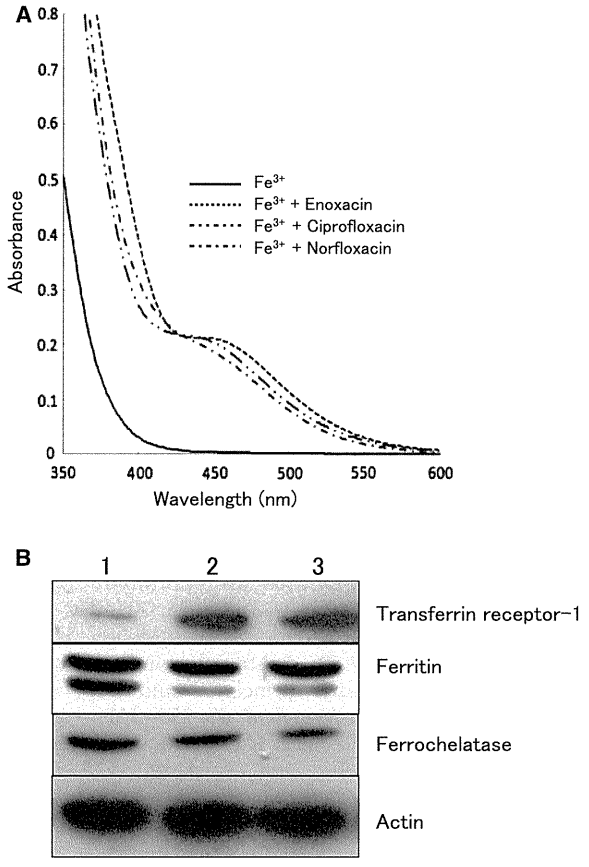

Fig. 5 Quinolone chemicals chelate with iron and affect the expression of iron-metabolizing proteins. (A) Spectroscopic scan of quinolone chemicals complexed with ferric ion. Ferric chloride (100 μM) was incubated without or with 100 μM enoxacin, 100 μM ciprofloxacin or 100 μM norfloxacin in phosphate-buffered saline at room temperature for 5 min. Absorption spectra of the formed iron–quinolone complexes were measured. Lower scan is a comparison of ferric chloride without quinolones. (B) Immunoblot analysis of transferrin receptor-1, ferritin and ferrochelatase in enoxacin- or ciprofloxacin-treated HeLa cells. The cells were treated without (lane 1) or with 100 μM enoxacin (lane 2) or 100 μM ciprofloxacin (lane 3) for 24 h, washed twice with phosphate-buffered saline, and then lysed. The cellular proteins were analysed by SDS-PAGE, and immunoblotting was performed using antibodies for transferrin receptor-1, ferritin, ferrochelatase and actin, as the primary antibodies.

observation was similar to that seen in case of desferrioxamine-iron complex (*23*). Immunoblot analysis revealed that the expression of transferrin receptor-1 was high and that of ferritin was low in enoxacin- and ciprofloxacin-treated cells compared with those of untreated cells. On the Basis of the fact that the alteration of expressions of both proteins occurs in a manner dependent on the level of intracellular iron (*19*), the treatment of cells with quinolones leads to iron deficiency. We (*20*) previously found that ferrochelatase is an iron-sulphur-containing enzyme and its expression was markedly decreased in iron-deficient cells. The decrease of the level of ferrochelatase was essential for ALA-PDT (*7*, *8*). The present data showed that the expression of ferrochelatase in enoxacin- and ciprofloxacin-treated cells was basically unchanged. Moreover, neither enoxacin nor ciprofloxacin inhibited iron-chelating ferrochelatase activity (Y. Ohgari & S. Taketani, unpublished results), suggesting that the iron deficiency in cells by the treatment with quinolones can be weak. Therefore, since the utilization of iron for ferrochelatase reaction *in vivo* is liable to drop by the treatment with quinolones, it is possible that the accumulation of protoporphyrin is, in contrast, increased.

We (*7*) previously reported that the low level of ferrochelatase in tumour cells could contribute to the ALA-induced accumulation of protoporphyrin and subsequent photodamage *in vitro*. The production of NO increased the ALA-induced photodamage by decreasing the levels of mitochondrial iron-containing enzymes including ferrochelatase and NADH dehydrogenase (*8*). Furthermore, the irradiation of epidermal cells led to a decrease of the level of ferrochelatase, which is mediated by the generation of reactive oxygen species (*24*). Thus, the ferrochelatase reaction is deeply related to mitochondrial functions. The present study showed that the level of ferrochelatase in enoxacin- or ciprofloxacin-treated cells was not decreased since these chemicals showed low cytotoxicity and caused partial iron deficiency. However, it is possible that quinolones target some unidentified proteins in mitochondria, which are related to iron metabolism, and then the utilization of iron to haem biosynthesis can be down-regulated.

In addition of the enhancement of *in vitro* ALA-induced photodamage by quinolones, we showed that Sn-protoporphyrin, an inhibitor of HO (*25*), increased the ALA-induced accumulation of protoporphyrin and photosensitivity. In humans, two isoforms of HO have been characterized: a constitutive expressed form, HO-2, and an inducible form, HO-1. The reduced expression of HO-1 mRNA by siRNA increased cell death upon ALA-PDT (*26*). Therefore, the decrease of HO function can contribute to the ALA-induced accumulation of protoporphyrin. We have showed that HO-1 is markedly induced not only by agents and chemicals that produce oxidative stress involving the generation of reactive oxygen species but also by the substrate haem (*27*, *28*). Then, through ALA-induced photodamage, HO-1 in ALA-treated cells was induced in time- and dose-dependent manners, and the induction of HO-1 was seen in the protoporphyrin-accumulated cells (*7*). It is considered that uncommitted haem in the cells is very dangerous for the maintenance of living systems, and reutilization of iron, including degradation of the haem, catalysed by HO, is essential for the homeostasis of iron in cells (*19*). Then, excess haem produced from ALA may induce HO-1. It was also considered possible that the accumulated protoporphyrin may generate reactive oxygen species via autoxidation (*29*), which leads to the induction of HO-1. This possibility was ruled out by the observations that the increased accumulation of protoporphyrin by Sn-protoporphyrin was cancelled by the exogenously added haemin or Fe-NTA. On the other hand, on the basis of the fact that HO degrades haem, producing iron, CO and biliverdin (*20*), the supply of iron for its reutilization is stopped by the inhibition of the HO reaction with Sn-protoporphyrin, leading to the increase in the

production of protoporphyrin. The present data revealed that enoxacin and Sn-protoporphyrin in combination exhibited a synergistic effect on ALA-induced accumulation of protoporphyrin as well as photodamage. Not only the decrease of reutilization of iron by HO but also the decrease of the utilization of mitochondrial iron for haem biosynthesis can further promote ALA-PDT. Nevertheless, it is still possible that there are other effects of quinolones on cellular metabolism.

Quinolones target several kinds of bacterial topoisomerases, resulting in inhibition of DNA synthesis (*13*, *14*), but several types of quinolones exhibit negative side effects, including significant phototoxicity (*30*). Quinolone-dependent phototoxicity is probably not due to the accumulation of protoporphyrin but instead to the generation of phototoxic metabolites of the drugs in cells, leading to DNA fragmentation and modification (*31*). The present data show that the augmentation of ALA-induced photodamage by quinolones is not responsible for the phototoxicity of quinolones, since the sole treatment with 100 μM enoxacin, ciprofloxacin or norfloxacin did not show photosensitivity because of the low concentration of drugs as well as a low dose of light (Fig. 1C). In addition, it is possible the phototoxicity of quinolones may be helpful to further augment the efficacy of ALA-PDT.

Quinolones were chosen as an attractive candidate agent for PDT because quinolones are widely used in clinical contexts, show relatively low toxicity (*13*), and can be easily administered orally with large volume of distribution and good tissue penetration (*14*). The concentration of quinolones used in this study was equivalent to that in blood of patients (10–250 μM) by clinically conventional therapy (*32*). These properties of quinolones are safer than those of iron-chelators including desferrioxamine. Other studies have recently shown their anti-tumour activity in a variety of human tumour cells by inhibiting the proliferation of tumour cells and promoting apoptosis (*33*, *34*). Furthermore, very recently, quinolone compounds were shown to enhance RNAi by interacting with trans-activating responsive region RNA-binding protein and may be useful as RNAi enhancers in the development of research tools and therapeutics (*35*). Taken together, the application of anti-bacterial agents, quinolones, may be extremely effective for ALA-PDT and their use in multiple combinations with other therapeutic reagents can optimize the treatment modalities for PDT of various tumour tissues.

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Conflict of interest
None declared.

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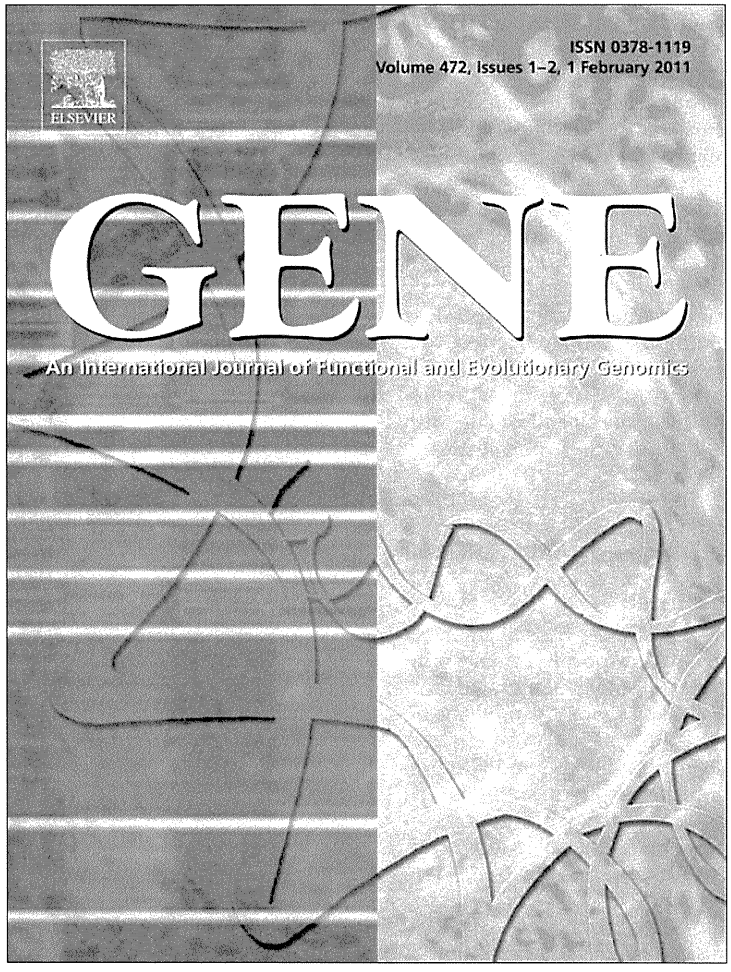

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Egr-1 regulates the transcriptional repression of mouse δ-aminolevulinic acid synthase 1 by heme

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ABSTRACT

δ-Aminolevulinic acid synthase 1 (ALAS1) is the first and rate-limiting enzyme in the heme biosynthesis. It has been well known that heme exerts a negative feedback control over the transcription of *ALAS1* gene to maintain intracellular heme at appropriate level. To clarify the mechanisms by which heme regulates the expression of ALAS1, we examined the promoter activity of the gene and identified the heme-responsive element (HRE) located in the proximal promoter of the mouse *ALAS1* gene. Reporter and EMSA assays revealed the sequence (GCGGGGGCG), as the site of repression by heme, at −301/−293 bp of the ALAS1 promoter. Subsequently, EMSA and ChIP assays showed that a transcription factor, early growth response 1 (Egr-1) and its major corepressors, NAB1 and NAB2 were found to bind to the ALAS1-HRE, and these bindings increased dependent on the level of intracellular heme. When Egr-1 and NAB1 in combination were expressed in the cells, decreases of the level of ALAS1 mRNA and intracellular level of heme were observed. These results suggest that Egr-1–NABs complex is involved in the regulation of the transcription of ALAS1 by heme, leading to the regulation of the heme biosynthesis.

1. Introduction

Heme biosynthesis in eukaryotic cells is composed of eight enzymatic steps, of which the first and the last three steps take place in the mitochondria, whereas the others in the cytoplasm. ALAS is the first and rate-limiting enzyme in the heme biosynthesis (Sassa, 1988). There are two isozymes for ALAS, namely, ALAS1 and ALAS2, encoded by separate genes. ALAS1 is ubiquitously expressed and provides heme for cytochromes and other hemoproteins (Furuyama et al., 2007). ALAS2 is expressed exclusively in erythroid cells and synthesizes heme specifically for hemoglobin (Kramer et al., 2000). It has been well known that heme negatively regulates ALAS1 expression by feedback mechanisms including reduction of transcription and translation, destabilization of mRNA and inhibition of mitochondrial transport of precursor protein, and it plays a crucial role for maintaining intracellular heme at appropriate level (Schuurmans et al., 2001; Furuyama et al., 2007). It is shown that PGC-1α via an insulin sensitive FOXO1 site within the promoter of ALAS1 can be involved in fasting response to the induction of ALAS1 (Handschin et al., 2005). The PGC-1α dependent transcriptional activity of ALAS1 gene seems to be reduced by a nuclear receptor Rev-erbα and β when heme binds to Rev-erbs after which recruit the corepressor NcoR (Yin et al., 2007; Wu et al., 2009). On the other hand, it is also shown that the circadian expression of ALAS1 can be controlled by the clock factor NPAS2–BMAL1 complex, which is regulated in a CO-heme-dependent manner (Kaasik and Lee, 2004). Thus various transcription factors are involved in transcriptional regulation of ALAS1 gene to various stimuli, but it is not still fully clarified how heme is involved in the transcriptional repression.

Egr-1 is a member of early growth response transcription factors that belong to immediately early genes that trigger de novo transcription of external stimuli (Svaren et al., 1996; Khachigian, 2006). Egr-1 binds to a GC-rich promoter motif to regulate the expression of various gene family including growth factor, cytokines and transcription factors (Blaschke et al., 2004). In contrast to the universal and immediate response of Egr-1 to mitogenic factors, Egr-1 also exhibits the suppressive gene activity via binding to or transactivation of major suppressor factors (Gashler et al., 1993; Svaren et al., 1996). This includes the interaction of Egr-1 with NAB1 and NAB2 transcriptional corepressors, whose complex deeply represses the activation of their target promoters (Blaschke et al., 2004; Kumbrink et al., 2005). Egr-1 is readily up-regulated by ischemia/reperfusion, hypoxia, hyperoxia and hemorrhagic shock, all inducers of ROS-mediated signaling and inflammation. Oxidative stress derived from heme in vascular smooth muscle cells caused the marked induction of Egr-1 expression (Hasan and Schafer, 2008;

Abbreviations: ALAS, δ-aminolevulinate synthase; PGC-1α, peroxisome proliferator-activated receptor γ coactivator 1 α; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; SA, succinylacetone; HRE, heme-responsive element; RT, reverse transcriptase; EMSA, electrophoretic mobility shift assays; Egr-1, early growth response 1; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis; NAB1/2, NGFI-A binding proteins 1/2; ChIP, chromatin immunoprecipitation.

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Rokosh, 2008), which may relate to cellular effects by increasing intracellular level of heme.

We tried to clarify the precise mechanism by which heme represses the transcription of the mouse *ALAS1* gene. Here we show the identification of a HRE in the mouse *ALAS1* gene promoter that is responsible for the repression by Egr-1 and its corepressors NAB1/2. Thus, Egr-1 complex newly functions as a heme-inducible repressor for the mouse *ALAS1* gene.

2. Materials and methods

2.1. Materials

[γ-^{32}P] ATP and poly (dI-dC) were purchased from GE Biosciences Co. (Buckinghamshire, UK). Restriction endonucleases and DNA modifying enzymes were obtained from Takara Co. (Kyoto, Japan) and Toyobo Co. (Tokyo Japan), respectively. The transfection reagent Hilymax was from Dojin Co. Ltd, (Tokyo, Japan). Antibodies for Egr-1, NAB1, NAB2 and actin were purchased from Santa Cruz Biotechnoloy Inc. (Santa Cruz, CA). All other chemicals were of analytical grade.

2.2. Cell cultures and treatment

Mouse embryonic fibroblast NIH3T3 and mouse hepatoma Hepa1-6 cells were grown in DMEM supplemented with 7% FCS and antibiotics. The cells were treated for 4–16 h in the medium supplemented with 7% FCS containing 10–50 μM hemin or 1 mM SA.

2.3. Plasmids

To construct the luciferase reporter plasmid with the *ALAS1* gene promoter, ALAS1 promoter region (−1241 to +39) was amplified by PCR using the sense primer (5'-TGATGATTTGGGTCAGG-3') and the antisense primer (5'-AACTCGAGCGGAGGACGCT-3'). The template named pANH III carrying the mouse *ALAS1* gene was a kind gift of Dr. O. Nakajima (Okano et al., 2010). Amplified DNA fragment was subcloned into *SacI*/*XhoI*-digested pGL3B (Promega), named as pGL-ALAS1p.

Reporter plasmid pGL-ALAS1(−302), pGL-ALAS1(−297), pGL-ALAS1(−293) and pGL-ALAS1(−62) were made as follows: PCR amplification was performed with antisense primer (5'-AAAA-GCTTCTCGAGCGGAGGACGCT-3') and the following sense primers

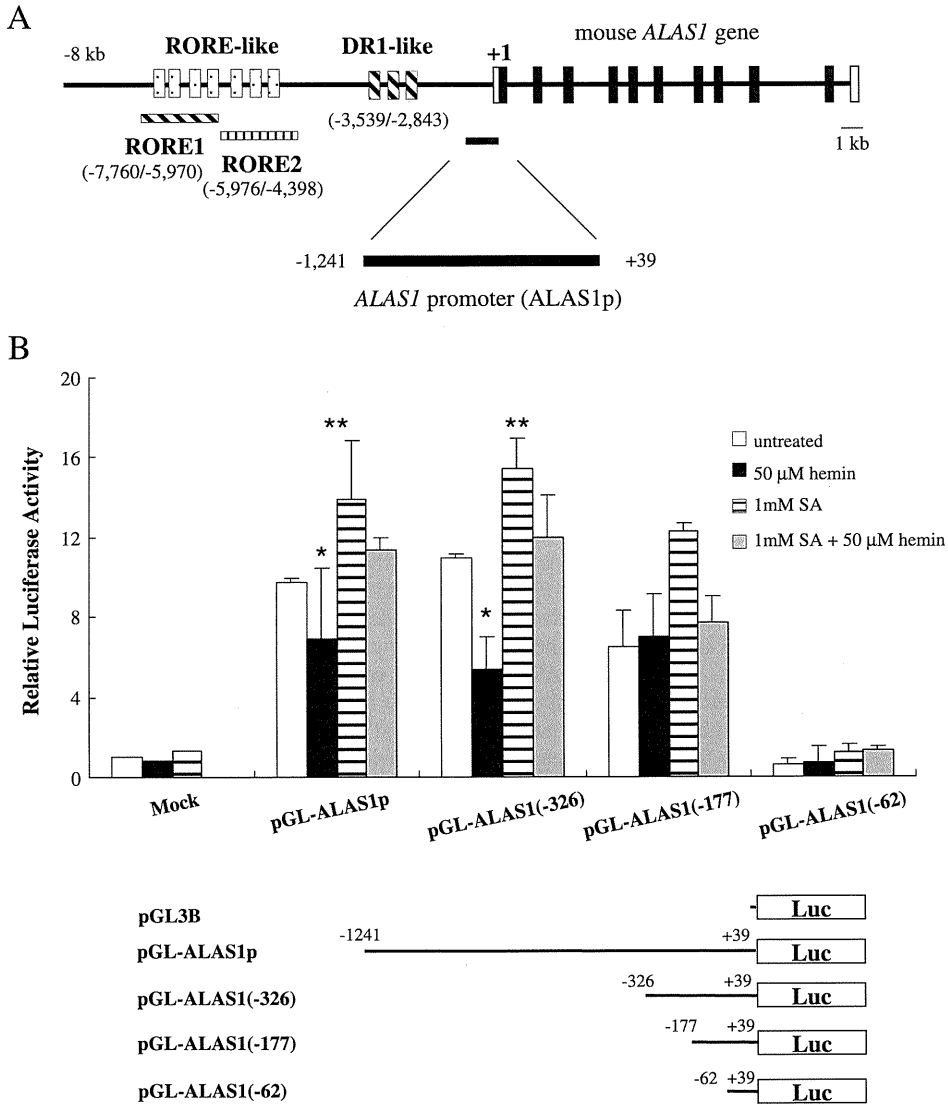


Fig. 1. Repression of the transcriptional activity of the mouse ALAS1 promoter region by heme. (A) Schematic representation of 5'-flanking region of the mouse *ALAS1* gene. (B) Effect of hemin and SA on the transcriptional activity of the mouse ALAS1 promoter. NIH3T3 cells were transiently transfected with pGL-ALAS1p, pGL-ALAS1 (−326), pGL-ALAS1 (−177) or pGL-ALAS1 (−62), followed by the treatment with 50 μM hemin and/or 1 mM SA for 16 h. Luciferase activity was measured and normalized to *Renilla* luciferase activity. The data are the average ± SEM for at least three independent experiments (*$P<0.05$, hemin treatment versus vehicle control; **$P<0.01$, SA treatment versus control).

(5'-AAGAGCTCAGCGGGGGCGCAGGAGT-3') for pGL-ALAS1(−302), (5'-AAGAGCTCGGGCGCAGGAGTCTGAC-3') for pGL-ALAS1(−297), (5'-AAGAGCTCGCAGGAGTCTGACGCAT-3') for pGL-ALAS1(−293), and (5'-AAGAGCTCCGCGGCGGTCACTCCGGC-3') for pGL-ALAS1(−62). Amplified fragments were subcloned into *Sac*I/*Hind*III-digested pGL3B. To construct pGL-ALAS1(−326) and pGL-ALAS1(-177), pGL-ALAS1p was digested with *Sma*I or *Aat*I/*Sma*I, the resulting fragments were self-ligated, and named as pGL-ALAS1(−326) and pGL-ALAS1(-177). pGL-ALAS1(−302)mtA and pGL-ALAS1(−302)mtB were made by PCR using the antisense primer (5'-AAAAGCTTCTCGAGCGGAG-GACGCT-3') plus the following mutagenic sense primers (5'-AAGAGCTCAGTGGGGGCGCAGGAGT-3') for mtA, and (5'-AAGAGCT-CAGCGTGGGCGCAGGAGT-3') for mtB. Amplified fragments were subcloned into *Sac*I/*Hind*III-digested pGL3B.

The reporter plasmids containing minimal promoter, pALAS1 HRE (×2) and pALAS1 HRE (×4) were constructed as follows: Double strand oligonucleotides containing ALAS1 HRE (5'-GCGG-GGGCGAATTTGCGGGGGCGTCA-3') were phosphorylated, and ligated into each other then to the *Pst*I site of pBluescript K/S$^+$. The clones with two and four tandem repeat of the consensus HRE were selected, the

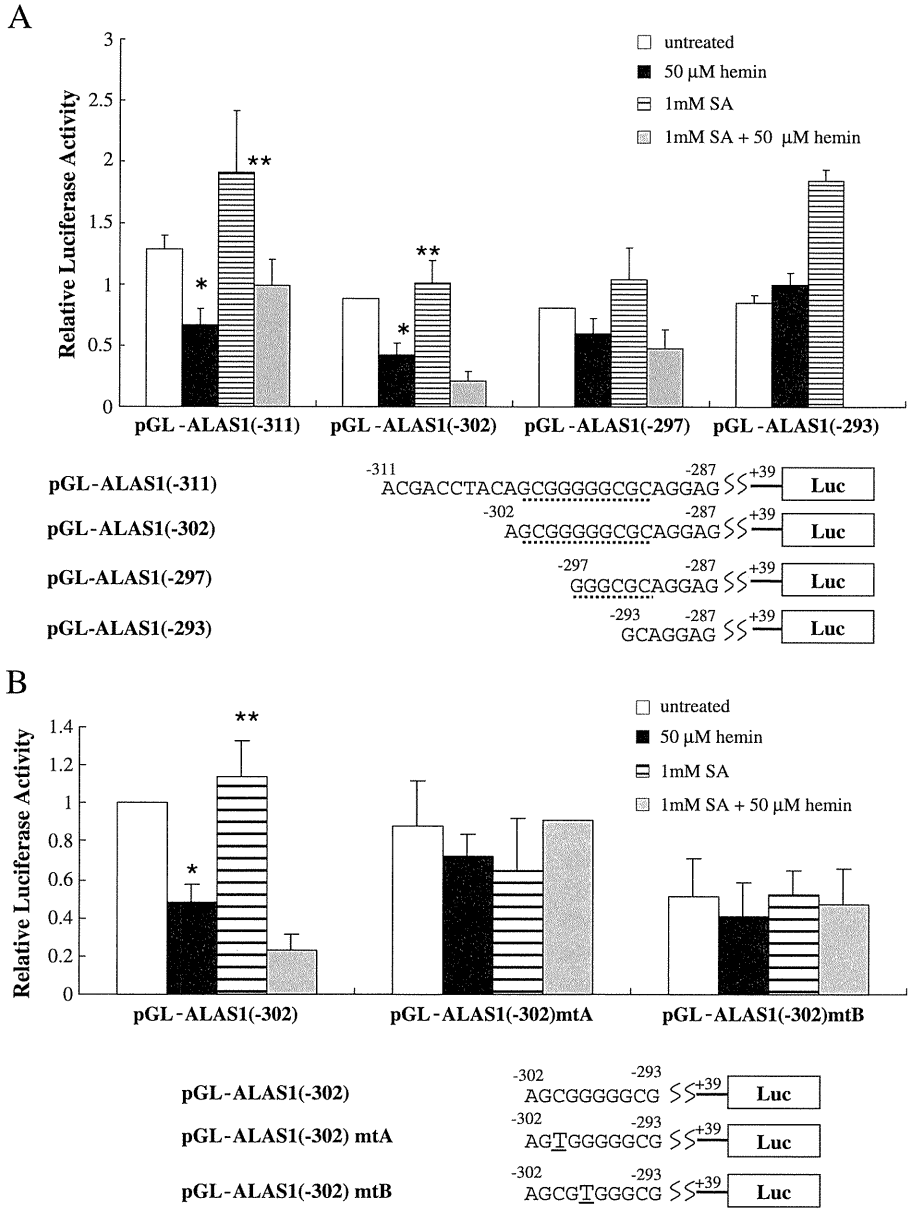
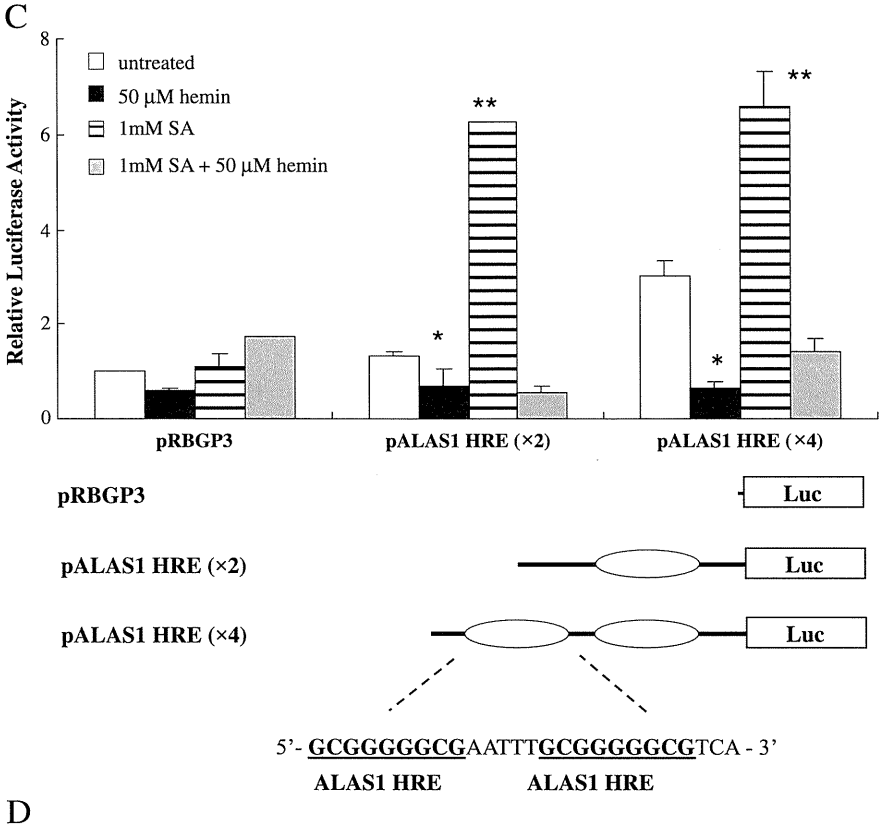
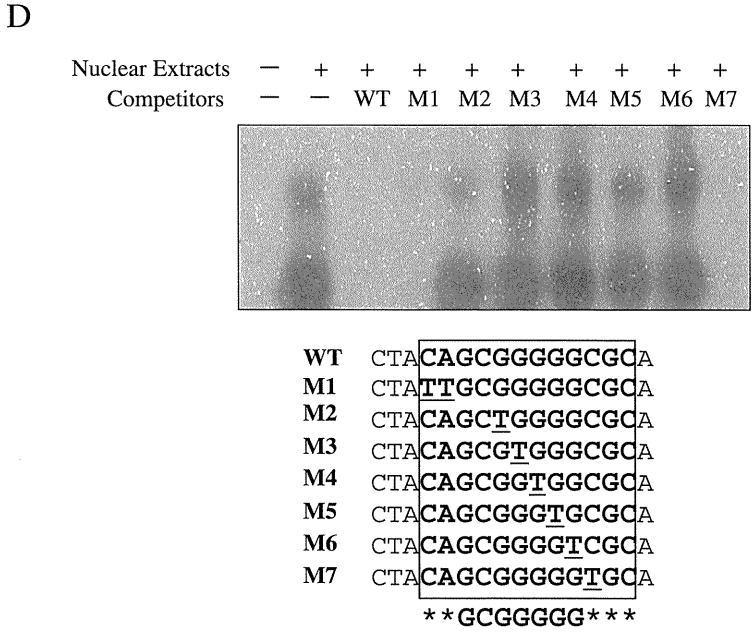


Fig. 2. Characterization of HRE in the mouse ALAS1 promoter. (A) Reporter activity of the mouse ALAS1 promoter in hemin- and SA-treated NIH3T3 cells. The cells were transfected with pGL-ALAS1 (−311), pGL-ALAS1 (−302), pGL-ALAS1 (−297), pGL-ALAS1 (−293), followed by the treatment with 50 μM hemin and/or 1 mM SA. Luciferase activity was measured and normalized to *Renilla* luciferase activity. The data are the average ± SEM for at least three independent experiments (*P<0.01, hemin treatment versus vehicle control; **P<0.05, SA treatment versus control). (B) Effect of mutations in the ALAS1-HRE on the transcriptional activity in hemin- and SA-treated NIH3T3 cells. Mutations in ALAS1-HRE were generated by site-directed mutagenesis. NIH3T3 cells were transfected with the indicated mutated constructs. The cells were treated without or with 50 μM hemin and/or 1 mM SA for 16 h. Luciferase activity was measured and normalized to *Renilla* luciferase activity. The data are the average ± SEM for at least three independent experiments (*P<0.01, hemin treatment versus vehicle control; **P<0.05, SA treatment versus control). (C) The reporter activity of the ALAS1-HRE. NIH3T3 cells were transfected with the ALAS1-HRE constructs as indicated. The cells were treated without or with 50 μM hemin and/or 1 mM SA for 16 h. Luciferase activity was measured and normalized to *Renilla* luciferase activity. The data are the average ± SEM of at least three independent experiments (*P<0.005, hemin treatment versus untreated control; **P<0.01, SA treatment versus untreated control). (D) EMSA of oligonucleotides containing the ALAS1-HRE with mutated competitors. EMSA was performed with nuclear extracts from untreated Hepa1-6 cells using ^{32}P-labeled ALAS1-HRE. Competitive assay was carried out by the addition of a 50-fold excess amount of mutated ALAS1-HRE oligonucleotides. An arrow indicates the position of a major competitive band. (E) ALAS1-HRE homologues found in the ALAS1 promoters of mouse, rat and human.





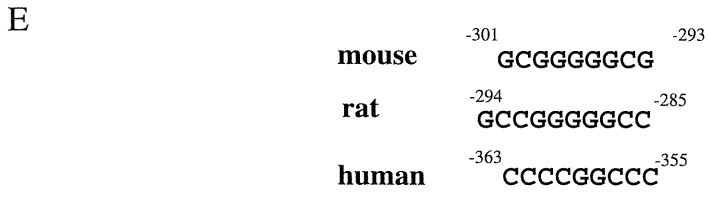


Fig. 2 (*continued*).

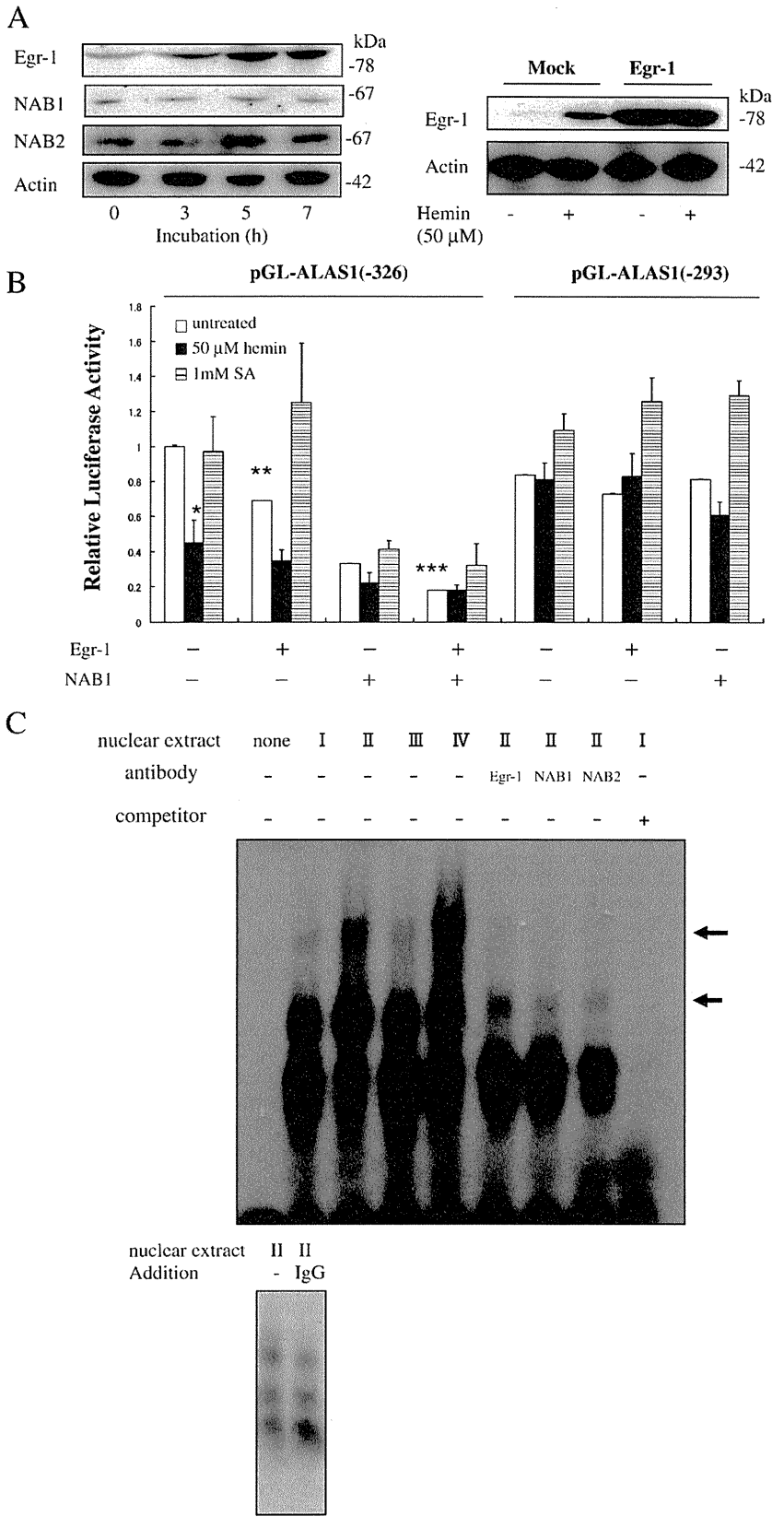
inserts were cut out with *Sma*I/*Kpn*I and inserted into the *Sma*I/*Kpn*I digested pRBGP3 (Tahara et al., 2004a,b).

For expression of mouse Egr-1 in mammalian cells, Egr-1 cDNA was obtained by PCR from the mouse liver cDNA library using primers 5'-AATCTAGAGCAGCGGCCAAGGCCGA-3' and 5'-AAAAGCTTCCCTTTAGCAAATTTCA-3'. The resulting amplicon was digested with *Xba*I and *Hind*III and inserted into *Xba*I/*Hind*III digested pCG-N-HA (Mizutani et al., 2002). The mouse NAB1 cDNA was amplified by PCR from mouse

kidney cDNA library using primers 5'-AAAAGCTTGCCACAGCCTTACC-TAGGA-3' and 5'- AATCTAGACTATCTTGAGTCTTCAGGC -3'. The resulting amplicon was digested with *Hind*III/*Xba*I and subcloned into *Hind*II/*Xba*I digested p3×FLAG-CMV-10 (SIGMA-ALDRICH, Tokyo, Japan). The sequence and insert orientation were confirmed by DNA sequence analysis.

2.4. Reporter gene assays

NIH3T3 cells were transfected with the reporter plasmids and pRL-CMV (Promega Co.), using a Hilymax reagent (Dojin. Co. Ltd. Tokyo, Japan) for 4 h, according to the manufacturer's recommendations. We also cotransfected these plasmids plus pCG-N-HA-Egr-1 or p3×FLAG-CMV-



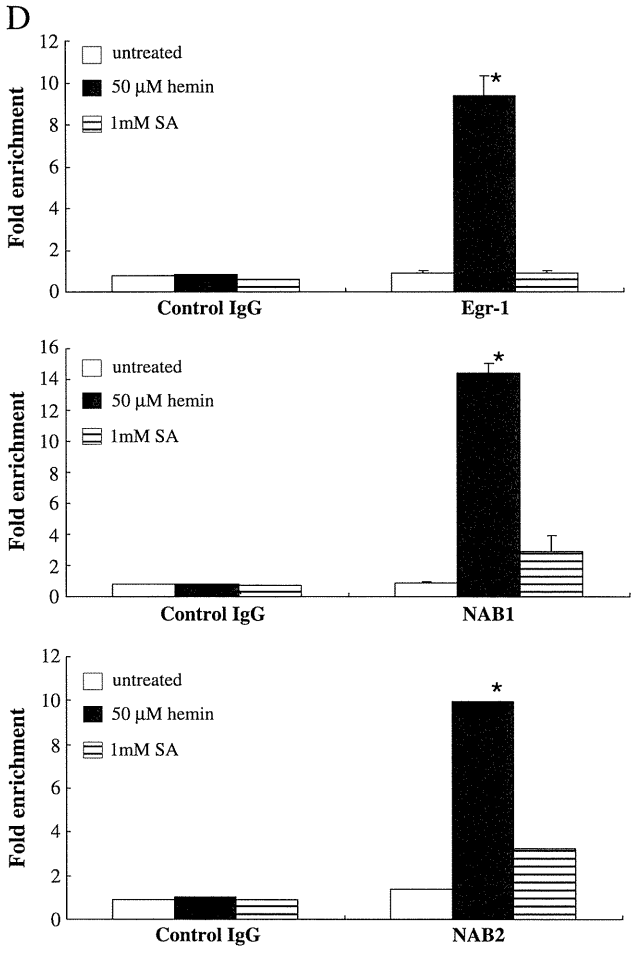


Fig. 3. Repression of the transcriptional activity of ALAS1-HRE by Egr-1, NAB1 and NAB2. (A) Left panel. Immunoblot analysis of the expression of the mouse Egr-1, NAB1 and NAB2 in hemin-treated NIH3T3 cells. The cells were treated in DMEM with 10 μM hemin for the indicated period. Right panel. The cells transfected with mock DNA and pCG-HA-Egr-1 were incubated for 16 h and then treated without or with 10 μM hemin for 5 h. The cellular proteins were separated by SDS-PAGE, followed by immunoblotting. The experiments were performed several times and the representative data are shown. (B) The transcriptional activity of Egr-1 and NAB1 for the ALAS1-HRE. NIH3T3 cells were transfected with pGL-ALAS1(−326) or pGL-ALAS1(−293) and plasmids carrying Egr-1 or NAB1. The cells were treated without or with 50 μM hemin and/or 1 mM SA for 16 h. Luciferase activity was measured and normalized to *Renilla* luciferase activity. The data are the average ± SEM for at least three independent experiments (*P<0.01, hemin treatment versus vehicle control; **P<0.01, Egr-1 overexpressing cells versus control cells; ***P<0.005, Egr-1 and NAB1 overexpressing cells versus control cells). (C) EMSA of the ALAS1-HRE oligonucleotides-binding activity in hemin- or SA-treated Hepa1-6 cells. EMSA was performed with nuclear extracts from the cells treated with 50 μM hemin and/or 1 mM SA using ^{32}P-labeled ALAS1-HRE. A 50-fold excess amount of unlabeled ALAS1-HRE oligonucleotide was used as a competitor. EMSA was carried out using the reaction mixture incubated with ^{32}P-labeled ALAS1-HRE at 4 °C for 10 min. The preincubation of nuclear extracts with antibodies for Egr-1, NAB1 or NAB2 was carried out at 4 °C for 20 min. Complexes were separated using 4% polyacrylamide gel. Nuclear extracts from untreated (I), and hemin (II)-, SA (III)- and hemin plus SA (IV)-treated cells were used. Arrows show the positions of the bands increasing by the hemin treatment (upper panel). Lower panel shows EMSA with nuclear extracts (II) preincubated with normal rabbit IgG (IgG). (D) Effect of hemin or SA on the occupancy of the ALAS1 promoter by Egr-1 (upper panel), NAB1 (middle panel) and NAB2 (lower panel). ChIP assay was performed with lysates from Hepa1-6 cells treated with 50 μM hemin and/or 1 mM SA for 16 h. Real-time PCR was performed to amplify the mouse ALAS1 promoter region containing ALAS1-HRE. Data are represented as fold enrichment compared to IgG control (*P<0.001, hemin treatment versus vehicle control).

10-NAB1. The cells were incubated with 50 μM hemin or 1 mM SA for 16 h. Dual-luciferase reporter assay system was used for measuring luciferase activity, and relative light units (RLU) represent *Firefly* luciferase normalized against *Renilla* luciferase activity (Tahara et al., 2004a,b).

2.5. Electrophoretic mobility shift assays (EMSA)

Nuclear extracts were prepared from cultured cells by the method of Schreiber et al. (1989). The radiolabeled probe CTACAGCGGGGGCGCA was phosphorylated at the 5'-end with [γ-^{32}P]ATP and T4 polynucleotide kinase and annealed with equimolar amount of CTACAGCGGGGGCGCA. EMSA was carried out as described previously (Gotoh et al., 2008). The reaction mixtures were separated on a 4% polyacrylamide. After electrophoresis, the gels were exposed to X-ray film at -80 °C. The oligonucleotides containing mutated sites used in competition experiments were as follows: 5'-CTATTGCGGGGGCGCA-3' and 5'-TGCGCCCCCGCAATAG-3'. (M1); 5'-CTACAGCTGGGGCGCA-3' and 5'-TGCGCCCCAGCTGTAG-3' (M2); 5'-CTACAGCGTGGGCGCA-3' and 5'-TGCGCCCACGCTGTAG-3' (M3); 5'-CTACAGCGGTGGCGCA-3' and 5'-TGCGCCACCGCTGTAG-3' (M4); 5'-CTACAGCGGGTGCGCA-3' and 5'-TGCGCACCCGCTGTAG-3' (M5); 5'-CTACAGCGGGGTCGCA-3' and 5'-TGCGACCCCGCTGTAG-3' (M6); and 5'-CTACAGCGGGGGTGCA-3' and 5'-TGCACCCCCGCTGTAG-3' (M7).

2.6. Reverse transcriptase (RT)-PCR analysis

Total RNA was isolated from the cells by the guanidium isothiocyanate method (Taketani et al., 2003). Single strand cDNA derived from the RNA was synthesized with the oligo(dT) primer, using ReveTra Ace (Toyobo, Co.), followed by PCR, using the indicated primers. The cDNAs obtained were analyzed using a 1.1% agarose gel. The DNA amount in the gel was quantified using Image J software. The primers were 5'-GGAACCATGCCTCCATGA-3' (forward) and 5'-GTTCTTAGCAGCATCGGC-3' (reverse) for ALAS1 and 5'-TGGGTGTGAACCACGAGA-3' (forward) and 5'-TTACTCCTTGGAGGCCATG-3' (reverse) for GAPDH. The amount of cDNA added to the reaction mixture was normalized by the intensity of GAPDH amplicon.

2.7. Immunoblotting

The lysates from NIH3T3 cells were subjected to SDS-PAGE and electroblotted onto PVDF membrane (Bio-Rad Laboratories, Hercules, CA). Immunoblotting was carried out with antibodies for Egr-1, NAB1, NAB2 and actin as the primary antibodies (Tahara et al., 2004a; Gotoh et al., 2008).

2.8. ChIP assay

Hepa1-6 cells (1×10^8) were cross-linked with 1% formaldehyde at room temperature for 15 min. Immunoprecipitation with anti-Egr-1, NAB1 and NAB2 antibodies, and control IgG was performed, followed by isolation of immunoprecipitates with protein A-Sepharose beads, as described (Tahara et al., 2004b; Gotoh et al., 2008). Primers 5'-CATGCAGCAAAGAAGGTCT-3' and 5'- ATATAGAGCCGGAGTGACC-3' were used to amplify the DNA fragment of the ALAS1 promoter (−455/−37) containing sequences of ALAS1 HRE (5'-GCGGGGGCG-3'). Real-time PCR was performed with a SYBR Green I kit (Toyobo Co.) and the Applied Biosystems 7500 real time PCR system.

2.9. Estimation of the intracellular level of heme

The level of heme in NIH3T3 cells (1×10^5) was estimated after conversion of heme to protoporphyrin, using a Hitachi MPF-4 fluorescence spectrophotometer (Taketani et al., 2003).

2.10. Statistics

Two-sample *t*-tests were used to compare the reporter activity, the amount of the protein–DNA complex and the level of ALAS1 mRNA between treated and untreated controls. Comparison of data from different treatment groups was conducted using one-way analysis of

variance (ANOVA). Analysis was performed using Microsoft Excel 2003 software.

3. Results

3.1. Identification of HRE in the mouse ALAS1 promoter

The expression of ALAS1 is known to be regulated by heme at a transcriptional level (Furuyama et al., 2007; Okano et al., 2010) and the level of ALAS1 mRNA in hemin-treated cells was markedly decreased (see Fig. 4A). To examine the heme-dependent transcriptional repression of the mouse *ALAS1* gene (Fig. 1A), we first carried out the reporter assay to find the heme-response in the distal region of the promoter of the mouse *ALAS1* gene. In the distal ALAS1 enhancer region (−3539/−2843 bp), two DR1-like sequences, the presumable binding sites of RXR (Gotoh et al., 2008) were found. We constructed the reporters containing these DR1-like sites, and transfected them into NIH3T3 cells. When we examined the luciferase reporter activity by the addition of hemin, virtually no change was observed (data not shown). We also found seven RORE-like sequences, the putative Rev-erbα binding site (Raghuram et al., 2007), from −8 to −4 kb upstream of the transcription start site and constructed two reporter plasmids containing −7760/−5970 and −5,976/−4,398 bp, but these reporters did not respond to heme even by coexpression of Rev-erbα (data not shown). Then we carefully examined the reporter activity within the proximal region of the mouse *ALAS1* gene promoter (−1241/+39). The luciferase activity of pGL-ALAS1p was decreased in hemin-treated cells, but slightly increased in the cells treated with SA, an inhibitor of heme biosynthesis, showing a heme-dependent repression (Fig. 1B). To find the heme-responsive region, sequential deletion of the promoter was carried out. We found that pGL-ALAS1(−302) exhibited a decrease by hemin and an increase by SA (Fig. 2A). The down regulation of the activity of pGL-ALAS1(−297) was weak or not observed with that of pGL-ALAS1 (−293), pGL-ALAS1(-177) and pGL-ALAS1(−62). To further examine the involvement of the region in heme-induced repression, we constructed the mutated reporter within the region −302/−293 and examined the effect of hemin on the transcriptional activity. Compared with the effect of hemin on the activities of pGL-ALAS1(−302), pGL-ALAS1(−302)mtA and pGL-ALAS1(−302)mtB showed the weak repression by hemin (Fig. 2B). These results indicated that the sequence (GCGGGGGCG) at −301/−293 corresponds to the HRE of the mouse *ALAS1* gene. To confirm the ALAS1-HRE (GCGGGGGCG), we connected tandem repeats of the double-stranded oligonucleotide to pRBGP3 carrying the minimal promoter. The resulting pALAS1 HRE (x2) and pALAS1 HRE (x4) showed the increase and decrease of the reporter activity in hemin- and SA-treated cells, respectively (Fig. 2C). Competitive assay of EMSA using ^{32}P-labeled HRE probe was carried out by the addition of an excess amount of mutated HRE fragments. As shown in Fig. 2D, the mutation of a single nucleotide inside of GCGGGGGC diminished the competition, indicated that the sequence of GGGGG in ALAS1-HRE was critically important in the heme repression. The GC-rich site corresponding to the putative ALAS1-HRE was found in the proximal promoter region of rat and human *ALAS1* gene (Fig. 2E).

3.2. Egr-1 and NAB1/2 complex binds to the ALAS1-HRE

Several transcription factors including Sp family and Egr-1 were found to bind to the GC-rich sequence of the gene promoter. Especially, Egr-1 functions in transcription of external stimuli and responds to oxidative stress such as ischemia/reperfusion, hypoxia, and heme. Then we examined the role of Egr-1 toward the heme repression through ALAS1-HRE. Immunoblot analysis showed that the expression of Egr-1 was rapidly increased by the treatment with hemin while those of NAB1 and NAB2, corepressors of Egr-1, did not change in hemin-treated cells (Fig. 3A). The analysis by RT-PCR

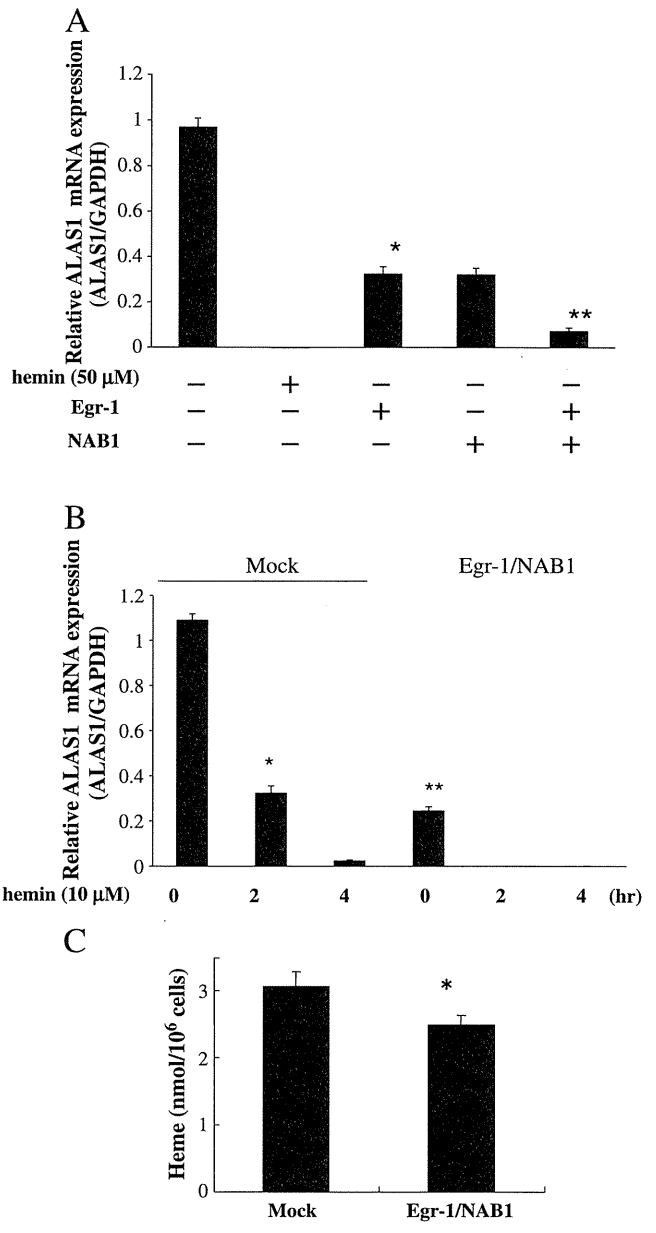


Fig. 4. Effect of the expression of Egr-1 and NAB1 on the levels of ALAS1 mRNA and intracellular heme. (A) RT-PCR analysis of the mouse ALAS1 mRNA in NIH3T3 cells overexpressing Egr-1 and NAB1. Cells were cotransfected with pCG-HA-Egr-1 and p3x-Flag-NAB1, followed by the treatment with 50 μM hemin for 16 h. RNA was isolated, and RT-PCR was carried out. The products were separated using an agarose gel. The DNA amount in the gel was quantified with Image J software (*P<0.01, Egr-1 overexpressing cells versus control cells; **P<0.001, Egr-1 and NAB1 overexpressing cells versus control cells). (B) Time course of the level of the ALAS1 mRNA in hemin-treated cells. The cells cotransfected with the indicated plasmid were cultured for 16 h and treated with 10 μM hemin in FCS-free medium. *P<0.01, hemin treatment versus control; **P<0.01, Egr-1 and NAB1 overexpressing cells versus control cells. (C) The intracellular level of heme in NIH3T3 cells overexpressing Egr-1 and NAB1. The cells were transfected with plasmids carrying Egr-1 and NAB1. After 24 h cultivation, the cells were washed twice with PBS and collected. The content of heme in cells was estimated after the conversion of heme to protoprphyrin. The data are the average ± SEM for five independent experiments (*P<0.01, treated versus vehicle control).

revealed that Egr-1 mRNA was increased by hemin treatment (data not shown), indicating that the induction of Egr-1 by heme is regulated at the transcriptional level. These results indicated that the expression of Egr-1 was controlled in response to the intracellular level of heme. The amount of Egr-1 in NIH3T3 cells was increased by the transfection with pCG-HA-Egr-1 (Fig. 3A). To investigate the involvement of Egr-1–NABs complexes in the heme-dependent

repression of the ALAS1 gene, reporter assay carrying ALAS1-HRE was carried out (Fig. 3B). When Egr-1 and NAB1 were overexpressed, the reporter activity of pGL-ALAS1(−326) decreased while that of pGL-ALAS1(−293) did not (Fig. 3B). The treatment of the cells over-expressing Egr-1 with hemin resulted in marked decrease of the reporter activity of pGL-ALAS1(−326), indicating that the activity is decreased dependent on the increase in the expression of Egr-1. We then conducted an EMSA of a synthetic oligonucleotide probe containing mouse ALAS1-HRE, using nuclear extracts of hemin- and SA-treated cells. Increases in the bindings corresponding to two bands to the nuclear extracts of hemin-treated cells were observed while the level of the binding slightly increased with the nuclear extracts of SA-treated cells (Fig. 3C). The upper bands disappeared upon preincubation of the nuclear extracts with anti-Egr-1, anti-NAB1 and anti-NAB2, but not with control IgG. These results indicated that the binding complex was composed of Egr-1 and NAB1/2. The status of the endogenous transcription complexes present on the ALAS1-HRE was determined by ChIP assay. The presence of the ALAS1-HRE in the chromatin immunoprecipitates was analyzed by semiquantitative PCR using specific pairs of primers spanning the ALAS1-HRE of the mouse *ALAS1* gene. As shown in Fig. 3D, Egr-1 was bound to ALAS1-HRE, and an increase in the binding of Egr-1 was observed when cells were treated with 50 μM hemin. The binding of NAB1/2 to ALAS1-HRE increased in parallel in the same cells. The increases of these factors were cancelled by treatment with 1 mM SA. These results, thereby, indicated that Egr-1 and NAB1/2 were involved in the repression of the expression of the mouse ALAS1 by heme.

3.3. Heme-dependent repression of the expression of ALAS1 gene via Egr-1 and NAB1

To verify the involvement of Egr-1 and NABs in the repression of the expression of the mouse ALAS1, we examined the expression of ALAS1 in pCG-HA-Egr-1 and p3x-Flag-NAB1-transfected cells. As shown in Fig. 4A, the level of ALAS1 mRNA was down-regulated by coexpressions of Egr-1 and NAB1. When the cells coexpressing Egr-1 and NAB1 were treated with 10 μM hemin, the expression of ALAS1 mRNA was rapidly decreased (Fig. 4B). Finally, the expression of Egr-1 and NAB1 led to a decrease of the intracellular level of heme (Fig. 4C). Taken together, the Egr-1–NABs complex occupied the ALAS1-HRE of the promoter of the mouse *ALAS1* gene in response to heme and reduced the expression of ALAS1.

4. Discussion

The present study showed that Egr-1 plays an important role in the regulation of heme biosynthesis and directly represses the expression of ALAS1 by heme. The expression of ALAS1 mRNA was markedly reduced when the content of heme in mouse NIH3T3 cells was increased by treatment with hemin. In contrast, exogenously added SA slightly increased the level of ALAS1 mRNA. These findings were similar to previous observations that the expression of ALAS1 in non-erythroid cells was reduced when these cells were cultured with hemin (Okano et al., 2010) and support the negative feedback effect of heme on heme biosynthesis (Furuyama et al., 2007). Using a promoter assay of the mouse *ALAS1* gene, the present study confirmed that the expression of ALAS1 mRNA is regulated by hemin at the transcriptional level. There are also many reports that heme reduces the expression of ALAS1 protein by the coordinate regulation of posttranslational events such as the stability of ALAS1 mRNA, the import of precursor ALAS1 into mitochondria and the enzyme activity (Lathrop and Timko, 1993; Munakata et al., 2004). We have shown for the first time that the heme-dependent repression of the expression of mouse ALAS1 at the transcriptional level occurred in the proximal promoter region of *ALAS1* gene where is interacted with Egr-1. The decrease of ALAS1 mRNA was dependent on the level of Egr-1, but the decrease by hemin treatment was greater than that in Egr-1 and NAB1 overexpressing cells (Figs. 3A and 4A), suggesting that heme not only negatively regulates the transcription of the ALAS1 gene but also reduces the stability of ALAS1 mRNA.

Other investigators (Fraser et al., 2003; Handschin et al., 2005; Peyer et al., 2007) have shown that several transcription factors including nuclear receptors CAR, PXR, FXR and HNF4α as well as the coactivator PGC-1α mediate the transcriptional regulation of ALAS1 to various stumuli such as fasting, or upon the exposure to endo- and xenobiotics. Since it is recently reported that ALAS1 expression is repressed by heme at a transcriptional level, mediating by recently identified nuclear receptors such as Rev-erbα (Yin et al., 2007; Wu et al., 2009), we tried to identify the site of the enhancer region involved of nuclear receptors in down-regulation of the expression of the mouse ALAS1 transcription, which is exerted by the end product heme. The reporters carrying the repeats of DR1 or RORE-like region (−8 to −2.8 kb) were constructed, and the effect of heme on the luciferase activity was examined. As far as we examined, we failed in the detection of the involvement of heme in the decrease of the transcriptional activity in these regions even by the expression of Rev-erbα. On the other hand, a heme-dependent decrease of the ALAS1 promoter activity was found in the proximal region (−301 to −293b), a finding consistent with the previous observations with the rat ALAS1 promoter (Varone et al., 1999).

The expression of ALAS1 is known to circadian, regulated by NPAS2, a heme-binding circadian factor and PGC-1α (Yin et al., 2007). In addition to repressive function of Rev-erbα (Wu et al., 2009), this feedback loop may control heme biosynthesis. However, this model seems to be an indirect regulation which is necessary for de novo protein synthesis. Additionally, the present study showed the direct effect of heme on the transcription of *ALAS1* gene, by regulating the binding of Egr-1 and NAB1/2 on the mouse ALAS1 promoter located at −301/−293. The EMSA with mutated competitors of mutants and reporter assays revealed that the sequence GCGGGGGCG was designated as mouse ALAS1-HRE. Interestingly, the possible ALAS1-HRE is found in the proximal region of the promoter of mammalian *ALAS1* gene. Varone et al. (1999) reported the GC rich regions which correspond to the putative AP-2 binding sites in proximal site of rat ALAS1 promoter, and these may contribute to the heme-dependent repression of the gene. Using chromatin immunoprecipitations, we found that the recruitments of Egr-1 and NAB1/2 to the ALAS1-HRE increased in hemin-treated cells. When we examined the involvement of AP-2 in the transcriptional activity of ALAS1-HRE, an increase in the activity by expression of AP-2α was observed (data not shown). Considering that the molecular and functional interactions of AP-2 with Egr-1 were found in the GC rich region of some genes (Imhof et al., 1999; Dabir et al., 2008), AP-2 and Egr-1 may share the HRE, and effectively compete with them. Therefore, in addition of contribution of Rev-erbα to the repressive effect on the enhancer region of *ALAS1* gene, Egr-1/NABs are involved in the heme-dependent repression at the proximal region of ALAS1 promoter.

There are several transcription factors such as Sp and Egr families recognizing GC-rich sequences (Imhof et al., 1999; Blaschke et al., 2004). Members of Egr family play diverse physiological roles and have negative and positive effects on growth. The NAB1 and NAB2, major transcriptional corepressors of Egr-1, directly interact with a conserved domain found in Egr-1 and repress the activation of the various target promoters (Houston et al., 2001; Kumbrink et al., 2005). We now demonstrated that the binding of Egr-1 as well as NAB1/2 complex to the ALAS1-HRE was increased by the treatment of the cells with hemin. We also found that overexpressions of Egr-1 and NAB1 resulted in decreases of the expression of ALAS1 mRNA and the intracellular level of heme. It is known that Egr-1 was induced by various stimuli including inflammation, ROS/oxidative stress, hypoxia and hyperoxia (Blaschke et al., 2004; Khachigian, 2006). Consistent with a recent finding that heme up-regulates the expression of Egr-1

(Hasan and Schafer, 2008), the present data showed that the level of Egr-1 was rapidly increased in hemin-treated cells (Fig. 3A), indicating a underlying mechanism for regulating the repressive function of Egr-1 and NAB1/2 complex in response to intracellular heme level. Thus, induced Egr-1 by heme provides down-regulation of the transcription of mouse ALAS1 via ALAS1-HRE. Moreover, since the expression of Egr-1 was regulated by multiple factors, it is possible that the Egr-1 binding site in the promoter of *ALAS1* gene can response to various factors as well as heme.

In summary, we have now identified the molecular mechanism of the heme-dependent transcriptional repression of ALAS1 via the regulation of Egr-1 and NABs. The exact functional correlates of the regulation of Egr-1 on the expression of ALAS1 are likely to be more complex, and the mechanisms involved in the increase in the recruitment of Egr-1 to the ALAS1-HRE by heme are unclear. These will require further investigations using in vitro and in vivo systems.

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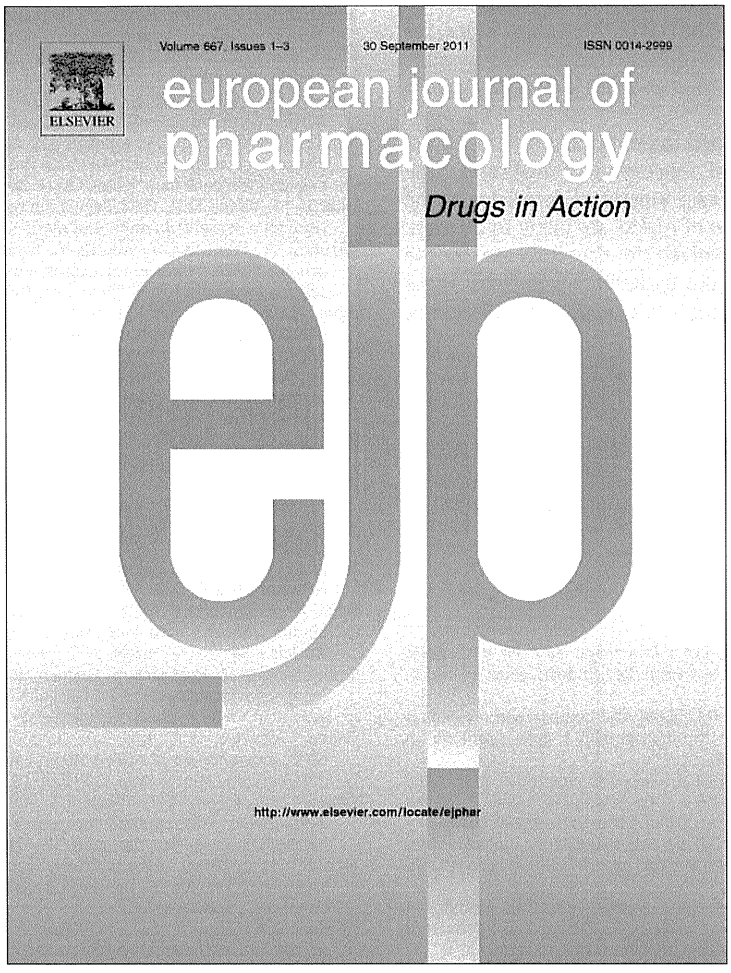

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Cytotrienin A, a translation inhibitor that induces ectodomain shedding of TNF receptor 1 via activation of ERK and p38 MAP kinase

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ABSTRACT

Cytotrienin A, a member of the triene-ansamycin family, was initially identified to be an inducer of apoptosis and recently shown to be an inhibitor of translation that interferes with eukaryotic elongation factor 1A function. In human lung carcinoma A549 cells, cytotrienin A was found to inhibit more strongly the cell-surface expression of intercellular adhesion molecule-1 (ICAM-1) induced by tumor necrosis factor (TNF)-α than the expression induced by interleukin (IL)-1α. Cytotrienin A induced the ectodomain shedding of TNF receptor 1 by TNF-α-converting enzyme (TACE). The TACE inhibitor TAPI-2 antagonized the selective inhibitory effect of cytotrienin A on inhibitor of nuclear factor-κB-α (IκBα) degradation as well as ICAM-1 expression in TNF-α-stimulated cells. The MEK inhibitor U0126 and the p38 MAP kinase inhibitor SB203580, but not the JNK inhibitor SP600125, prevented the ectodomain shedding of TNF receptor 1 induced by cytotrienin A and reversed the inhibitory effects of cytotrienin A on the TNF-α-induced IκBα degradation. In the presence of both U0126 and SB203580, cytotrienin A inhibited TNF-α- and IL-1α-induced ICAM-1 expression at almost equivalent concentrations. Thus, our present results demonstrate that cytotrienin A is a translation inhibitor that triggers ribotoxic stress response and selectively inhibits the TNF-α-induced ICAM-1 expression by inducing the ectodomain shedding of TNF receptor 1 via the activation of ERK and p38 MAP kinase.

1. Introduction

Pro-inflammatory cytokines mainly induce the activation of the nuclear factor-κB (NF-κB) signaling pathway that leads to the expression of a variety of genes essential for inflammation and carcinogenesis (Karin and Greten, 2005). Upon stimulation with tumor necrosis factor (TNF)-α and interleukin (IL)-1, TNF receptor 1 and IL-1 receptor recruit different sets of adaptor proteins required for the activation of inhibitor of NF-κB (IκB) kinase that phosphorylates the N-terminal serine residues of IκB (Bhoj and Chen, 2009; Hayden and Ghosh, 2008). Phosphorylated IκB is ubiquitinated and immediately undergoes proteasomal degradation. In unstimulated cells, the NF-κB heterodimer forms a complex with IκB and localizes in the cytosol. Upon IκB degradation, the NF-κB heterodimer becomes free and translocates to the nucleus where it activates the transcription of target genes, such as intercellular adhesion molecule-1 (ICAM-1) (Roebuck and Finnegan, 1999). It has been shown that various types of natural and synthetic small molecules specifically block the NF-κB signaling pathway (Kataoka, 2009).

TNF-α-converting enzyme (TACE), also referred to as a disintegrin and metalloprotease 17 (ADAM17), is a cell-surface metalloproteinase required for the ectodomain shedding of cytokines (e.g., TNF-α), growth factors (e.g., TGF-α), receptors (e.g., TNF receptor 1), and other cell-surface proteins (Seals and Courtneidge, 2003). Ectodomain shedding produces soluble ligands and receptors and thus plays a crucial role in the regulation of inflammatory responses. It has been reported that extracellular signal-regulated kinase (ERK) and p38 mitogen-activated protein (MAP) kinase phosphorylate the cytoplasmic tail of TACE at threonine 735 and thereby regulate TACE-mediated ectodomain shedding (Díaz-Rodríguez et al., 2002; Liu et al., 2009; Soond et al., 2005; Xu and Derynck, 2010).

Cytotrienin A (Fig. 1A), a member of the triene-ansamycin family, has been initially identified as an inducer of apoptosis in human leukemia HL-60 cells (Kakeya et al., 1997; Zhang et al., 1997). Recently, cytotrienin A was shown to inhibit translation elongation by interfering with eukaryotic elongation factor (eEF) 1A function (Lindqvist et al., 2010). In our effort to identify anti-inflammatory agents, we found that mycotrienin II, a structural analog of cytotrienin A, inhibits ICAM-1 expression induced by inflammatory cytokines (Yamada et al., 2011). In addition, we found that cytotrienin A inhibits more strongly ICAM-1 expression induced by TNF-α than the expression induced by IL-1α. In this study, we further investigated the molecular mechanism underlying the selective inhibition of the

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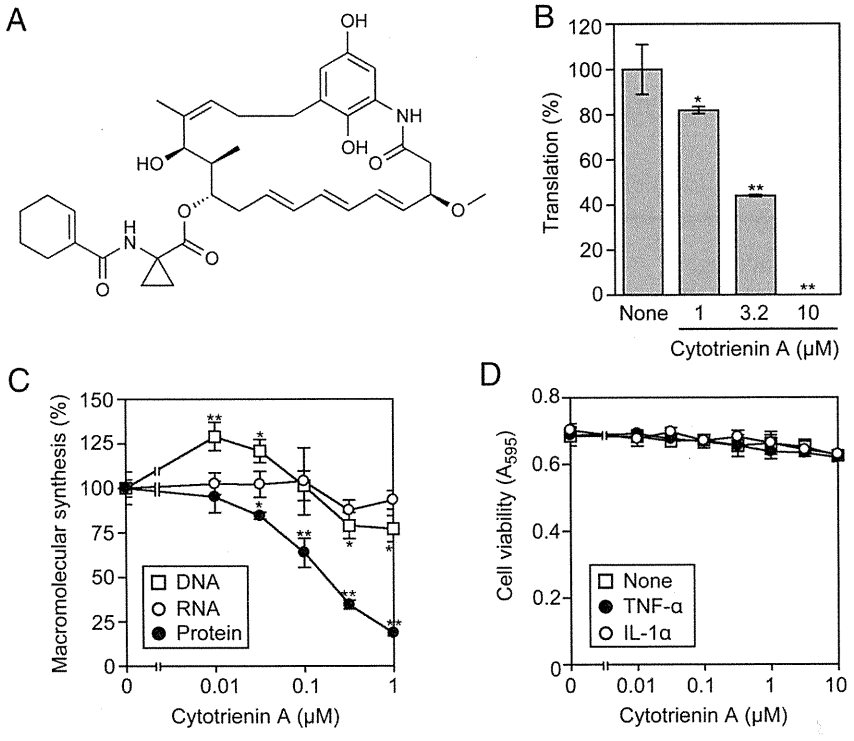

Fig. 1. Cytotrienin A directly inhibits protein synthesis. (A) Structure of cytotrienin A. (B) Luciferase mRNA was translated by the Rabbit Reticulocyte Lysate System in the presence of various concentrations of cytotrienin A. Luciferase activity (%) is shown as means ± S.D. (n = 3). *P<0.05 and ** P<0.01, compared with control. (C) A549 cells were pretreated with various concentrations of cytotrienin A for 30 min and pulse-labeled with [^{3}H]thymidine (open squares), [^{3}H]uridine (open circles), or [^{3}H]L-leucine (filled circles) for 1 h in the presence or cytotrienin A. Radioactivity incorporated into acid-insoluble fractions was measured. Radioactivity (%) is shown as means ± S.D. (n = 3). *P<0.05 and ** P<0.01, compared with control. (D) A549 cells were incubated with various concentrations of cytotrienin A for 1 h and then incubated with TNF-α (2.5 ng/ml, filled circles) or IL-1α (0.25 ng/ml; open circles) or without cytokines (open squares) for 6 h in the presence of cytotrienin A. Cell viability (A_{595}) is shown as means ± S.D. (n = 3).

TNF-α-induced ICAM-1 expression by cytotrienin A. Our present results demonstrate that cytotrienin A is a translation inhibitor that induces the TACE-mediated ectodomain shedding of TNF receptor 1 via the activation of ERK and p38 MAP kinase.

2. Materials and methods

2.1. Cell culture

Human lung carcinoma A549 cells (Health Science Research Resources Bank, Osaka, Japan) were maintained in RPMI 1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% (v/v) heat-inactivated fetal calf serum (JRH Biosciences, Lenexa, KS, USA) and penicillin–streptomycin mixed solution (Nacalai Tesque Inc., Kyoto, Japan).

2.2. Reagents

Recombinant human TNF-α and human IL-1α were kindly provided by Dainippon Pharmaceutical (Osaka, Japan). Cytotrienin A was prepared as described previously (Hayashi et al., 2008). The proteasome inhibitor MG-132 (benzyloxycarbonyl-L-leucyl-L-leucyl-L-leucinal) and the TACE inhibitor TAPI-2 (*N*-(*R*)-(2-(hydroxyaminocarbonyl) methyl)-4-methylpentanoyl-L-*t*-butyl-glycyl-L-alanine 2-aminoethyl amide) were purchased from Peptide Institute, Inc. (Osaka, Japan). The MAP kinase/ERK kinase (MEK) inhibitor U0126 (1,4-diamino-2, 3-dicyano-1,4-bis[2-aminophenylthio]butadiene; Wako Pure Chemical Industries, Ltd., Osaka, Japan), the c-Jun N-terminal kinase (JNK) inhibitor SP600125 (1,9-pyrazoloanthrone; Sigma-Aldrich Co., St. Louis, MO, USA), and the p38 MAP kinase inhibitor SB203580 (4-(4-fluorophenyl)-2-(4-methysulfinylphenyl)-5-(4-pyridyl)1H-imidazole; Cayman Chemical Co., Ann Arbor, MI, USA) were commercially obtained.

2.3. Antibodies

Antibodies to β-actin (AC-15; Sigma-Aldrich), ICAM-1 (clone 15.2; Leinco Technologies, Inc., St. Louis, MO, USA), IκBα (clone 25; BD Biosciences), and phospho-IκBα (Ser32/36) (5A5; Cell Signaling Technology, Inc., Danvers, MA, USA) and TNF receptor 1 (H-5; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) were commercially obtained.

2.4. Assay for translation

Luciferase mRNA was translated into its protein product by the Rabbit Reticulocyte Lysate System (Promega Co., Madison, WI, USA) at 30 °C for 90 min. Luciferase activity in the reaction mixtures was measured by mixing with the substrate solution (0.25 mM luciferin, 0.8 mM ATP, 1 mM DTT, 6.75 mM $MgCl_2$, 18.75 mM Tris-phosphate (pH 7.8), 0.75% Triton X-100, 0.75% bovine serum albumin, 11.25% glycerol) and relative light units were immediately measured with a Lumitester K-100 Luminometer (Hamamatsu Photonics K.K., Hamamatsu, Japan).

2.5. Assay for macromolecular synthesis

A549 cells were pulse-labeled with [4,5-^{3}H]L-leucine (41.66 TBq/mmol; Moravek Biochemicals, Inc., Brea, CA, USA), [methyl-^{3}H] thymidine (2.37 TBq/mmol; MP Biomedicals, LLC., Santa Ana, CA, USA), or [5-^{3}H]uridine (0.626 TBq/mmol; Moravek Biomedicals), and washed three times with phosphate-buffered saline (PBS). Then, the labeled cells were lysed with 0.25 M NaOH for 15 min. Macromolecules

were precipitated by 1 h incubation on ice in the presence of 5% trichloroacetic acid and then collected by centrifugation (10,000 xg, 5 min). After washing once with 5% trichloroacetic acid, radioactivity was measured with a 1900CA TRI-CARB® liquid scintillation analyzer (Packard Instrument Co., Meriden, CT, USA).

2.6. Assay for cell viability

A549 cells were pulsed with 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT, 500 μg/ml) for 2 h. Resultant MTT formazan was solubilized with 5% sodium dodecyl sulfate (SDS) overnight. Absorbance at 595 nm was measured with a Model 680 microplate reader (Bio-Rad Laboratories, Hercules, CA, USA).

2.7. Assay for cell-surface expression of ICAM-1

A549 cells were washed twice with PBS and fixed with 1% paraformaldehyde–PBS for 15 min. The cells were washed twice with PBS and incubated with 1% bovine serum albumin (Sigma-Aldrich)–PBS overnight. Fixed cells were incubated with mouse anti-human ICAM-1 IgG antibody (clone 15.2) for 60 min, followed by washing three times with 0.02% Tween 20–PBS. The cells were further incubated with horseradish peroxidase (HRP)-linked anti-mouse IgG antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) for 60 min, followed by washing three times with 0.02% Tween-20–PBS. The cells were incubated with the substrate solution (0.2 M sodium citrate (pH 5.3), 0.1% *o*-phenylenediamine dihydrochloride, 0.02% H_2O_2) for 20 min at 37 °C. Absorbance at 415 nm was measured with the Model 680 microplate reader.

2.8. Preparation of cell lysates and Western blotting

A549 cells were washed once with PBS and lysed with Triton X-100 lysis buffer (50 mM Tris–HCl (pH 7.4), 1% Triton X-100, the protease inhibitor mixture Complete™ (Roche Diagnostics, Mannheim, Germany), 2 mM DTT, 2 mM orthovanadate). Postnuclear lysates were collected by centrifugation (10,000 xg, 5 min). The culture medium was centrifuged (10,000 xg, 5 min) to remove cell debris and insoluble materials. Proteins were then precipitated with chloroform/methanol. Protein samples (30 μg/lane) were separated by SDS-PAGE and transferred onto Hybond-ECL nitrocellulose membranes (GE Healthcare, Piscataway, NJ, USA). The transferred membranes were blocked with 4% skim milk in 0.5% Tween 20–PBS overnight, and then incubated with primary antibodies and HRP-linked secondary antibodies (Jackson ImmunoResearch). Protein bands were detected by analysis using ECL Western blotting detection reagents (GE Healthcare).

2.9. Statistical analysis

Statistical significance was assessed by one-way ANOVA followed by the Tukey test for multiple comparisons. Differences of $P<0.05$ were considered to be statistically significant.

3. Results

3.1. Cytotrienin A is an inhibitor of translation

Cytotrienin A at concentrations of 1 to 10 μM directly inhibited translation in the cell-free system based on rabbit reticulocyte lysate (Fig. 1B). In human lung carcinoma A549 cells, cytotrienin A at the 50% inhibitory concentration (IC_{50}) value of around 0.1 μM significantly decreased cellular protein synthesis, but weakly inhibited or did not inhibit DNA and RNA syntheses (Fig. 1C). Cytotrienin A at concentrations of up to 10 μM did not decrease cell viability (Fig. 1D). These results indicate that cytotrienin A selectively inhibits cellular protein synthesis without affecting the viability of A549 cells.

3.2. Cytotrienin A inhibits more strongly TNF-α-induced ICAM-1 expression than IL-1α-induced ICAM-1 expression

A549 cells express cell-surface ICAM-1 in an NF-κB-dependent manner upon stimulation with pro-inflammatory cytokines (Holden et al., 2004). In this study, A549 cells were preincubated with cytotrienin A for 1 h and then incubated with TNF-α or IL-1α for 6 h. In the absence of cytotrienin A, TNF-α and IL-1α induced the cell-surface expression of ICAM-1 at similar levels. We found that cytotrienin A inhibited the TNF-α- and IL-1α-induced ICAM-1 expression in a dose-dependent manner and the IC_{50} values were 0.8 μM and 9.2 μM, respectively (Fig. 2). However, the fact that cytotrienin A did not decrease cell viability even in the presence of TNF-α (Fig. 1D) excludes the possibility that cytotrienin A sensitizes TNF-α-induced cell death and thereby nonspecifically inhibits ICAM-1 expression. Therefore, it seems likely that cytotrienin A inhibits TNF-α-induced ICAM-1 expression by targeting the TNF-α-dependent NF-κB signaling pathway.

3.3. Cytotrienin A inhibits IκBα phosphorylation and degradation induced by TNF-α, but not IL-1α

In the NF-κB signaling pathway, TNF-α and IL-1α induce the rapid phosphorylation of IκBα by IκB kinase and phosphorylated IκBα immediately undergoes proteasomal degradation. Cytotrienin A was found to inhibit the TNF-α-induced IκBα degradation (Fig. 3A). It has been shown that the proteasome inhibitor MG-132 prevents IκBα degradation, thus allowing the accumulation of phosphorylated IκBα (Takada et al., 2009). Cytotrienin A also prevented the constitutive and TNF-α-induced IκBα phosphorylation in the presence of MG-132 (Fig. 3A). However, cytotrienin A did not prevent the IL-1α-induced IκBα degradation and phosphorylation (Fig. 3B). These results suggest that cytotrienin A specifically targets the NF-κB signaling pathway upstream of IκBα phosphorylation upon TNF-α stimulation.

3.4. Cytotrienin A induces ectodomain shedding of TNF receptor 1 by TACE

TNF-α binds to two different receptors known as TNF receptor 1 and TNF receptor 2. A549 cells express only TNF receptor 1 on the cell surface (Ogura et al., 2008a). To investigate whether cytotrienin A downregulates the expression of TNF receptor 1, A549 cells were incubated with various concentrations of cytotrienin A for 1 h. Cytotrienin A decreased the intracellular level of full-length TNF receptor 1 (55 kDa) and conversely increased the amount of a small

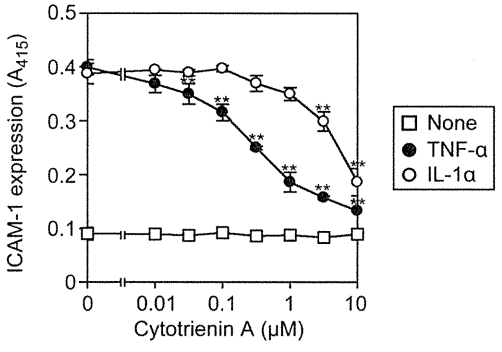

Fig. 2. Cytotrienin A inhibits more strongly TNF-α-induced cell-surface ICAM-1 expression than IL-1α-induced cell-surface ICAM-1 expression. A549 cells were pretreated with various concentrations of cytotrienin A for 1 h and then incubated with TNF-α (2.5 ng/ml; filled circles) or IL-1α (0.25 ng/ml; open circles) or without cytokines (open squares) for 6 h in the presence of cytotrienin A. ICAM-1 expression (A_{415}) is shown as means ± S.D. ($n=3$). ** $P<0.01$, compared with control.

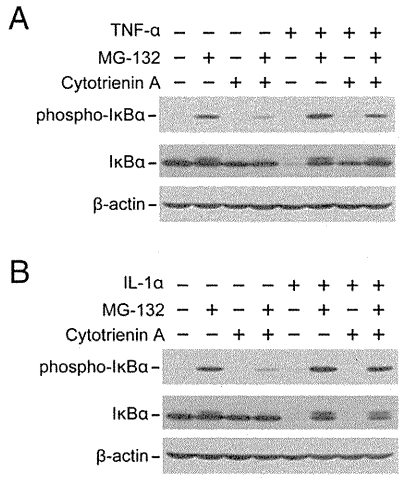

Fig. 3. Cytotrienin A inhibits IκBα phosphorylation and degradation induced by TNF-α, but not that induced by IL-1α. (A and B) A549 cells were pretreated with (+) or without (−) cytotrienin A (1 μM) in the presence (+) or absence (−) of MG-132 (20 μM) for 1 h and then treated with (+) or without (−) TNF-α (2.5 ng/ml; A) or IL-1α (0.25 ng/ml; B) for 15 min in the presence (+) or absence (−) of cytotrienin A or MG-132. Cell lysates were analyzed by Western blotting.

fragment (around 30 kDa) reactive to anti-TNF receptor 1 antibody in the culture medium (Fig. 4A). As observed previously (Ogura et al., 2008a), this fragment was most likely to be soluble TNF receptor 1 containing extracellular domain produced by proteolytic cleavage. The TACE inhibitor TAPI-2 suppressed the elevation of soluble TNF receptor 1 as well as the reduction of intracellular TNF receptor 1 in cytotrienin-A-treated cells (Fig. 4B). These results indicate that cytotrienin A induces the ectodomain shedding of TNF receptor 1 by TACE.

We further addressed whether the ectodomain shedding of TNF receptor 1 is responsible for the inhibition of the TNF-α-induced IκBα degradation by cytotrienin A. In the presence of TAPI-2, the TNF-α-induced IκBα degradation was no longer inhibited by cytotrienin A (Fig. 4C). Moreover, cytotrienin A inhibited the TNF-α- and IL-1α-induced ICAM-1 expression at relatively similar concentrations in the presence of TAPI-2 (Fig. 4D). Thus, these data indicate that the ectodomain shedding of TNF receptor 1 induced by cytotrienin A is responsible for the preferential inhibition of TNF-α-induced IκBα degradation and ICAM-1 expression by cytotrienin A.

3.5. Cytotrienin A induces ectodomain shedding of TNF receptor 1 via ERK and p38 MAP kinase

In response to various stimuli, ERK and p38 MAP kinase regulate TACE-mediated ectodomain shedding (Díaz-Rodríguez et al., 2002; Liu et al., 2009; Soond et al., 2005; Xu and Derynck, 2010). To determine which members of the MAP kinase superfamily are necessary for the TACE-mediated ectodomain shedding of TNF receptor 1 in A549 cells exposed to cytotrienin A, specific inhibitors of the MAP kinase superfamily were used as blocking agents. In cytotrienin-A-treated cells, the MEK inhibitor U0126 and the p38 MAP kinase inhibitor SB203580 prevented the release of soluble TNF

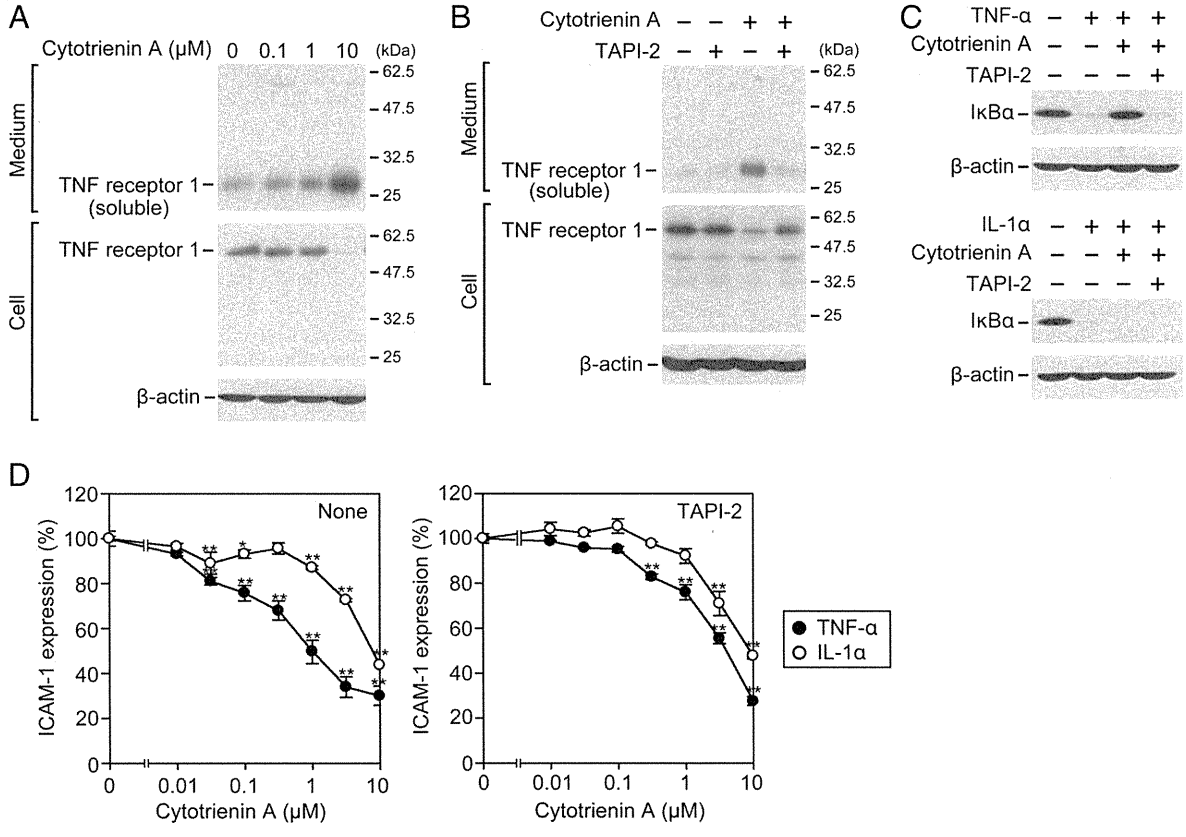

Fig. 4. Cytotrienin A induces the ectodomain shedding of TNF receptor 1 by TACE. (A and B) A549 cells were incubated with various concentrations of cytotrienin A for 1 h (A). A549 cells were preincubated with (+) or without (−) TAPI-2 (25 μM) for 30 min and then incubated with (+) or without (−) cytotrienin A (1 μM) for 1 h in the presence (+) or absence (−) of TAPI-2 (B). The cell lysates (Cell) and the culture medium (Medium) were analyzed by Western blotting. (C) A549 cells were pretreated with (+) or without (−) TAPI-2 (25 μM) for 30 min, then treated with (+) or without (−) cytotrienin A (1 μM for TNF-α and 10 μM for IL-1α) for 1 h and stimulated with (+) or without (−) TNF-α (2.5 ng/ml) or IL-1α (0.25 ng/ml) for 15 min in the presence (+) or absence (−) of TAPI-2 or cytotrienin A. Cell lysates were analyzed by Western blotting. (D) A549 cells were pretreated with or without TAPI-2 (25 μM) for 30 min, then treated with various concentrations of cytotrienin A for 1 h and incubated with TNF-α (2.5 ng/ml; filled circles) or IL-1α (0.25 ng/ml; open circles) for 6 h in the presence or absence of TAPI-2 or cytotrienin A. ICAM-1 expression (%) is shown as means ± S.D. (n = 3). *P<0.05 and ** P<0.01, compared with control.

receptor 1 markedly but not completely, whereas the JNK inhibitor SP600125 was inactive (Fig. 5A). In accord with these data, pretreatment with either U0126 or SB203580, but not SP600125, reversed the blockade of the TNF-α-induced IκBα degradation by cytotrienin A at concentrations of 1 to 10 μM (Fig. 5B). In the presence of either U0126 or SB203580, the selective inhibitory effects of cytotrienin A on the TNF-α- and IL-1α-induced ICAM-1 expression became less evident (Fig. 5C). Moreover, cytotrienin A inhibited ICAM-1 expression induced by TNF-α and IL-1α at almost equivalent concentrations when A549 cells were treated with the combination of U0126 and SB203580 (Fig. 5C). This effect was consistent with the observation that the ectodomain shedding of TNF receptor 1 induced by cytotrienin A was completely suppressed by the combined treatment with U0126 and SB203580 (Fig. 5A).

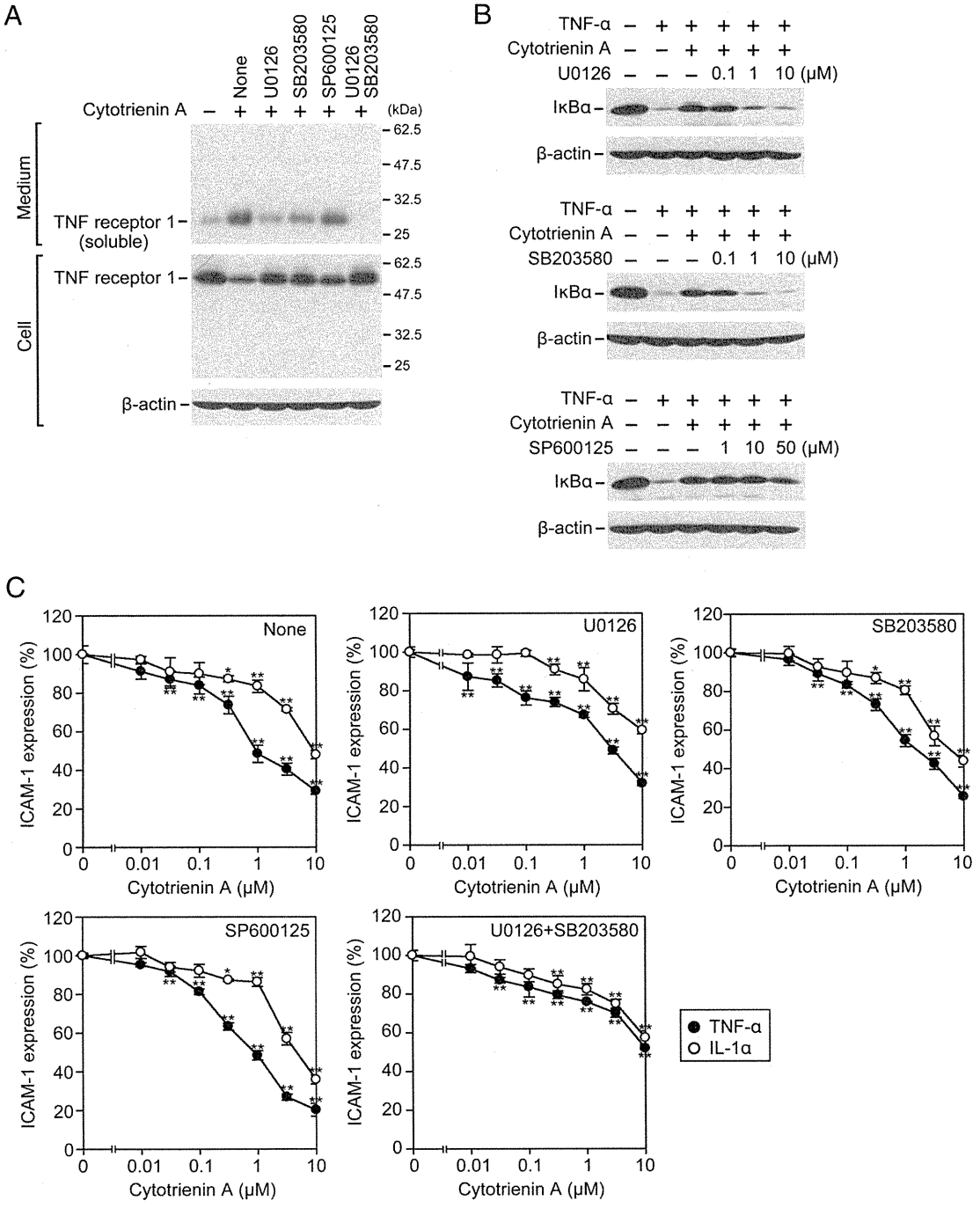

Fig. 5. ERK and p38 MAP kinase are necessary for the ectodomain shedding of TNF receptor 1 induced by cytotrienin A. (A) A549 cells were preincubated with or without U0126 (10 μM), SB203580 (10 μM), SP600125 (10 μM) or U0126 (10 μM) plus SB203580 (10 μM) for 1 h and incubated with (+) or without (−) cytotrienin A (1 μM) for 1 h in the presence or absence of the protein kinase inhibitors. The cell lysate (Cell) and the culture medium (Medium) were analyzed by Western blotting. (B) A549 cells were pretreated with various concentrations of U0126, SB203580 or SP600125 for 1 h, then treated with (+) or without (−) cytotrienin A (1 μM) for 1 h and stimulated with (+) or without (−) TNF-α (2.5 ng/ml) for 15 min in the presence (+) or absence (−) of cytotrienin A or the protein kinase inhibitors. Cell lysates were analyzed by Western blotting. (C) A549 cells were pretreated with or without U0126 (10 μM), SB203580 (10 μM), SP600125 (10 μM) or U0126 (10 μM) plus SB203580 (10 μM) for 1 h, then treated with various concentrations of cytotrienin A for 1 h and incubated with TNF-α (2.5 ng/ml; filled circles) or IL-1α (0.25 ng/ml; open circles) for 6 h in the presence or absence of cytotrienin A or the protein kinase inhibitors. ICAM-1 expression (%) is shown as means ± S.D. (n = 3). *P<0.05 and ** P<0.01, compared with control.

4. Discussion

Cytotrienin A, a member of the triene-ansamycin family, was recently shown to be a translation inhibitor that targets eEF1A function (Lindqvist et al., 2010). Cytotrienin A was found to inhibit more strongly the TNF-α-induced cell-surface ICAM-1 expression than the IL-1α-induced expression and to selectively block the TNF-α-dependent NF-κB signaling pathway. Due to the fact that cytotrienin A blocks translation, it was reasonably speculated that cytotrienin A would prevent the cytokine-induced ICAM-1 expression at the translation stage. However, the molecular mechanism by which cytotrienin A exerts a strong inhibitory effect on the NF-κB signaling pathway as well as the ICAM-1 expression upon TNF-α stimulation remained unclear. In this study, we have demonstrated that cytotrienin A induces the ectodomain shedding of TNF receptor 1 and thereby diminishes responsiveness to TNF-α possibly via the downregulation of cell-surface TNF receptor 1.

In addition to mycotrienin II and its structural derivatives (Yamada et al., 2011), cytotrienin A inhibited more strongly the TNF-α-induced ICAM-1 expression than the IL-1α-induced ICAM-1 expression. Thus, it seems that these inhibitory profiles are common to the triene-ansamycin group compounds that show the ability to inhibit translation (Lindqvist et al., 2010; Yamada et al., 2011; Yamamoto et al., 2011). Similar inhibitory profiles were also observed with other structurally different translation inhibitors, i.e., acetoxycycloheximide and anisomycin (Sugimoto et al., 2000; Yamada et al., 2011). We have previously shown that acetoxycycloheximide induces the ectodomain shedding of TNF receptor 1 by TACE (Ogura et al., 2008a). As cytotrienin A inhibited the TNF-α- and IL-1α-induced ICAM-1 expression at similar concentrations only in the presence of the TACE inhibitor TAPI-2, these observations clearly indicate that the TACE-mediated ectodomain shedding of TNF receptor 1 is responsible for the selective inhibitory effects of cytotrienin A on the TNF-α-induced ICAM-1 expression and the IκBα degradation.

It has been shown that cytotrienin A triggers the activation of JNK, p38 MAP kinase, and p36 myelin basic protein (MBP) kinase (Kakeya et al., 1998; Watabe et al., 2000). Some types of translation inhibitors, such as acetoxycycloheximide and anisomycin, are known to induce ribotoxic stress response, which leads to the activation of MAP kinase superfamily, and to regulate various cellular responses (Chinen et al., 2010; Iordanov et al., 1997; Kadohara et al., 2005; Ogura et al., 2008b; Shifrin and Anderson, 1999; Sidhu and Omiecinski, 1998). In this study, we have shown that either the MEK inhibitor or the p38 MAP kinase inhibitor reduces the ectodomain shedding of TNF receptor 1 induced by cytotrienin A. Therefore, we conclude that cytotrienin A is a translation inhibitor that can trigger ribotoxic stress response.

It has been reported that ERK and p38 MAP kinase phosphorylate the cytoplasmic tail of TACE and induce the TACE-mediated ectodomain shedding by regulating intracellular localization, maturation, and proteolytic function (Díaz-Rodríguez et al., 2002; Liu et al., 2009; Soond et al., 2005; Xu and Derynck, 2010). The TACE-mediated ectodomain shedding of TNF receptor 1 in cytotrienin-A-treated cells was reduced by either the MEK inhibitor or the p38 MAP kinase inhibitor and suppressed completely by their combined treatment. Therefore, it seems that the ERK pathway and the p38 MAP kinase pathway complementarily regulate the TACE-mediated ectodomain shedding of TNF receptor 1. In agreement with this notion, cytotrienin A inhibited the TNF-α- and IL-1α-induced ICAM-1 expression at almost equivalent concentrations only when both ERK and p38 MAP kinase were pharmacologically blocked.

The concentrations of cytotrienin A required to inhibit the IL-1α-induced ICAM-1 expression were much higher than those required to inhibit cellular protein synthesis. This characteristic effect was conserved in mycotrienin II (Yamada et al., 2011), but might not be common in other structurally different translation inhibitors, because we observed that cycloheximide prevented cellular protein synthesis and the IL-1α-induced ICAM-1 expression at similar concentrations (Sugimoto et al., 2000). It is a well-established fact that eEF1A is indispensable for the translation of all polypeptide-encoded mRNAs (Lamberti et al., 2004; Mateyak and Kinzy, 2010). Therefore, the inhibitory effect of cytotrienin A may be influenced by unidentified intracellular factors and/or conditions other than eEF1A in intact living cells.

Translation inhibitors, such as cycloheximide, are frequently used to sensitize tumor cells to TNF-α-induced cell death, largely by preventing the expression of NF-κB-responsive anti-apoptotic proteins, such as c-FLIP (Aggarwal, 2003; Kataoka, 2005). Due to the fact that the ectodomain shedding of TNF receptor 1 is induced by cytotrienin A or acetoxycycloheximide, translation inhibitors are not always effectively sensitize cells to TNF-α-induced cell death. In fact, we have previously shown that cycloheximide, regarded as a weak inducer of ribotoxic stress response, downregulates cell-surface TNF receptor 1 expression at high concentrations and potentiates TNF-α-induced caspase-8 activation only when metalloproteinase inhibitors are present (Ogura et al., 2008a). Therefore, in addition to the c-FLIP level, the expression of the cell-surface TNF receptor 1 and related proteins should be evaluated when translation inhibitors are used to promote susceptibility to TNF-α in various types of cells.

In conclusion, our data reveal that cytotrienin A is a translation inhibitor that can induce the ectodomain shedding of TNF receptor 1 via the activation of ERK and p38 MAP kinase. Triene-ansamycin compounds have been shown to exert several biological activities, including antitumor activity. Thus, the ability of these compounds to block translation as a molecular target and thereby induce ribotoxic stress response may account for the molecular basis of some biological activities that have been not previously understood.

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