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Bile Acids Regulate RANTES Gene Expression through Its Cognate NF-κB Binding Sites

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ABSTRACT

Regulated upon activation, normal T-cells expressed and secreted (RANTES) mainly migrates memory type CD4+ T-lymphocytes to inflamed tissues. In this study, we examined effects of bile acids on RANTES gene expression in human hepatoma cells. Upon stimulation with hydrophobic bile acids, RANTES proteins were clearly increased. Semiquantitative RT-PCR analysis revealed that chenodeoxycholic acid (CDCA) induced RANTES mRNA expression. Moreover, RANTES was transcriptionally induced in two hepatoma cell lines by CDCA, presumably via its cognate NF-κB binding sites in the RANTES promoter. Electrophoretic mobility shift assay revealed that hydrophobic bile acids induced DNA-binding activity of NF-κB. Additionally, the magnitude of inducibility was closely associated with the hydrophobicity of bile acids. In conclusion, we might indicate that bile acids induced RANTES gene expression in human hepatoma cells, possibly suggesting that bile acids play an important role in migration of inflammatory cells by RANTES to the liver in patients with primary biliary cirrhosis.

Key Words:

RANTES; bile acid; chenodeoxycholic acid; NF-κB.

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Primary biliary cirrhosis (PBC) is a chronic cholestatic liver disease characterized by gradual inflammatory destruction of intrahepatic bile ducts with later portal fibrosis and ultimately cirrhosis and infiltration of large numbers of T lymphocytes, into the affected portal tracts (1, 2). Several studies have suggested that most of these infiltrating T lymphocytes are CD4+ helper type (3, 4). Especially, it is reported that liver-infiltrating CD4+ T lymphocytes of patients with PBC are mainly memory type of CD4+ T lymphocytes (5, 6). In addition, α-naphthylisothiocyanate-treated rats characterized by cholestasis have portal inflammation centered on damaged bile ducts, an influx of CD4+ T lymphocytes in portal areas (7). Thus, it is possible that cholestasis itself is related to infiltrate CD4+ T lymphocytes to the liver in patients with PBC.

It is well known that endogenous bile acids including CDCA, cholic acid, deoxycholic acid (DCA), and lithocholic acid (LCA) accumulate in the liver during cholestasis such as PBC (8, 9). In contrast, ursodeoxycholic acid (UDCA), which is a 7β isomer of CDCA, is considered to be a nonhepatotoxic hydrophilic bile acid that may reverse the potential hepatotoxicity of endogenous bile acids (10), and is often used in patients with cholestasis to improve liver dysfunction (11-13). Notably, a beneficial effect of UDCA in patients with PBC has been demonstrated in double-blind controlled studies (14-16). In those studies, it is shown that treatment with UDCA improves biochemical parameters, together with most histological features except liver fibrosis in patients with PBC (14-16). Terasaki et al reported that administration of UDCA reduced the hepatocellular infiltration of activated T lymphocytes in patients with PBC (17). Therefore, these clinical reports might show that intrahepatic bile acids affect the infiltration of T lymphocytes to the liver. However, experimental evidence is completely lacking to confirm these hypotheses.

RANTES, which is one of the CC chemokines, mainly migrates memory type of CD4+ T lymphocytes to inflamed tissues (18, 19) and is produced by fibroblasts, T lymphocytes, monocytes, and endothelial cells (20). Furthermore, Rowell et al reported that

hepatic RANTES was increased in patients with PBC (21). Thus, it is suggested that liver-infiltrating CD4+ T lymphocytes from patients with PBC are accumulated by RANTES. However, the mechanism involved in increased production of RANTES in PBC is yet unknown. We presented here direct evidence for the fact that RANTES was transcriptionally induced in two human hepatoma cell lines by treatment with CDCA, presumably via its cognate NF-κB sites in the RANTES promoter. Furthermore, the magnitude of inducibility was closely correlated with the hydrophobicity of bile acids.

MATERIALS AND METHODS

Cell culture - The human hepatoma cell line HLE was provided by Japanese Cancer Research Resources Bank (JCRB) (22). Cells were cultured in the minimum essential medium (MEM) 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.

Bile acids - UDCA and CDCA were kindly gifted from Tokyo Mitsubishi Co. (Tokyo, Japan), and DCA and LCA were purchased from Sigma Chemical Co. (St. Louis). These bile acids were dissolved in ethanol. Gaschromatography demonstrated that composition of all bile acids was a purity of at least 99.5%. Potency of hydrophobicity indices of bile acids was already described by Heuman et al (23), and the ascending order is as follows: UDCA, CDCA, DCA, and LCA. CDCA was used at final concentrations of 10 μM to 100 μM, because the serum concentration of total bile acids in patients with cholestasis was reported to be around 10 μM to 100 μM (24-27).

Plasmid construct- RANTES promoter-luciferase reporter plasmid was a kind gift from Dr. Alan M. Krensky (Stanford University School of Medicine). This construct includes the 961 nucleotides of the region immediately upstream of the transcriptional start

site and the complete RANTES 5' untranslated region to the KpnI site. 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 the 961-b 5'-flanking sequence of the RANTES gene by oligonucleotide-directed, site-specific mutagenesis according to instructions delivered with the site-directed mutagenesis kit (Promega). Mutant sequences utilized for the κB1 and the κB2 were GGAAACTtaC and GGtaATGCCC, respectively. Lower case letters represent mutant nucleotides.

ELISA for RANTES- HLE cells were grown to confluence in 60 mm collagen-coated culture dishes and treated with bile acids. 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).

RNA extraction and semiquantitative RT-PCR analysis- Total RNA was extracted from hepatoma cells according to the method of Chomczynski and Sacchi (28), which includes a single step of acid guanidium thiocyanate (GTC) 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) as described previously (29). The following primers were used: for GAPDH, sense 5'-ACATCGCTCAGACACCATGG-3', antisense 5'-GTAGTTGAGGTCAATGAAGGG-3'; for RANTES, sense 5'-GCTGTCATCCTCATTGCTAC-3', antisense 5'-TCCATCCTAGCTCATCTCCA-3'. 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- Whole cell extracts were prepared as described

previously (29). 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 14,000 rpm for 20 min, the supernatant was used as a whole cell extract.

Electrophoretic mobility shift assay (EMSA)- Equal amounts of whole cell extracts (10 μg of protein) were subjected to an EMSA by using a ^{32}P-end-labeled double-stranded oligonucleotide probe corresponding to the NF-κB binding region of the RANTES promoter (5'-gatcATTTTGGAAACTCCCCTTAGGGGATGCCCCTCAA-3'). 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 TBE buffer. The gel was dried and subjected to autoradiography. Oligonucleotides were synthesized and end-labeled by the Klenow fragment. For cold oligonucleotide competition assays, a 50-fold molar excess of unlabeled DNA was added to the binding reaction mixture as indicated prior to the addition of the labeled probe. For antibody supershift-blocking assays, the antibody reagents were added to the binding reaction mixture 15 min into the incubation.

Transfection and luciferase assays- Transient transfection was performed as described previously (30). In brief, HLE cells were plated in 6-well plastic dishes (IWAKI Glass, Funabashi, Japan) to 30%~50% confluency and were washed three times with PBS and medium was replaced with Opti-MEM medium (Life Technologies, Inc., Grand Island, NY). The plasmid mixtures were mixed with 4 μl of Trans-IT lipofection reagent (Life Technologies, Inc.) and added to the culture. After 6 h of incubation, the medium was replaced with fresh medium supplemented with 10% FCS and cells were treated by CDCA for 24 h. After normalization of transfection efficiency by β-galactosidase expression, luciferase activity was determined by Lumat LB9501 (Berthold Japan, Tokyo, Japan).

RESULTS

Production of antigenic RANTES by CDCA- To examine the effect of CDCA on RANTES production in HLE cells, ELISA was performed. Conditioned media were collected from cells treated for 48 h with the indicated concentrations of CDCA. As shown in Fig. 1A, CDCA significantly increased RANTES protein in a dose-dependent manner. Cell viability was intact in the medium containing all CDCA concentrations (data not shown). Moreover, time course of RANTES production by 100 μM of CDCA for the indicated times was shown in Fig. 1B. After 48 h-culture in the presence of 100 μM of CDCA, the level of RANTES protein in conditioned media was significantly increased when compared with that before CDCA treatment (Fig. 1B). Next, to test the effect of CDCA on RANTES mRNA expression, semiquantitative RT-PCR analysis was performed. As shown in Fig. 2, 100 μM of CDCA time-dependently increased steady-state levels of RANTES mRNA in HLE cells. In another human hepatoma cell line HepG2, we also observed this positive regulatory effect of CDCA on RANTES mRNA expression (data not shown).

Roles of NF-κB in CDCA-induced RANTES promoter activity- To test effects of CDCA on RANTES gene expression, luciferase assay was performed using the reporter plasmid. According to dosage, CDCA clearly increased RANTES gene expression in HLE cells (Fig. 3B, lanes 1-4). In addition, RANTES gene expression was up-regulated by CDCA in HepG2 cells (data not shown). Thus, CDCA induced RANTES expression at the transcriptional level in human hepatoma cells. Furthermore, to define more precisely the roles of κB sites in CDCA-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 presented 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 CDCA inducibility, suggesting that both sites were required for CDCA induction of RANTES (Fig. 3B, lanes 5 - 12). These results might indicate CDCA induced RANTES gene expression through its cognate NF-κB binding sites in the RANTES promoter.

Rank order of bile acids on RANTES protein-inducibility- We next investigated the quantitative difference in RANTES protein-inducibility by various bile acids. ELISA revealed that 50 μM of UDCA, CDCA, DCA, and LCA apparently increased the expression of RANTES protein (Fig. 4). The order of RANTES protein-inducibility was UDCA<CDCA<DCA< LCA in HLE cells (Fig. 4). In addition, inducibility of RANTES protein was clearly correlated with the hydrophobicity of the corresponding bile acids (Fig. 5).

Induction of DNA-binding activity of NF-κB by bile acids- To determine the effects of bile acids on DNA-binding activity of NF-κB, EMSA were performed. The κB-binding activity of the protein extracts was analyzed using an oligonucleotide encoding the NF-κB binding region in the RANTES promoter. HLE cells were incubated in medium containing 50 μM of indicated bile acids for 10 h. DNA-binding activity of NF-κB was clearly induced by treatment with CDCA, DCA and LCA (Fig. 6, lanes 3-5). By contrast, DNA-binding activity of NF-κB induced by UDCA was very weak (Fig. 6, lane 2). The order of NF-κB-inducibility was also associated with the hydrophobicity of the corresponding bile acids. Moreover, successful competition was observed using unlabeled NF-κB probe (Fig. 6, lanes 6 compared with lane 5), whereas an unrelated oligonucleotide was ineffective (Fig. 6, lanes 7 compared with lane 5). Additionally, LCA-induced

DNA-binding activity of NF-κB was clearly blocked and supershifted by anti-p65 and p50 antibodies, respectively (Fig. 6, lanes 8, 9 compared with lane 5). These data suggested that bile acids-induced NF-κB consisted of the components of p65 and p50 heterodimers.

DISCUSSION

In the present study, we found that endogenous bile acids induced RANTES expression in cultured human hepatoma cells. Moreover, bile acid-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 (31). 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 (31). In the present study we asserted that mutation on NF-κB binding sites markedly reduced CDCA inducibility, possibly indicating that NF-κB is a potent inducer of RANTES expression in response to CDCA. In fact, we found that hydrophobic bile acids such as CDCA, DCA, and LCA clearly induced DNA-binding activity of NF-κB. In addition, we also presented that specific mutations of either NF-κB binding site of the RANTES promoter resulted in loss of CDCA-induced transcriptional activity in human hepatoma cells. 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 (32). Thus, we might show that two NF-κB binding sites in the RANTES promoter played an important role in the functional transactivation in human hepatoma cells as well as in activated T lymphocytes.

In addition, we have already presented in previous reports that CDCA up-regulated major histocompatibility complex (MHC) class I mRNA on hepatocytes through protein

kinase C-mediated pathway (33, 34). Recently it was demonstrated that α- and ζ-protein kinase C isoforms directly phosphorylated IκB kinase β and activated NF-κB (35), and that calphostin C inhibited NF-κB activation via these protein kinase C (36). Given these reports, it is possible that CDCA induced NF-κB activation and RANTES expression in hepatoma cells through α- and ζ-protein kinase C. On the other hand, although we have observed similar effects of CDCA on RANTES expression in two different hepatoma cell lines, we cannot rule out the possibility that RANTES expression is rather differently regulated in non-malignant cells. For further confirmation, studies using primary hepatocyte culture would be clearly merited.

It has been demonstrated that bile acids have various effects on cell membranes and transcriptional regulation (33, 34, 37-39). Quist et al have reported that histamine release from mast cell is highly correlated with the hydrophobicity of bile acids (37). We have also reported that inducibility of MHC class I mRNA is correlated with the hydrophobicity of bile acids (34). Furthermore, bile acids transcriptionally repress steady-state cholesterol 7α-hydroxylase mRNA levels according to their relative hydrophobicity (38). In addition, bile acid hydrophobicity was reported to be correlated with induction of apoptosis in the human colon cancer cell line HCT116 (39). Thus, those reports and our study strongly support the notion that the hydrophobicity itself of bile acids may be related to, at least, some of their biological effects including both MHC class I and RANTES gene expression. Recently, bile acids have been shown to bind and activate the orphan nuclear receptor, farnesoid X receptor (FXR) (40-42). FXR binds to DNA as a heterodimer with retinoid X receptor, recognizing an inverted hexanucleotide repeat separated by a single base (an IR-1 motif) (43). Although no IR-1 element has been identified in the RANTES promoter, further study should examine whether RANTES gene expression is induced by bile acids via FXR activation.

In summary, we might indicate that bile acids induced RANTES gene expression through two NF-κB binding sites in the RANTES promoter in human hepatoma cells,

possibly indicating that bile acids play an important role in migration of inflammatory cells by RANTES to the liver in patients with PBC.

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Figure Legends

Fig. 1. Effect of CDCA on antigenic RANTES production in human hepatoma cells. A: Dose-dependency. ELISA was performed using conditioned media as described in *Materials and Methods*. Conditioned media were collected after treatment with the indicated concentrations of CDCA for 48 h. Levels of RANTES protein in unstimulated cells were taken as 1.0. Experiments were performed quadruplicately. Results were presented as mean + SE of three independent experiments. B: Time course. ELISA was performed using conditioned media as described in *Materials and Methods*. Conditioned media were collected after treatment with 100 μM of CDCA for the indicated times. Levels of RANTES protein in unstimulated cells were taken as 1.0. Experiments were performed quadruplicately. Results were presented as mean + SE of three independent experiments.

Fig. 2. Effect of CDCA on RANTES mRNA expression. Semiquantitative RT-PCR analysis was performed using total RNA in HLE cells as described in *Materials and Methods*. Cells were treated with 100 μM of CDCA for the indicated times. 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. CDCA induced RANTES gene expression through two cis-acting elements of NF-κB binding sites in 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 were shown with an X. Mutation of κB1 or κB2 site was shown as pmκB1 or pmκB2, respectively. B: Transcriptional effect of CDCA on RANTES gene expression. Cells were transfected by lipofection with 2 μg of pN, pmκB1 and pmκB2. After transfection, cells were incubated

with indicated concentrations of CDCA. After 24 h, 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 (lane 1). Results were mean + SE of three independent experiments.

Fig. 4. Effects of various bile acids on antigenic RANTES production in human hepatoma cells. Cells were cultured in the absence or presence of 50 μM of various bile acids for 48 h, and ELISA was performed. Levels of RANTES protein in unstimulated cells were taken as 1.0. Experiments were performed quadruplicately. Results were presented as mean + SE of three independent experiments.

Fig. 5. Correlation between the RANTES mRNA-inducibility and hydrophobicity of bile acids. Cells were cultured in the absence or presence of 50 μM of various bile acids for 48 h, and ELISA was performed. Levels of RANTES protein in unstimulated cells were taken as 1.0. Experiments were performed quadruplicately. Results were presented as mean + SE of three independent experiments. Potency of hydrophobicity indices of bile acids was described by Heuman et al (10).

Fig. 6. DNA-binding activity of NF-κB was induced by bile acids in HLE cells. EMSA using a ^{32}P-end-labeled double-stranded oligonucleotide probe corresponding to the NF-κB binding region in the RANTES promoter was performed as described in *Materials and Methods*. Cells were treated with 50 μM of indicated bile acids for 10 h. Specific NF-κB band was shown as closed triangles. Unbound labeled oligonucleotide was presented as open triangles. Data was representative of three similar experiments. For competition, EMSA was performed using specific (SC) or nonspecific (NC) oligonucleotide on extracts obtained following 10 h of 50 μM of LCA. For supershift analysis, EMSA was performed using anti-p65 or p50 antibody on extracts obtained following 10 h of 50 μM of LCA.

Fig. 1A

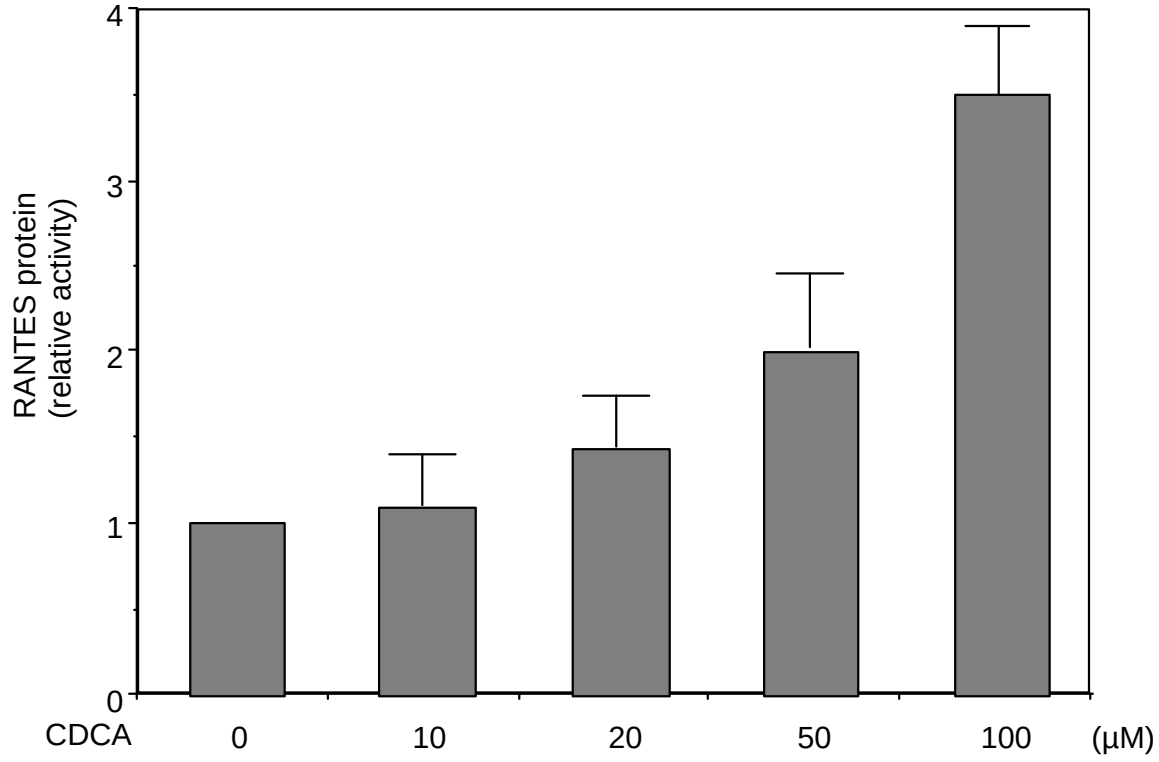

RANTES protein
(relative activity)
4
3
2
1
0
CDCA
0
10
20
50
100
(μM)

Fig. 1B

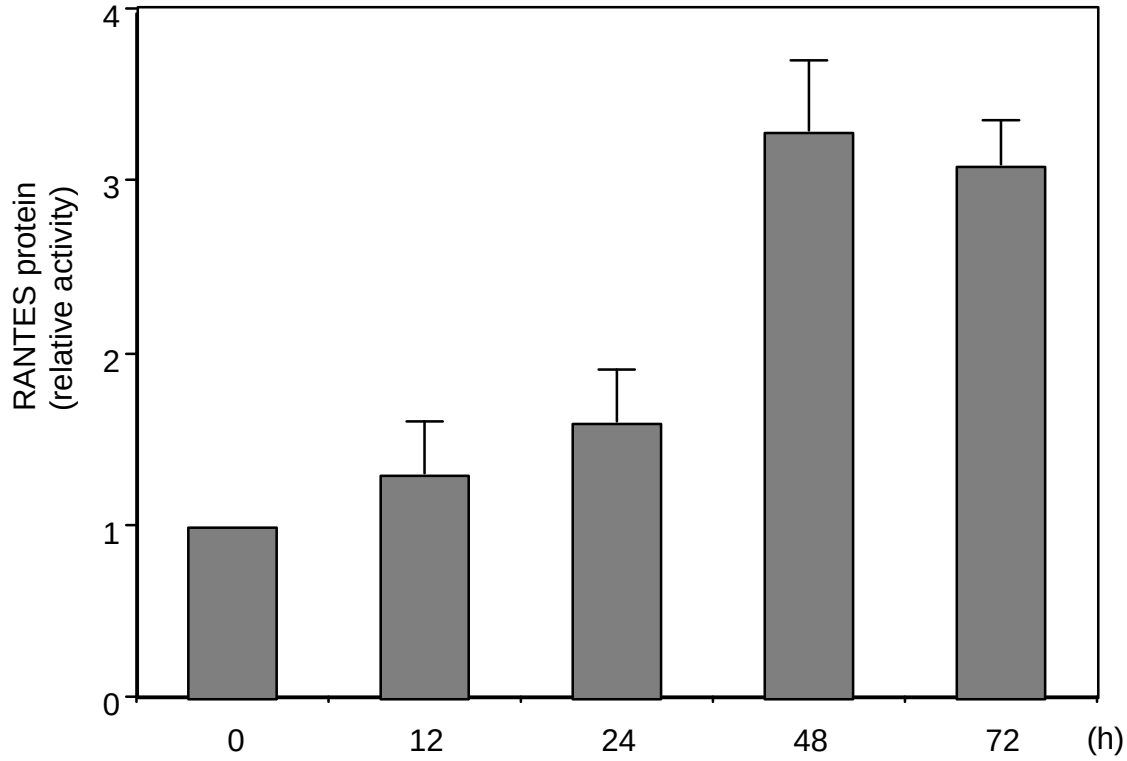


Fig. 2

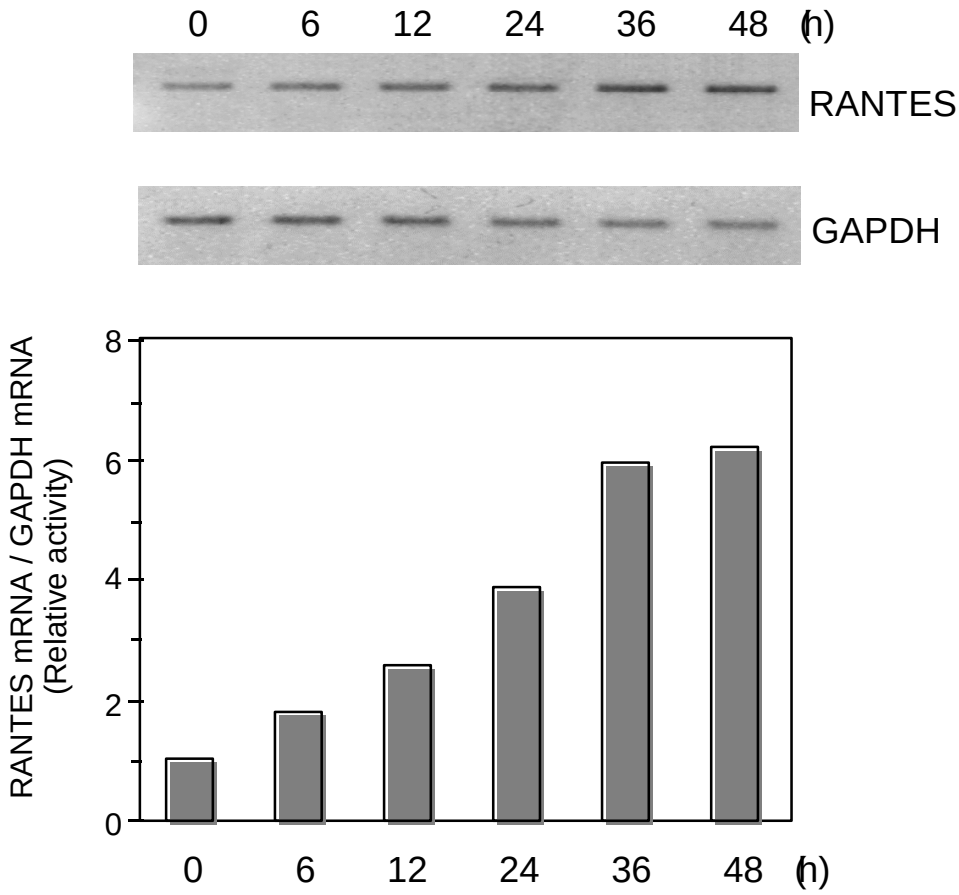


Fig. 3A

RANTES promoter-construct plasmids

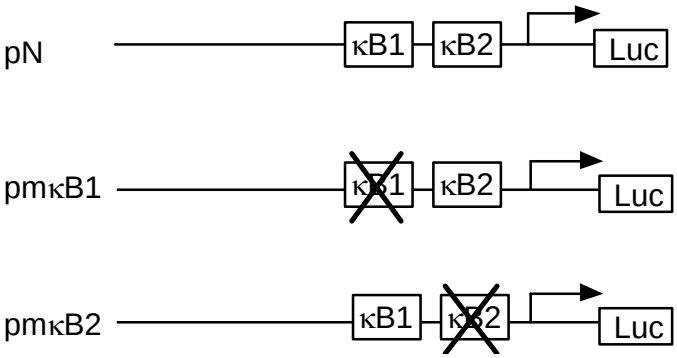


Fig. 3B

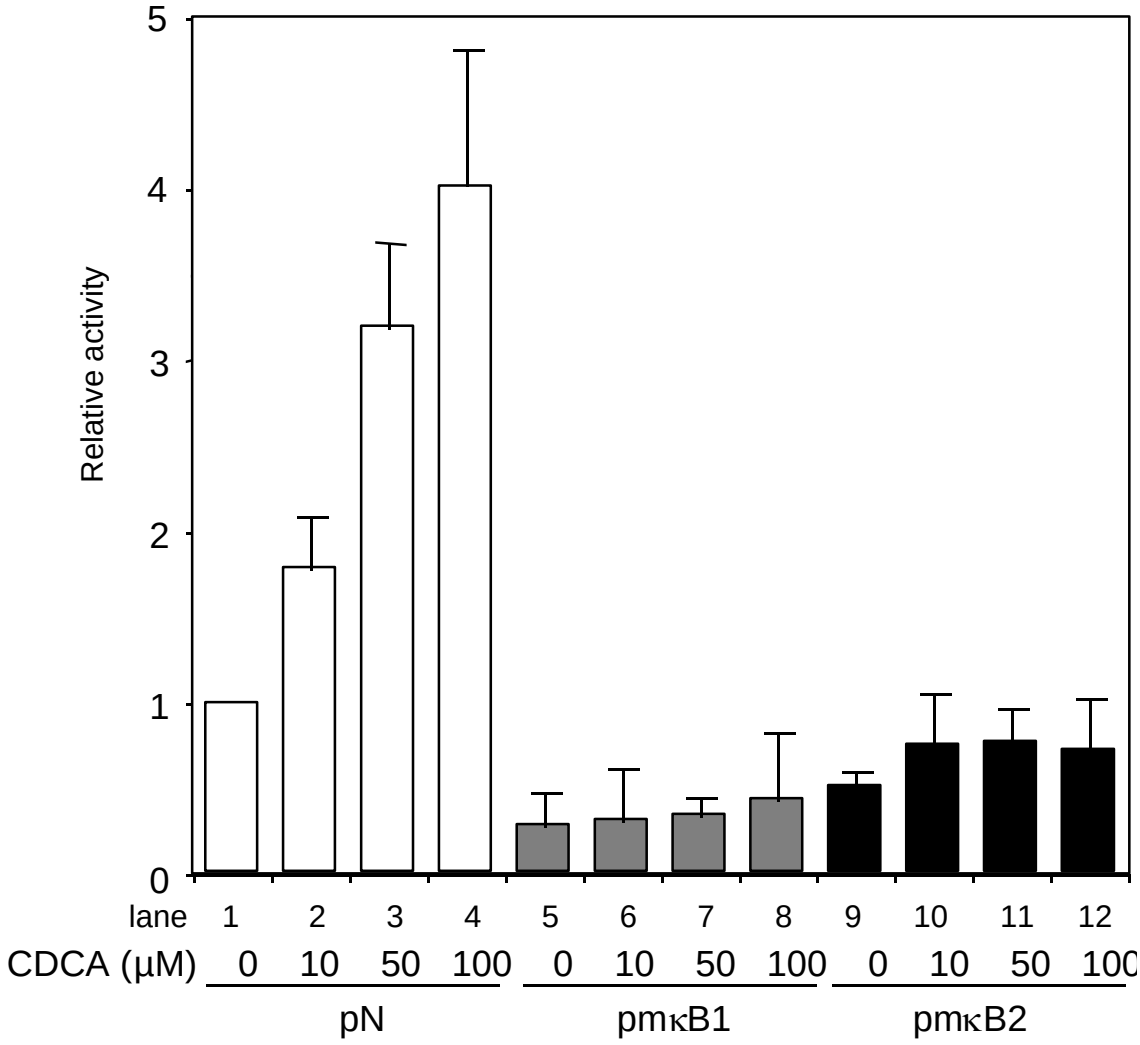

Relative activity
0
1
2
3
4
5
lane 1 2 3 4 5 6 7 8 9 10 11 12
CDCA (μM) 0 10 50 100 0 10 50 100 0 10 50 100
pN
pmκB1
pmκB2

Fig. 4

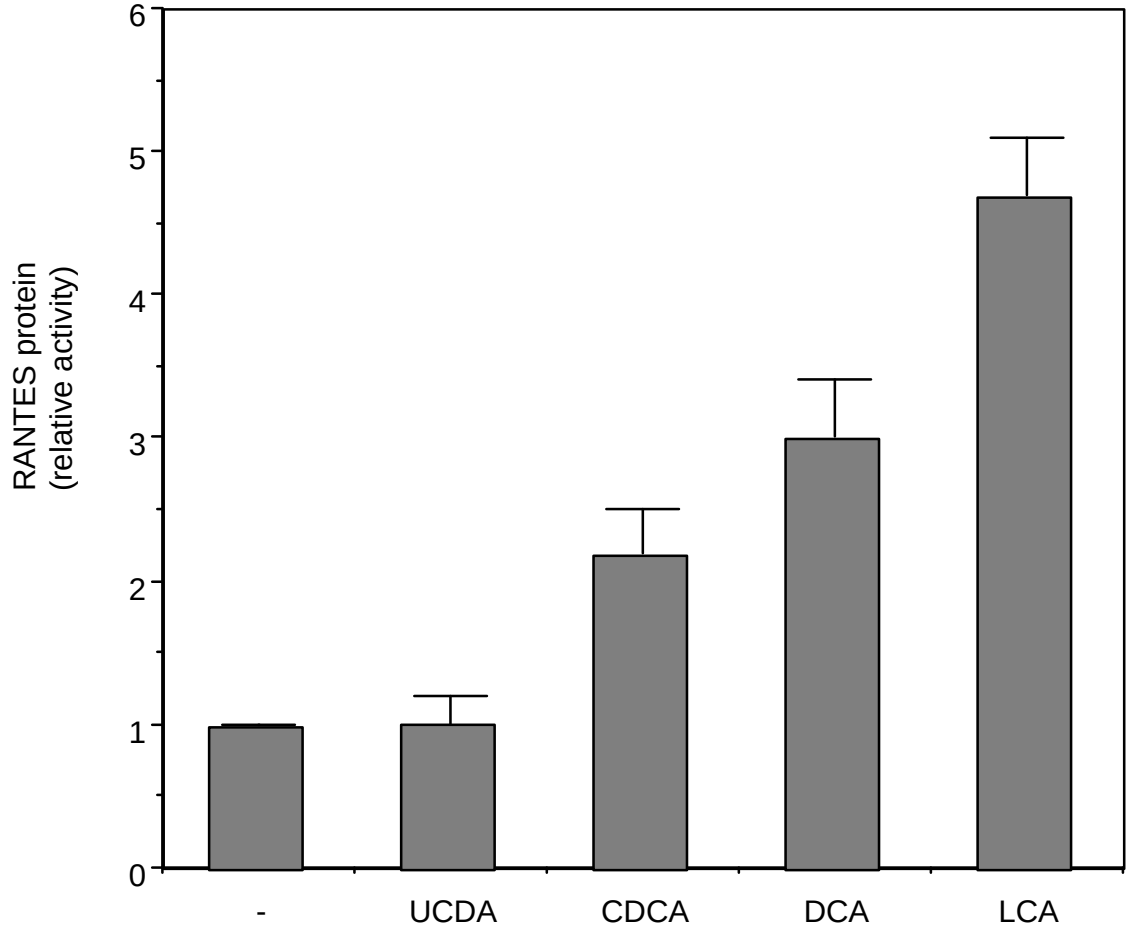

RANTES protein
(relative activity)
6
5
4
3
2
1
0
-
UCDA
CDCA
DCA
LCA

Fig. 5

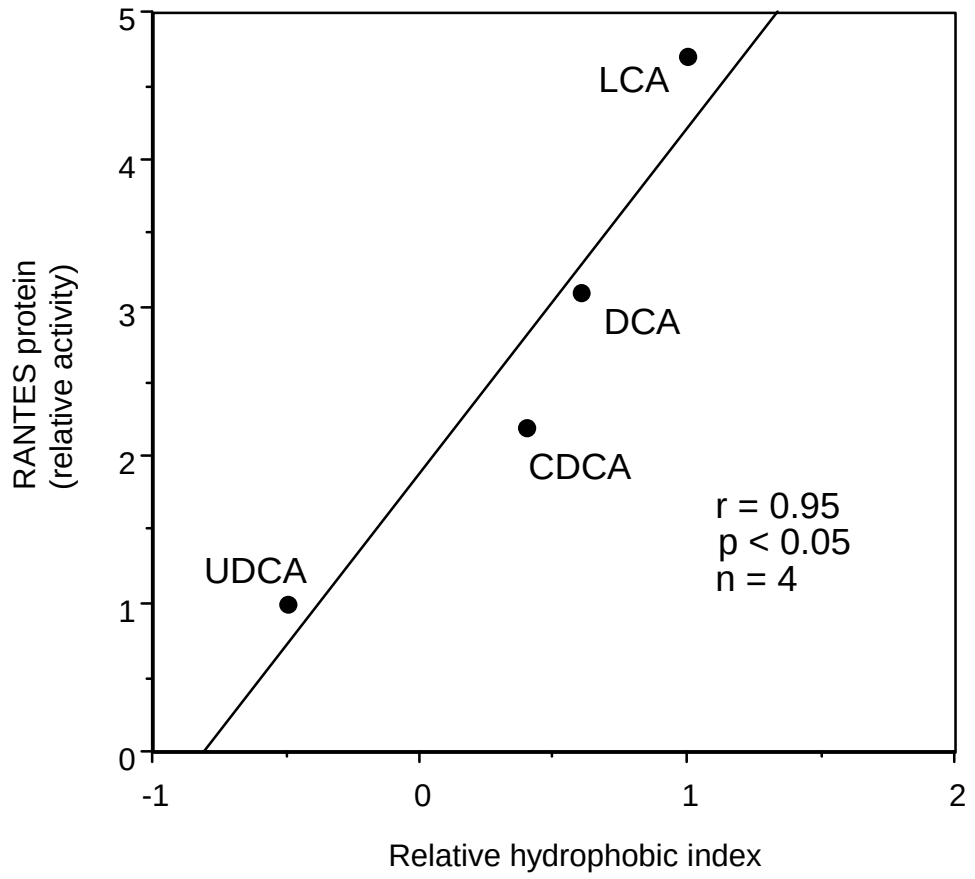
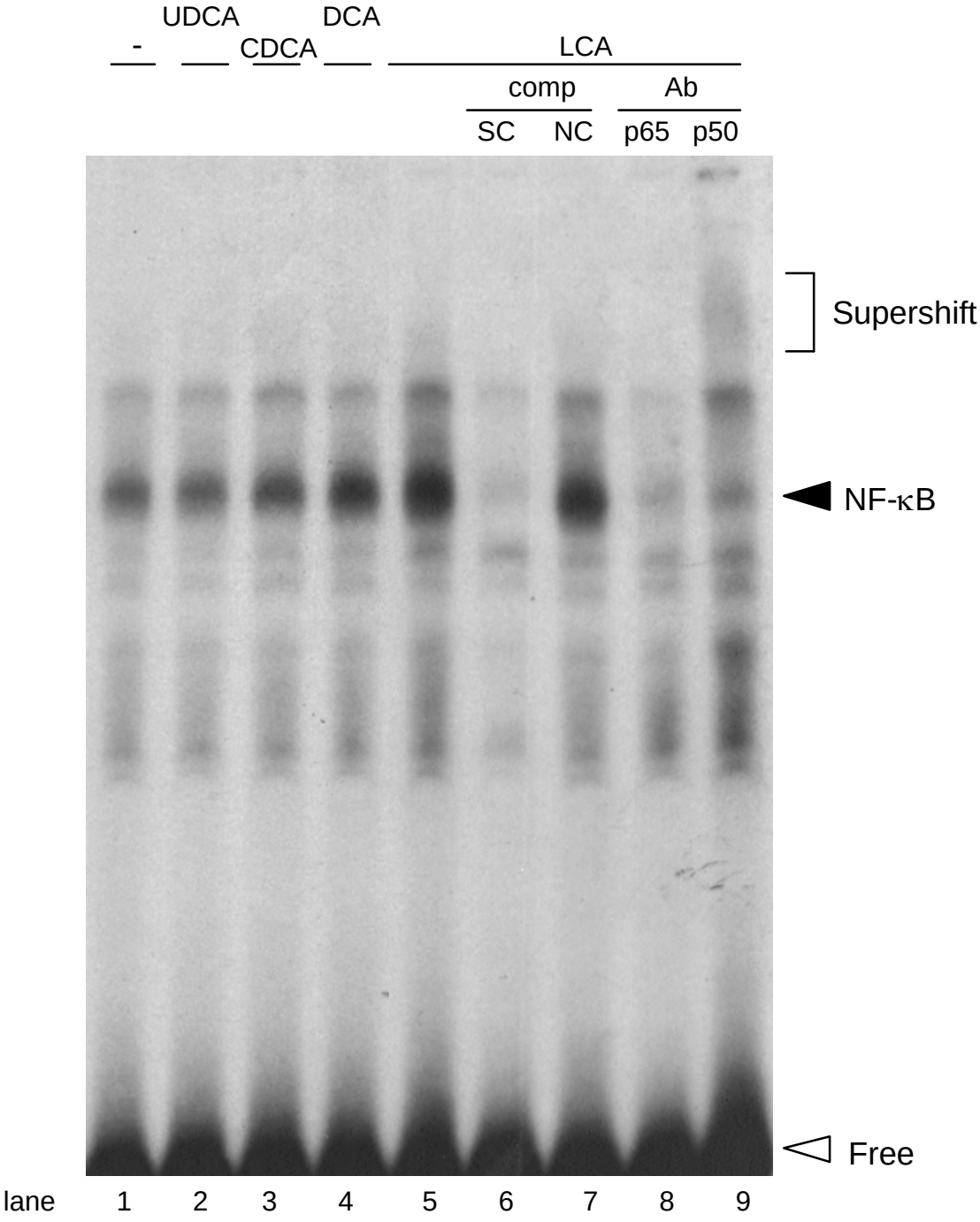

RANTES protein
(relative activity)
5
4
3
2
1
0
-1
0
1
2
LCA
DCA
CDCA
UDCA
r = 0.95
p < 0.05
n = 4
Relative hydrophobic index

Fig. 6


UDCA
DCA
-
CDCA
LCA
comp
Ab
SC
NC
p65
p50
Supershift
NF-κB
Free
lane
1
2
3
4
5
6
7
8
9