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Full-length papers

Inhibition of TNF-α-induced RANTES expression in human hepatocyte-derived cells by fibrates, the hypolipidemic drugs

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Abstract

Increased concentrations and activity of plasma cytokines produced by monocytes, macrophages, and hepatocytes in patients with alcoholic liver diseases, correlate with the clinical course of liver diseases and are of prognostic value. Especially, high levels of circulating tumor necrosis factor (TNF)-α have been found to correlate with increased mortality in alcoholic hepatitis. Moreover, hepatic RANTES was increased in patients with alcoholic hepatitis. Thus, TNF-α-induced RANTES expression may have a critical role in cell-mediated liver injury associated with alcoholic hepatitis. Fibrates are widely used in the treatment of hyperlipidemia and lower triglyceride levels in patients with hyperlipidemia. Recently, several groups reported that bezafibrate, one of fibrates, is effective in primary biliary cirrhosis treatment. Additionally, it is reported that bezafibrate is effective in the treatment not only of primary biliary cirrhosis but also of chronic hepatitis C and tamoxifen-induced non-alcoholic steatohepatitis. We, here, presented that bezafibrate and fenofibrate repressed TNF-α-induced protein production and mRNA expression of RANTES in human hepatocyte-derived cells. Luciferase assay showed that bezafibrate and fenofibrate inhibited RANTES gene expression in response to TNF-α. Moreover, bezafibrate repressed TNF-α-induced DNA-binding activity of NF-κB. Thus, fibrates reduced TNF-α-induced NF-κB activation and RANTES expression, possibly suggesting that fibrates might be inhibitory agents of migration of inflammatory cells by RANTES to the liver in patients with alcoholic liver diseases. In line of these results, it might be possible that fibrates are therapeutic agents in alcoholic liver diseases.

Keywords: RANTES; Chemokine; Tumor necrosis factor-α; NF-κB; Fibrate

1. Introduction

Alcoholic liver diseases are the dominant precursor lesion in those subjects consuming excessive quantities of alcohol who eventually develop from fatty liver to cirrhosis [1-4]. Increased concentrations and activity of plasma cytokines produced by monocytes, macrophages, and hepatocytes in patients with alcoholic liver disease, correlate with the clinical course of liver disease and are of prognostic value [5-8]. Especially, high levels of circulating tumor necrosis factor (TNF)-α have been found to correlate with increased mortality in alcoholic hepatitis [6, 9-11]. Moreover, Rowell et al reported that hepatic RANTES was increased in patients with alcoholic hepatitis [12]. In experimental alcoholic liver disease in rats, RANTES was elevated in hepatocytes [13]. RANTES mainly migrates T lymphocytes to inflamed tissues [14,15] and is produced by fibroblasts, T lymphocytes, monocytes, and endothelial cells [16]. In addition to those cells, we have found that bile acids transcriptionally induced RANTES expression in human hepatoma cells [17]. Thereafter, it is reported that RANTES is induced by TNF-α in T lymphocytes, pulmonary vascular endothelial cells, bronchial epithelial cells, and granuloma cells from human preovulatory follicle [18-21]. Collectively, several evidences suggest that TNF-α-induced RANTES expression may have a critical role in cell-mediated liver injury associated with alcoholic hepatitis. In fact, immunohistochemical studies of alcoholic cirrhotic livers have indicated that both CD4 and CD8 T lymphocytes can be detected in expanded portal tracts and in periseptal areas associated with interface hepatitis and progressive fibrosis [22].

Fibrates are widely used in the treatment of hyperlipidemia and lower triglyceride levels in patients with hyperlipidemia [23,24]. Recently, several groups reported that bezafibrate, one of fibrates, is effective in primary biliary cirrhosis treatment [25-29]. In their reports, bezafibrate is more profitable than ursodeoxycholic acid in patients with primary biliary cirrhosis. Additionally, it is reported that bezafibrate is effective in the

treatment not only of primary biliary cirrhosis but also of chronic hepatitis C and tamoxifen-induced non-alcoholic steatohepatitis [30,31]. These fibrates promote β-oxidation and suppress acetyl CoA carboxylase activity in the liver [32]. In addition to these pharmacological effects, fibrates activate the peroxisome proliferator-activated receptor (PPAR) α, a member of the nuclear hormone receptor superfamily [33]. These PPARα are reported to be involved in cell proliferation and inflammatory response as well as lipid metabolism [34,35]. We have also reported that fibrates transcriptionally reduced bile acid-induced RANTES expression in human hepatoma cells, at least in part through inhibition of both DNA-binding activity and transcriptional activation of NF-κB [36]. However, nobody investigated the effects of fibrates on TNF-α-induced RANTES expression in hepatocytes. We, here, presented that bezafibrate and fenofibrate repressed TNF-α-induced RANTES expression in human hepatocyte-derived cells, possibly suggesting that fibrates might be inhibitory agents of migration of inflammatory cells by RANTES to the liver in patients with alcoholic liver diseases.

2. Materials and Methods

2.1. Cell culture and Chemical Reagents

Human hepatoma cell line HLE was provided by Japanese Cancer Research Resources Bank [37]. Cells were cultured in the minimum essential medium supplemented with 20% fetal calf serum (FCS), 100 μg/ml penicillin, and 100 U/ml streptomycin at 37 ^{o}C, in a humidified atmosphere of 5% CO_2 in air. Human primary hepatocyte cells were purchased from Applied Cell Biology Research Institute (Kirkland, WC, USA) and cultured in the CS-C Serum-Free Medium Kit (Applied Cell Biology Research Institute). Recombinant human TNF-α was purchased from Boehringer Mannheim Corporation

(Indianapolis, IN, USA) and dissolved in distilled water. Bezafibrate and fenofibrate were kindly gifted from Kissei (Tokyo, Japan) and Kaken pharmaceutical Co. Ltd (Tokyo, Japan), respectively and dissolved in dimethyl sulfoxide.

2.2. Enzyme-linked immunoassay (ELISA) for RANTES

HLE cells were grown to confluence in 60 mm collagen-coated culture dishes and treated with two fibrates in presence or absence of TNF-α. The supernatants were collected and analyzed for RANTES content. Levels of RANTES were measured using an RANTES monoclonal antibody sandwich ELISA employing two anti-RANTES antibodies recognizing different, non-competing determinants according to the instructions delivered with the Quantikine Human RANTES Immunoassay (R&D systems, Minneapolis, MN, USA).

2.3. RNA extraction and reverse transcriptase-polymerase chain reaction (RT-PCR) analysis

Total RNA was extracted from HLE cells according to the method of Chomczynski and Sacchi [38], which includes a single step of acid guanidium thiocyanate and phenol/chloroform extraction. RNA was quantified spectrophotometrically. Synthesis of the first strand of cDNA and PCR analysis were performed according to instructions delivered with the RNA PCR Kit (AMV) Ver.2 (TaKaRa, Tokyo, Japan) as described previously [17]. In brief, 500 nanograms of total RNA were subjected to first-strand cDNA synthesis in a 20 μl reaction containing 10 mM Tris-HCl (pH. 8.3), 50 mM KCl, 5 mM $MgCl_2$, 1 μM of each dNTP, in presence of 2.5 μM random 9 mer nucleotides, 20 U RNase inhibitor and 5 U avian myeloblastosis virus reverse transcriptase. After completion of first-strand cDNA synthesis, the reaction was stopped by heat inactivation (5 min, 99°C).

For RT-PCR analysis, cDNA amounts equivalent to 500 ng of total RNA were subjected to PCR amplification in a 50 μl reaction containing 10 mM Tris-HCl (pH. 8.3), 50 mM KCl, 5 mM $MgCl_2$, 200 μM of each dNTP, 20 μM of each primer, and 2.5 U of TaKaRa Taq DNA polymerase. For RANTES mRNA, samples from HLE cells were amplified at 94°C for 5 min, at 56°C for 90 seconds and at 72°C for 120 seconds, followed by 28 cycles at 94°C for 30 seconds, at 56°C for 90 seconds, and at 72°C for 120 seconds. The following primers were used for RANTES, sense 5'-GCTGTCATCCTCATTGCTAC-3', antisense 5'-TCCATCCTAGCTCATCTCCA-3'. For GAPDH mRNA, samples from HLE cells were amplified at 94°C for 5 min, at 56°C for 90 seconds and at 72°C for 120 seconds, followed by 23 cycles at 94°C for 30 seconds, at 56°C for 90 seconds, and at 72°C for 120 seconds. The following primers were used for GAPDH, sense 5'-ACATCGCTCAGACACCATGG-3', antisense 5'-GTAGTTGAGGTCAATGAAGGG-3'. Samples of 10 μl of the PCR products were electrophoresed through 1.5% agarose gels and visualized by ethidium bromide. Then, PCR was performed at different cycle numbers for each primer set to ensure that the assay was in the linear range for each molecule tested.

2.4. Reporter plasmid and luciferase enzyme assays

RANTES promoter-luciferase reporter plasmid was a kind gift from Dr. A. M. Krensky (Stanford University School of Medicine) and described previously [17]. HLE cells were plated in 6-well plastic dishes (IWAKI Glass, Funabashi, Japan) and washed three times with PBS, then medium was replaced with Opti-MEM medium (Life Technologies, Inc., Grand Island, NY). Plasmid mixtures were mixed with 4 μl of Trans-IT lipofection reagent (Life Technologies, Inc.) and added to the culture. After 6 h, the medium was replaced with fresh medium supplemented with 10% FCS and cells were treated with TNF-α and fibrates for 24 h. After normalization of transfection efficiency by

β-galactosidase expression, luciferase enzyme activity was determined by Lumat LB9501 (Berthold Japan, Tokyo, Japan).

2.5. Preparation of whole cell extracts and electrophoretic mobility shift assay (EMSA)

Whole cell extracts were prepared as described previously [39]. Briefly, HLE and human primary hepatocyte cells were washed twice with PBS and incubated in 20 mM HEPES (pH. 7.9), 350 mM NaCl, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.1 mM EGTA, 1% Nonidet P-40, 0.5 mM dithiothreitol (DTT), and 0.4 mM 4-(2-amino-ethyl)benzenesulfonyl fluoride hydrochloride (Boehringer Mannheim) on ice at 15 min. After centrifugation at 10,000 g for 20 min, the supernatant was used as a whole cell extract. Equal amounts of whole cell extracts (10 μg of protein) were incubated with 30,000 cpm of ^{32}P-labeled H_2k oligonucleotide probe for binding NF-κB. Reactions were performed in 20 μl of binding buffer containing 20 mM HEPES (pH. 8.4), 60 mM KCl, 4% Ficoll, 5 mM DTT, 1 μg of bovine serum albumin, and 2 μg of poly(dI-dC), for 20 min at 30 °C. The reaction mixture was loaded on a 4% polyacrylamide gel and run in 1X Tris-borate-EDTA buffer. The gel was dried and subjected to autoradiography.

2.6. Statistical analysis

Levels of significance for comparisons between samples were determined using Student's-*t* test distribution. Results were expressed as mean ± SE.

3. Results

3.1. Fibrates inhibited TNF-α-induced RANTES production in HLE cells

To exam effects of fibrates on TNF-α-induced RANTES production in HLE cells, we used two fibrates, bezafibrate and fenofibrate. For the measurement of antigenic RANTES protein, conditioned media were collected from cells treated with bezafibrate or fenofibrate in addition to TNF-α. As shown in Fig. 1A, no remarkable changes of RANTES protein production were shown by 100 μM of bezafibrate or fenofibrate alone for 48 h (lane 2, 3). By contrast, TNF-α significantly increased RANTES protein production in a dose-dependent manner (Fig.1A, lane 4 – 6 compared with lane 1). Moreover, TNF-α-induced RANTES proteins in conditioned media were clearly decreased by 100 μM of either bezafibrate or fenofibrate (Fig. 1A, lane 7, 8). In addition, inhibitory effects of bezafibrate and fenofibrate on TNF-α-induced RANTES production were in a dose-dependent manner, and the IC_{50} values were 12 μM and 9 μM, respectively (Fig. 1B). Cell viability was intact in the medium containing indicated concentrations of TNF-α (data not shown). In contrast, cell viability was completely intact in the medium containing 300 μM of bezafibrate and fenofibrate (data not shown). Thus, we thought that the TC_{50} values for bezafibrate and fenofibrate were more than 300 μM. Next, to examine effects of fibrates on TNF-α-induced RANTES mRNA expression, RT-PCR was performed. 100 μM of two fibrates, bezafibrate and fenofibrate, failed to change RANTES mRNA expression in absence of TNF-α (data not shown). By contrast, TNF-α up-regulated RANTES mRNA expression (Fig. 2, lane 2). Additionally, 100 μM of bezafibrate or fenofibrate clearly reduced TNF-α-induced RANTES mRNA (Fig. 2, lane 3, 4). Thus, we might show that fibrates diminished TNF-α-induced RANTES production.

3.2. Fibrates down-regulated TNF-α-induced RANTES gene expression in HLE cells

To test effects of fibrates on RANTES gene expression induced by TNF-α, luciferase enzyme assay was performed using the reporter plasmid. As shown in Fig. 3,

bezafibrate or fenofibrate did not change the basal levels of RANTES gene expression (lane 2-5). However, TNF-α-induced RANTES gene expression was significantly down-regulated by bezafibrate in a dose-dependent manner (Fig. 3 lane 6-9). In addition, 100 μM of fenofibrate also reduced RANTES gene expression at the basal level (Fig. 3, lane 10 compared with lane 6). These results might indicate that fibrates down-regulated TNF-α-induced RANTES production at the transcriptional level.

3.3. Fibrates decreased TNF-α-induced DNA-binding activity of NF-κB in human primary hepatocyte and HLE cells

To study effects of fibrates on TNF-α-induced DNA-binding activity of NF-κB in human primary hepatocyte and HLE cells, EMSA was performed. As shown in Fig. 4A, 100 μM of bezafibrate inhibited DNA-binding activity of NF-κB induced by 10 ng/ml of TNF-α for 1 h in human primary hepatocyte cells (lanes 1 - 3). In contrast, bezafibrate alone did not influence NF-κB activation in human primary hepatocyte cells (Fig. 4A, lane 4). Thereafter, 100 μM of fenofibrate also repressed DNA-binding activity of NF-κB in HLE cells (data not shown). These results might infer that fibrates inhibited both NF-κB activation and RANTES production in response to TNF-α. Next, to identify the Rel proteins associated with TNF-α-induced NF-κB-DNA complex, competition and supershift analyses were performed. We used whole cell extracts from human primary hepatocyte cells activated by 10 ng/ml of TNF-α for 1 h. Upper and lower bands were successfully competed using unlabeled NF-κB probe (Fig. 4B, lane 2 compared with lane 1), whereas an unrelated oligonucleotide was ineffective (Fig. 4B, lane 3). Moreover, upper band was clearly supershifted by anti-p65 and p50 antibodies, and lower band was supershifted by anti-p50 antibody but not by anti-p65 antibody (Fig. 4B, lanes 4, 5 compared with lane 1). In contrast, anti-c-Rel antibody did not supershifted both bands (Fig. 4B, lane 6). Therefore, TNF-α-induced NF-κB-DNA complexes consisted of p65 and p50 heterodimer.

4. Discussion

In the present study, we found that fibrates inhibited TNF-α-induced RANTES production and NF-κB activation in human hepatocyte-derived cells, possibly suggesting that this pharmacological effect of fibrates might be a therapeutic basis in patients with alcoholic liver diseases. Nelson et al demonstrated that multiple *cis*-acting elements interspersed within the RANTES promoter sequence contribute to promoter activity upon cell activation [40]. The upstream sequence of the RANTES gene contains a number of putative *cis*-acting elements for transcription factors such as activator protein (AP)-1, NF-interleukin 6, and NF-κB [40]. In addition, we have presented that NF-κB is a potent inducer of RANTES expression in response to bile acids in hepatocytes [17]. Several investigators also showed that TNF-α induction of RANTES was associated with activation of NF-κB in human pancreatic cancer cells and astrocytic cells [18, 41-43]. However, Ammit et al reported that TNF-α-induced RANTES gene expression is mediated via activation of AP-1 and NF-AT [44,45]. In hepatocytes, we showed that fibrates inhibited DNA-binding activity of NF-κB. Thus, it is possible that fibrates inhibited TNF-α-induced protein production and mRNA of RANTES via NF-κB activation. In fact, our previous data revealed that PPARα overexpression inhibited NF-κB -driven RANTES gene transcription [36]. Moreover, Delerive et al. have reported that PPARα physically interacts with p65 via its Rel homology domain which mediates homo- and heterodimerization and interaction with inhibitor of NF-κB in human aortic smooth muscle cells [46]. Alternatively, inhibitory effects might occur through competitive binding of transcriptional coactivators by PPARα or by PPARα-induced transcription factors. Moreover, longer exposure to fibrates was found to induce IκBα mRNA and protein expression in primary smooth muscle cells and hepatocytes [47]. Future study should investigate these inhibitory effects of fibrates via

PPARα on TNF-α-induced RANTES expression in human hepatocytes.

Specific genetic polymorphisms have been detected in patients with alcoholic liver disease, most notably mutations in the TNF promoter and mutations in alcohol-metabolizing enzyme systems [48]. Under these condition, it is suggested that TNF-α production by peripheral blood monocytes and Kupffer cells in patients with alcoholic hepatitis is easily increased, suggesting that such patients may have a lower threshold for TNF release in the presence of endotoxin [49]. Additionally, alcohol-induced TNF-α is related to hepatocyte-apoptosis and has an important role in toxic- and cell-mediated hepatic injury [50,51].

In summary, we indicate that fibrates decreased TNF-α-induced RANTES gene expression in human hepatocyte-derived cells, possibly at least in part, through inhibition of NF-κB activation, suggesting that fibrates are inhibitory agents of migration of inflammatory cells by RANTES to the liver in patients with alcoholic liver diseases.

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Legend for Figures

Fig. 1

Effect of fibrates on TNF-α-induced RANTES protein in HLE cells. ELISA was performed using conditioned media as described in *Materials and methods*. Conditioned media were collected after treatment with the indicated concentration of TNF-α and/or of bezafibrate and fenofibrate for 48 h. Experiments were performed quadruplicately. Results were presented as mean ± SE of three independent experiments. A: Effects of fibrates. *, $p < 0.05$ v. s. lane 1 and †, $p < 0.05$ v. s. lane 6. B: Dose-dependency. *, $p < 0.05$ v. s. 0 μM of bezafibrate or fenofibrate.

Fig. 2

Effect of fibrates on TNF-α-induced RANTES mRNA expression. RT-PCR analysis was performed using total RNA in HLE cells as described in *Materials and methods*. Cells were treated with 10 ng/ml of TNF-α and 100 μM of bezafibrate or fenofibrate for 24 h. GAPDH was used as an internal control. (Bottom) Densitometric quantification of RANTES mRNA / GAPDH mRNA ratio. RANTES mRNA / GAPDH mRNA ratio in unstimulated cells was taken as 1.0.

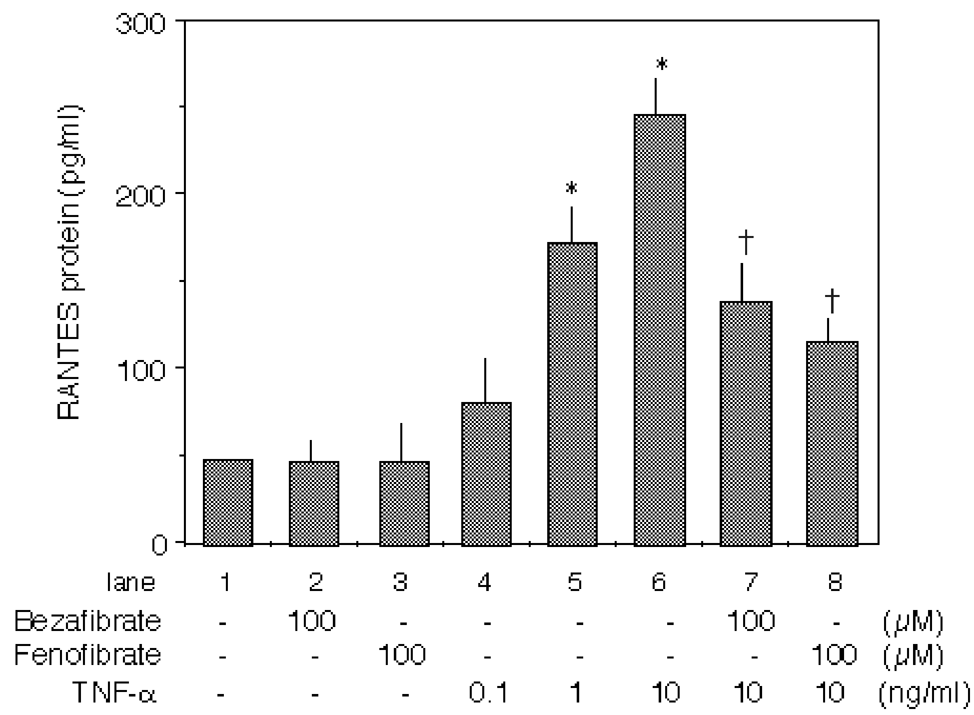
Fig. 3

Effects of bezafibrate and fenofibrate on TNF-α-induced RANTES gene expression. Cells were transfected by lipofection with 1 μg of a reporter plasmid containing the RANTES promoter. After transfection, cells were co-incubated with 10 ng/ml of TNF-α and/or the indicated concentration of bezafibrate and 100 μM of fenofibrate. After 24 h, cellular extracts were prepared for luciferase enzyme assay. Experiments were performed quadruplicately. Levels of luciferase activity of a reporter plasmid alone in unstimulated cells were taken as 1.0 (lane 1). Results were mean ± SE of three independent experiments.

*, $p < 0.05$ v. s. lane 6.

Fig. 4

Effect of bezafibrate on TNF-α-induced DNA-binding activity of NF-κB in human primary hepatocyte cells. A: Effect of bezafibrate on TNF-α-induced NF-κB activation. Cells were treated with 10 ng/ml of TNF-α and/or 100 μM of bezafibrate for 1 h. After preparation of whole cell extract, EMSA was performed using H_2k oligonucleotides for a probe as described in *Materials and methods*. Specific NF-κB bands and free DNA were shown as closed and open triangles, respectively. Data was representative of three similar experiments. B: Competition and supershift analysis. For competition (comp), EMSA was performed using specific (SC) unlabelled NF-κB probe or nonspecific (NC) oligonucleotides on extracts obtained following 1 h of 10 ng/ml of TNF-α. For supershift analysis, EMSA was performed using anti-p65, p50 or c-Rel antibody (Ab) on extracts obtained following 1 h of 10 ng/ml of TNF-α. Specific NF-κB band (p65/p50 or p50/p50) and free DNA are shown as closed and open triangles, respectively. Data is representative of three similar experiments.


RANTES protein (pg/ml)
300
200
100
0
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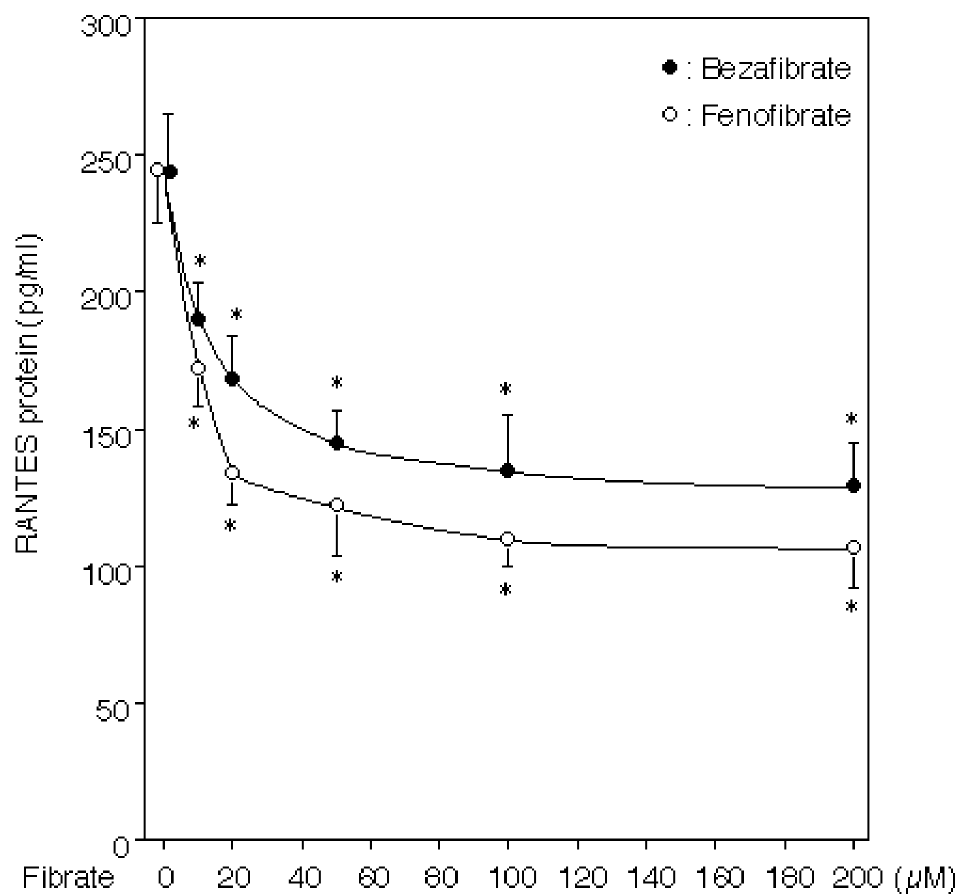
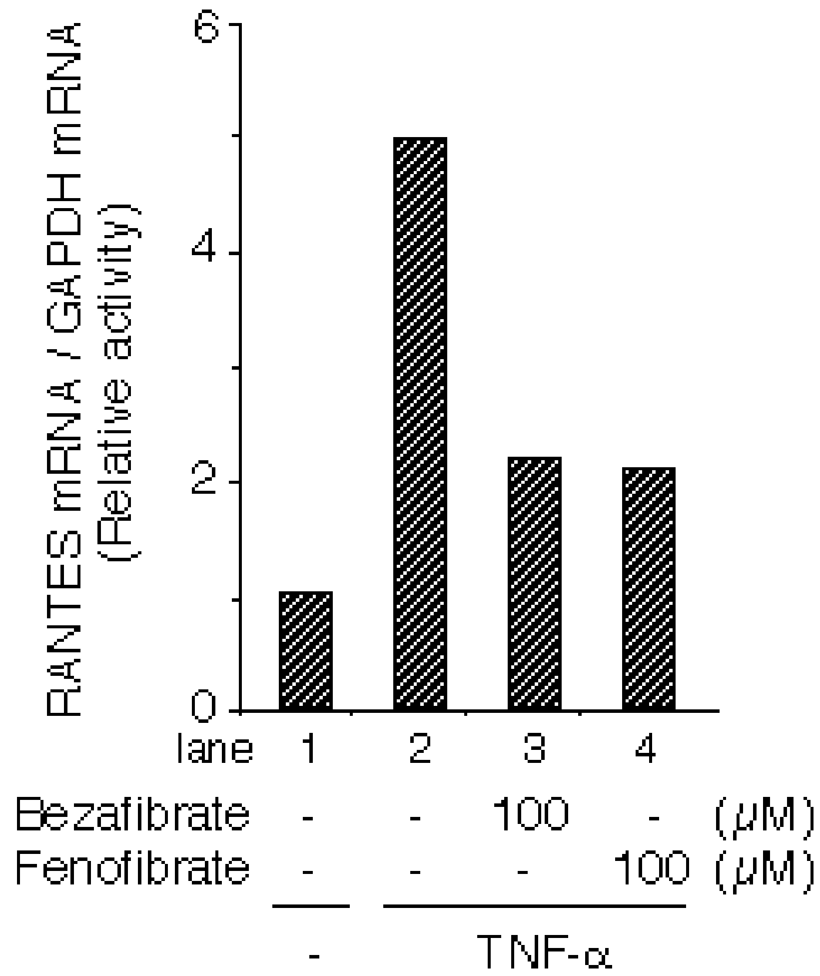
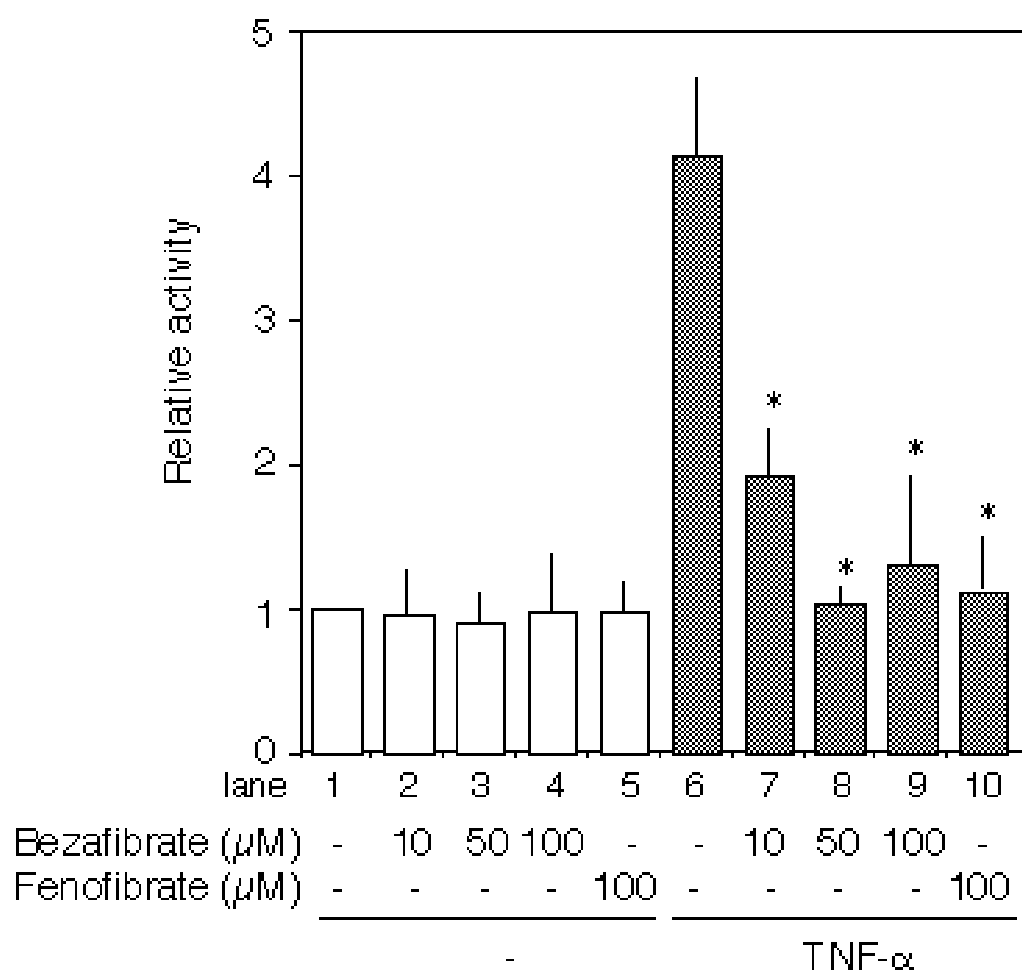
lane 1 2 3 4 5 6 7 8
Bezafibrate - 100 - - - - 100 - (μM)
Fenofibrate - - 100 - - - - 100 (μM)
TNF-α - - - 0.1 1 10 10 10 (ng/ml)



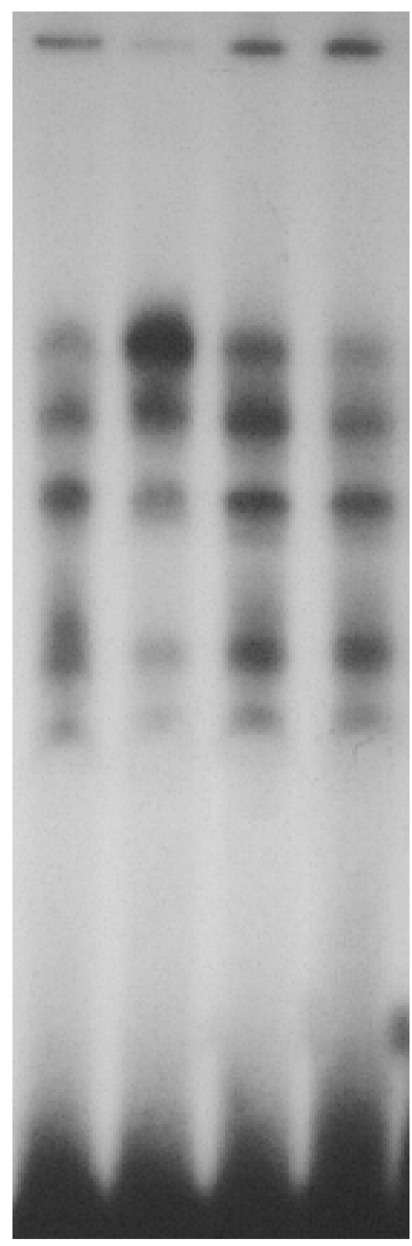
300
250
200
150
100
50
0
RANTES protein (pg/ml)
● : Bezafibrate
○ : Fenofibrate
*
Fibrate
0
20
40
60
80
100
120
140
160
180
200
(μM)

RANTES
GAPDH

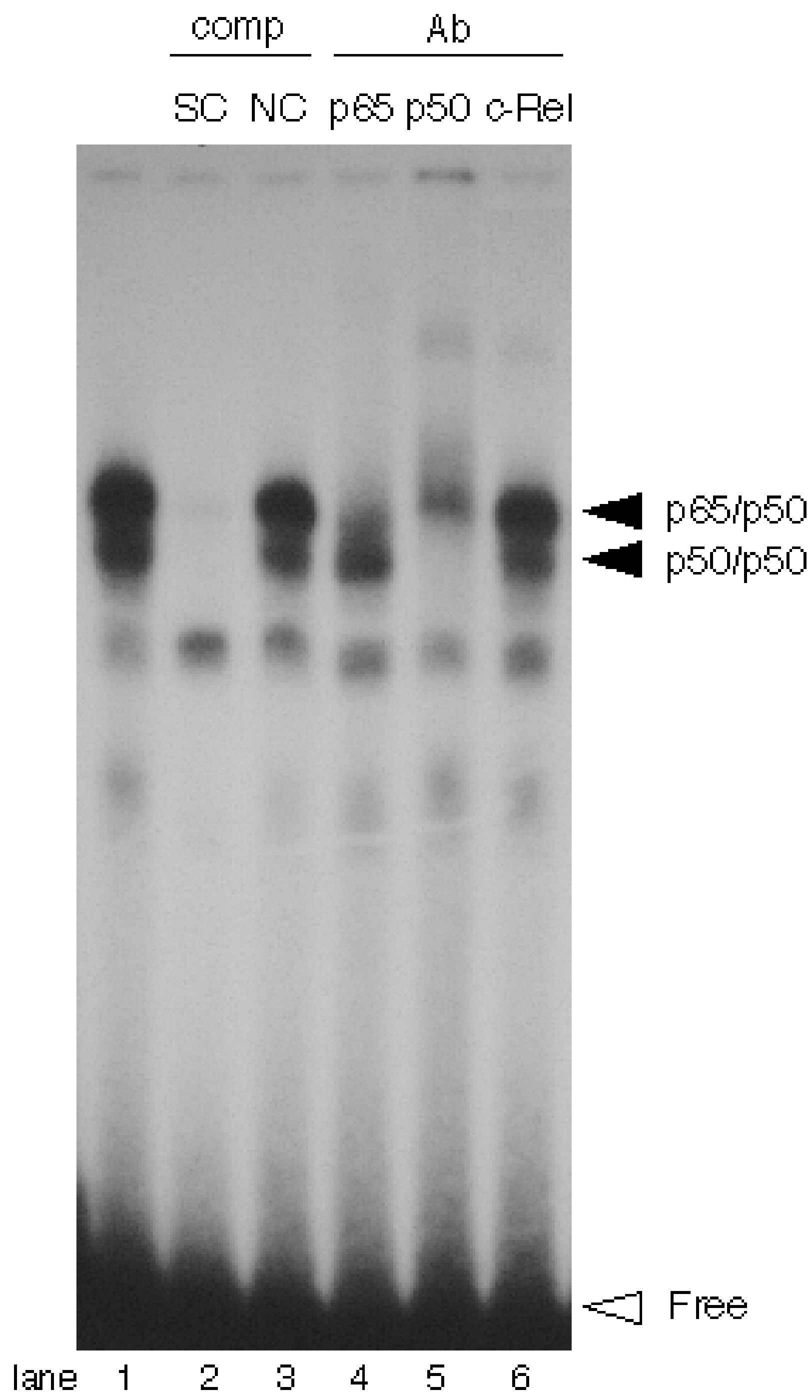

RANTES mRNA / GAPDH mRNA
(Relative activity)
6
4
2
0
lane 1 2 3 4
Bezafibrate - - 100 - (μM)
Fenofibrate - - - 100 (μM)
- TNF-α



Relative activity
5
4
3
2
1
0
*
*
*
*
lane 1 2 3 4 5 6 7 8 9 10
Bezafibrate (μM) - 10 50 100 - - 10 50 100 -
Fenofibrate (μM) - - - - 100 - - - - 100
-
TNF-α



TNF-α
Bezafibrate
-
-
+
-
+
+
-
+
NF-κB
Free
lane
1
2
3
4


comp
Ab
SC
NC
p65
p50
c-Rel
p65/p50
p50/p50
Free
lane
1
2
3
4
5
6