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TNF-α induced RANTES chemokine expression via activation of NF-κB and p38 MAP kinase: roles of TNF-α in alcoholic liver diseases.

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Short title; TNF-α induced RANTES via NF-κB and p38

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Abstract

Background/Aims: Increased concentration of plasma TNF-α correlates with the clinical course of alcoholic liver diseases. In addition, hepatic RANTES which migrates CD4 T lymphocytes to liver is increased in patients with alcoholic hepatitis. We investigated that roles of TNF -α on RANTES expression in hepatocytes.

Methods: HLE cells were treated with TNF-α in the presence, or absence of several inhibitors. Enzyme-linked immunoassay and reverse transcriptase-polymerase chain reaction were performed for the measurement of protein production and mRNA of RANTES, respectively. Moreover, DNA-binding activity of NF-κB was investigated using electrophoretic mobility shift assay. To exam effects of TNF-α on RANTES gene expression, luciferase assay was performed.

Results: TNF-α clearly up-regulated RANTES expression in a time-dependent fashion and induced DNA-binding activity of NF-κB. Moreover, TNF-α -induced RANTES expression was completely inhibited by SB203580, but not calphostin C and wortmannin. Luciferase assay showed that TNF-α increased RANTES gene expression and mutation of NF-κB binding sites in the RANTES promoter ablated TNF-α inducibility.

Conclusions: We presented that RANTES was transcriptionally induced in human hepatoma cells by treatment with TNF-α via activation of NF-κB and p38 MAP kinase, presumably suggesting that TNF-α-induced expression of RANTES plays important roles in cell-mediated liver injury in alcoholic liver diseases.

Key words; TNF-α, RANTES, NF-κB, p38, MAP kinase, hepatocyte,

(Introduction part)

Alcoholic hepatitis is the dominant precursor lesion in those subjects consuming excessive quantities of alcohol who eventually develop cirrhosis (1-4). Increased concentrations and activity of plasma cytokines produced by monocytes, macrophages, and hepatocytes, including interleukin-1 and tumor necrosis factor (TNF)-α in patients with alcoholic liver diseases, correlate with the clinical course of liver disease and are of prognostic value (5-8). Especially, high levels of circulating TNF-α have been found to correlate with increased mortality in alcoholic hepatitis (6, 9-11). Moreover, the production of TNF-α by isolated monocytes is increased in patients with fulminant hepatic failure and severe alcoholic hepatitis (12,13). Therefore, several lines of clinical evidence in patients suggest that TNF-α may have a critical role in the pathogenesis of liver injury associated with alcoholic hepatitis.

Regulated upon activation, normal T-cells expressed and secreted (RANTES), which is one of the CC chemokines, mainly migrates T lymphocytes to inflamed tissues (14, 15) and is produced by fibroblasts, T lymphocytes, monocytes, and endothelial cells (16). Furthermore, Rowell et al reported that hepatic RANTES was increased in patients with alcoholic hepatitis (17). In experimental alcoholic liver disease in rats, RANTES was elevated in hepatocytes (18). 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 (19). CD8 T lymphocytes have a tendency to occupy the peripheral areas of the infiltrates in portal tracts, whereas CD4 T lymphocytes are more centrally located (19). Furthermore, CD8 T lymphocytes lead to hepatic necrosis. Thus, it is suggested that liver-infiltrating T lymphocytes from patients with alcoholic liver diseases are accumulated by RANTES and accelerates hepatic injury.

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 (20-23). However, whether TNF-α directly induced RANTES in hepatocytes is yet unknown. We presented here direct evidence for the fact that RANTES was transcriptionally induced in human hepatoma cells by treatment with TNF-α, presumably via its cognate NF-κB sites in the RANTES promoter. Furthermore, TNF-α-induced RANTES production in hepatocytes required p38 mitogen-activated protein (MAP) kinase activation. Our data might indicate that TNF-α-induced expression of RANTES plays important roles in cell-mediated liver injury in alcoholic liver diseases.

Materials and Methods

Cell culture

Human hepatoma cell line HLE was provided by Japanese Cancer Research Resources Bank (JCRB) (24). The HLE cells were cultured in the minimum essential medium (MEM) supplemented with 20% fetal calf serum (GIBCO BRL, New York, U.S.A.) supplemented with 100 μg/ml penicillin, and 100 U/ml streptomycin at 37 ^{o}C, in a humidified atmosphere of 5% CO_2 in air.

Ligands

Recombinant human TNF-α was purchased from Boehringer Mannheim Corporation (Indianapolis, IN) and was dissolved in distilled water supplemented with 0.1 % human serum albumin. Protein kinase C inhibitor calphostin C was purchased from Wako Chemical (Tokyo, Japan) and dissolved in dimethyl sulfoxide. Phosphatidylinositol (PI)3 kinase inhibitor wortmannin and p38 MAP kinase inhibitor SB203580 were purchased from Calbiochem-Behring Co. (La Jolla, CA) and Sigma chemical CO (St. Louis, MO), respectively and dissolved in ethanol.

Measurement of RANTES protein

Aliquots of cultured medium were frozen at - 70 ^{o}C until assayed. Levels of RANTES were measured with a specific enzyme-linked immunoassay (ELISA) kit (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions. Sensitivity of the assay was as follows: RANTES, 5 pg / ml.

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 (25), which includes a single step of acid guanidium thiocyanate (GTC) and

phenol/chloroform extraction. RNA was quantitated spectrophotometrically. Synthesis of the first strand of cDNA and PCR analysis were performed according to the instructions delivered with the RNA PCR Kit (AMV) Ver.2 (TaKaRa). 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^{o}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^{o}C for 5 min, at 56^{o}C for 90 seconds and at 72^{o}C for 120 seconds, followed by 28 cycles at 94^{o}C for 30 seconds, at 56^{o}C for 90 seconds, and at 72^{o}C for 120 seconds. The following primers were used: for RANTES, sense 5'-GCTGTCATCCTCATTGCTAC-3', antisense 5'-TCCATCCTAGCTCATCTCCA-3'; 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.

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

Whole cell extracts were prepared as described previously (26). Briefly, 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 ^{o}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.

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 (27). The κB1 site at positions -53 to -44 and the κB2 site at positions -39 to -30 of the RANTES promoter were respectively mutated in 5'-flanking sequence of the RANTES gene by oligonucleotide-directed, site-specific mutagenesis according to instructions delivered with the site-directed mutagenesis kit (Promega). The mutant sequences utilized for the κB1 and the κB2 were GGAAACTtaC and GGtaATGCCC, respectively. Lower case letters represent mutant nucleotides. 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 for 24 h. After normalization of transfection efficiency by β-galactosidase expression, luciferase enzyme activity was determined by Lumat LB9501 (Berthold Japan, Tokyo, Japan).

Results

Production of antigenic RANTES by TNF-α

To examine the effect of TNF-α on RANTES production in HLE cells, ELISA was performed. Conditioned media were collected from cells treated with 10 ng/ml of TNF-α for the indicated times. As shown in Fig. 1A, TNF-α significantly increased RANTES protein in a time-dependent manner. After 24 h-culture in the presence of 10 ng/ml of TNF-α, the l. Cell viability was intact in the medium containing 10 ng/ml of TNF-α (data not shown). Next, to test the effect of TNF-α on RANTES mRNA expression, RT-PCR analysis was performed. As shown in Fig. 1B, 10 ng /ml of TNF-α time-dependently increased steady-state levels of RANTES mRNA in HLE cells. At 12 h incubation of TNF-α, RANTES mRNA expression reached a plateau in HLE cells (Fig. 1B). Thus, we showed that TNF-α clearly induced RANTES production in human hepatoma cells.

Induction of DNA-binding activity of NF-κB by TNF-α

To determine the effect of TNF-α on NF-κB activation in HLE cells, EMSA were performed. Whole cell extracts were prepared from HLE cells following 0.5 to 24 h incubations with TNF-α (10 ng/ml). The κB-binding activity of the protein extracts was analyzed using an oligonucleotide encoding the NF-κB site of the major histocompatibility complex class I promoter. Here, DNA-binding activity of NF-κB was strongly induced by TNF-α and was maximal at 1 h (Fig. 2A, lane 4). Moreover, TNF-α continuously activated DNA-binding activity of NF-κB until 10 hours tested (Fig. 2A, lanes 3 - 7). In addition, TNF-α-induced mobility shifts were abrogated by prior heat inactivation (95^{o}C, 10 min) (data not shown). To identify Rel proteins associated with TNF-α-induced NF-κB-DNA complexes, competition and supershift analyses were performed. Whole cell extracts from HLE cells activated by TNF-α (10 ng/ml) were used. Successful competition was observed using unlabeled NF-κB probe (Fig. 2B, lane 2), whereas an unrelated oligonucleotide was

ineffective (Fig. 2B, lane 3). Moreover, TNF-α-induced DNA-binding activity of NF-κB was clearly supershifted by anti-p65 and p50 antibodies, but not by anti-c-Rel antibody (Fig. 2B, lanes 4 - 6 compared with lane 1).

Roles of NF-κB in TNF-α-induced RANTES promoter activity

To test effects of TNF-α on RANTES gene expression, luciferase assay was performed using the reporter plasmid. According to dosage, TNF-α increased RANTES gene expression (Fig. 3B, lanes 1-3). Thus, TNF-α induced RANTES expression at the transcriptional level. Next, to define more precisely the roles of κB sites in TNF-α-controlling RANTES expression, each cis-acting element of NF-κB binding sites was mutated individually. The RANTES gene upstream sequence contains the two putative NF-κB binding sites (κB1 and κB2; -44 and -30 relative to the transcription start site, respectively). Figure 3A presents mutations of individual *cis*-acting elements of NF-κB binding sites. The base vector for these constructions was the wild-type pN construct (Fig. 3A, upper). Mutation of either κB1 or κB2 ablated TNF-α inducibility, suggesting that both sites were required for TNF-α induction of RANTES (Fig. 3B, lanes 4 - 9). These results might indicate that TNF-α induced RANTES gene expression through NF-κB activation.

Roles of p38 MAP kinase in TNF-α-induced RANTES production

To clarify roles of protein kinase C, PI_3 kinase, and p38 MAP kinase in TNF-α-induced RANTES production, we used calphostin C, wortmannin, and SB203580 and performed ELISA and RT-PCR analysis. SB203580 distinctly reduced both TNF-α-induced protein and mRNA of RANTES, though calphostin C could not inhibit TNF-α-effects (Fig. 4A and 4B). In contrast, TNF-α-induced RANTES production was more increased by wortmannin at the levels of protein and mRNA (Fig. 4A and 4B). Moreover, wortmannin alone significantly induced RANTES protein production in a dose-dependent fashion (Fig. 4A,

lane 4, 5). Collectively, these results might show that TNF-α up-regulated RANTES production in HLE cells via p38 MAP kinase.

Discussion

In the present study, we found that TNF-α induced RANTES expression in cultured human hepatoma cells. Moreover, TNF-α-induced RANTES expression might be through its cognate NF-κB binding sites in the RANTES promoter. Functional studies indicate that multiple *cis*-acting elements interspersed within the RANTES promoter sequence contribute to promoter activity upon cell activation (28). The upstream sequence of the RANTES gene contains a number of putative *cis*-acting elements for transcription factors such as activator protein-1, nuclear factor-interleukin 6, and NF-κB (28). In addition, we asserted that mutation on NF-κB binding sites markedly reduced TNF-α inducibility, possibly indicating that NF-κB is a potent inducer of RANTES expression in response to TNF-α. Nelson et al have already demonstrated that both NF-κB binding sites are critically required for the basal activity of the RANTES promoter in activated T lymphocytes (29). Moreover, we have also previously shown that chenodeoxycholic acid-induced RANTES expression required both NF-κB binding sites in the RANTES promoter (27). Thus, we might confirm that two NF-κB binding sites in the RANTES promoter played an important role in the functional transactivation in response to TNF-α in human hepatoma cells.

p38 MAP kinase was originally identified in pre-B lymphocytes transfected CD14 and macrophages in response to endotoxin and hyperosmolarity (30). In addition to these stimuli, p38 MAP kinase is also activated by physicochemical stresses, cytokines (31,32). Our results showed that TNF-α-induced RANTES production was completely inhibited by p38 MAP kinase inhibitor SB203580 in a dose-dependent manner. Hashimoto et al have also reported that abrogation of p38 MAP kinase activity by SB203580 repressed TNF-α-induced p38 MAP kinase activity and RANTES production in human pulmonary vascular endothelial cells (21). However, they did not refer to NF-κB activation in response to TNF-α. p38 MAP kinase is known to regulate various transcription factors, such as

ATF-2 and NF-κB, which in turn modulate the transactivation capacity of the transcription enhancers (33-35). Moreover, TNF-α is known to activate p38 MAP kinase in many kinds of cells (reviewed in 36,37), though it is not investigated whether TNF-α induced p38 MAP kinase in hepatocytes. Furthermore, it was already known that TNF-α activates IκB kinase (IKK) β and induced NF-κB through degradation of IκBα (reviewed in 38,39). Several investigators have demonstrated that NF-κB activation is due to dependent- or independent-pathway of p38 MAP kinase (40-42). Goebeler et al presented that NF-κB-dependent expression of monocyte chemoattractant protein-1 in primary endothelial cells was regulated by both IKK-mediated IκB phosphorylation and p38-dependent transcriptional activity of p65 (40). In cardiac myocytes, TNF-α-activation of p38 MAP kinase induced IKKβ (41). On the other hand, it is demonstrated that NF-κB is not a direct target for the p38 MAP kinase pathway in osteoblasts, since neither TNF-α-induced DNA-binding activity of NF-κB nor TNF-α-induced IκBα degradation was modulated by SB203580 (42). We presented in this study that inhibitions of either NF-κB binding to DNA or p38 MAP kinase clearly repressed TNF-α-induced RANTES production in hepatocytes. Further study should examine whether RANTES gene expression is induced by collaboration of NF-κB and p38 MAP kinase.

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 (43). 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-α release in the presence of endotoxin (13). Interestingly, it is reported that alcohol consumption alone does not lead to the development of marked liver necrosis (44). Thus, it is suggested that increased TNF-α induces RANTES gene expression in hepatocytes and secreted RANTES from hepatocytes accumulates T lymphocytes into liver.

In the presence of liver-infiltrated CD4 T lymphocytes, cytotoxic CD8 T lymphocytes are activated and able to attack hepatocytes directly (45). Collectively, TNF-α-induced production of RANTES in liver has an important role in cell-mediated hepatic injury in alcoholic liver diseases.

In summary, we presented that TNF-α induced protein and mRNA expression of RANTES in human hepatoma cells though activation of NF-κB and p38 MAP kinase, possibly suggesting that TNF-α play a pivotal role in migration of inflammatory cells by RANTES to the liver in patients with alcoholic liver diseases.

Acknowledgements

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References

1. Scherlock S. Alcohol-related liver disease. Br Med Bull 1982;38:67-70.
2. Lieber CS, DeCarli LM, Sorrell MF. Experimental methods of ethanol administration. Hepatology 1989;10:501-10.
3. Hall PD. Pathological spectrum of alcoholic liver disease. Alcohol 1994;suppl2:303-13.
4. Lieber CS. Alcoholic liver injury: pathogenesis and therapy in 2001. Pathol Biol 2001;49:738-52.
5. McClain CJ, Cohen DA, Dinarello CA, Cannon JG, Shedlofsky SI, Kaplan AM. Serum interleukin-1 (IL-1) activity in alcoholic hepatitis. Life Sci 1986;39:1479-85.
6. Bird GL, Sheron N, Goka AK, Alexander GJ, Williams RS. Increased plasma tumor necrosis factor in severe alcoholic hepatitis. Ann Intern Med 1990;112:917-20.
7. Tilg H, Wilmer A, Vogel W, Herold M, Nolchen B, Judmaier G, et al. Serum levels of cytokines in chronic liver diseases. Gastroenterology 1992;103:264-74.
8. Daniluk J, Szuster-Ciesielska A, Drabko J, Kandefer-Szerszen M. Serum cytokine levels in alcohol-related liver cirrhosis. Alcohol 2001;23:29-34.
9. Felver ME, Mezey E, McGuire M, Mitchell MC, Herlong HF, Veech GA, et al. Plasma tumor necrosis factor α predicts decreased long-term survival in severe alcoholic hepatitis. Alcohol Clin Exp Res 1990;14:255-9.
10. Khoruts A, Stahnke L, McClain CJ, Logan G, Allen JI. Circulating tumor necrosis factor, interleukin-1 and interleukin-6 concentrations in chronic alcoholic patients. Hepatology 1991;13:267-76.
11. Menon KV, Gores GJ, Shah VH. Pathogenesis, diagnosis, and treatment of alcoholic liver disease. Mayo Clin Proc 2001;76:1021-9.
12. Muto Y, Nouri-Aria KT, Meager A, Alexander GJ, Eddleston AL, Williams R. Enhanced tumour necrosis factor and interleukin-1 in fulminant hepatic failure. Lancet

1988;2:72-4.

13. McClain CJ, Cohen DA. Increased tumor necrosis factor production by monocytes in alcoholic hepatitis. Hepatology 1989;9:349-51.
14. Schall TJ, Bacon K, Toy KJ, Goeddel DV. Selective attraction of monocytes and T lymphocytes of the memory phenotype by cytokine RANTES. Nature 1990;347:669-71.
15. Ward SG, Bacon K, Westwick J. Chemokines and T lymphocytes: more than an attraction. Immunity 1998;9:1-11.
16. Schall TJ. Biology of the RANTES/SIS cytokine family. Cytokine 1991;3:165-83.
17. Maltby J, Wright S, Bird G, Sheron N. Chemokine levels in human liver homogenates: associations between GROα and histopathological evidence of alcoholic hepatitis. Hepatology 1996;24:1156-60.
18. Nanji AA, Jokelainen K, Rahemtulla A, Miao L, Fogt F, Matsumoto H, et al. Activation of nuclear factor κB and cytokine imbalance in experimental alcoholic liver disease in the rat. Hepatology 1999;30:934-43.
19. Chedid A, Mendenhall CL, Moritz TE, French SW, Chen TS, Morgan TR, et al. Cell-mediated hepatic injury in alcoholic liver disease. Veterans Affairs Cooperative Study Group 275. Gastroenterology 1993;105:254-66.
20. Moriuchi H, Moriuchi M, Fauci AS. Nuclear factor-κB potently up-regulates the promoter activity of RANTES, a chemokine that blocks HIV infection. J Immunol 1997;158:3483-91.
21. Hashimoto S, Gon Y, Asai Y, Asai Y, Machino T, Jibiki I, et al. p38 MAP kinase regulates RANTES production by TNF-α-stimulated human pulmonary vascular endothelial cells. Allergy 1999;54:1168-72.
22. Hashimoto S, Matsumoto K, Gon Y, Maruoka S, Kujime K, Hayashi S, et al. p38 MAP kinase regulates TNFα-, IL-1α- and PAF-induced RANTES and GM-CSF production

by human bronchial epithelial cells. Clin Exp Allergy 2000;30:48-55.

23. Machelon V, Nome F, Emilie D. Regulated on activation normal T expressed and secreted chemokine is induced by tumor necrosis factor-α in granulosa cells from human preovulatory follicle. J Clin Endocrinol Metab 2000;85:417-24.
24. Doi I. Establishment of a cell line and its clonal sublines from a patient with hepatoblastoma. Gann 1976; 67:1-10.
25. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem;162:156-9.
26. Hirano F, Chung M, Tanaka H, Maruyama N, Makino I, Moore DD, et al. Alternative splicing variants of IκBβ establish differential NF-κB signal responsiveness in human cells. Mol Cell Biol 1998;18:2596-607.
27. Hirano F, Kobayashi A, Hirano Y, Nomura Y, Fukawa E, Makino I. Bile acids regulate RANTES gene expression through its cognate NF-κB binding sites. Biochem Biophys Res Commun 2001;288:1095-101.
28. Nelson PJ, Kim HT, Manning WC, Goralski TJ, Krensky AM. Genomic organization and transcriptional regulation of the RANTES chemokine gene. J Immunol 1993;151:2601-12.
29. Nelson PJ, Ortiz BD, Pattison JM, Krensky,AM. Identification of a novel regulatory region critical for expression of the RANTES chemokine in activated T lymphocytes. J Immunol 1996;157:1139-48.
30. Han J, Lee JD, Bibbs L, Ulevitch RJ. A MAP kinase targeted by endotoxin and hyperosmolarity in mammalian cells. Science 1994;265:808-11.
31. Lee JC, Laydon JT, McDonnell PC, Gallagher TF, Kumar S, Green D, et al. A protein kinase involved in the regulation of inflammatory cytokine biosynthesis. Nature 1994;372:739-46.
32. Raingeaud J, Gupta S, Rogers JS, Dickens M, Han J, Ulevitch RJ, et al.

Pro-inflammatory cytokines and environmental stress cause p38 mitogen-activated protein kinase activation by dual phosphorylation on tyrosine and threonine. J Biol Chem 1995;270:7420-26.

33. Brinkman BM, Telliez JB, Schievella AR, Lin LL, Goldfeld AE. Engagement of tumor necrosis factor (TNF) receptor 1 leads to ATF-2- and p38 mitogen-activated protein kinase-dependent TNF-α gene expression. J Biol Chem 1999;274:30882-6.
34. Vanden Berghe W, Plaisance S, Boone E, De Bosscher K, Schmitz ML, Fiers W, et al. p38 and extracellular signal-regulated kinase mitogen-activated protein kinase pathways are required for nuclear factor-κB p65 transactivation mediated by tumor necrosis factor. J Biol Chem 1998;273:3285-90.
35. Hanafusa H, Ninomiya-Tsuji J, Masuyama N, Nishita M, Fujisawa J, Shibuya H, et al. Involvement of the p38 mitogen-activated protein kinase pathway in transforming growth factor-β-induced gene expression. J Biol Chem 1999;274:27161-7.
36. Wajant H, Grell M, Scheurich P. TNF receptor associated factors in cytokine signaling. Cytokine Growth Factor Rev 1999;10:15-26.
37. Vanden Berghe W, Vermeulen L, De Wilde G, De Bosscher K, Boone E, Haegeman G. Signal transduction by tumor necrosis factor and gene regulation of the inflammatory cytokine interleukin-6. Biochem Pharmacol 2000;60:1185-95.
38. Chen G, Goeddel DV. TNF-R1 signaling: a beautiful pathway. Science 2002;296:1634-5.
39. Cao Z, Tanaka M, Regnier C, Rothe M, Yamit-hezi A, Woronicz JD, et al. NF-κB activation by tumor necrosis factor and interleukin-1. Cold Spring Harb Symp Quant Biol 1999;64:473-83.
40. Goebeler M, Gillitzer R, Kilian K, Utzel K, Brocker EB, Rapp UR, et al. Multiple signaling pathways regulate NF-κB-dependent transcription of the monocyte chemoattractant protein-1 gene in primary endothelial cells. Blood 2001;97:46-55.

41. Craig R, Larkin A, Mingo AM, Thuerauf DJ, Andrews C, McDonough PM, et al. p38 MAPK and NF-κB collaborate to induce interleukin-6 gene expression and release. Evidence for a cytoprotective autocrine signaling pathway in a cardiac myocyte model system. J Biol Chem 2000;275:23814-24.
42. Chae HJ, Chae SW, Chin HY, Bang BG, Cho SB, Han KS, et al. The p38 mitogen-activated protein kinase pathway regulates interleukin-6 synthesis in response to tumor necrosis factor in osteoblasts. Bone 2001;28:45-53.
43. Grove J, Daly AK, Bassendine MF, Day CP. Association of a tumor necrosis factor promoter polymorphism with susceptibility to alcoholic steatohepatitis. Hepatology 1997;26:143-6.
44. Cao Q, Batey RG, Pang GT, Clancy RL. Ethanol-alatered liver associated T cells mediated liver injury in rats administered concanavalin (Con A) or lipopolysaccharide (LPS). Alcohol Clin Exp Res 1999;23:1660-7.

Figure Legends

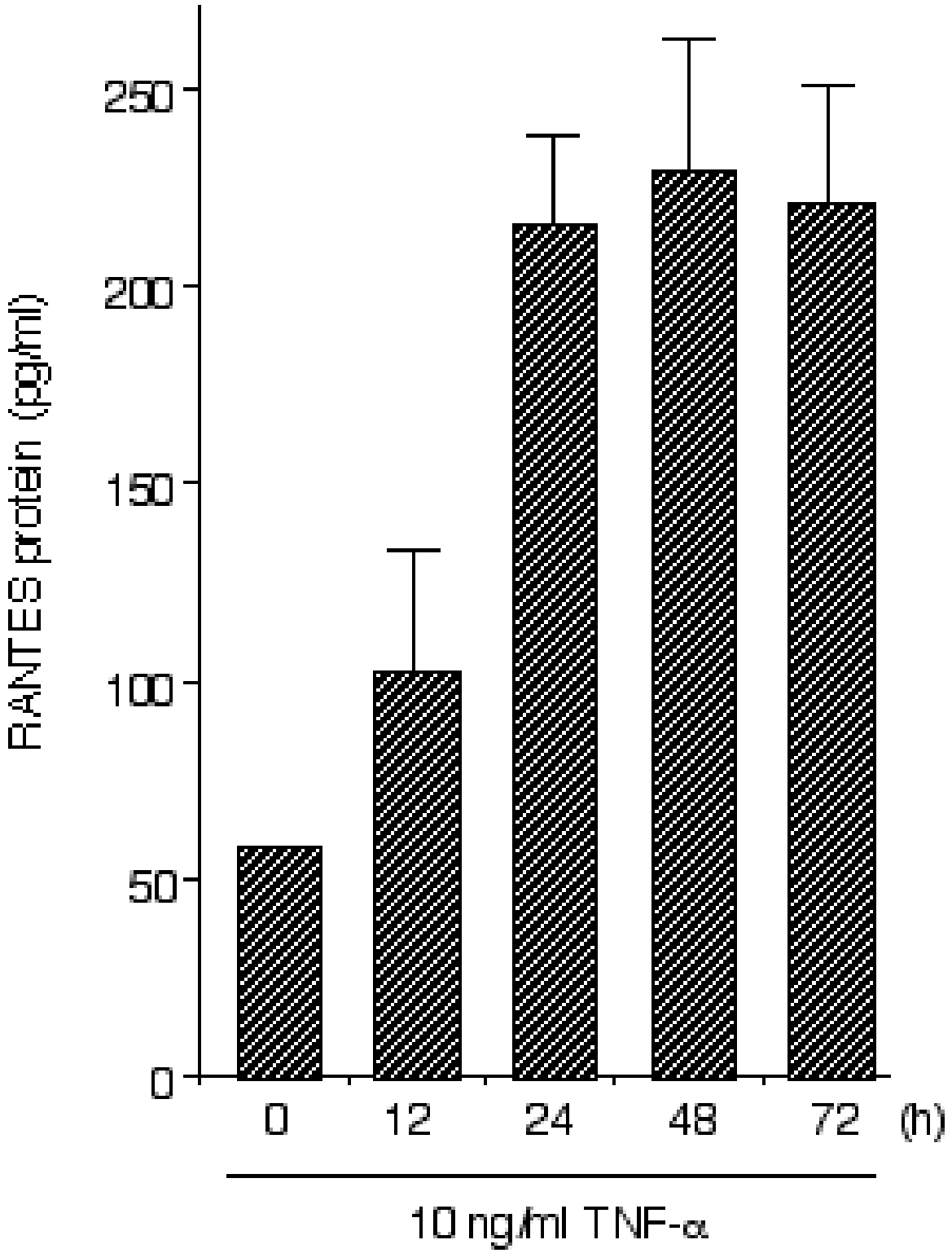
FIGURE 1. Effect of TNF-α on RANTES production in HLE cells. A: RANTES protein in conditioned media. ELISA was performed as described in *Materials and Methods*. Conditioned media were collected after treatment with 10 ng/ml of TNF-α for indicated times. Experiments were performed quadruplicately. Results were presented as mean + SE of three independent experiments. B: RANTES mRNA. RT-PCR analysis was performed using total RNA in HLE cells as described in *Materials and Methods*. GAPDH was used as an internal control. (Bottom) Densitometric quantification of RANTES mRNA / GAPDH mRNA ratio.

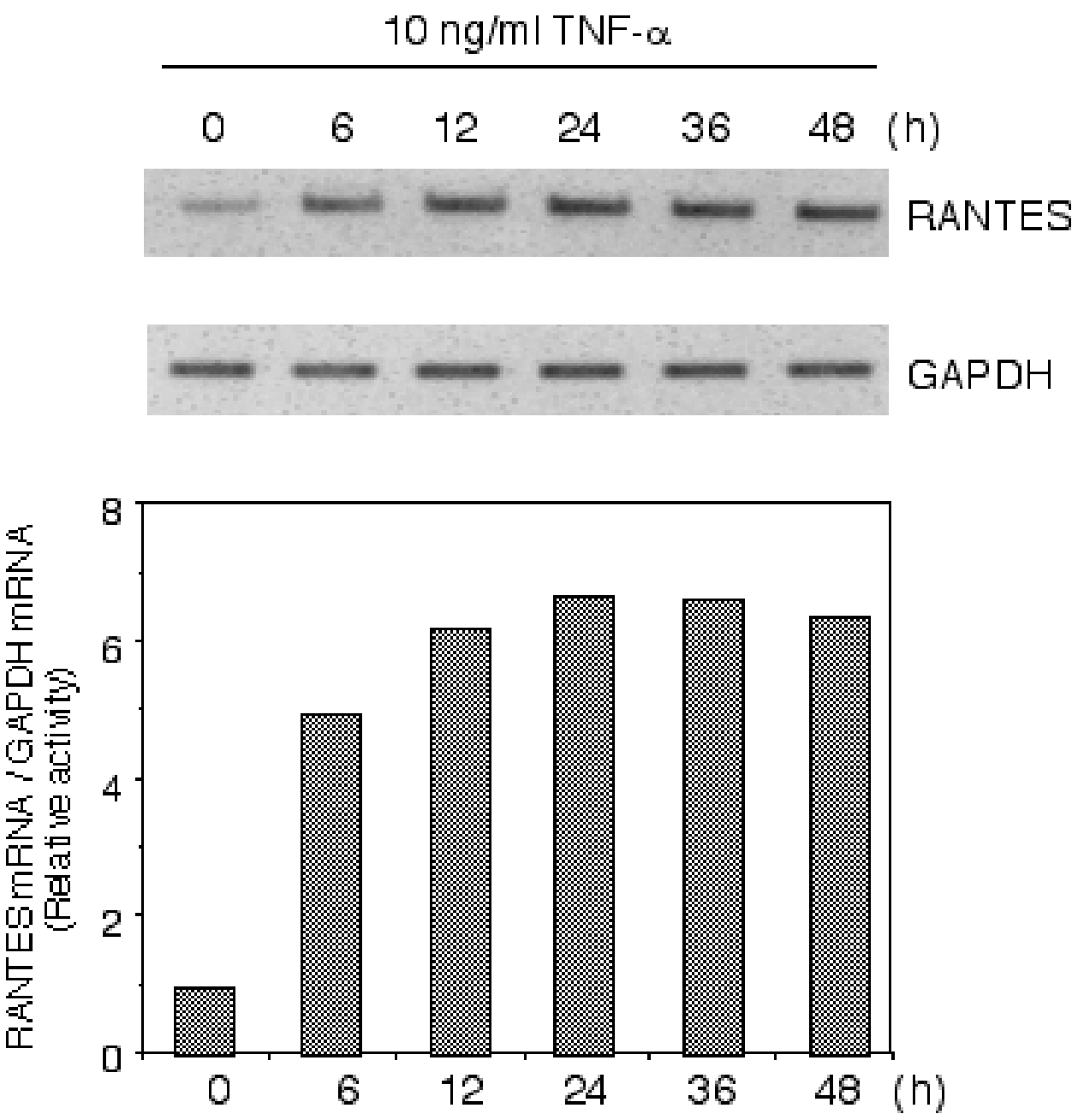
FIGURE 2. DNA-binding activity of NF-κB was induced by TNF-α in HLE cells. EMSA using H_2k oligonucleotide for a probe was performed as described in *Materials and Methods*. A: Time course. HLE cells were treated with 10 ng/ml of TNF-α for indicated times. Specific NF-κB bands were shown as closed triangles. Unbound labeled oligonucleotides were presented as open triangles. Data was representative of three similar experiments. B: Competition and supershift analysis. For competition, EMSA was performed using specific (SC) or nonspecific (NC) oligonucleotides on extracts obtained following 1 h of 10 ng/ml TNF-α. For supershift analysis, EMSA was performed using anti-p65, p50, or c-Rel antibody on extracts obtained following 1 h of 10 ng/ml of TNF-α. Specific NF-κB bands (p65 and p50) were shown as closed triangles. Unbound labeled oligonucleotides were presented as open triangles. Data is representative of three similar experiments.

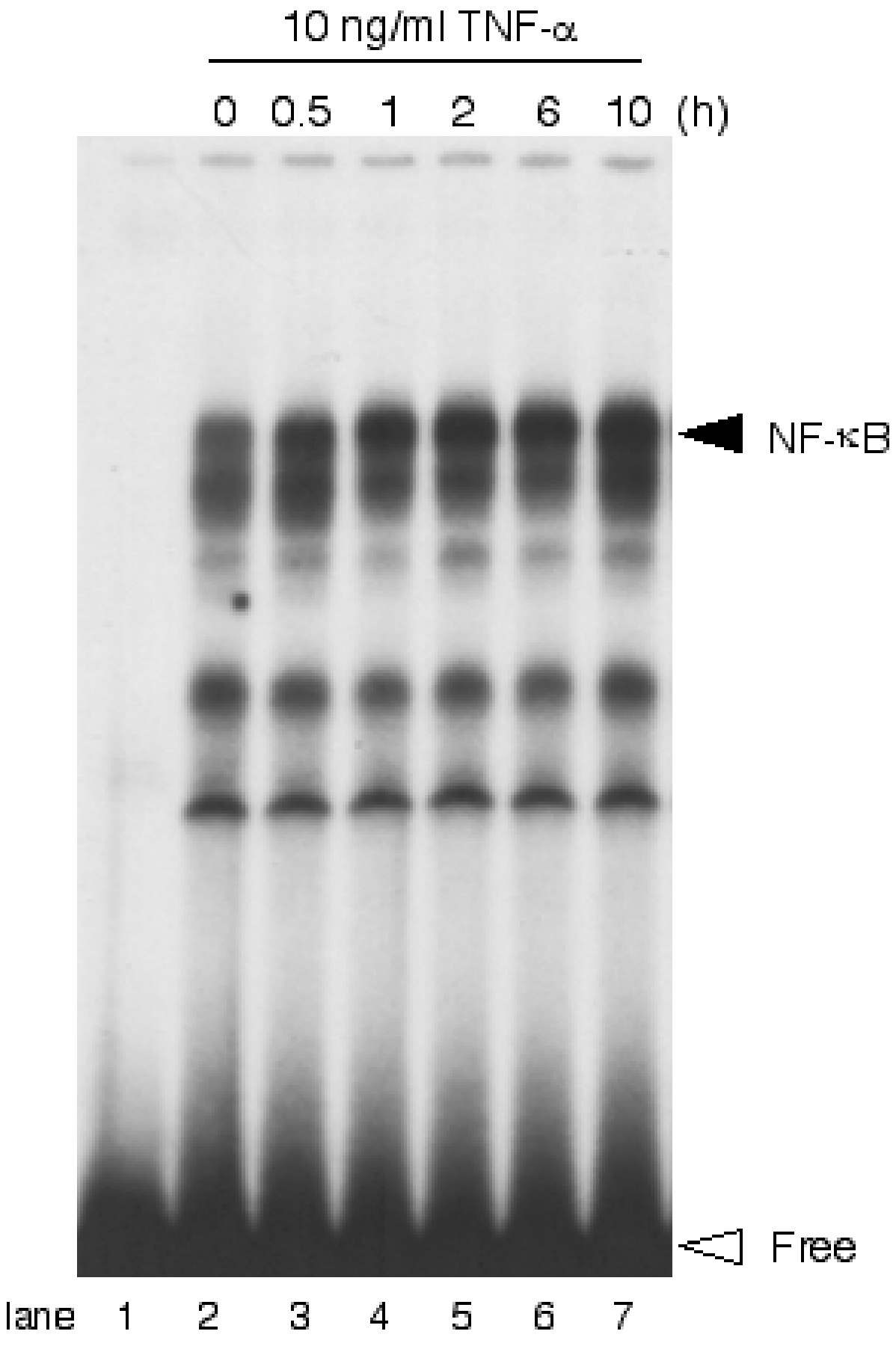
FIGURE 3. TNF-α induced RANTES gene expression through two *cis*-acting elements of κB sites on the RANTES promoter. A: Schematic maps of the RANTES reporter

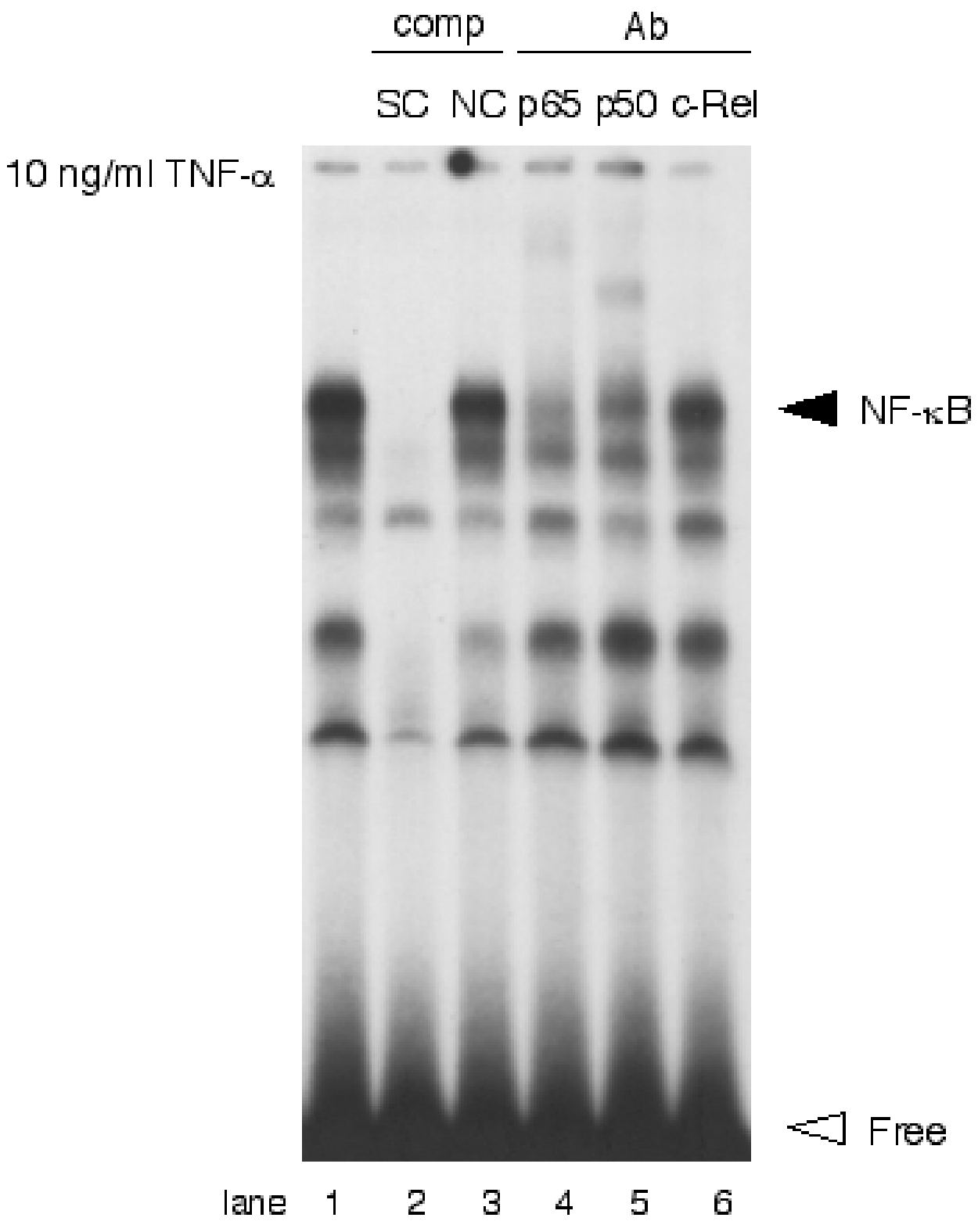
constructions. Two NF-κB binding sites were shown as κB1 and κB2. Here, pN was wild type RANTES promoter construct plasmid. Mutated sites are shown with an X. Mutation of κB1 or κB2 site is shown as pmκB1 or pmκB2, respectively. B: Transcriptional effect of TNF-α on RANTES gene expression. Cells were transfected with 1 μg of pN, pmκB1 and pmκB2. After transfection, cells were incubated with indicated concentrations of TNF-α. After 24 hours, cellular extracts were prepared for luciferase enzyme assay. Experiments were performed quadruplicately. Levels of luciferase activity of pN in unstimulated cells were taken as 1.0. Results were mean + SE of three independent experiments.

FIGURE 4. Effect of calphostin C, wortmannin and SB203580 on TNF-α-induced RANTES production in HLE cells. A: RANTES protein in conditioned media. ELISA was performed as described in *Materials and Methods*. Conditioned media were collected after treatment with 10 ng/ml of TNF-α for 24 h in the presence or absence of calphostin C, wortmannin and SB203580. Experiments were performed quadruplicately. Results were presented as mean + SE of three independent experiments. B: RANTES mRNA. RT-PCR analysis was performed using total RNA in HLE cells as described in *Materials and Methods*. GAPDH was used as an internal control. Cells were treated with 10 ng/ml of TNF-α for 24 h in the presence or absence of calphostin C, wortmannin and SB203580. (Bottom) Densitometric quantification of RANTES mRNA / GAPDH mRNA ratio.

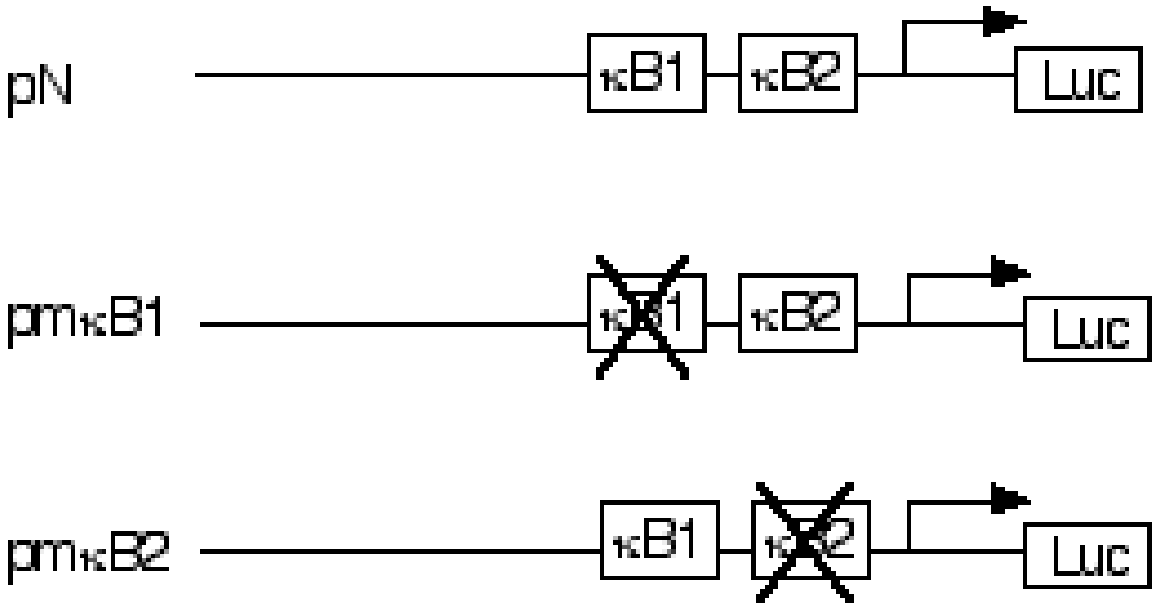

RANTES protein (pg/ml)
250
200
150
100
50
0
0
12
24
48
72
(h)
10 ng/ml TNF-α

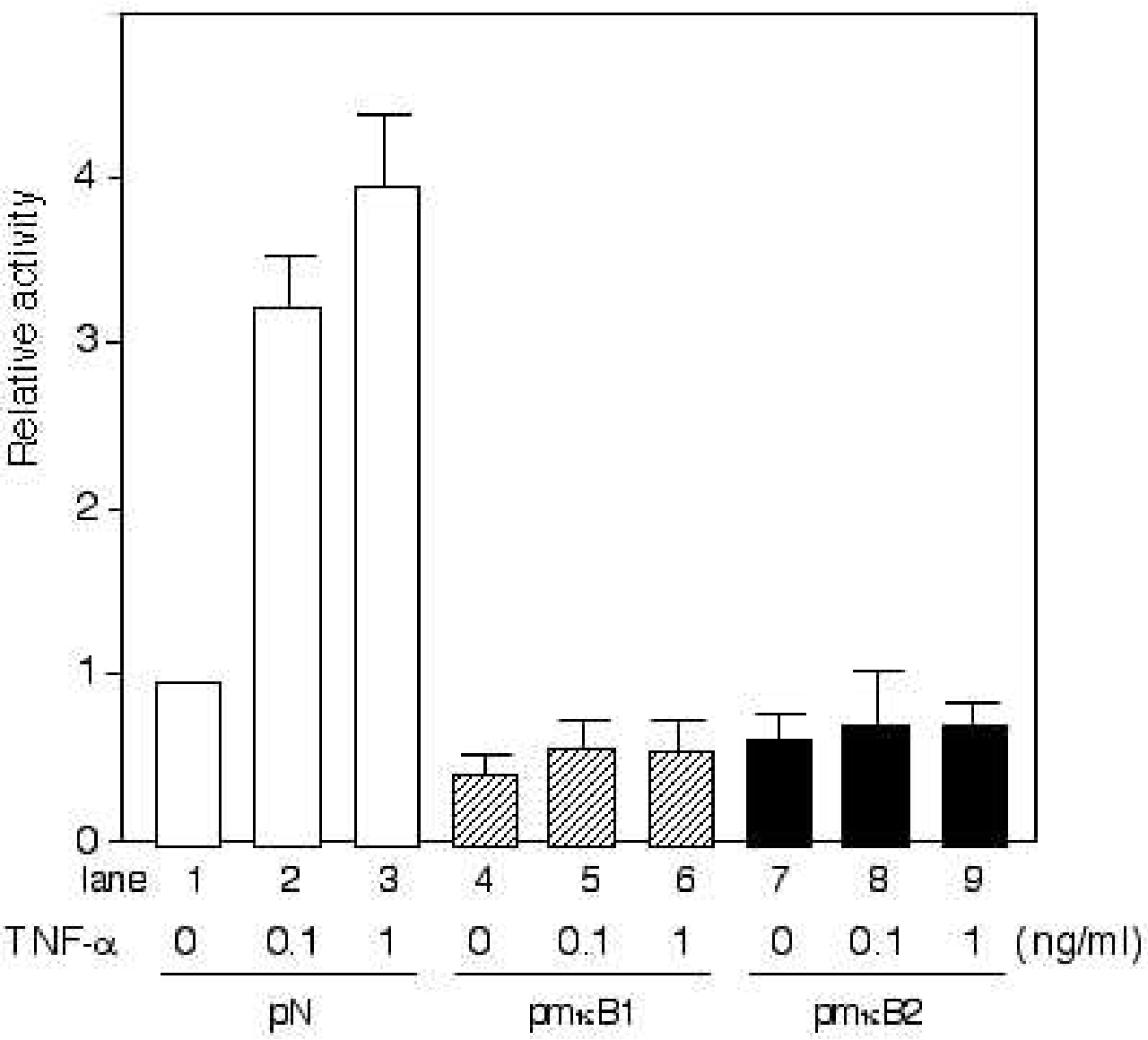

10 ng/ml TNF-α
0
6
12
24
36
48
(h)
RANTES
GAPDH
RANTES mRNA / GAPDH mRNA
(Relative activity)
0
2
4
6
8
0
6
12
24
36
48
(h)

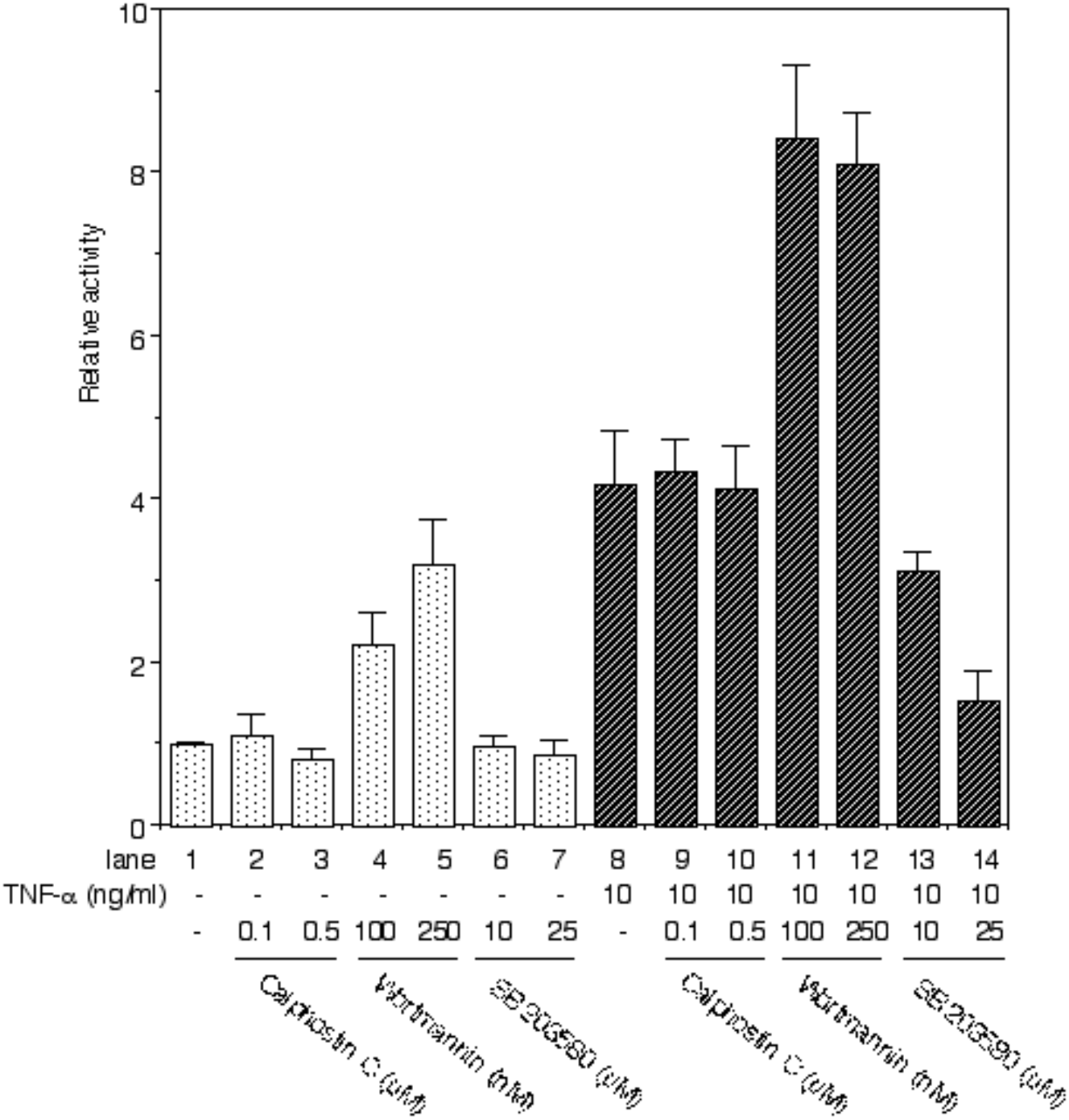

10 ng/ml TNF-α
0 0.5 1 2 6 10 (h)
NF-κB
Free
lane 1 2 3 4 5 6 7

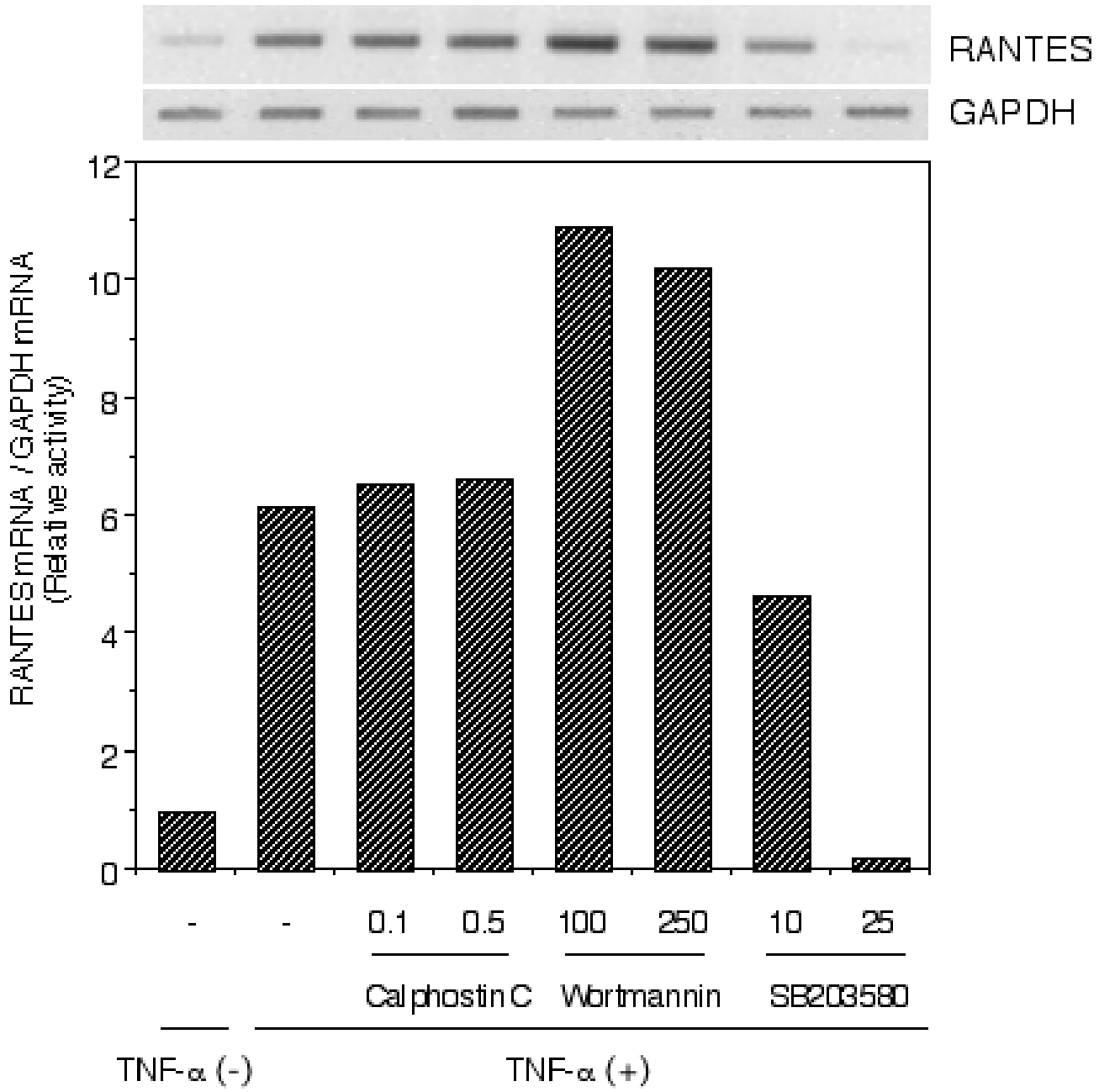

comp
Ab
SC
NC
p65
p50
c-Rel
10 ng/ml TNF-α
NF-κB
Free
lane 1 2 3 4 5 6

RANTES promoter-construct plasmids




RANTES-Luc
Relative activity
0
1
2
3
4
lane 1 2 3 4 5 6 7 8 9
TNF-α 0 0.1 1 0 0.1 1 0 0.1 1 (ng/ml)
pN
pmκB1
pmκB2


Relative activity
10
8
6
4
2
0
lane 1 2 3 4 5 6 7 8 9 10 11 12 13 14
TNF-α (ng/ml) - - - - - - - 10 10 10 10 10 10 10
- 0.1 0.5 100 250 10 25 - 0.1 0.5 100 250 10 25
Calphostin C (uM)
Wortmannin (nM)
SB 203580 (uM)
Calphostin C (uM)
Wortmannin (nM)
SB 203580 (uM)


RANTES
GAPDH
RANTES mRNA / GAPDH mRNA
(Relative activity)
12
10
8
6
4
2
0
-
-
0.1
0.5
100
250
10
25
Calphostin C
Wortmannin
SB203580
TNF-α (-)
TNF-α (+)