厚生労働科学研究研究費補助金

厚生労働特別研究事業

多 施設共同臨床研究：

自己骨髄細胞を用いた肝臓再生療法の開発に関する研究

平成17年度　総括研究報告書

主任研究者　　沖田　極

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厚生労働科学研究費補助金（厚生労働特別研究事業）
総括研究報告書
多施設共同臨床研究：自己骨髄細胞を用いた肝臓再生療法の開発（LRCT study）
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研究要旨

骨髄細胞が肝細胞（Hepatology 2000）、腸管上皮細胞に分化することの発見（渡辺ら、Nature Med 2002）により骨髄中に幹細胞が存在することが明らかになった。我々は（自己骨髄細胞を用いた肝臓再生療法）の臨床応用を進める基盤モデルとして骨髄細胞から肝細胞への分化評価モデル（GFP/CCl4 モデル）の開発し、骨髄細胞が持続的肝障害の肝硬変時に肝臓に遊走され肝細胞へ分化・増殖することを明らかにした（JB 2003、特公 2003-70377）。さらに骨髄細胞移植により肝機能と生存率の回復、また肝線維化の改善を発見した（Hepatology 2004, Hepatology Alert 記事とし世界に発信された）。基礎研究を基盤に約２年の月日をかけ臨床研究の準備を進め、引き続き平成 15 年 11 月 14 日に国内初の（自己骨髄細胞を用いた肝臓再生療法）の臨床研究を開始した。自己骨髄細胞を用いた肝臓再生療法は安全に施行でき、かつ基礎研究と同様に骨髄移植による肝不全患者の肝機能の改善が明らかになってきた。平成 16 年度末までに８例の症例に施行した。さらに平成 17 年度に、多施設共同臨床研究を推進することでさらに早期に多数の症例での検討が可能になり、エビデンスを持った治療法の実用化の道筋がつけられると考え研究を推進した。その結果、平成 17 年末までにさらに６症例に対して臨床研究を行い、うち１例は平成 18 年２月 10 日に山形大学医学部において、山口大学チームと山形大学チームで共同で、山口大学でのプロトコールの技術移転を行い実際に施行した。これにより山口大学医学部以外の施設においても臨床研究が可能な体制を整えた。自己骨髄細胞を用いた肝臓再生療法の効果については、長期６ヶ月観察可能な症例９症例に観察において、血清アルブミン値、Child-Pugh Score, 血清総蛋白値の有意な改善を認めた。また山形大学で施行した症例においても血清アルブミン値、ICG15 分値の有意な改善効果が明らかになっており、本治療法の開発をさらに推進することは非常に意義があることが明らかになった。

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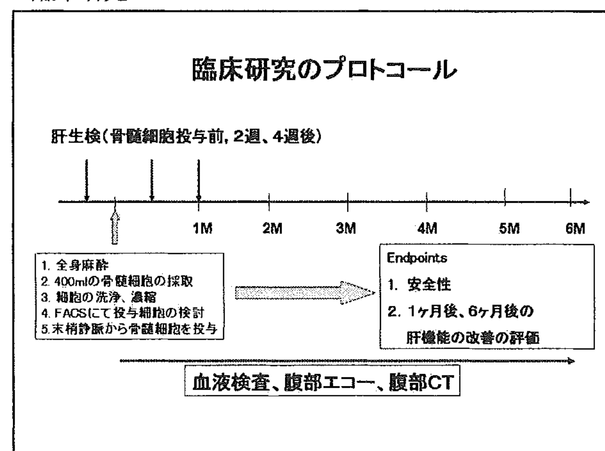
A．研究目的

『自己骨髄細胞を用いた肝臓再生療法』の早期実用化のためには、平成 16 年度までに施行した PhaseI 臨床研究により開発した基盤技術（実際の臨床研究のプロトコール）を、他の施設においても施行可能にすることが、全国の肝不全患者によりよい治療法を提供するために不可欠である。本研究では旭川医科大学、山形大学、東京医科歯科大学、山口大学の多施設による臨床研究実施体制（グループ名：Liver Regeneration with Cell Transplantation (LRCT) Study Group）の確立と、このLRCT study group による『自己骨髄細胞を用いた肝臓再生療法』の臨床研究を多施設にて実施することを目的とする。この臨床研究は多施設共同臨床研究であり、山口大学で開発した臨床研究プロトコールが多施設においても実施可能なものとし推進する。

今回の多施設共同臨床研究を推進によりすることで、より早期に多数の症例の臨床研究が実施可能となり、本治療法の臨床上の有効性のエビデンスがより迅速に明確化すると考える。その結果、『自己骨髄細胞を用いた肝臓再生療法』が、肝移植に代わる次世代のスタンダードな治療法として実用化され、肝不全治療に多大な貢献をすることが期待される。

B．研究方法

臨床研究



臨床研究の対象は（75歳以下、非代償性肝硬変症, ビリルビンの値は３mg/dl以下、肝癌はコントロールされていること、また心肺機能正常）が基本的な適

応である。実際のプロトコールは，全身麻酔下にて、

自己骨髄細胞を400ml採取する。採取した骨髄液は濃縮器セルプロセッサーCytomateを使用し骨髄細胞を濃縮・洗浄し、患者の末梢静脈より投与する。その後経時的に血液検査を施行し，肝再生についてモニターしていく。また画像的にも腹部エコー、ＣＴ検査等にて，肝再生の促進の有無について評価する。First Endpointは１ヶ月後，Second Endpointは６ヶ月後の肝機能に対する改善について評価していく。腹水の状況次第であるが、可能なかぎり患者の同意の上，エコー下肝生検を行い肝臓の状態について，組織学的検討をしていく。その際にAFP, c-kit, HNF4, CD34, CD45, HHM抗体を用い免疫染色を行い，実際に骨髄細胞投与により肝再生が誘導されるかを評価する。肝線維化の改善の有無については，血清ヒアルロン酸、４型コラーゲン、３型プロコラーゲンペプタイドを測定し評価していく。この具体的な臨床研究が山口大学を含め旭川医科大学、山形大学、東京医科歯科大学の多施設共同で臨床研究ができる体制の確立を目指す。グループ名はLiver Regeneration with Cell Transplantation (LRCT) Study Groupとし、実際に臨床研究を推進していく。今年度は現在までに施行した8例にあわせ、合計20例を目標に臨床研究を推進し、有効性について評価し、自己骨髄細胞を用いた肝臓再生療法の早期実用化を目指していく。

（倫理面への配慮）

本臨床研究は、ヘルシンキ宣言、臨床研究に関する倫理指針（厚生労働省告示第255号）、申請者が所属する機関の倫理規定を遵守する。基盤になるPhaseI臨床研究は山口大学医学部倫理委員会で平成14年1月に臨床研究の承認を得て、準備を行い平成15年11月より現在8例に実施され、副作用もない。多施設研究推進にあたっては研究参加各大学でも個別に大学の倫理委員会において新たに申請し承認をえる予定である。また臨床研究に参加する患者に対し、臨床研究について十分に説明し、インフォームドコンセントを得て実施していく。すでに実施経験がある山口大学の臨床研究プロトコールを使うことで多施設研究行えると考える。また分離した骨髄細胞には，分離後遺伝子，サイトカイン等は加えない。このため自己血輸血とほぼ同様の安全性で行えると考えられる。さらに臨床研究を進めるにあたり、これまでと同様に臨床研究担当者は各都道府県の医師会保険を通じ賠償保険額を増額し、臨床研究にて生じる不測の状況の対処することにする。

C．研究結果

１．平成16年11月からの実施症例

施行症例（14症例）

Pt. (etiology)	BMT
1. 69, M (HBV)	H15.11.14
2. 59, M (HBV)	H16. 2.10
3. 57, M (alcohol+C)	H16. 2.27
4. 65, F (unknown)	H16. 3.05
5. 69, M (HCV)	H16. 5.07
6. 63, M (HCV)	H16. 5.21
7. 55, M (HBV)	H16. 9.24
8. 56, M (HCV)	H17. 1.14
9. 60, M (HCV)	H17. 7.29
10. 60, M (HCV)	H17. 8.19
11. 60, M (HCV+HBV)	H17.10.21
12. 59, M (alcohol)	H18. 2.10
13. 59, F (HCV)	H18. 2.24
14. 62, F (HCV)	H18. 3.10

２．６ヶ月以上長期観察可能であった症例経過

（最初の10症例の経過、１例は経過観察中のアルコール摂取のため脱落例とした。）

施行症例のうち長期に経過観察可能であった症例について、骨髄細胞投与後、血清アルブミン値、総蛋白値、Child-Pugh Scoreの有意な改善効果が明らかになった（$p<0.05$）

多施設臨床研究の状況、山形大学での実施症例（平成18年2月10日）

山口大学で行っている方法を、山形大学医学部において個別に倫理委員会にかけ、承認後LRCT studyの一環として、準備を進め、山口大学チーム、山形大学チームで平成18年2月10日、骨髄細胞を用いた再生療法を実施した。

実施症例は、59才男性、アルコール性肝硬変症であった。血液検査上、血清アルブミン値2.5g/dLから3.5g/dLの上昇、また肝線維化の指標のICG R15値が、術前40%であったものが、術後33%に改善した。

再生療法を見学した大学（信州大学、昭和大学、韓国Yonsei大学）

本治療法に対する興味は高く、第9,10例（韓国Yonsei大学）、第13例（信州大学）、第14例（昭和大学）から医師が派遣され、実際の治療法について見学した。この実際の治療の見学は、さらに来年度以降、本治療法の開発を推進する上において重要なことになると考えられた。

D．考察

平成17年度に、多施設共同臨床研究を推進することでさらに早期に多数の症例での検討が可能にな

り、エビデンスを持った治療法の実用化の道筋がつけられると考え研究を推進した。その結果、平成17年末までにさらに6症例に対して臨床研究を行い、うち1例は平成18年2月10日に山形大学医学部において、山口大学チームと山形大学チームとの共同で、山口大学でのプロトコールの技術移転を行い実際に施行した。これにより山口大学医学部以外の施設においても臨床研究が可能な体制を整えた。自己骨髄細胞を用いた肝臓再生療法の効果については、長期6ヶ月観察可能な症例9症例の観察において、血清アルブミン値、Child-Pugh Score, 血清総蛋白値の有意な改善を認めた。また山形大学で施行した症例においても血清アルブミン値、ICG15分値の有意な改善効果が明らかになっており、本治療法の開発をさらに推進することは非常に意義があることが明らかになった。

E．結論

今年度の研究により、実際に山口大学で施行した臨床研究（自己骨髄細胞を用いた肝臓再生療法）のプロトコールを実際に山形大学で施行した。技術移転を実際に行えたことにより、技術移転に関するノウハウを得ることができた。今後さらに、LRCTの参加施設で研究を推進するとともに、LRCT参加施設以外の医師が見学した施設において、今後は本治療法の技術移転は可能になっていくと考える。また長期観察例において、肝機能改善効果があきらかになってきており、本治療法の臨床上の有効性のエビデンスがより迅速に明確化できると考える。今後『自己骨髄細胞を用いた肝臓再生療法』が、肝移植に代わる次世代のスタンダードな治療法として実用化され、肝不全治療に多大な貢献をすることが期待される。

F．健康危険情報
特になし

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（平成１７年１０月５日）
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H．知的財産権の出願・登録状況（予定を含む。）

１．特許取得
坂井田　功、寺井　崇二　肝再生用骨髄細胞画分
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３．その他
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研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Shen J, Sakaida I, Uchida K, Terai S, Okita K	Leptin enhances TNF-alpha production via p38 and JNK MAPK in LPS-stimulated Kupffer cells.	Life Sci.	77(13)	1502-1515	2005
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沖田　極、寺井　崇二、坂井田　功	医学と医療の最前線　骨髄幹細胞移植による肝疾患の治療	日本内科学会誌	94(4)	163-168	2005
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寺井　崇二、坂井田　功	骨髄細胞移植による肝再生	医学のあゆみ	216(7)	546-547	2006
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Leptin enhances TNF-α production via p38 and JNK MAPK in LPS-stimulated Kupffer cells

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Abstract

Leptin is now recognized as a proinflammatory cytokine and thought to be a progressive factor for non-alcoholic steatohepatitis (NASH). Here we showed the effects of leptin on the production of TNF-α(tumor necrosis factor-alpha) by Kupffer cells (KCs) with signal transduction. Leptin enhanced TNF-α production accompanied by a dose-dependent increase of MAPK activity in lipopolysaccharide (LPS)-stimulated KCs. SB203580 and JNK inhibitor I, specific inhibitors of P38 and JNK, inhibited TNF-α production in KCs but PD98059, an inhibitor of the ERK pathway, did not affect TNF-α production by KCs. Recombinant constitutively active adenovirus (Ad)-MKK6 and-MKK7 increased TNF-α production in KCs with activation of P38 and JNK without any change by Ad-MEK1 delivery. On the other hand, KCs isolated from the Zucker rat (fa/fa), a leptin receptor-deficient rat, showed reduced production of TNF-α on stimulation with LPS. The delivery of Ad-MKK6 and-MKK7, but not Ad-MEK1, increased TNF-α production in KCs of Zucker rats with activation of P38 and JNK. Addition of leptin to normal rats increased LPS-induced hepatic TNF-α production in vivo and leptin receptor-deficient Zucker rats showed reduced hepatic TNF-α production on addition of LPS in vivo. These findings indicate that P38 and JNK pathways are involved in the signal transduction of leptin enhancement of LPS-induced TNF-α production.

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Keywords: Leptin; TNF-α; MAPK; MAPK inhibitor; Recombinant adenovirus

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Introduction

KCs, the resident macrophages of the liver, are located in the hepatic sinusoids. KCs are the first cells to respond to LPS and considered to be involved in the clearance of gut-derived bacteria and bacterial toxin. High portal levels of LPS can lead to a pronounced secretion of inflammatory mediators, including IL-1 and TNF-α, by KCs and ultimately to endotoxin-induced liver injury (Freudenberg et al.,1982; Hartung et al.,1997; Winwood and Arthur, 1993).

Leptin, the product of the *ob* gene, is a 16-kDa secreted protein mainly produced by adipose tissue, serving as a satiety factor for the hypothalamus. The amount of leptin mRNA and serum level of leptin is highly correlated with body fat volume (Zhang et al., 1994; Pellymounter et al., 1995; Halaas et al., 1995; Campfied et al., 1995). In addition to regulating body weight, leptin also influences reproductive, hematopoietic and immune systems in which its receptors are expressed (Coiffi et al., 1996; Montgomery et al., 1990; Bennett et al., 1996; Gainsford et al., 1996; Chehab et al., 1996; Barash et al., 1996). Leptin receptors have been found in various tissues and organs including the hypothalamus, lung, liver, kidney, testis (Tartaglia et al., 1995; Lee et al., 1996), and adipose tissue, as well as in hematopoietic cells and macrophages (Ahn and Krebs, 1990). To investigate the role of leptin, we used the Zucker rat (fa/fa) which has a mutation in the leptin receptor and deficiency of leptin signaling exhibiting hyperphagia and obesity.

Because leptin is considered to be a member of the cytokine family based on its structure (Tartaglia et al., 1995; Zhang et al., 1997), its signal transduction may be similar to that of other cytokines. Many cytokines stimulate MAPK (mitogen-activated protein kinase), which in turn phosphorylates a number of nuclear transcription factors and cytoplasmic proteins involved in the regulation of macromolecular synthesis and mitogenesis (Blenis, 1993; Hill and Treisman, 1995; Treisman, 1994). Thus we examined the effect of leptin on LPS-induced TNF-α production in KCs with the involvement of MAPK signal transduction.

Experimental procedures

Experimental animals

Male Zucker (fa/fa) rats and control rats, 8 weeks of age and weighing 150–200 g (Nippon SLC Co. Ltd., Shizuoka, Japan), were housed in a specific pathogen-free room under controlled temperature, humidity and lighting with free access to standard chow and water.

In vitro

Isolation and culture of KCs

KCs were isolated from rats according to a method described previously (Kayano et al., 1995), with some modification. After cannulation with a sterile 18-gauge catheter into the portal vein, the liver was perfused with Ca^{2+}, Mg^{2+}-free Hanks balanced salt solution (HBSS) containing 0.01% EDTA (Wako Pure Chemical Industries Ltd. Tokyo, Japan) at 37 °C (pH, adjusted to 7.3). The flow rate was adjusted to 25ml/min. After 2 min, the solution was replaced with prewarmed (37 °C) HBSS containing 0.015% collagenase (Shinda Zerachin Inc., Osaka, Japan), 0.5 mmol $CaCl_2$ and 0.2% bovine serum albumin (BSA) (Sigma, St. Louis, MO) and the pH was adjusted to 7.3. After 5 min of perfusion, the liver was

excised and disaggregated in 100 ml of HBSS containing 0.2% BSA at 4 °C. The total cell suspension was then passed through a nylon mesh. Hepatocytes were removed by repeated differential centrifugation (45 $\times g$ for 2 min and 50 $\times g$ for 1.5 min) to obtain highly purified nonparenchymal cells.

KCs were separated from whole nonparenchymal cells using a centrifugal elutriation rotor (model J6-MC rotor, Beckman Instruments, Palo Alto, CA). The whole nonparenchymal cell suspension in HBSS containing 0.2% BSA was loaded onto an elutriation rotor at a flow rate of 18.5 ml/min and a rotor speed of 2500 rpm at 4 °C. The cell suspension of KCs was finally collected at a flow rate of 22.5ml/min and a rotor speed of 1500 rpm. After centrifugation at 500 $\times g$ for 5 min, pelleted KCs were resuspended in RPMI 1640 medium (Nissui Pharm. Co. Ltd., Tokyo, Japan) buffered with $NAHCO_3$, containing 10% heat-inactivated (56 °C, 30 min) fetal calf serum (FCS) (Commonwealth Serum Laboratories, Melbourne, Australia) and supplemented with 100 U/ml of penicillin and 100 U/ml of streptomycin (Gibco Laboratories, Life Technologies Inc., NY). KCs were plated on tissue culture plates and incubated at 37 °C and in 5% CO_2 for 15 min. Then the culture was washed with RPMI 1640 medium to remove hepatocytes.

Cell viability was always over 95%, as determined with the trypan blue exclusion test. Cell purity was more than 90%, as determined by non-specific esterase staining.

Experimental protocol

After incubation for 18 h, the medium was replaced and isolated KCs from Zucker or control rats were treated with 500 ng/ml of LPS (Sigma Chemical Co., St. Louis, MO) and various concentrations of rat recombinant leptin (Techene Corporation, Minneapolis). MAPK activity was measured 3 min after leptin was added by western blotting and TNF-α was measured 6 h after LPS and leptin were added by ELISA. With the experiments using specific inhibitors of ERK, p38, and JNK (Cuenda et al., 1995; Barr et al., 2002; Kultz et al., 1998), cells were loaded with PD98059 (10 μM) (EMD Biosciences Inc., CA) or SB203580 (10 μM) (EMD Biosciences Inc., CA) or JNK inhibitor I (0.2 μM) (EMD Biosciences Inc., CA) for 2 h before the addition of 500 ng/ml of LPS with or without leptin (50 nM). Three minutes or 6 h later, MAPK activity and TFN-α production were measured. PD98059 and SB203580 were dissolved in DMSO (dimethyl sulfoxide) and JNK inhibitor I was dissolved in sterilized water. The final concentration of DMSO in the medium was 0.1% (V/V).

Adenovirus was added 18 h after the isolation of KCs, and the activity of MAPK and production TNF-α were measured 24 h after the infection.

Western blot analysis of activated MAPK

The cultures were collected for Western blotting to detect three activated members of the MAPK family, phospho-ERK, -p38 and -JNK (Denhardt, 1996; Kyriakis, 1999; Han et al., 1994). After being washed twice in phosphate-buffered saline (PBS), the cells were resuspended in 250 μl of cell lysis buffer containing 2.5 μl of 100 mM phenylsulfonyl fluoride (PMSF) (Roche Diagnostic GmbH, Mannheim, Germany). After homogenization by the cell disruptor, samples were centrifuged at 7300 $\times g$ for 10 min in an Eppendorf centrifuge and supernatants were collected. Total protein (80 μg) was loaded onto a readygels J (Bio-Rad Laboratories, CA) and blotted on a membrane after electrophoresis. The membrane was blocked for 1 h by Tris-buffered saline (20 mM Tris–HCl, 150 mM NaCl, pH 7.5) containing 0.2% I-block (Tropix Inc., MA) and 0.1% Tween 20 (Kanto Chemical Co. Inc., Tokyo,

Japan). The primary antibody phospho-p38, phospho-ERK or phospho-JNK (Cell Signaling Technology, Massachusetts) was diluted 1 : 500 in Tris-buffered saline. The blots were shaken by a shaking mixer for 1 h at room temperature. After 4 washes with Tris-buffered saline for 5 min, the secondary antibody antirabbit Ig (Biosciences Inc., CA) was added in a 1 : 5000 dilution in Tris-buffered saline and blots were shaken for 1 h at room temperature. After 4 washes, the ECL (Biosciences Inc., CA) system was used to visualize immunoreactive bands.

TNF-α bioassay

TNF-α levels in KC supernatants were determined using a "sandwich" enzyme-linked immunosorbent assay (ELISA) by rat TNF-α ELISA kit (Biosource International Inc., CA), according to the manufacturer's instructions.

Recombinant adenoviral vector

Recombinant viruses were grown in human embryonic kidney (293) cells, purified and dialyzed before storage at − 80 °C. A plaque assay of 293 cells was used to quantify the viral stock.

Recombinant adenoviral vectors were kind gifts from Dr. Aoki (Yamaguchi University, Japan) (Aoki et al., 2002). The cDNA of constitutively active MEK1 {MEK1(DN3-S218-E-S222D)} was a gift from Dr.Guan (University of Michigan, Ann Arbor, MI.). The cDNA of constitutively active MKK6 was obtained by RT-PCR and by substituting Ser151 and Thr155 to Glu and Asp, respectively, by site directed mutagenesis. The cDNA of constitutively active MKK7 was obtained by RT-PCR and by substituting Ser271 and Thr275 to Glu and Asp, respectively, by site directed mutagenesis. Both the constitutively active MKK6 and constitutively active MKK7 were tagged with FLAG epitope. The recombinant-deficient adenovirus lacZ (β-galactosidase) was used as a control. Isolated KCs from control or Zucker rats were infected with the recombinant adenovirus at various concentrations 18 h after the isolation. Infection efficiency was examined using the control vector, lacZ-adenovirus. After infection for 24 h at various multiplicities, the cytotoxic effects of adenovirus on the viability were examined. The infection at a multiplicity of 5.6×10^5 pfu did not affect the viability (data not shown). Thus we infected KCs of Zucker and control rats with Ad-MEK1 or -MKK6 or -MKK7 at a multiplicity of 5.6×10^5 pfu in subsequent experiments.

In vivo

Zucker and control rats treated with 0 and 100 μg/kg of leptin (i.p.) were injected with 500 μg/kg of LPS (i.p.). After 1 h, the animals were killed under ether anesthesia to harvest the liver. Each group consisted of 8 rats. The livers were disrupted in phosphate-buffered saline (PBS) and homogenized. After centrifugation for 10 min at 12,000 ×*g*, the supernatant was collected to assay for TNF-α by ELISA.

Statistical analysis

Results are expressed as the mean ± SD, and the data obtained were evaluated by ANOVA as appropriate. The level of significance was set at 5% for each analysis.

Results

In vitro

Effect of leptin on production of TNF-α

The LPS-induced TNF-α production was enhanced by leptin in a dose-dependent manner with a significant effect at more than 50 nM (6026 ± 1191 pg/5×10^5 cells) compared with LPS alone (2880 ± 138 pg/5×10^5 cells) in isolated KCs of control rats (Fig. 1). However, leptin did not increase TNF-α production without LPS (data not shown).

Effect of leptin on MAPK activation

The activation of ERK, p38 and JNK was dependent on time up to the peak at 3 min after addition of leptin (data not shown). Thus we examined the effect of leptin on the activation of these pathways at this time point. Activation of ERK, p38 and JNK was seen in a dose-dependent manner on the addition of leptin at up to 50 nM (Fig. 2).

Effect of MAPK inhibitor on TNF-α production

KCs were pretreated with MAPK inhibitors resulting in the inhibition of phosphorylated ERK, p38 and JNK. Addition of leptin did not affect the inhibition of ERK, p38 or JNK phosphorylation (Fig. 3).

As shown in Fig. 4, SB203580 significantly ($P < 0.01$) reduced the production of TNF-α (1240 ± 266 pg/5×10^5 cells) compared with that of DMSO alone as control (2286 ± 184 pg/5×10^5 cells). Also, JNK inhibitor I (1440 ± 92 pg/5×10^5 cells) reduced significantly ($p < 0.05$) the production of TNF-α compared with the control (2186 ± 141 pg/5×10^5 cells). However, PD98059, a MEK inhibitor, did not change the LPS-induced TNF-α production of KCs. In any case, TNF-α level was not increased by leptin when KCs were pretreated with MAPK inhibitors.

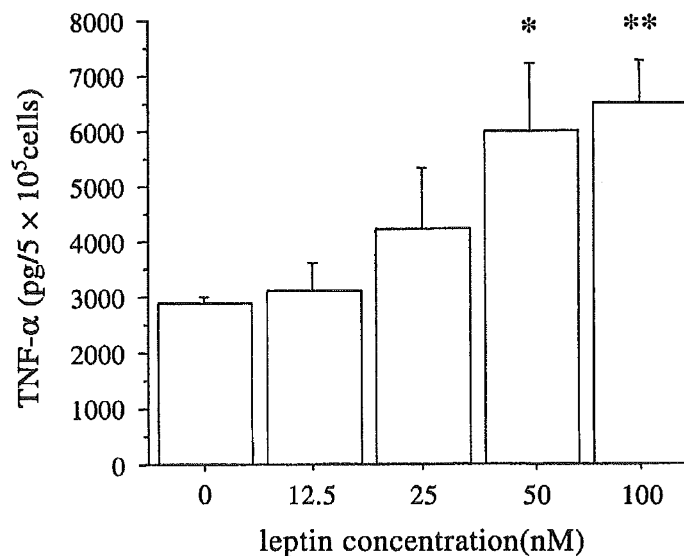

Fig. 1. Eighteen hours after the isolation of KCs from control rats, the medium was replaced and 500 ng/ml of LPS was added with various concentrations of rat leptin. TNF-α in the medium was measured after a 6-h culture. Values indicate the mean ± SD for triplicate determinations and the figure shows a result representative of three independent experiments. $^{*}P < 0.04$, $^{**}P < 0.03$ vs. LPS alone.

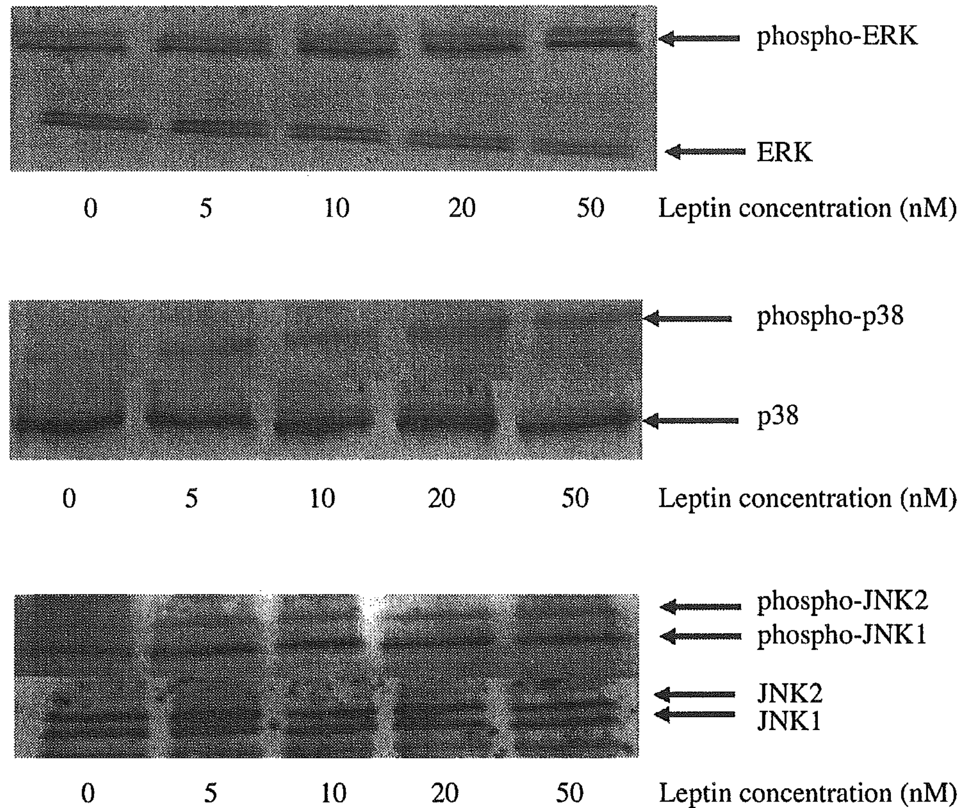

Fig. 2. Three minutes after addition of leptin in Fig. 1, MAPK activity was examined by Western blot analysis. The figure shows an example representative of three independent experiments.

Infection of KCs with recombinant adenoviruses and its effect on TNF-α production

As shown in Fig. 5, infection of Ad-MEK1,-MKK6 and -MKK7 which are upstream of ERK, p38 and JNK increased the activity of ERK, p38 and JNK of KCs. The production of TNF-α significantly increased in KCs treated with Ad-MKK6 (4066 ± 196 pg/5×10^5 cells, $P<0.002$) or Ad-MKK7 (3700 ± 100 pg/5×10^5 cells, $P<0.006$) compared with that of Ad-lacZ (2226 ± 331 pg/ 5×10^5 cells). However, activation of ERK with Ad-MEK1 did not increase TNF-α production of KCs. (Fig. 6).

The same experiments were conducted using KCs of Zucker rats and the same results were obtained. As shown in Fig. 7, the level of TNF-α significantly increased in KCs treated with Ad-MKK6 (2780 ± 670 pg/5×10^5 cells, $P<0.05$) or Ad-MKK7 (3200 ± 323 pg/5×10^5 cells, $P<0.02$) compared to that of Ad-lacZ (1386 ± 218 pg/5×10^5 cells) but again Ad-MEK1 did not change TNF-α production of KCs.

The difference in TNF-α production by KCs of Zucker and control rats

TNF-α production by KCs in control rats treated with LPS showed a significant increase compared to that in Zucker rats (3953 ± 267 pg/5×10^5 cells vs.2623 ± 200 pg/5×10^5 cells, $P<0.05$) (Fig. 8).

In vivo

The concentration of TNF-α in liver increased significantly in control rats compared to Zucker rats after the addition of LPS(52690 ± 2610 pg/mg protein vs. 41310 ± 3790 pg/mg protein, $P<0.03$) (Fig. 9).

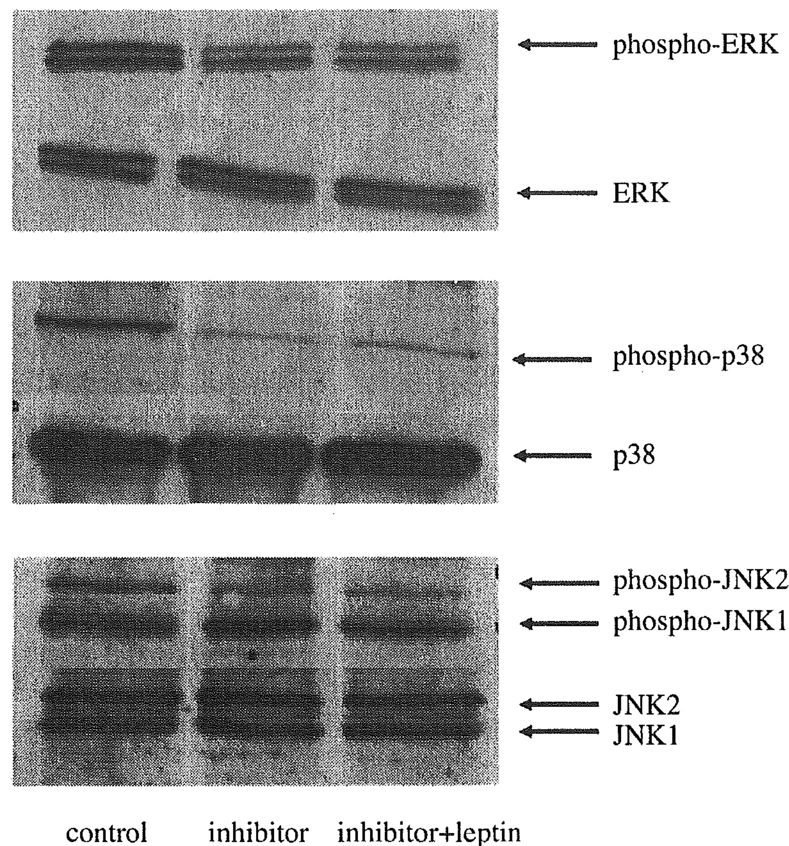

Fig. 3. Eighteen hours after isolation of KCs from control rats, the medium was replaced and KCs were incubated with either PD98059 (10 μM), SB203580 (10 μM) or JNK inhibitor I (0.2 μM) for 2 h before the addition of 500 ng/ml of LPS with or without 50 nM leptin. Three minutes later, Western blotting was performed to examine MAPK activity. The figure shows an example representative of three independent experiments.

Also administration of leptin to rats significantly enhanced TNF-α production in the liver of control rats (44700 ± 3459 vs. 33650 ± 3786, P<0.05) (Fig. 10).

Discussion

In the present study, we demonstrated that leptin enhanced hepatic TNF-α production by KCs when administered with LPS both in vitro and in vivo. TNF-α plays a critical role physiologically and causes severe damage to the host organism when produced in excess. *Extensive study has already proven that LPS-induced liver injury is mediated by TNF-α* (Diehl, 2000; Hartung et al., 1997; Mayeux, 1997). Although many types of cells are capable of TNF-α production, KCs are thought to be the principal hepatic source of TNF-α after LPS challenge. KCs synthesize large amounts of TNF-α when exposed to gut-derived endotoxin or bacterial LPS (Ulevitch and Tobias, 1995; Nathan et al., 1980). Our results showed that addition of leptin increases and leptin receptor-deficiency reduces LPS-induced TNF-α production of Kupffer cells in vitro as well as in vivo. Thus these findings suggest that leptin augments production of a toxic mediator, TNF-α, thereby exacerbating the liver inflammatory response following exposure to LPS. Because leptin is an adipocyte-derived hormone, the serum leptin level, as well as TNF-α level, actually increased in most patients with hepatic steatosis and/or NASH (non-alcoholic

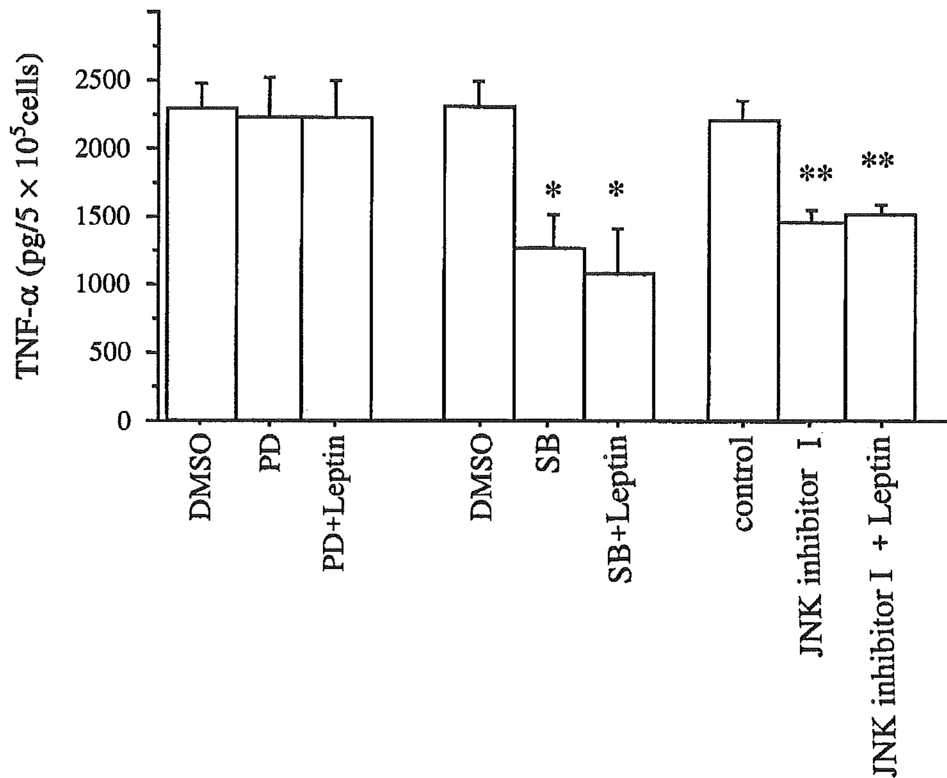

Fig. 4. TNF-α in the medium was measured 6 h after the addition of LPS performed as in Fig. 3. Values indicate the mean ± SD for triplicate determinations. The figure shows a result representative of three independent experiments. *$P<0.01$ vs. DMSO alone, **$P<0.05$ vs. control.

steatohepatitis) (Winkler et al., 1998; Uygun et al., 2000). The results imply that *the increased basal expression of leptin leads to overproduction of TNF-α, which may play a significant role in the pathogenesis of NASH as a "second hit" following the development of simple steatosis as previously reported (Diehl, 1999, 2004;* James and Day, 1999; *Solga and Diehl, 2003). Thus the overproduction of TNF-α induced by leptin may lead NASH to the progressive disease ultimately resulting in liver cirrhosis and hepatocellular carcinoma (Diehl et al., 2005; Honda et al., 2002; Ikejima et al., 2002; Sakaida et al., 2003; Younossi et al., 2002).*

In addition, we found that the activity of MAPK was increased by leptin in LPS-stimulated KCs of normal rats. Both the structure of leptin and that of its receptor suggest that leptin might be classified as a cytokine. The structure of leptin has similarities to the long-chain helical cytokine family which includes IL-2, IL-6 and growth factor, and the structure of the leptin receptor is closely related to that of gp130, a signal-transducing component of IL-6, CNTF, and LIF (Tartaglia et al., 1995; Zhang et al., 1997). These cytokines as well as other diverse extracellular stimuli including osmotic shock, stress and elevated temperature cause the activation of phosphorylation cascades involving MAPK (Hill and Treisman, 1995; Freshney et al., 1994). MAPK has been revealed to be an important group of regulators of a broad range of genes involved in cellular responses to inflammatory and stress signals (Cobb and Goldsmith, 1995). A study of cytokine-induced TNF-α production by astrocytes has shown the involvement of p38 MAPK (Bhat et al., 1998). These findings imply that leptin may exert its effect in a manner similar to these cytokines and MAPK may be involved in leptin signal transduction in activated KCs.

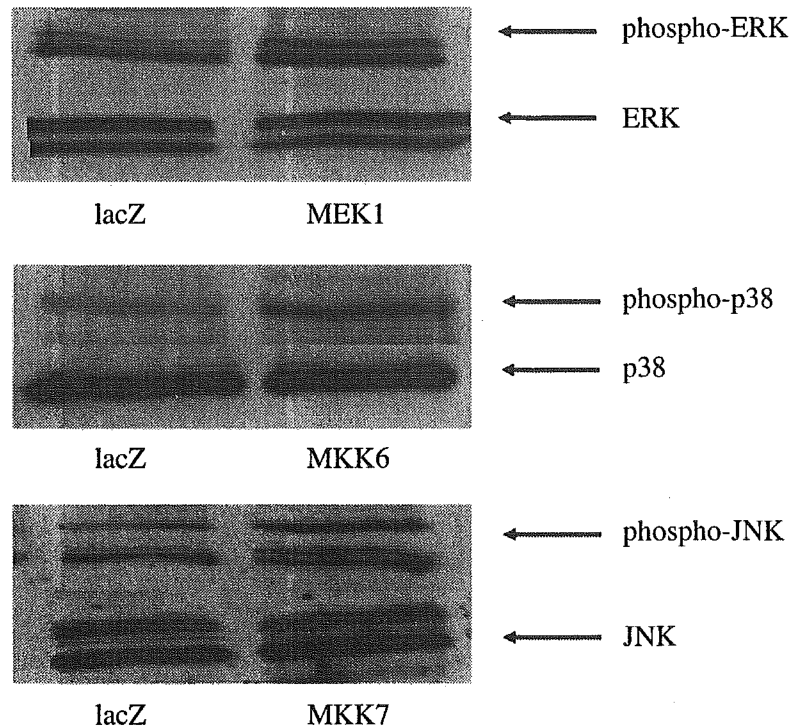

Fig. 5. Eighteen hours after the isolation of KCs from control rats, the medium was replaced and KCs were incubated with either Ad-MEK1, Ad-MKK6, Ad-MKK7 or Ad-lacZ for 24 h. MAPK activity was examined by Western blot analysis. The figure shows an example representative of three independent experiments.

Our results indicate that the production of TNF-α was inhibited by SB203580 and JNK inhibitor I, but not by PD98059. *Furthermore, in any case, TNF-α level was not increased by leptin when KCs were pretreated with MAPK inhibitors.* Some research also showed that TNF-α production is affected by p38 and JNK inhibitor, but not by ERK inhibitor in various cells (Corrigall et al., 2004; Hua et al., 2002; Cameron et al., 2003). We then infected KCs from normal rats with recombinant constitutively active Ad-MEK1,-MKK6, and-MKK7, upstream of ERK, p38 and JNK. Adenovirus has advantages as a

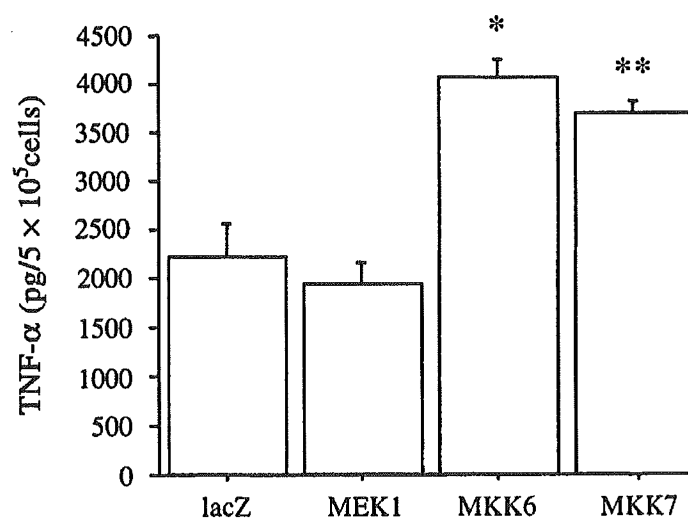

Fig. 6. TNF-α in the medium was measured 24 h after the adenovirus infection shown in Fig. 5. Values indicate the mean ± SD in triplicate and the figure shows a result representative of three independent experiments. *P<0.002 vs. lacZ, **P<0.006 vs. lacZ.

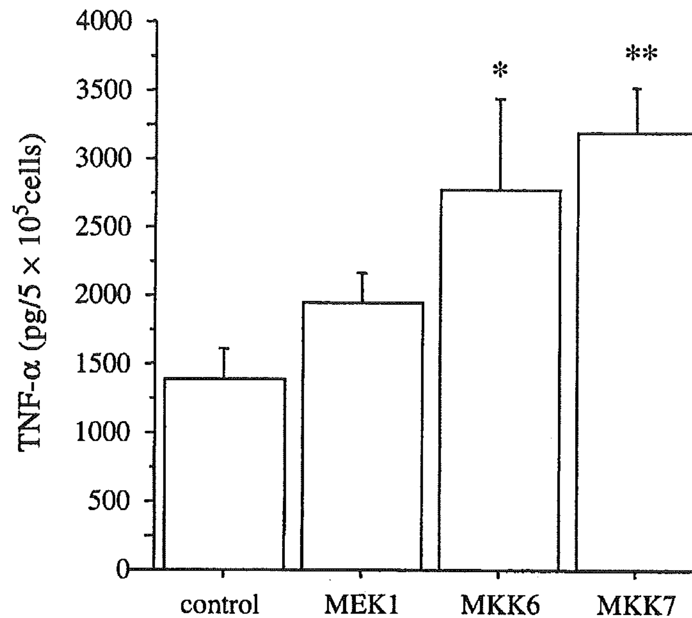

Fig. 7. The same experiment as in Fig. 6 was performed using isolated Kupffer cells of leptin receptor-deficient Zucker (*fa*/*fa*) rats. Values indicate the mean ± SD for triplicate determinations and the figure shows a result representative of three independent experiments. $*P<0.05$ vs. lacZ, $**P<0.02$ vs. lacZ.

vector system in terms of producing a high titer recombinant virus and high transduction efficiency, as well as its ability to transfer the gene of interest. The protein kinases MEK1, MKK6 and MKK7 (MAPK kinase) have been found to be the very specific upstream regulators of ERK, p38, and JNK, respectively (Denhardt, 1996; Kyriakis, 1999; Goedert et al., 1997). To examine the role of MAPK, we used the recombinant adenovirus (Ad)-MEK1, MKK6, and MKK7 to activate ERK, p38, and JNK. In this study, Ad-MEK1,-MKK6, and-MKK7 directly activate ERK, p38 and JNK through efficient transduction of the gene. The production of TNF-α was significantly increased in KCs infected with Ad-MKK6 or Ad-MKK7 rather than Ad-lacZ as a control without any change by Ad-MEK1. Our findings clearly

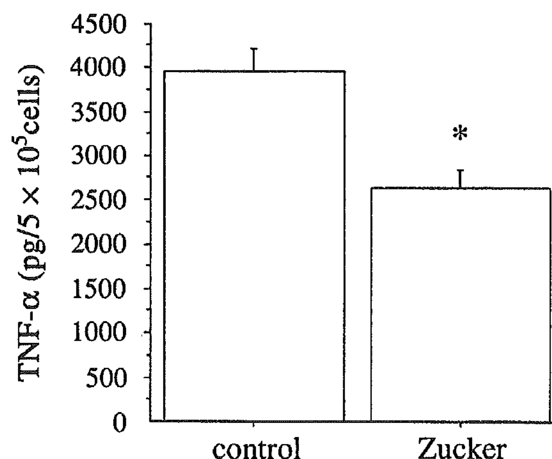

Fig. 8. Eighteen hours after the isolation of KCs from control and Zucker rats, the medium was replaced and 500 ng/ml of LPS was added. TNF-α in the medium was measured after a 6-h culture. Values indicate the mean ± SD for triplicate determinations and the figure shows a result representative of three independent experiments. $*p<0.05$.

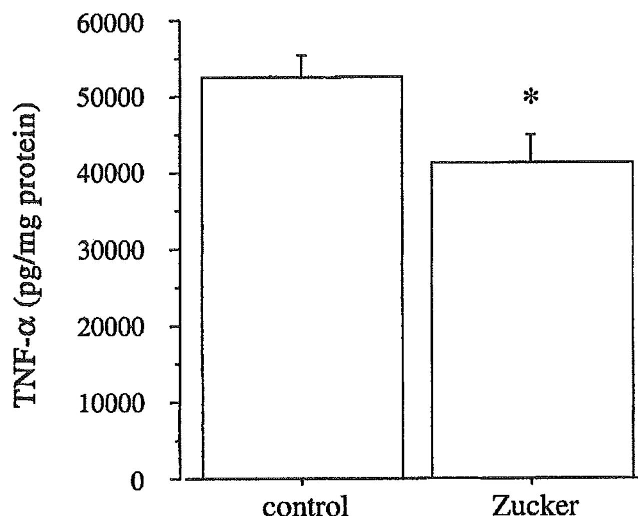

Fig. 9. Control and Zucker rats ($n=8$ each) were injected (i.p) with 500 μg/kg of LPS. One hour later, rats were killed and TNF-α in the liver was measure. *$P<0.03$.

demonstrate that the p38 and JNK MAPK pathways are involved in the enhancement of LPS-induced TNF-α production by leptin in KCs.

A clear definition of leptin signal transduction including upstream activators and downstream targets of the p38 and JNK MAPK pathways remains unknown. Regarding the downstream targets, a possible mechanism is through the regulation of an essential transcription factor, NF-κB. NF-κB plays an important role in the transcriptional regulation of proinflammatory cytokines including TNF-α and IL-1β (Bergmann et al., 1998; Gu et al., 2004). However, more studies are needed to clarify whether NF-κB is the target of MAPK activated by leptin.

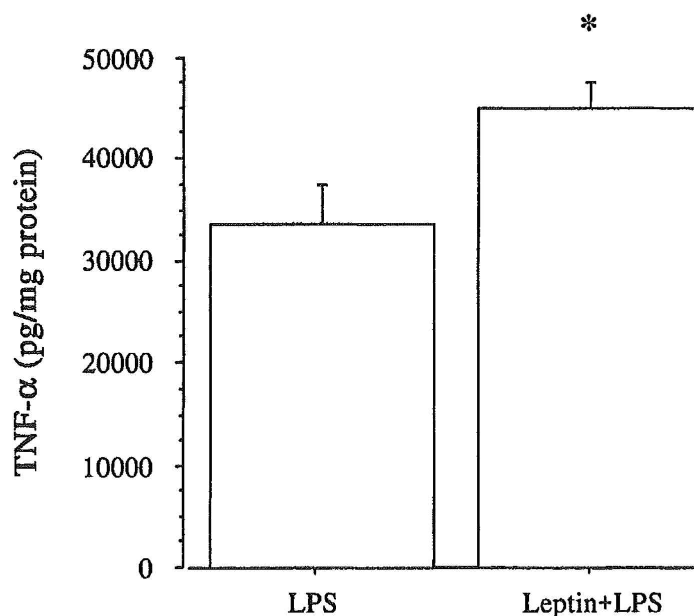

Fig. 10. Control rats ($n=8$ each) with or without 100 μg/kg of leptin were injected (i.p) 1 h later with 500 μg/kg of LPS. After an hour, rats were killed and TNF-α in the liver was measured. *$P<0.05$.

In conclusion, our findings support that p38 and JNK MAPK, but not ERK, are involved in leptin signal transduction and enhance LPS-induced TNF-α production in KCs.

Acknowledgement

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