CYP1A1* and h*CYP1A2* mRNA levels were cell type-dependent. In hepatoma cells such as HuH-7 and HepG2 cells, h*CYP1A1*, but not h*CYP1A2*, mRNA levels were increased in response to LXRα ligands. In contrast, h*CYP1A2*, but not h*CYP1A1*, mRNA levels were increased after LXRα ligand treatment in primary human hepatocytes. At this moment, the reason for the discrepancy remains unknown. It is well known that *CYP1A2* is highly expressed in adult livers of mammals and *CYP1A1* is barely expressed in the liver. In contrast, *CYP1A1*, but not *CYP1A2* is highly expressed in the extrahepatic tissues. In HuH-7 and HepG2 cells, both h*CYP1A1* and h*CYP1A2* are expressed. These facts suggest that an as-yet-unidentified factor(s) regulates the cell-specific expression of these genes. Such factor(s) may also influence the hLXRα-mediated expression of h*CYP1A1* and h*CYP1A2*.

The LXRα-responsive ER8 motifs are located within the XRE cluster, which is found to be critical for the AhR-mediated expression of both h*CYP1A1* and h*CYP1A2* (Ueda et al., 2006; Yoshinari et al., 2008), and one of the ER8 motifs (ER8$_1$) is found to be the CAR-responsive motif. In our previous study, CAR and AhR synergistically transactivated h*CYP1A1* and h*CYP1A2* expression (Yoshinari et al., 2010). In consistent with this, LXRα synergistically transactivated both genes with AhR. In contrast, LXRα and CAR did not show any synergistic effect on the transcription of h*CYP1A1* and h*CYP1A2*. This is probably because these two receptors share their binding motifs and have similar ability to transactivate both h*CYP1A1* and h*CYP1A2*, as shown in this study. Taken together, LXRα may function as a positive regulator of h*CYP1A1* and h*CYP1A2* expression independent of AhR but compete with CAR for the transcription of these genes.

During the course of our present experiments, Shibahara et al. reported that hLXRα transactivated h*CYP1A1* in HepG2 cells (Shibahara et al., 2011), which is consistent with our present results. Their results of chromatin immunoprecipitation assays indicate that the receptor binds to a region from −446 to −607 of h*CYP1A1* in response to LXRα agonist T0901317 (Shibahara et al., 2011). Consistently, two ER8 motifs found in our study are included within this region. It is thus strongly suggested that this region is critical

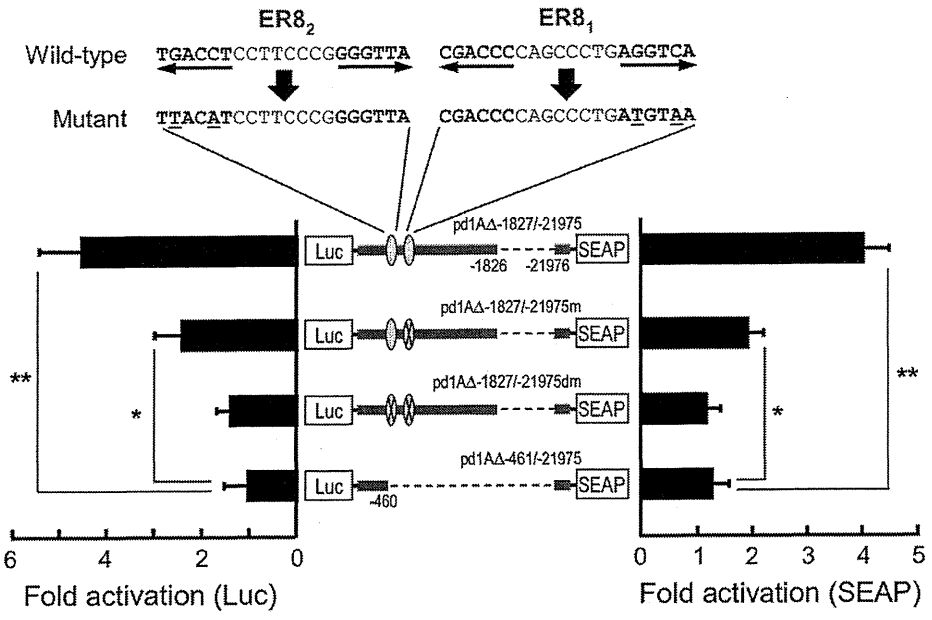


Fig. 5. Role of two ER8 motifs in the hLXRα-mediated transcription of h*CYP1A* genes. Schematic structures of the reporter plasmids used are shown in the middle. X indicates mutation. Wild-type and mutated sequences of ER8$_1$ and ER8$_2$ are also shown above. Reporter assays were performed as in Figs. 1 and 3. Data are shown as the ratio of reporter activities of hLXRα-transfected/24HC-treated cells against those of mock-transfected/vehicle-treated cells for each reporter construct (the mean ± S.D. (n = 3–4)). *P < 0.05, **P < 0.01 versus pd1AΔ-461/-21975 (one-way ANOVA followed by Dunnett's post hoc test).

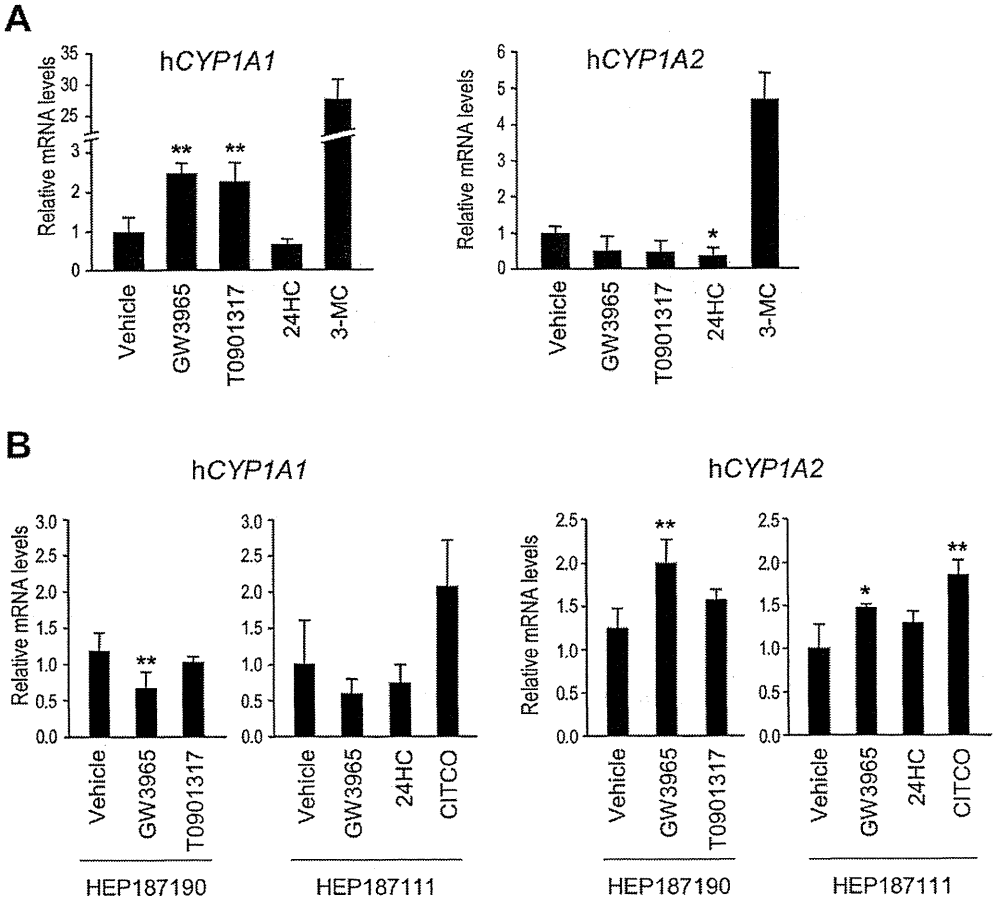


Fig. 6. Changes in mRNA levels of h*CYP1A1* and h*CYP1A2* in HuH-7 cells and primary human hepatocytes. Relative mRNA levels of h*CYP1A1*, h*CYP1A2* and *GAPDH* in HuH-7 cells (A) and primary human hepatocytes (B) were determined in duplicate or triplicate as described in Section 2.5. h*CYP1A1* and h*CYP1A2* mRNA levels were normalized with those of *GAPDH*. In A, statistical analyses were performed only among the cells treated with vehicle or LXRα ligands. *P < 0.05, **P < 0.01 versus vehicle-treated group (one-way ANOVA followed by Dunnett's post hoc test).

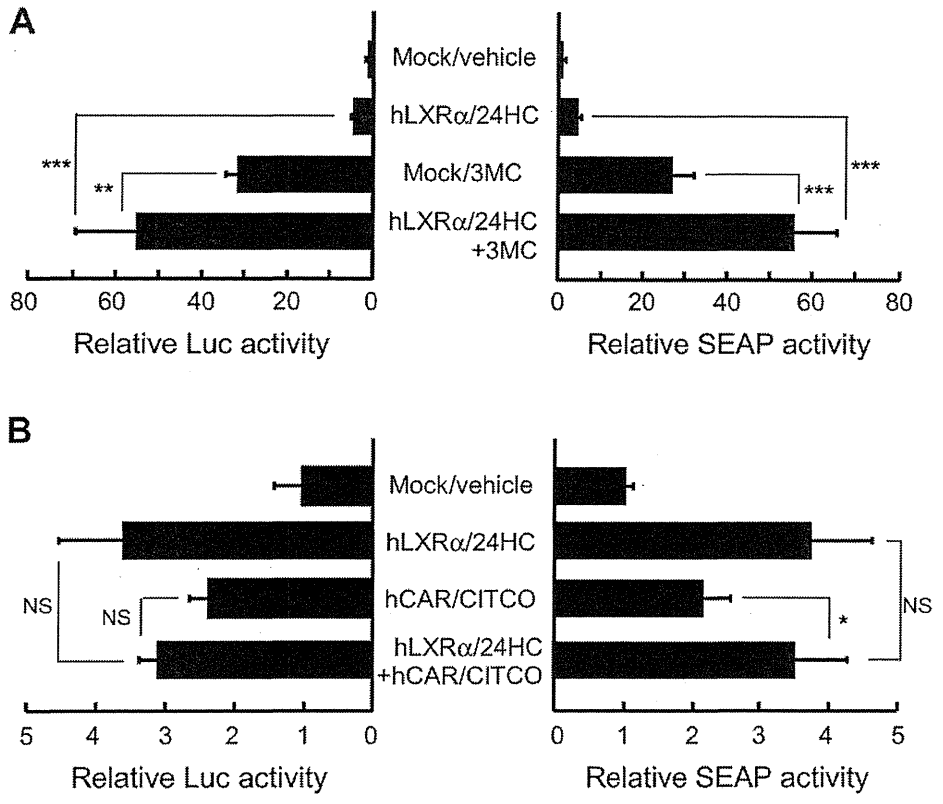


Fig. 7. Co-regulation of h*CYP1A* genes by LXRα, AhR and CAR. (A) HuH-7 cells (3×10^4 cells in 48-well plate) were transfected with pd-1A1/1A2 (300 ng), phRL-tk (10 ng) and either control or hLXRα expression plasmid (20 ng). Eight hours after transfection, cells were treated with 10 μM 24HC, 1 μM 3MC or vehicle (0.2% DMSO) for 40 h. (B) HuH-7 cells (3×10^4 cells in 48-well plate) were transfected with pd1AΔ-1827/-21975 (300 ng), phRL-tk (10 ng) and either control, hLXRα or hCAR expression plasmid (20 ng). Total amount of expression plasmid was adjusted to 40 ng with control plasmid. Eight hours after transfection, cells were treated with vehicle (0.2% DMSO), 10 μM 24HC, 0.3 μM CITCO or both ligands for 40 h. Reporter activities were determined and relative reporter activities are shown as in Fig. 1. Data are shown as the mean ± S.D. ($n = 4$). The significance of differences between the groups indicated was analyzed with one-way ANOVA followed by Bonferroni's multiple comparison test. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. NS, not significant.

for the hLXRα-mediated expression of both h*CYP1A1* and h*CYP1A2*. Yet, difference is observed for the hLXRα binding sequence between their and our studies. We have identified two ER8 motifs as LXRα binding sites while Shibahara et al. demonstrated that LXRα binds to a DR4 motif located in a region from −452 to −467 in EMSAs (Shibahara et al., 2011). Because the DR4 motif and $ER8_2$ share a common AGGTCA nuclear receptor binding half-site, we cannot rule out a possibility that both motifs function as LXRα-responsive elements in hepatocytes. In the meantime, the mutation of the DR4 motif did not completely eliminate LXRα-response of the h*CYP1A1* reporter construct (Shibahara et al., 2011). Because this mutated reporter construct still contained $ER8_1$, $ER8_1$ might mediate the residual LXRα-responsiveness.

LXRα is expected as a potential target of anti-arteriosclerotic drugs because it regulates the transcription of several genes involved in reverse cholesterol transport (Repa and Mangelsdorf, 2002). Thus, combinational use of these LXRα-activating drugs with drugs that are metabolized by CYP1A1 and/or CYP1A2 such as clozapine and theophilline could lead to drug-drug interactions. Recently, a proton pump inhibitor lansoprazole has been reported as an LXRα ligand (Cronican et al., 2010). In our dual-reporter system, this drug transactivated both h*CYP1A1* and h*CYP1A2* expression (Yoshinari et al., 2008). Since lansoprazole is long known to induce CYP1A1 and CYP1A2 through the activation of AhR probably without direct binding to the receptor (Curi-Pedrosa et al., 1994; Kikuchi et al., 1996; Krusekopf et al., 2003), this drug might be a dual activator of AhR and LXRα. Nonetheless, it is worth considering a possibility of LXRα-associated drug-drug interactions through inducing CYP1A enzymes in future studies and drug development processes.

Physiological relevance of the LXRα-mediated regulation of h*CYP1A1* and h*CYP1A2* expression remains unclear. Recombinant hCYP1A2 has been reported to catalyze the conversion of cholesterol into 4β-hydroxycholesterol, an LXRα ligand (Honda et al., 2011). Both CYP1A1 and CYP1A2 metabolize endogenous cholesterol-derived compounds such as estrogens (Badawi et al., 2001; Lee et al., 2003). Intriguingly, LXRα is reported to regulate the expression of human SLUT1E1 (Gong et al., 2007), which metabolizes estrogens into the corresponding sulfates. Thus, the LXRα-mediated regulation of h*CYP1A1* and h*CYP1A2* might be associated with homeostasis of cholesterol and its related compounds as in the case of estrogens and SULT1E1.

Conflict of interest statement

None.

Acknowledgements

We thank Ms. Noriko Sato for her technical assistance in cell culture and qRT-PCR. This study was supported in part by Grant-in-Aid from Ministry of Education, Culture, Sports, Sciences and Technology of Japan [22390027], a grant form Ministry of Health, Labour and Welfare, and a grant from The Mochida Memorial Foundation for Medical and Pharmaceutical Research.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.toxlet.2012.09.021.

References

Badawi, A.F., Cavalieri, E.L., Rogan, E.G., 2001. Role of human cytochrome P450 1A1, 1A2, 1B1, and 3A4 in the 2-, 4-, and 16alpha-hydroxylation of 17beta-estradiol. Metabolism: Clinical and Experimental 50, 1001–1003.

Chiang, J.Y., Kimmel, R., Stroup, D., 2001. Regulation of cholesterol 7alpha-hydroxylase gene (CYP7A1) transcription by the liver orphan receptor (LXRalpha). Gene 262, 257–265.

Chisaki, I., Kobayashi, M., Itagaki, S., Hirano, T., Iseki, K., 2009. Liver X receptor regulates expression of MRP2 but not that of MDR1 and BCRP in the liver. Biochimica et Biophysica Acta 1788, 2396–2403.

Collins, J.L., Fivush, A.M., Watson, M.A., Galardi, C.M., Lewis, M.C., Moore, L.B., Parks, D.J., Wilson, J.G., Tippin, T.K., Binz, J.G., Plunket, K.D., Morgan, D.G., Beaudet, E.J., Whitney, K.D., Kliewer, S.A., Willson, T.M., 2002. Identification of a nonsteroidal liver X receptor agonist through parallel array synthesis of tertiary amines. Journal of Medicinal Chemistry 45, 1963–1966.

Cronican, A.A., Fitz, N.F., Pham, T., Fogg, A., Kifer, B., Koldamova, R., Lefterov, I., 2010. Proton pump inhibitor lansoprazole is a nuclear liver X receptor agonist. Biochemical Pharmacology 79, 1310–1316.

Curi-Pedrosa, R., Daujat, M., Pichard, L., Ourlin, J.C., Clair, P., Gervot, L., Lesca, P., Domergue, J., Joyeux, H., Fourtanier, G., et al., 1994. Omeprazole and lansoprazole are mixed inducers of CYP1A and CYP3A in human hepatocytes in primary culture. Journal of Pharmacology and Experimental Therapeutics 269, 384–392.

Denison, M.S., Nagy, S.R., 2003. Activation of the aryl hydrocarbon receptor by structurally diverse exogenous and endogenous chemicals. Annual Review of Pharmacology and Toxicology 43, 309–334.

Gnerre, C., Schuster, G.U., Roth, A., Handschin, C., Johansson, L., Looser, R., Parini, P., Podvinec, M., Robertsson, K., Gustafsson, J.A., Meyer, U.A., 2005. LXR deficiency and cholesterol feeding affect the expression and phenobarbital-mediated induction of cytochromes P450 in mouse liver. Journal of Lipid Research 46, 1633–1642.

Gong, H., Guo, P., Zhai, Y., Zhou, J., Uppal, H., Jarzynka, M.J., Song, W.C., Cheng, S.Y., Xie, W., 2007. Estrogen deprivation and inhibition of breast cancer growth in vivo through activation of the orphan nuclear receptor liver X receptor. Molecular Endocrinology 21, 1781–1790.

Honda, A., Miyazaki, T., Ikegami, T., Iwamoto, J., Maeda, T., Hirayama, T., Saito, Y., Teramoto, T., Matsuzaki, Y., 2011. Cholesterol 25-hydroxylation activity of CYP3A. Journal of Lipid Research 52, 1509–1516.

Janowski, B.A., Grogan, M.J., Jones, S.A., Wisely, G.B., Kliewer, S.A., Corey, E.J., Mangelsdorf, D.J., 1999. Structural requirements of ligands for the oxysterol liver X receptors LXRalpha and LXRbeta. Proceedings of the National Academy of Sciences of the United States of America 96, 266–271.

Janowski, B.A., Willy, P.J., Devi, T.R., Falck, J.R., Mangelsdorf, D.J., 1996. An oxysterol signalling pathway mediated by the nuclear receptor LXR alpha. Nature 383, 728–731.

Kikuchi, H., Kato, H., Mizuno, M., Hossain, A., Ikawa, S., Miyazaki, J., Watanabe, M., 1996. Differences in inducibility of CYP1A1-mRNA by benzimidazole compounds between human and mouse cells: evidences of a human-specific signal transduction pathway for CYP1A1 induction. Archives of Biochemistry and Biophysics 334, 235–240.

Krusekopf, S., Roots, I., Hildebrandt, A.G., Kleeberg, U., 2003. Time-dependent transcriptional induction of CYP1A1, CYP1A2 and CYP1B1 mRNAs by H+/K+-ATPase inhibitors and other xenobiotics. Xenobiotica 33, 107–118.

Lee, A.J., Cai, M.X., Thomas, P.E., Conney, A.H., Zhu, B.T., 2003. Characterization of the oxidative metabolites of 17beta-estradiol and estrone formed by 15 selectively expressed human cytochrome p450 isoforms. Endocrinology 144, 3382–3398.

Lu, T.T., Repa, J.J., Mangelsdorf, D.J., 2001. Orphan nuclear receptors as eLiXiRs and FiXeRs of sterol metabolism. Journal of Biological Chemistry 276, 37735–37738.

Meyer Zu Schwabedissen, H.E., Bottcher, K., Chaudhry, A., Kroemer, H.K., Schuetz, E.G., Kim, R.B., 2010. Liver X receptor alpha and farnesoid X receptor are major transcriptional regulators of OATP1B1. Hepatology 52, 1797–1807.

Repa, J.J., Mangelsdorf, D.J., 2002. The liver X receptor gene team: potential new players in atherosclerosis. Nature Medicine 8, 1243–1248.

Repa, J.J., Turley, S.D., Lobaccaro, J.A., Medina, J., Li, L., Lustig, K., Shan, B., Heyman, R.A., Dietschy, J.M., Mangelsdorf, D.J., 2000. Regulation of absorption and ABC1-mediated efflux of cholesterol by RXR heterodimers. Science 289, 1524–1529.

Schultz, J.R., Tu, H., Luk, A., Repa, J.J., Medina, J.C., Li, L., Schwendner, S., Wang, S., Thoolen, M., Mangelsdorf, D.J., Lustig, K.D., Shan, B., 2000. Role of LXRs in control of lipogenesis. Genes and Development 14, 2831–2838.

Shibahara, N., Masunaga, Y., Iwano, S., Yamazaki, H., Kiyotani, K., Kamataki, T., 2011. Human cytochrome P450 1A1 is a novel target gene of liver X receptor alpha. Drug Metabolism and Pharmacokinetics 26, 451–457.

Swales, K., Negishi, M., 2004. CAR, driving into the future. Molecular Endocrinology 18, 1589–1598.

Ueda, R., Iketaki, H., Nagata, K., Kimura, S., Gonzalez, F.J., Kusano, K., Yoshimura, T., Yamazoe, Y., 2006. A common regulatory region functions bidirectionally in transcriptional activation of the human CYP1A1 and CYP1A2 genes. Molecular Pharmacology 69, 1924–1930.

Verreault, M., Senekeo-Effenberger, K., Trottier, J., Bonzo, J.A., Belanger, J., Kaeding, J., Staels, B., Caron, P., Tukey, R.H., Barbier, O., 2006. The liver X-receptor alpha controls hepatic expression of the human bile acid-glucuronidating UGT1A3 enzyme in human cells and transgenic mice. Hepatology 44, 368–378.

Willy, P.J., Umesono, K., Ong, E.S., Evans, R.M., Heyman, R.A., Mangelsdorf, D.J., 1995. LXR, a nuclear receptor that defines a distinct retinoid response pathway. Genes and Development 9, 1033–1045.

Yoshinari, K., Ueda, R., Kusano, K., Yoshimura, T., Nagata, K., Yamazoe, Y., 2008. Omeprazole transactivates human CYP1A1 and CYP1A2 expression through the common regulatory region containing multiple xenobiotic-responsive elements. Biochemical Pharmacology 76, 139–145.

Yoshinari, K., Yoda, N., Toriyabe, T., Yamazoe, Y., 2010. Constitutive androstane receptor transcriptionally activates human CYP1A1 and CYP1A2 genes through a common regulatory element in the 5′-flanking region. Biochemical Pharmacology 79, 261–269.