Suppressive Effects of Leflunomide on Leptin-Induced Collagen I Production Involved in Hepatic Stellate Cell Proliferation

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In this manuscript, we showed that following a fibrogenic stimulus of leptin, hepatic stellate cells (HSCs) underwent a complex activation process characterized by increased proliferation and excessive deposition of type I collagen. Studies with special chemical inhibitors demonstrated that this process involved Janus protein tyrosine kinase (JAK)/signal transducer and activator of transcription (STAT), mitogen-activated protein kinases (MAPK), and phosphatidylinositol 3-linase (PI3K)/Protein kinase B (AKT) signal pathways. Leflunomide pretreatment significantly inhibited the deposition of type I collagen in HSCs and the proliferation of primary HSC by interrupting the three proliferative signal transduction pathways *in vitro*, which was indicated by [^{3}H]thymidine incorporation and cell cycle analysis. Furthermore, leptin-induced cyclin D1 protein expression, which correlates well with HSC proliferation, was also significantly inhibited by leflunomide. On the other hand, leflunomide also prevented leptin-induced Kupffer cell (KC) activation and HSC collagen synthesis induced by KC-conditioned medium (KCCM). Collectively, these results provided a novel insight into the mechanisms by which leflunomide may exert in liver fibrosis. Exp Biol Med 232:427–436, 2007

Key words: leptin; hepatic stellate cells; proliferation; signal pathways; Kupffer cells; leflunomide; liver fibrosis

Introduction

Liver fibrosis is the common consequence of chronic liver injury of any etiology. Advanced liver fibrosis disrupts the normal liver architecture, characterized with an excessive deposition of extracellular matrix proteins, of which type I collagen predominates. Hepatic stellate cells (HSCs) play a crucial role in liver fibrosis. Following liver injury, HSCs undergo transdifferentiation from a quiescent vitamin A–storing cell to an activated myofibroblastic phenotype with an increase in DNA synthesis (1) and expression of α-smooth muscle actin (α-SMA) (2), the process of which is a seminal event in hepatic fibrogenesis.

Leptin was initially identified by Friedman and colleagues in 1994 as the product of the obese gene. It is a 16-kDa nonglycosylated protein, mainly synthesized in adipose cells. Apart from its effects on energy homeostasis, recently, leptin has been shown to be critical in the development of hepatic fibrosis. In the chemical-induced hepatotoxicity models, such as carbon tetrachloride (CCl_4) and thioacetamide, the absence of circulating leptin or appropriate leptin signal transduction prevents liver fibrosis (3). Plasma leptin levels were reported to be increased in patients with alcoholic cirrhosis (4) and nonalcoholic steatohepatitis (5). Studies also showed that not only CCl_4 treatment upregulated the collagen I gene and protein expression (6), but in culture-activated rat HSC, rat HSC-T6, and human HSC-LX-1 cell lines, leptin was also found to stimulate collagen I promoter activity, upregulate collagen I mRNA, and increase collagen I protein production (7). Furthermore, leptin would be a factor responsible for converting steatosis into fibrosis (8). All of these results suggest a possible involvement of leptin in the pathogenesis of liver fibrosis.

Recently, the molecular mechanisms of leptin in the pathogenesis of liver fibrosis have been substantiated. Firstly, upon leptin interaction with its long functional receptor (ObRb), it has the capacity to activate the Janus protein tyrosine kinase (JAK)/signal transducer and activator of transcription (STAT) pathways (9). Then, tyrosine-phosphorylated STATs are translocated to the nucleus, where they mediate gene activation important for energy homeostasis. In addition to STATs, different pathways were

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known to be involved in leptin receptor signaling. For example, leptin has been found to activate mitogen-activated protein kinases (MAPKs) in different systems (10) and phosphatidylinositol 3-kinase (PI3K) (11), mediating a proliferative response. Both extracellular regulated kinase (ERK)-1 and ERK-2 can be phosphorylated after 10 mins of incubation with human leptin in human peripheral blood mononuclear cells (12). Activated PI3K generates several phosphoinositols, leading to Protein kinase B (AKT) activation by phosphorylation at Thr308 and Ser473 by phosphoinositide-dependent kinase-1; the serine/threonine kinase AKT was known to influence various cellular processes and cell survival (13). In addition, inhibiting both PI3K and AKT blocks HSC proliferation and type I collagen synthesis (14).

Inflammation and activation of HSCs are two important events in the process of liver pathogenesis. Attenuation of liver inflammation and inhibition of HSC activation are two crucial goals for intervention in the hepatic fibrogenesis cascade. Kupffer cells (KCs), the resident macrophages in the liver, have been implicated in this liver injury because they can produce and release numerous mediators, many of which further activate or modulate the effects of nearby cells involved in the inflammatory process (15). Leptin was reported to augment both inflammatory and profibrogenic responses in the liver caused by hepatotoxic chemicals (16). It can modulate peritoneal machrophage function and regulate proinflammatory response. It enhances proinflammatory cytokines production in murine peritoneal macrophages and monocyte, the effect of which is comparable to that of LPS and PMA (17). Furthermore, except for HSCs that have a functional leptin receptor, KCs can also be activated by leptin to release TGF-β via their functional leptin receptor (3). These findings not only underscore the critical importance of KCs in regulating liver inflammation, but indicate that leptin may amplify proinflammatory responses in KCs and exert an indirect effect on the activation of HSCs.

Recent evidence suggests that the anti-inflammatory and immunoregulatory effects of leflunomide can suppress IL-1 and TNF-α selectively in T lymphocyte/monocyte activation and inhibit the activation of nuclear factor κB, a potent mediator of inflammation when stimulated by inflammatory agents (18–20). It is metabolized to its active form, A771726, which exerts the immunosuppressive activity (21). It was also reported to block cell cycle progression by downregulating cell signaling in the G0/G1 phase (22) and inhibit *de novo* pyrimidine synthesis showing an antiproliferative effect (23). Additionally, at a higher concentration, it mainly inhibits protein tyrosine kinase–initiating signaling (24), which plays an important role in the activation of HSCs (25). Based on these results and the immunological dysfunction in hepatic fibrosis, the principal aim of this study, therefore, is to examine whether leflunomide inhibits leptin-induced KC activation, especially on leptin-induced HSC proliferation and associated signal transduction pathways.

Materials and Methods

Reagents. Polyclonal antibodies against ERK1/2, phospho-ERK1/2 (Thr202/Tyr204), rabbit polyclonal p-$STAT_3$ (Tyr-705), $STAT_3$, β-actin, anti-α-smooth muscle actin (α-SMA), anticollagen type I monoclonal, horseradish peroxidase–conjugated goat anti-mouse IgG antibody, and ^{32}P-end-labeled consensus oligonucleotide m67-SIE were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Antibody against cycline D1, polyclonal phospho-specific AKT (Ser473), and AKT were purchased from Cell Signaling Technology Inc. (Beverly, MA). Recombinant murine leptin, LY29004, PD098059, and AG490 were obtained from Sigma Chemical (St. Louis, MO). Collagenase, proteinase E, DNase I, Nycodenz, ELISA kits, Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and powdered 1640 medium were obtained from Gibco (Grand Island, NY). Leflunomide and its active metabolite, A771726, were kindly donated by Cinkate Co. (Shanghai, China).

Animals and Treatment. Twenty Sprague-Dawley rats, weighing 180–220 g (from Anhui Medical University Laboratory Animal Center, Hefei, China), were divided into two groups (control group, carbon tetrachloride/leptin treatment group). All rats were housed in a controlled environment with free access to food and water. Rats were given an intraperitoneal (ip) injection of 50% carbon tetrachloride (CCl_4) (1 ml/kg, ip) and recombinant murine leptin (1 mg/kg, ip) twice a week except for the control group, which was given an injection of saline (2 ml/kg) for 12 weeks. At end of the experiment, rats were used for HSC and KC isolation.

HSC and KC Isolation and Culture. Sprague-Dawley rats weighing 350–450 g were used for isolation of HSCs and KCs by sequential *in situ* perfusion with collagenase and protease as described before (26) with slight modifications. Briefly, the liver was perfused with Geyes Balanced Salt Solution (GBSS) for 4 mins (20 ml/min) at 37°C. This was followed by perfusion of an enzyme digest containing 47.9 U/ml) collagenase and 1.3 mg/ml protease dissolved in GBSS for 7.5 mins (20 ml/min). After perfusion, the liver was harvested, cut into small pieces, and incubated in 90 ml of an enzyme digest (32.5 U/ml collagenase, 0.6 mg/ml protease and Dnase I 11 μg/ml) for 30 mins at 37°C. After passing through a filter, cells were washed twice with GBSS, and HSCs were obtained by centrifugation over 12% (w/v) Nycodenz gradient for 20 mins at 1400 *g*. After centrifugation, HSCs were collected from the interface, washed with GBSS, and cultured in DMEM containing 15% fetal calf serum (FCS), 2 m*M* glutamine, and 1% antibiotic solution under an atmosphere containing 5% CO_2 at 37°C. KCs were collected under the interface, washed with GBSS, and cultured at a concen-

tration of 1×10^6 cells/ml, in PRMI 1640 supplemented with 10% FBS, 2 m*M* glutamine, and 1% antibiotic solution under an atmosphere containing 5% CO_2 at 37°C. To separate KCs from endothelial cells, cells were plated on tissue culture plates at 37°C and 5% CO_2 for 30 mins followed by a single wash step discarding the nonadherent endothelial cells. The media of KCs were replaced 6 hrs after seeding and at every 48 hrs thereafter; HSCs were replaced 24 hrs after seeding and at every 48 hrs thereafter. HSCs and KCs were identified by immunocytochemistry (27). HSCs isolated from normal livers and cultured in serum-free medium for 3 days were referred to as quiescent HSCs in this study.

ELISA Analysis. For cytokines (TGF-β, TNF-α, and IL-6) releasing in KCs, KCs were cultured in six-well plates at a density of 4×10^5 cells per well for 24 hrs. Then medium was removed and incubated in serum-free medium for another 24 hrs. Two hours before challenged with 75 ng/ml leptin for 6 hrs, KCs were treated with or without A771726 (0.1, 1.0, 10 μ*M*), 50 μ*M* JAK Inhibitor AG490, 30 μ*M* ERK1/2 Inhibitor PD098095, or 25 μ*M* PI3 kinase Inhibitor LY294002. Supernatants were collected, and released cytokines were measured by ELISA following the manufacturer's instructions. For collagen type I secretion in HSC culture media, it was measured using ELISA method as previously described (28). Anti-rat type I collagen antibody was used as the primary antibody, and peroxidase-conjugated goat antibody against rabbit IgG was used as the secondary antibody.

Analysis of DNA Synthesis. DNA synthesis was assessed by [^{3}H]thymidine or [^{3}H]proline radioimmunoassay kit (Institute of Nuclear Research, Beijing, China) following the manufacturer's instructions. Briefly, primary HSCs (5.5×10^4) were seeded in 24-well plates in growth medium containing 15% FBS. After 24 hrs, the medium was changed to 0.2% FBS, and the cells were incubated for another 24 hrs to reduce cell proliferation. Two hours before stimulation, HSCs were treated with A771726 (0.1, 1.0, 10 μ*M*), 50 μ*M* JAK Inhibitor AG490, 30 μ*M* ERK1/2 Inhibitor PD098095, or 25 μ*M* PI3 kinase Inhibitor LY294002. Afterward, 75 ng/ml recombinant murine leptin or 50 μl KC-conditioned medium (KCCM) was added and incubated for 24 hrs with 1 μCi/ml [^{3}H]thymidine or 1 μCi/ml [^{3}H]proline present. At the end of the incubation, 10% trichloroacetic acid was added, and the cells were maintained on ice for 15 mins. After being washed with D-Hanks, HSCs were harvested, lysed, and counted with a liquid scintillation counter. Results were expressed as counts per min (cpm) from triplicate experiments.

Cell Cycle Analysis (FACS). Primary HSCs (5.5×10^4) were seeded in 24-well plates in growth medium containing 15% FBS, then serum-starved (0.2% FBS) for an additional 24 hrs. Cells were subsequently treated with A771726 (0.1, 1.0, 10 μ*M*), 50 μ*M* JAK Inhibitor AG490, 30 μ*M* ERK1/2 Inhibitor PD098095, or 25 μ*M* PI3 kinase Inhibitor LY294002 for 2 hrs. Afterward, 75 ng/ml leptin was added, and the cells were incubated for 24 hrs. For cell cycle analysis, cells were scraped and washed twice with PBS. Then, cells were fixed with 70% ice-cold ethanol, followed by the incubation of the freshly prepared nuclei staining buffer (0.1% Triton X-100 in PBS, 200 μg/ml RNase, and 20 g/ml PI) for 30 mins at room temperature. Cell cycle state was assessed by using a Becton-Dickinson FACScan (Becton-Dickinson, San Jose, CA). Histograms generated by FACS were analyzed by ModFit Cell Cycle Analysis software (Verity, Topsham, ME) to determine the percentage of cells in each phase (G1, S, and G2/M).

Preparation of KC-Conditioned Medium (KCCM). KCs isolated from carbon tetrachloride/leptin treatment group were grown in 24-well tissue culture plates for a 72-hr recovery period and stimulated with 75 ng/ml leptin for 48 hrs. Conditioned medium was collected and filtered with a 0.45-μm membrane filter and stored at −70°C until use.

Western Blot Analysis. Culture-treated HSCs were collected by rubber policeman, washed in PBS, and lysed in ice-cold RIPA buffer (10 μ*M* Tris-HCl [pH 8.0], 100 m*M* NaCl, 1 m*M* EDTA, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, and 10 μl/ml protease inhibitor cocktail) for 30 mins on ice. The lysates were sonicated for 2 secs to shear the DNA to reduce its viscosity, followed by centrifugation at 14,000 rpm for 30 mins at 4°C. The supernatant was harvested, and protein concentrations were measured using the Bradford method (Bio-Rad Laboratories, Hercules, CA). Protein samples were heated at 95°C for 5 mins, and 20-μg proteins were applied to a 10% SDS-polyacrylamide gel (7.5% SDS-polyacrylamide gels were used for collagen analysis) and transblotted to nitrocellulose membranes (Schleicher&Schuell, Dassel, Germany). For detection of collagen I secretion, prior to electrophoresis, some samples were digested at room temperature for 30 mins with pepsin (1000 U; Sigma Chemical) at pH 2.5 or with collagenase (7.5 U; Sigma Chemical) as controls for antibody specificity. Membranes were stained with 0.5% Ponceau S to ensure equal protein loading and transfer. After blocking with 5% milk in TBS-T (25 m*M* Tris-HCl [pH 8.0], 144 m*M* NaCl, 0.1% Tween 20) for 1 hr, membranes were incubated with the following primary antibodies: *p*-$STAT_3$ (1:1000), *p*-p44/p42 (1:1000), *p*-AKT (1:1000), rabbit anti-collagen type I (1:1000), cyclin D1 (1:1000), then followed by the secondary antibody, horseradish peroxidase–conjugated goat anti-rabbit IgG antibody (1:2000) for 1 hr at room temperature. Equal protein loading was controlled by immunoblot of the corresponding nonphosphorylated $STAT_3$, ERK, and AKT using rabbit polyclonal antibodies against the respective proteins. Immunodetected proteins were visualized using ECL assay kit (Amersham Biosciences, Buckinghamshire, UK) and band intensities were quantitated by personal densitometer scan v1.30 using Image Quant software version 3.3 (Molecular Dynamics, Sunnyvale, CA).

Preparation of Nuclear Extracts. Nuclear extracts

were prepared according to standard methods described previously (29). After the indicated treatments, HSCs were rinsed three times with ice-cold PBS containing 1 m*M* Na_3VO_4 and 5 m*M* NaF. The cells were pelleted at 1500 rpm for 10 mins at 4°C twice. The pellet was resuspended in a buffer containing 10 m*M* HEPES (pH 7.9), 10 m*M* KCl, 1.5 m*M* $MgCl_2$, 1 m*M* EDTA, 1 m*M* EGTA, 1 m*M* Na_3VO_4, 1 m*M* $Na_4P_2O_7$, 20 m*M* NaF, 1 m*M* DTT, 0.5 m*M* PMSF, and 1 μg/ml each of leupeptin, antipain, pepstatin, and chymostatin. The lysate was vortexed vigorously for 1 min, and the nuclei were pelleted (14,000 *g* for 30 secs). The pellet was then resuspended in 20 m*M* HEPES (pH 7.9), 420 m*M* NaCl, 1.5 m*M* $MgCl_2$, 25% glycerol, 1 m*M* EDTA, 1 m*M* EGTA, 1 m*M* Na_3VO_4, 1 m*M* $Na_4P_2O_7$, 20 m*M* NaF, 1 m*M* DTT, 0.5 m*M* PMSF, and 1 μg/ml each of leupeptin, antipain, pepstatin, and chymostatin. The mixture was then spun at 14,000 *g* for 10 mins. Protein concentration of the nuclear extract was then determined, and the supernatant were stored at –80°C.

Electrophoretic Mobility-Shift Assays (EMSA). Nuclear extracts (12 μg) were incubated with ^{32}P-end-labeled oligonucleotide m67-SIE probe (30) (30,000 cpm, 5 fmol) in binding buffer for 30 mins at 25°C. Final binding reactions (20 μl) contained 14 m*M* HEPES (pH 7.9), 85 m*M* NaCl, 10 m*M* KCl, 0.3 m*M* $MgCl_2$, 1.25 m*M* DTT, 0.25 m*M* EDTA, 0.2 m*M* EGTA, 15% glycerol, 50 μg/ml poly(dIdC)·poly(dI-dC), and 250 μg/ml acetylated bovine serum albumin. The samples were then separated on 5% polyacrylamide gel (50 m*M* Tris, 380 m*M* glycine, 10% glycerol). After electrophoresis, the gel was dried and autoradiographed.

Statistical Analysis. Student's *t* test was used for determination of statistical significance as appropriate. Statistical values of $P < 0.05$ were considered to be statistically significant. Data were presented as the mean ± SE.

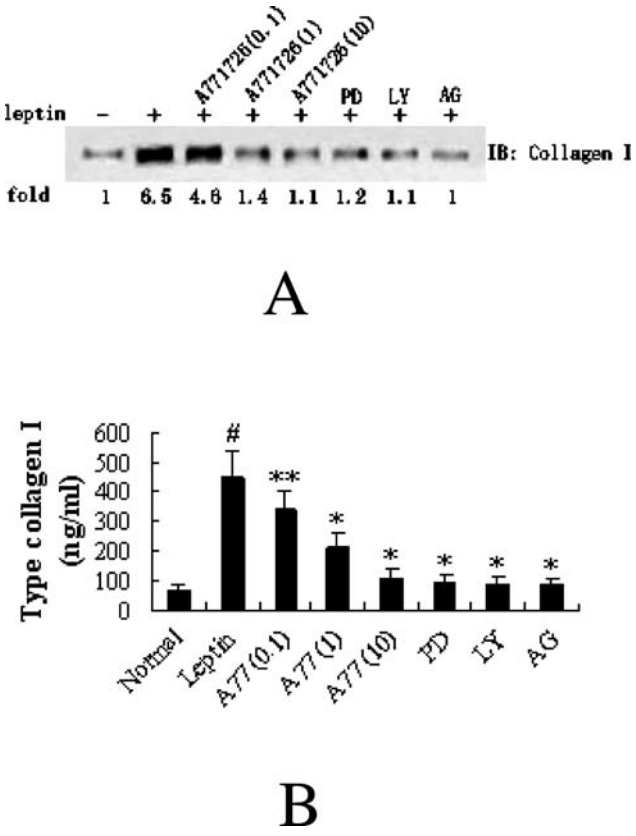

Figure 1. Effect of leflunomide on leptin-induced type I collagen production in HSCs. Primary HSCs (5.5×10^4) were seeded in 24-well plates in growth medium containing 15% FBS for 24 hrs, then serum-starved for an additional 24 hrs. Subsequently, HSCs were either left alone as a control or preincubated with A771726 (0.1, 1, 10 μ*M*), PD098095 ("PD" at 30 μ*M*), LY294002 ("LY" at 25 μ*M*), or AG490 ("AG" at 50 μ*M*) for 2 hrs before being stimulated with leptin (75 ng/ml) for 24 hrs. (A) Western blot analysis of type I collagen protein expression in HSCs. (B) ELISA analysis of type I collagen protein secretion in HSC culture media. Results are the mean ± SE from three independent experiments. $^{\#}P < 0.01$ versus control values; $^{**}P < 0.05$, $^{*}P < 0.01$ versus leptin.

Results

Effect of Leflunomide on Leptin-Induced Type I Collagen Production in HSCs. Hepatic stellate cells play a crucial role in liver fibrosis, as they are responsible for excessive deposition of extracellular matrix proteins, of which type I collagen predominates (31). To assess the effect of leflunomide on leptin-induced extracellular matrix production, Western blot and ELISA analysis were performed for type I collagen in HSCs and HSC-cultured media, respectively. Pretreatment with A771726 led to a dose-dependent suppression of intracellular (Fig. 1A) and extracellular type I collagen protein accumulation (Fig. 1B), which was confirmed by each other. This effect was also significantly inhibited by three specific kinase inhibitors, although the inhibiting extent was different.

Effect of Leflunomide on Leptin-Induced HSC Proliferation and Cell Cycle Phase. To elucidate whether leflunomide inhibits leptin-induced HSC proliferation, we assessed HSC growth by [^{3}H]thymidine incorporation *in vitro*. As data show (Fig. 2A), leptin treatment resulted in a higher proliferation compared with untreated HSCs. Data showed more than a 3-fold increases in [^{3}H]thymidine incorporation. Preincubation with A771726 significantly reduced [^{3}H]thymidine incorporation in a dose-dependent manner. Similar results were found on cell cycle progression (Fig. 2B). Pretreatment with three specific kinase inhibitors also significantly prevented leptin-induced HSC proliferation.

Effect of Leflunomide on Leptin-Induced HSC Cycle–Related D1 Proteins. It known that proliferation of mammalian cells is controlled at specific stages in the cell cycle. D-type cyclins play a critical role in HSC cycle progression, especially at early transition from G1 to S-phase (32). Leflunomide was reported to induce down-regulation of cell signaling in the G0/G1 phase at higher doses (22). The present study showed that that cyclin D1 content was increased nearly 10-fold by leptin compared with serum-free conditions at 2 hrs (Fig. 3A). Pretreatment

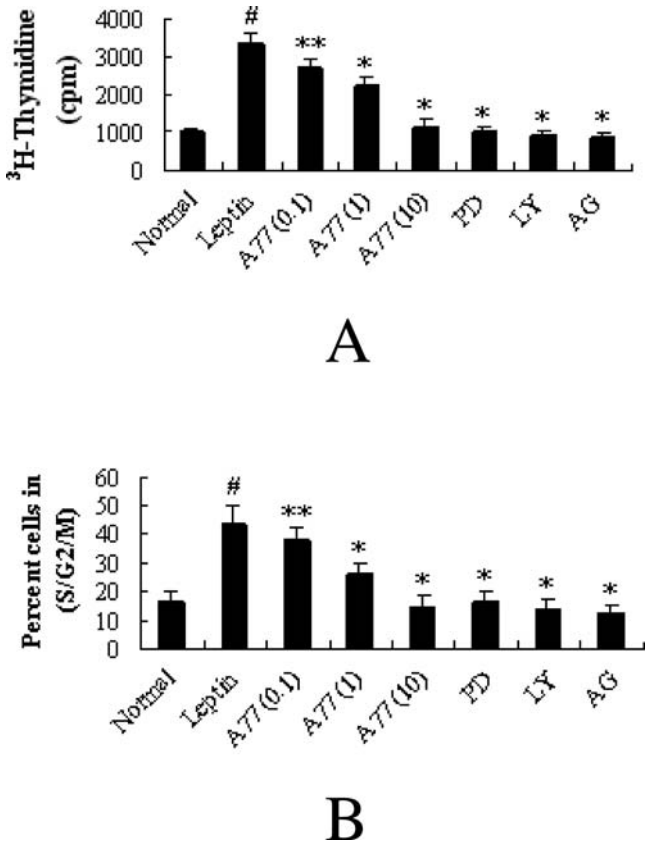

Figure 2. Effect of leflunomide on leptin-induced HSC proliferation and cell cycle phase. Primary HSCs (5.5×10^4) were seeded in 24-well plates in growth medium containing 15% FBS for 24 hrs, then serum-starved for an additional 24 hrs. Subsequently, HSCs were either left alone as a control or preincubated with A771726 (0.1, 1, 10 μM), PD098095 ("PD" at 30 μM), LY294002 ("LY" at 25 μM), or AG490 ("AG" at 50 μM) for 2 hrs before being stimulated with leptin (75 ng/ml) for 24 hrs. (A) HSC proliferation *in vitro* was determined by [^{3}H]thymidine incorporation. (B) The distribution of HSC in the cell cycle was determined by flow cytometry using PI-stained nuclei. Results are the mean ± SE from three independent experiments. $^{\#}P < 0.01$ versus control values; $^{**}P < 0.05$, $^{*}P < 0.01$ versus leptin.

with A771726 significantly attenuated the levels of cyclin D1 protein expression (Fig. 3B). Pretreatment with three specific kinase inhibitors showed the same effects as leflunomide.

Effect of Leflunomide on Leptin-Induced $STAT_3$ Activation in HSCs. A major transduction pathway for leptin's tyrosine kinase signaling is the $STAT_3$ phosphorylation-dependent activation (33). Leflunomide mainly inhibits protein tyrosine kinase-initiating signaling at a higher concentration (24). In the present study we showed that leptin treatment resulted in significant phosphorylation of $STAT_3$, first detected at 10 mins, and reached a maximum at 30 mins (Fig. 4A). The magnitude of this effect was decreased after 30 mins and returned to control levels at 24 hrs. A771726 (at 10 μM) and AG490 (50 μM) pretreatment almost completely blocked $STAT_3$ activation (Fig. 4B) and STATs dimerization to the nucleus (Fig. 4C).

Effect of Leflunomide on Leptin-Induced ERK1/2 and AKT Activation in HSCs. Leptin can lead to activation of MAPK pathway (10) and enhance AKT phosphorylation in endothelial cells, isolated vessels, and several other tissues (33). Previously, leflunomide was reported to inhibit MAP kinase and PI3 kinase activities in AIS cells (cloned from the A431 human epidermoid cell line and selected to be responsive to EGF; Ref. 34). To examine whether leflunomide inhibits MAP kinase and PI3 kinase activities in HSCs, ERK1/2, and AKT phosphorylation was conducted by Western blot. Results indicated that leptin increased both ERK1/2 (Fig. 5A) and AKT (Fig. 5B) phosphorylation markedly, which was maximal at 2 hrs and 30 mins, respectively, and both were detected up to 24-hr time points. A771726 pretreatment significantly suppressed ERK1/2 and AKT kinase phosphorylation, but pretreatment with PD98059 (30 μM) and LY294002 (25 μM) showed complete inhibitory effect on ERK1/2 and AKT kinase activation, respectively (Fig. 5C and D).

Effect of Leflunomide on Leptin-Induced Kupffer Cell Activation. In liver injury, KCs are considered to be the main source of proinflammatory cytokines and chemokines. Leptin, in addition to the stimulating effect on monocyte activation and proliferation, is able to enhance cytokine production in rat KCs via their functional leptin receptor (3). To assess whether leflunomide inhibits leptin-induced cytokine secretion in KCs, we analyzed the levels of TGF-β, TNF-α, and IL-6 cytokines by ELISA. As shown in Figure 6, A771726 pretreatment significantly inhibited proinflammatory and profibrogenic cytokine secretion into the culture (Fig. 6).

Effects of Leflunomide on HSC Collagen Synthesis Stimulated by KC-Conditioned Medium (KCCM). KCs were reported to involve in the pathogenesis of liver cirrhosis (35). Recruitment and activation of KCs are prominent features of liver injury for man and animals. When KCs are activated, it amplifies proinflammatory responses in the liver and exerts an indirect effect on the activation of HSC. Several results indicated that signals from injured KCs might contribute to the so-called activated HSC phenotype (36, 37). To determine the role of KCs on HSC activation and whether leflunomide directly suppresses HSC collagen synthesis, we applied an *in vitro* cultured model (37) in which HSC collagen synthesis is provoked by exposure to KCCM, which recapitulates some profibrogenic situations of the chronic liver disease. As data show, HSC collagen synthesis increased strikingly when exposed to KCCM as assessed by incorporation of [^{3}H]proline. Preincubation of HSC with A771726 significantly reduced the increase of [^{3}H]proline incorporation (Fig. 7). Treatment of the cells with three specific kinase inhibitors also reduced the [^{3}H]proline incorporation significantly.

Discussion

During liver fibrogenesis, leptin is required for increased activated HSC proliferation, impedance of HSC

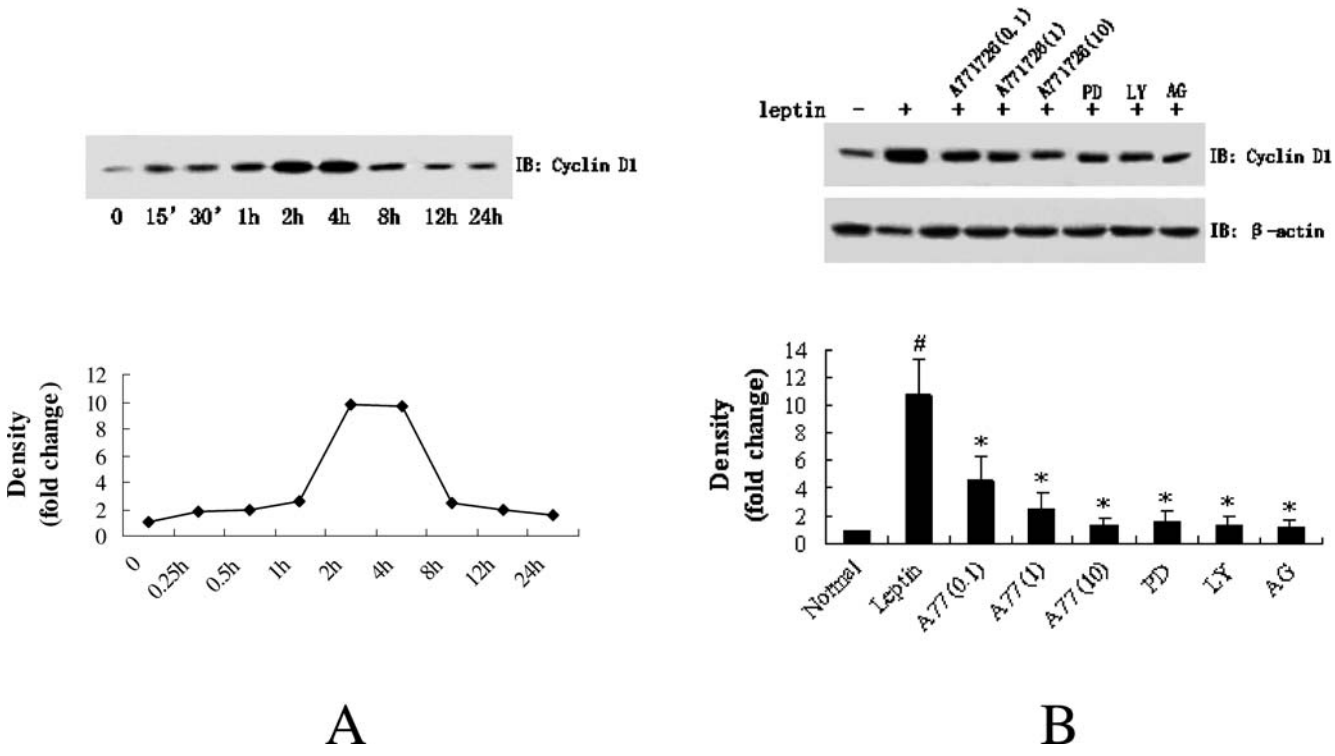

Figure 3. Effect of leflunomide on leptin-induced HSC cycle related D1 proteins. Primary HSCs (5.5×10^4) were seeded in 24-well plates in growth medium containing 15% FBS for 24 hrs, then serum-starved for an additional 24 hrs. Subsequently, HSCs were either left untreated as a control or preincubated with A771726 (0.1, 1, 10 μM), PD098095 ("PD" at 30 μM), LY294002 ("LY" at 25 μM), or AG490 ("AG" at 50 μM) for 2 hrs before being stimulated with leptin (75 ng/ml) for 2 hrs. (A) Time course of cyclin D1 expression stimulated by leptin in HSCs. (B) Effects of A771726, PD098095, LY294002, or AG490 on leptin-induced cyclin D1 protein expression. For Figure 3A and B, cell extracts (20 μg) were subjected to Western blot analysis using antibodies against cyclin D1. The band intensity of cyclin D1 was quantified by densitometry and normalized to β-actin. Densitometry values in the histograms were expressed as -fold change relative to the control, which was assigned a value of 1. Results are the mean ± SE from three independent experiments. $^{\#}P < 0.01$ versus control values; $^{*}P < 0.01$ versus leptin.

death by apoptosis, and increased synthesis of type I collagen (7, 38). One hypothesis suggests that leptin acts on the KCs (in which functional receptor was detected) to release mediators that, in turn, stimulate fibrogenesis in activated hepatic stellate cells (3, 39). Another view holds that leptin acts directly on HSC and triggers specific signal transduction systems, which alter collagen gene expression (40). Moreover, leptin administration can exacerbate thioacetamide-induced liver fibrosis in mice (41). A simultaneous injection of leptin enhanced acute CCl_4-induced necroinflammatory and subsequent fibrotic changes in the hepatic lobules, such as the steady-state messenger RNA (mRNA) levels of alpha1(I) procollagen, the expression of α-SMA and transforming growth factor β1 (TGF-β1) mRNA were all potentiated when leptin was injected together with CCl_4 (16). In addition, leptin was also found to have the capacity to stimulate TIMP-1 gene expression and to increase TIMP-1 protein production in activated human LX-2 HSC (42), which is a major determinant factor of collagen degradation in liver fibrosis. All these indicate that leptin plays an important role in the activation of KC and HSC, which subsequently leads to major alterations in both the quantity and composition of extracellular matrix during hepatic fibrogenesis.

Recent evidence indicates that leflunomide is a novel anti-inflammatory agent with antiproliferative properties. It can inhibit T-cell and B-cell proliferation (43) and minimize the cell response to diverse stimuli, such as TNF-α, H_2O_2, LPS, and PMA (20). Furthermore, this effect of leflunomide is not cell-type specific (20). Therefore, to explore the mechanisms of leflunomide on intervention of leptin-induced collagen production in HSCs, both KC, and HSC activation and the relationships between them are focused in the present study. We revealed that the suppressive effect of leflunomide on leptin-induced HSC collagen production was attributable to its anti-inflammatory action against KC activation and its antiproliferative properties against HSC proliferation. These findings may help to explain why leflunomide showed suppressive effects on leptin-induced collagen production in HSCs.

HSCs play a pivotal role in liver fibrosis and are considered as the therapeutic target for the treatment of hepatic fibrosis. When HSC activation, two major events occur. First, HSCs increase the synthesis and deposition of extracellular matrix proteins, especially type I collagen protein. Second, the proliferation rate of HSC increases. It reported that leptin was a potent HSC mitogen and dramatically inhibited stellate cell apoptosis (38). Lefluno-

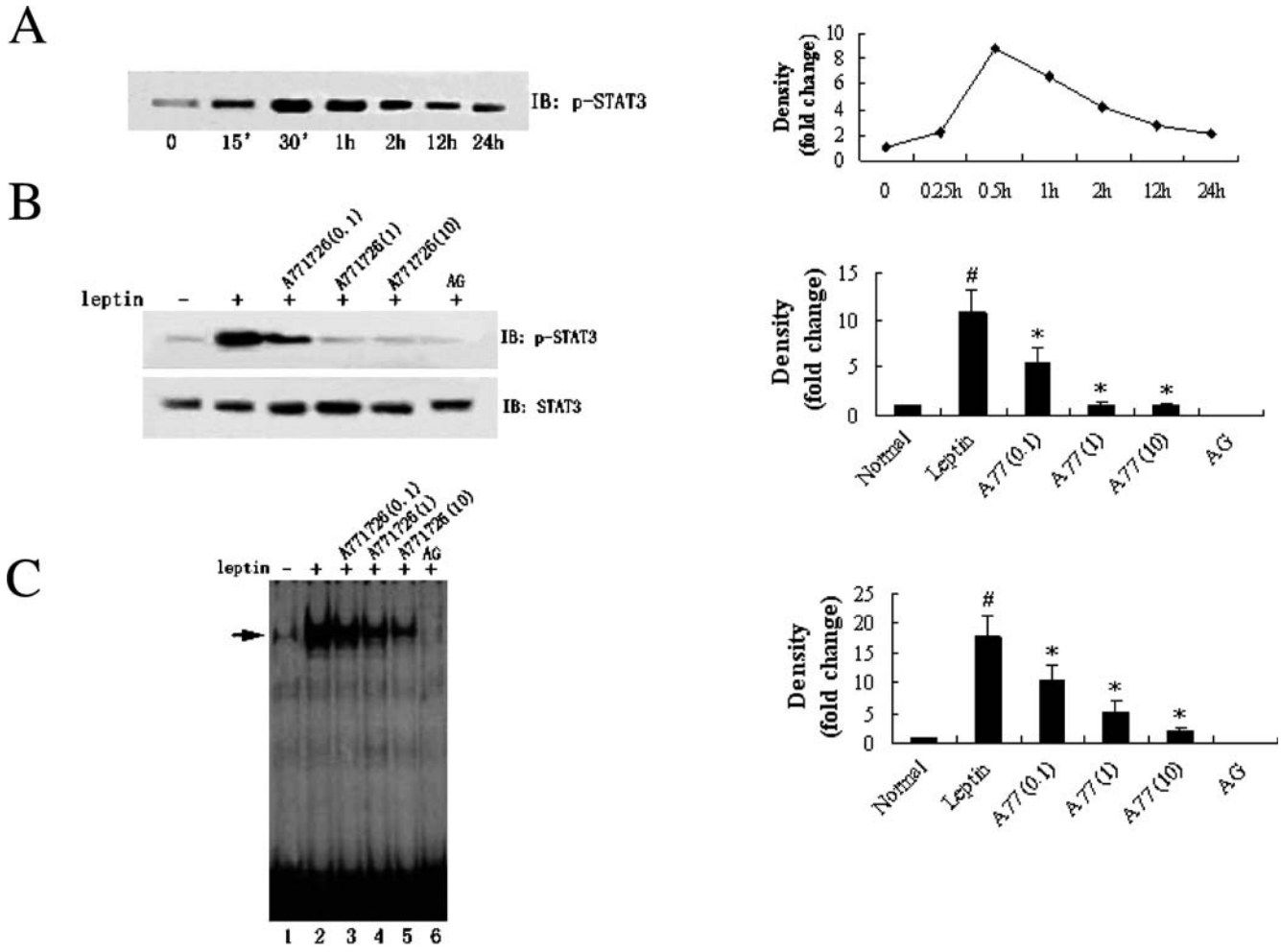

Figure 4. Effect of leflunomide on leptin-induced $STAT_3$ activation in HSCs. Primary HSCs (5.5×10^4) were seeded in 24-well plates in growth medium containing 15% FBS for 24 hrs, then serum-starved for an additional 24 hrs. Subsequently, HSCs were either left alone as a control or preincubated with A771726 (0.1, 1, 10 μM) or AG490 ("AG" at 50 μM) for 2 hrs before being stimulated with leptin (75 ng/ml) for 30 mins. (A) Time course of $STAT_3$ phosphorylation stimulated by leptin. (B) Effects of A771726 and AG490 on leptin-induced $STAT_3$ phosphorylation. For Figure 4A and B, cell extracts (20 μg) were subjected to Western blot analysis using phospho-specific antibodies. The band intensities of phosphorylation of $STAT_3$ were normalized to that of the corresponding nonphosphorylated. (C) Effects of A771726 and AG490 on leptin-induced STATs protein DNA binding activity. Nuclear extracts (12 μg) were analyzed by EMSA using m67 probe. The arrow indicated the protein-DNA complex. For Figure 4A–C, densitometry values in the histograms were expressed as -fold change relative to the control, which was assigned a value of 1. Results are the mean $\pm$ SE from three independent experiments. $^{\#}P < 0.01$ versus control values; $^{*}P < 0.01$ versus leptin.

mide can downregulate cell signaling in the G0/G1 phase (22) and inhibit the expression of the early proliferative immediate genes such as c-fos and c-jun (34). Results in this paper support that leflunomide showed significant inhibition on leptin-induced HSC proliferation as indicated by interfering with leptin-induced three proliferative signaling pathways. Furthermore, leptin-induced cyclin D1 protein expression, which correlates well with HSC proliferation, was also significantly inhibited by leflunomide (32). All these results may suggest that leflunomide can reduce the pool of HSC and hence deposit of extracellular matrix such as collagen in fibrotic liver.

Recently, a role for leptin's signal transduction system in the molecular pathogenesis of mammalian cells has been substantiated in different systems. It signals from ObRb (functional receptor) through the JAK/STAT pathway in the hypothalamic nuclei and other various cell types (9). It activates extracellular signal-regulated kinase (ERK1/2) (10) and phosphatidylinositol 3-kinase (PI3K) kinases family members (11), mediating a proliferative response. Furthermore, leptin-ObRb complex formation leads to the induction of tyrosine phosphorylation through its association with JAK_2 (9), which also leads to ERK and AKT phosphorylation directly (44). Leflunomide inhibits several protein tyrosine kinases, including those of the Src family (45), the Janus kinase family (24), and epidermal growth factor receptor kinase (46). Additionally, it is also a potent inhibitor of MAPK (47). Treatment of AIS cells in culture by a tyrosine kinase inhibitor, leflunomide, leads to the inhibition of MAP kinanse and PI3 kinase activities (34).

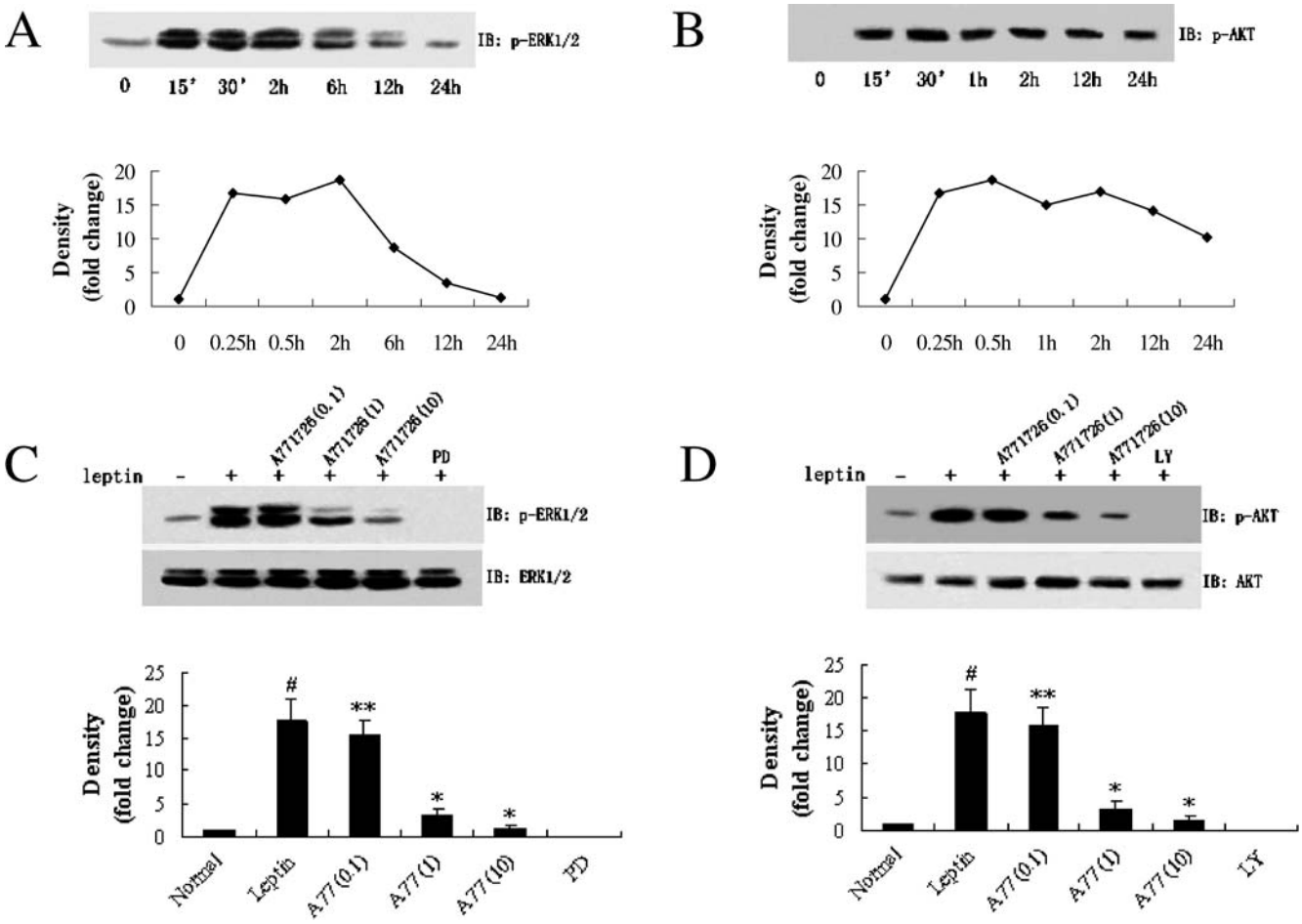

Figure 5. Effect of leflunomide on leptin-induced ERK1/2 and AKT activation in HSCs. Primary HSCs (5.5×10^4) were seeded in 24-well plates in growth medium containing 15% FBS for 24 hrs, then serum-starved for an additional 24 hrs. Subsequently, HSCs were either left alone as a control or preincubated with A771726 (0.1, 1, 10 μM), PD098095 ("PD" at 30 μM), or LY294002 ("LY" at 25 μM) for 2 hrs before being stimulated with leptin (75 ng/ml) for 2 hrs. (A and B) Time course of phosphorylated ERK1/2 and AKT kinase. (C and D) Effects of A771726, PD098095, or LY294002 on the leptin-induced activation of ERK1/2 and AKT. For Figure 5A–D, cell extracts (20 μg) were subjected to Western blot analysis using phospho-specific antibodies. The band intensities of phosphorylation of ERK1/2 and AKT were normalized to that of the corresponding nonphosphorylated. Densitometry values in the histograms were expressed as -fold change relative to the control, which was assigned a value of 1. Results are the mean ± SE from three independent experiments. $^{\#}P < 0.01$ versus control values; $^{**}P < 0.05$, $^{*}P < 0.01$ versus leptin.

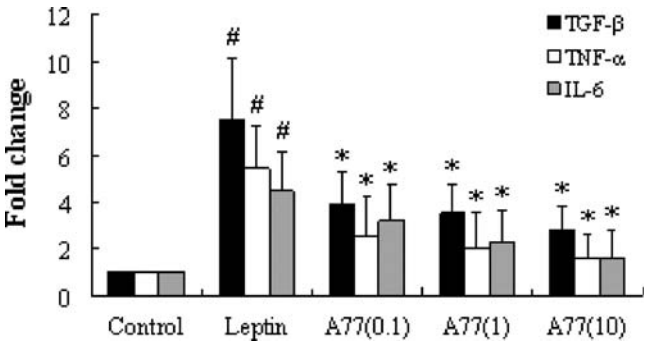

Figure 6. Effect of leflunomide on leptin-induced cytokine secretion in Kupffer cells. KCs isolated from carbon tetrachloride/leptin treatment group were grown in 24-well tissue culture plates for a 72-hr recovery period and preincubated with A771726 (0.1, 1, 10 μM) for 2 hrs, then exposed to 75 ng/ml of leptin for 6 hrs. Cell medium was collected, and the levels of TNF-α, TGF-β, and IL-6 were measured by ELISA. Results are the mean ± SE from three independent experiments. $^{\#}P < 0.01$ versus control; $^{*}P < 0.01$ versus leptin.

We found here that leflunomide showed significant suppression on leptin-induced $STAT_3$, ERK1/2, and AKT kinase activation in cultured HSCs, which may suggest that leflunomide's inhibitory effect on HSC proliferation mainly because of its ability to interrupt tyrosine phosphorylation of intracellular proteins against leptin stimulation.

An inflammatory response is known to contribute to the pathogenesis of liver injury and disease. Several models of hepatocellular injury demonstrated that activation of KC, the resident inflammatory cells of the liver, was crucial for the development of hepatocellular necrosis. For example, CCl_4-induced hepatic fibrosis (36) was dramatically decreased by $GdCl_3$, a known inhibitor of KC activity. Moreover, conditioned medium and coculture experiments further predict a role for KCs in fibrogenesis (36, 37). When KCs are activated, they amplifies proinflammatory responses in the liver and exert an indirect effect on the

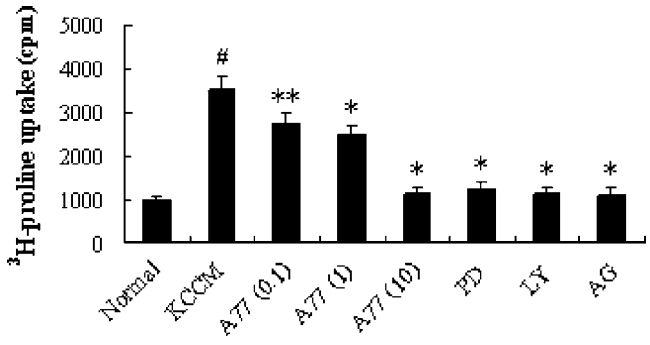

Figure 7. Effect of leflunomide on KCCM-induced HSC collagen synthesis determined by [^{3}H]proline incorporation. KCs isolated from carbon tetrachloride/leptin treatment group were grown in 24-well tissue culture plates for a 72-hr recovery period and stimulated with 75 ng/ml leptin for 48 hrs. Then conditioned medium was collected. Primary HSCs (5.5×10^4) were seeded in 24-well plates in growth medium containing 15% FBS for 24 hrs, then serum-starved for an additional 24 hrs. Subsequently, HSCs were either left alone as a control, or preincubated with A771726 (0.1, 1, 10 μM), PD098095 ("PD" at 30 μM), LY294002 ("LY" at 25 μM), or AG490 ("AG" at 50 μM) for 2 hrs before being stimulated with KCCM (50 μL) for 24 hrs. Results (mean $\pm$ SE) are represented as counts per minute (cpm) from triplicate experiments. $^{\#}P < 0.01$ versus control values; $^{**}P < 0.05$, $^{*}P < 0.01$ versus leptin.

activation of HSC (48). Leptin was reported to augment both inflammatory and profibrogenic responses in the liver because, except for HSC, KC also has a functional receptor, which can also be activated by leptin to release mediators (3). Leflunomide is a novel immunosuppressive and anti-inflammatory agent currently being tested for treatment of autoimmune diseases and transplant rejection. Its anti-inflammatory effect may be related to its ability to suppress tumor necrosis factor and interleukin-1 production during the cell-cell contact activation between T lymphocytes and monocytes (24, 49). We previously also reported that leflunomide could attenuate hepatocyte injury by inhibiting KC activation (50). In the present study, we further confirmed that KCs, as a focused target, can be suppressed by leflunomide to reduce the proinflammatory cytokine secretion and HSC collagen synthesis, which may suggest that the inhibitory effect of leflunomide on HSC collagen production probably acted indirectly through reducing hepatic necrosis and inflammation.

In summary, this paper not only provided a clear physiological and biological evidence for the role of the leflunomide on leptin-induced HSC and KC activation, but it also provided a novel insight into the mechanisms by which leflunomide may exert in liver fibrosis. Acting as an anti-inflammatory agent and direct inhibition of HSC proliferation are two possible mechanisms for the suppressive effect of leflunomide on leptin-induced collagen production in HSCs. To further elucidate the underlying mechanisms of leflunomide in liver injury, additional studies beyond the scope of this study are necessary such as induction of HSC apoptosis.

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