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Transcriptional Regulation of IL-8 by Iron Chelator in Human Epithelial Cells is Independent From NF-κB but Involves ERK1/2- and p38 Kinase-Dependent Activation of AP-1

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Abstract We have shown that the bacterial iron chelator, deferoxamine (DFO), triggers inflammatory signals including the production of CXC chemokine IL-8, in human intestinal epithelial cells (IECs) by activating the ERK1/2 and p38 kinase pathways. In this study we investigated the mechanisms involved in IL-8 generation by DFO, focusing on the transcription factors involved and the roles of both mitogen-activated protein kinases (MAPKs) in the transcription factor activation. Treatment of human epithelial HT-29 cells with DFO markedly up-regulated the expression of the essential components of the transcription factor AP-1 at a transcriptional level, while it minimally affected the expression of the NF-κB subunits. DFO also induced AP-1-dependent transcriptional activity in HT-29 cells, and this activity was further augmented by the wild-type c-Jun transfection. In contrast, the AP-1 activity by DFO was markedly decreased by the dominant-negative c-Jun transfection. Electrophoretic mobility shift assays revealed that DFO increases the specific binding of AP-1 but not of NF-κB. Such AP-1 binding and transcriptional activities were blocked by the inhibitors of the ERK1/2 and p38 kinase pathways, suggesting that both mitogen-activated protein kinases (MAPKs) lie upstream of AP-1. Besides its action on AP-1, DFO also induced the specific binding of other transcription factors such as CREB and Egr-1. In summary, our results indicate that iron chelator-induced IL-8 generation in IECs involves activation of ERK1/2 and p38 kinase and downstream activation of AP-1. A possible link between iron status and two additional transcription factors, that is, CREB and Egr-1, rather than NF-κB, was also suggested. J. Cell. Biochem. 102: 1442–1457, 2007.

Key words: iron chelator; human intestinal epithelial cells; interleukin-8; AP-1; NF-κB

The intestinal epithelium, a complex physical and biochemical barrier, participates in innate immune responses. Initiation and maintenance of the inflammatory response in the intestinal epithelium requires intercellular regulatory molecules. One such messenger is interleukin (IL)-8, a key component of the innate immune response. IL-8 production is upregulated in intestinal epithelial cells (IECs) upon infection by microbial pathogens or stimulation by pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α) and IL-1β [Warhurst et al., 1998; Dwinell et al., 2003] which in turn attracts various inflammatory cells including neutrophils. Neutrophils are known to synthesize and secrete lactoferrin, a member of transferrins, and thereby regulate iron homeostasis and modulate the inflammatory processes in the infected tissues [Cavestro et al.,

Eun-Young Choi and Zee-Yong Park contributed equally to this work.

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2002; Legrand et al., 2004; Valenti et al., 2004; Ward and Conneely, 2004].

Inflammatory responses in the intestinal epithelium are caused by mainly bacteria and injury. The intestinal epithelium is always exposed to bacteria which have potential to initiate inflammatory responses. Pathogens entering the intestine require host iron in order to sustain their survival and proliferation [Weinberg, 1978; Neilands, 1981]. In fact, highly virulent strains possess exceptionally powerful mechanisms for obtaining host iron from healthy hosts [Weinberg, 1989]. An excellent example is bacterial siderophores which take iron from transferrins produced by a variety of host species [Weiss et al., 1994; Saleppico et al., 1996; Dlaska and Weiss, 1999; Tanji et al., 2001]. Therefore, maintaining iron availability is crucial to the survival of both eukaryotes and prokaryotes. In this regard, it is not surprising that iron chelators alter immune status and regulate inflammatory processes [Weiss et al., 1994; Saleppico et al., 1996; Dlaska and Weiss, 1999; Tanji et al., 2001]. For example, iron chelator deferoxamine (DFO) induces transcription of NO synthase (NOS) and increases the release of IL-1β in human alveolar macrophages [O'Brien-Ladner et al., 1998; Dlaska and Weiss, 1999]. DFO has been known to upregulate cyclooxygenase-2 (COX-2) and prostaglandin E2 (PGE2) production in the human macrophage cell line U937 [Tanji et al., 2001]. DFO also induces leukemic cell differentiation, potentially by hypoxia-inducible factor-1α that augments transcriptional activity of CCAAT/enhancer-binding protein-α [Jiang et al., 2005].

In good agreement with these lines, of interest was the finding that iron chelator could also cause IL-8 release in both human IECs and peripheral blood mononuclear cells (PBMCs) as a single stimulus [Choi et al., 2004; Lee et al., 2005]. These evidences suggest that, in addition to the bacterial products or cell-wall components, direct chelation of host Fe by infected bacteria may also contribute to the evocation of host inflammatory responses. DFO-induced IL-8 production was dependent on both p38 and extracellular signal-regulated kinase-1/2 (ERK1/2) activation, but independent of nuclear factor (NF)-κB activation [Choi et al., 2004].

There are various motifs within the 5′-flanking region of the human IL-8 promoter with the potential to bind a number of important transcription factors in a cell and stimulus-dependent manner. Although we previously observed that DFO-induced IL-8 production is independent of NF-κB, we paid little attention to the roles of other transcription factors that have been postulated to have binding sites on the 5′-flanking region of the human IL-8. Previous work by many groups has shown that NF-κB, activating protein-1 (AP-1), NF-IL6, cAMP response element-binding (CREB) protein, and early growth response protein (Egr-1) are involved in production of chemokines including IL-8 [Andoh et al., 1999; Jaramillo and Olivier, 2002; Kikuchi et al., 2002; Giri et al., 2003; Kumar et al., 2003; Zhu et al., 2003]. In addition, some of these transcription factors have been shown to be regulated by ERK1/2 and p38 kinase pathways [Kumar et al., 2003; Hoffmann et al., 2005]. Therefore, the aim of the present study is to determine transcription factors that are activated and involved in iron-chelator-induced IL-8 production in human IECs. We have also considered whether MAPKs could account for the DFO-induced IL-8 production at the transcription level.

MATERIALS AND METHODS

Reagents and Antibodies

DFO, mimosine (MIM), ferric citrate (FC), 4α-phorbol 12-β-myristate-13-acetate (PMA), pyrrolidine dithiocarbamate (PDTC), alkaline phosphatase-labeled monoclonal mouse anti-rabbit IgG, recombinant human IL-8, and *p*-nitrophenyl phosphate tablets were purchased from Sigma Chemical Company (St. Louis, MO). Caffeic acid phenethyl ester (CAPE), SB202190 and PD98059 were purchased from Calbiochem (La Jolla, CA) and Z-Leu-Leu-Leu-CHO (MG132) was from Biomol (Plymouth, PA). Human TNF-α and polyclonal goat anti-human IL-8 were purchased from R&D Systems, Inc. (Minneapolis, MN), and polyclonal rabbit anti-human IL-8 was from Endogen (Woburn, MA). All reagents and media for tissue culture experiments were tested for their LPS contents using a colorimetric *Limulus* amoebocyte lysate assay (detection limit 10 pg/ml; Sigma). The reagents and media that were free from the LPS contamination (<10 pg/ml) were used for further experiments. Antibodies against phosphorylated CREB and total CREB were purchased from Cell Signaling Technology

(Beverly, MA). HRP-conjugated anti-rabbit IgG was from Amersham Biosciences (Little Chalfont, UK).

Cell Culture

HT-29 human colon epithelial cells were obtained from American Type Culture Collection (ATCC) and grown in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, penicillin G (100 IU/ml), streptomycin (100 μg/ml), and L-glutamine (2 mM). Cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2 and 95% air. Log-phase cells were seeded at $0.2–10 \times 10^6$ in a 24-well, 12-well, 6-well plate, or 100 mm culture dish and used for various experimental purposes.

IL-8 Measurement

HT-29 cells were seeded at 2×10^5 in 24-well plates (Nunclon™, Denmark) and grown until formation of confluent monolayers. The cells were incubated for 16 h in a fresh medium containing stimuli as indicated. The supernatants were collected, cleared by centrifugation and kept at −20°C until evaluation by ELISA. To measure the IL-8 concentrations in the cell culture supernatants, 96-well microtiter plates (MaxiSorp™, Nunc, Denmark) were coated with 0.2 μg/well goat anti-human IL-8 antibodies (R&D Systems) in 50 μl PBS at 4°C overnight. All further steps were carried out at room temperature. After washing three times with PBS, non-specific binding sites were blocked by incubation with 150 μl PBS + 1% BSA/0.05% Tween-20/well for 2 h. After three washes with PBS, 50 μl of samples or IL-8 standards were added and incubated for 2 h. As a second antibody, 0.05 μg/well polyclonal rabbit anti-human IL-8 (Endogen) was added and incubated for 2 h. As a third antibody, alkaline phosphatase-labeled monoclonal mouse anti-rabbit IgG (Sigma) was diluted in 50 μl PBS + 0.1% BSA/0.05% Tween-20 to 1:50,000 and incubated for 2 h. Finally, alkaline phosphatase substrate *p*-nitrophenyl phosphate (Sigma) was added at a concentration of 1 mg/ml in 0.1 M glycine buffer, pH 10.4, containing 1 mM $MgCl_2$ and 1 mM $ZnCl_2$. After overnight incubation, plates were read at 405 nm on a microplate reader (Molecular Devices Corp., Sunnyvale, CA). The detection limit of the ELISA was 30 pg/ml.

RNA Isolation and RT-PCR

HT-29 cells were grown in 6-well plate and were incubated for the indicated time periods in a fresh medium containing stimuli, as indicated in the figures. After discarding the growth medium, total RNA was isolated using easy-Blue™ (Intron Biotechnology, Korea), following the manufacturer's instructions. Reverse transcription of the RNA was performed using AccuPower® RT PreMix (Bioneer, Korea). Thereafter, the RT-generated DNA (2–5 μl) was amplified using AccuPower® PCR PreMix (Bioneer) as described previously [Choi et al., 2004]. The primers used for cDNA amplification were listed in Table I. PCR conditions were as follows: an initial denaturation at 94°C for 5 min; 25 cycles being carried out for the mRNA levels of c-Fos, c-Jun, JunB, c-Rel, and GAPDH and 30 cycles for p65, each cycle with 30 s of denaturation at 94°C, 30 s of annealing at 56–65°C and 30 s of extension at 72°C; and a final dwell at 72°C for 7 min. The expected PCR products were 193 bp for c-Fos, 200 bp for c-Jun, 322 bp for JunB, 427 bp for p65, 389 bp for c-Rel and 306 bp for GAPDH. PCR products were resolved on a 1.5% agarose gel and stained with ethidium bromide. Densitometric values of the bands corresponding to the target genes were determined using UN-SCAN-IT gel™ image analysis software (Silk Scientific, Inc., Orem, UT) and statistically analyzed.

Real-Time Quantitative RT-PCR

For some experiments, the expression levels of IL-8 mRNA were evaluated by real-time RT-PCR. Total RNA was isolated and cDNA was synthesized as described above. PCR amplification was performed in DNA Engine Opticon® for continuous fluorescence detection system (MJ Research, Waltham, MA) in a total volume of 20 μl containing 1 μl of cDNA/control and gene specific primers using DyNAmo™ SYBR Green qPCR kit (MJ Research). Each PCR reaction was performed in triplicate using the following conditions: 94°C 30 s, 62°C 30 s, 72°C 30 s, plate read (detection of fluorescent product) for 40 cycles followed by 7 min extension at 72°C. Melting curve analysis was done to characterize the dsDNA product by slowly raising the temperature (0.2°C/s) from 65°C to 95°C with fluorescence data collected at 0.2°C intervals. The levels of IL-8 mRNA normalized for GAPDH were expressed as fold changes

TABLE I. Gene Specific Primers and Probes for the RT-PCR and EMSA

ODNs	Gene name	Primer or probe sequences (5′ → 3′)	References
Primer sequences for the RT-PCR	c-Fos	F: ATGTTCTCGGGCTTCAACGCAGA	Marden et al. [2003]
	Jun B	R: CAGTGACCGTGGGAATGAAGTTGG	Marden et al. [2003]
		F: CTGGCCGACCCCTACCGGAGT	
		R: CATCTTGTGCAGATCGTCCAGGG	
	c-Jun	F: GCCTCCAAGTGCCGAAAAAG	Hayez et al. [2004]
		R: CAAAATGTTTGCAACTGCTGC	
	p65	F: AGCGCATCCAGACCAACAACAACC	Takao et al. [2003]
		R: CCGCCGCAGCTGCATGGAGACAC	
	c-Rel	F: CCCCACGCTCAGGCAATACAAACC	Takao et al. [2003]
		R: GGCGCCTGCTGACATACTGGAAGA	
	GAPDH	F: CGGAGTCAACGGATTTGGTCGTAT	Choi et al. [2004]
		R: AGCCTTCTCCATGGTGGTGAAGAC	
Probe sequences for the EMSA	NF-κB/IL-8	GCAAATCGTGGAATTTCCTCTGAC	Kikuchi et al. [2002]
	AP-1/IL-8	AGTGTGATGACTCAGGTTTGCCC	Kikuchi et al. [2002]
	NF-IL6/IL-8	TACTCCGTATTTGATAAGGAACAAATA	Kikuchi et al. [2002]
	Egr-1	GGATCCAGCGGGGGCGAGCGGGGGCGA	Giri et al. [2003]
	CREB	AGAGATTGCCTGACGTCAGAGAGCTAG	Giri et al. [2003]

ODNs, oligodeoxynucleotides; F, forward; R, reverse.

relative to that of the untreated controls. The fold change in gene expression was calculated using the following equation: Fold change $= 2^{-\Delta\Delta CT}$, where $\Delta\Delta C_T = (C_{T,Target} - C_{T,GAPDH})_{Time\ x} - (C_{T,Target} - C_{T,GAPDH})_{Time\ 0}$, in which Time x is any time point and Time 0 represents the 1× expression of the target gene of untreated cells, which was normalized to GAPDH [Livak and Schmittgen, 2001].

Cell Viability Assay

Cell viability was examined by MTT assay as described previously [Ben Trivedi et al., 1990]. A stock solution of MTT was prepared in phosphate-buffered saline (PBS), diluted in RPMI1640 medium and added to cell-containing wells at a concentration of 0.5 mg/ml after removing the culture medium. The plates were then incubated for 4 h at 37°C in 5% CO_2. At the end of incubation, the medium was aspirated, and the formazan product was solubilized with dimethyl sulfoxide (DMSO). Absorbency was measured on a multi-scan reader with a 570 nm wavelength filter. All experiments were performed at least three times.

Transient Transfection and Luciferase Assay

HT-29 cells were seeded at a density of 2×10^5 cells per well in a 24-well plate 1 day prior to transfection. The cells were transfected with Lipofectamine™ 2000 reagent (Invitrogen) according to the manufacturer's protocol. The cells were incubated for 6 h with transfection reagents and 0.8 μg NF-κB-, IL-8- (obtained from Dr. J.-S. Chun, Kwangju Institute of Science and Technology, Korea), or AP-1-(purchased from Clontech) promoter-luciferase reporter constructs. In some experiments, cells were transfected with either wild-type (WT) c-Jun or dominant-negative (DN) c-Jun vector (obtained from Prof. D.-K. Kim, Chonbuk National University, Korea), together with AP-1- or IL-8 promoter-luciferase reporter constructs. After the transfection period, the medium was changed, and the cells were allowed to recover overnight before treatment. The cells were then treated as indicated. Twenty-four hours later, cells were washed with PBS and were lysed in 70 μl/well of lysis reagent. Luciferase activity was assayed using Luciferase Assay System (Promega, Madison, WI), according to the manufacturer's instructions.

Electrophoretic Mobility Shift Assay (EMSA)

HT-29 cells (1×10^7) were cultured on 100-mm dishes and were treated as indicated. The nuclear fractions for EMSA were prepared by pipetting the cell pellets in a buffer containing 1 M HEPES, 1 M $MgCl_2$ and 0.5% Triton X-100, followed by repetitive pipetting in a buffer containing 1 M HEPES, 0.5 M EDTA, 1 M $MgCl_2$, 0.1 M DTT, 2.5 M KCl, 10% glycerol, and protease inhibitors. Protein concentrations of nuclear fractions were determined by Bradford assay. Oligonucleotides used for the EMSA

study were listed in Table I. Probes were labeled using [γ-^{32}P]ATP (Perkin Elmer) and T4 polynucleotide kinase (Takara, Japan). The DNA binding reaction mixture contained nuclear proteins (8–10 μg), ^{32}P-labeled double-stranded oligonucleotide probe (~20,000 cpm), and 2 μg poly(dI-dC). In competition assays, 100× unlabeled probes were added prior to the addition of the labeled probes. The mixture was incubated in a buffer containing 1 M HEPES, 0.5 M EDTA, 1 M $MgCl_2$, 0.1 M DTT, 10% glycerol, and protease inhibitors at room temperature for 20 min and then loaded on a 5% polyacrylamide gel in 0.5× TBE buffer followed by electrophoresis. The gel was vacuum-dried and exposed to X-ray film at −70°C for 18 h. Densitometric values of the bands were determined using UN-SCAN-IT gel™ image analysis software (Silk Scientific).

Western Blotting

For analysis of the phosphorylated or total protein levels of MAPKs and CREB, stimulated cells (5×10^5/well) were rinsed twice with ice-cold phosphate-buffered saline and then lysed in ice-cold lysis buffer (50 mM Tris-HCl, pH 7.4, containing 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, 0.1% deoxycholate, 5 mM sodium fluoride, 1 mM sodium orthovanadate, 1 mM 4-nitrophenyl phosphate, 10 μg/ml of leupeptin, 10 μg/ml of pepstatin A, and 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride). Cell lysates were centrifuged at 15,000 rpm for 20 min at 4°C, and the supernatant was mixed with a one-fourth volume of 4× SDS sample buffer, boiled for 5 min, and then separated through a 12% SDS–PAGE gel. After electrophoresis, proteins were transferred to a nylon membrane by means of Trans-Blot SD semi-dry transfer cell (Bio-Rad, Hercules, CA). The membrane was blocked in 5% skim milk (1 h), rinsed, and then incubated with primary antibody (for phosphorylated MAPs or CREB) in TBS containing 0.05% Tween-20 (TBS-T) and 3% skim milk overnight at 4°C. Excess primary antibody was then removed by washing the membrane four times in TBS-T; the membrane was then incubated with 0.1 μg/ml peroxidase-labeled secondary antibody (against rabbit) for 1 h. Following three washes in TBS-T, bands were visualized by ECL™ Western Blotting Detection reagents and exposed to X-ray film. Densitometric values of the bands were determined using UN-SCAN-IT gel™ image analysis software (Silk Scientific).

Statistics

The mean values were calculated from data taken from at least three (usually four or more) separate experiments conducted on separate days. Where significance testing was performed, an independent *t*-test (Student's; two populations) was used. A P value less than 0.05 was considered to indicate statistical significance.

RESULTS

Iron Chelator Induces IL-8 Promoter Activity

As previously reported by our group [Choi et al., 2004], HT-29 cells release IL-8 after iron chelator stimulation. We chose a concentration of 0.2 mM of DFO for our present experiments as our previous studies showed that this concentration causes maximal IL-8 release. We conclude that the target of DFO is specific for the cellular iron pool as MIM, an iron chelator structurally distinct from DFO, induced IL-8 mRNA expression as well as IL-8 release in HT-29 cells; in addition, IL-8 induction by DFO was prevented by the addition of FC (Fe^{3+}, 0.5 mM) (Fig. 1A,B).

In our previous study, we also reported that DFO does not affect NF-κB-dependent transcriptional activity in terms of IL-8 production, but instead it acts by post-transcriptional modifications of IL-8 transcripts [Choi et al., 2004]. However, because IL-8 promoter region contains a number of important transcription factors other than NF-κB, in the present study we wished to determine whether DFO acts transcriptionally and whether its efficacy on IL-8 induction is potentially regulated by other transcription factors such as AP-1, NF-IL-6, and CREB. To test this, a vector containing the wild-type IL-8 promoter was transfected into HT-29 cells, and the cells were then treated with DFO (0.2 mM, 24 h). As shown in Figure 1C, luciferase activity was slightly but significantly increased in cells treated with DFO (2.2-fold increased compared to the control), and this activity was almost completely abolished in the presence of FC, thereby suggesting that iron chelator also acted on transcriptional level. This result further suggests that there is an

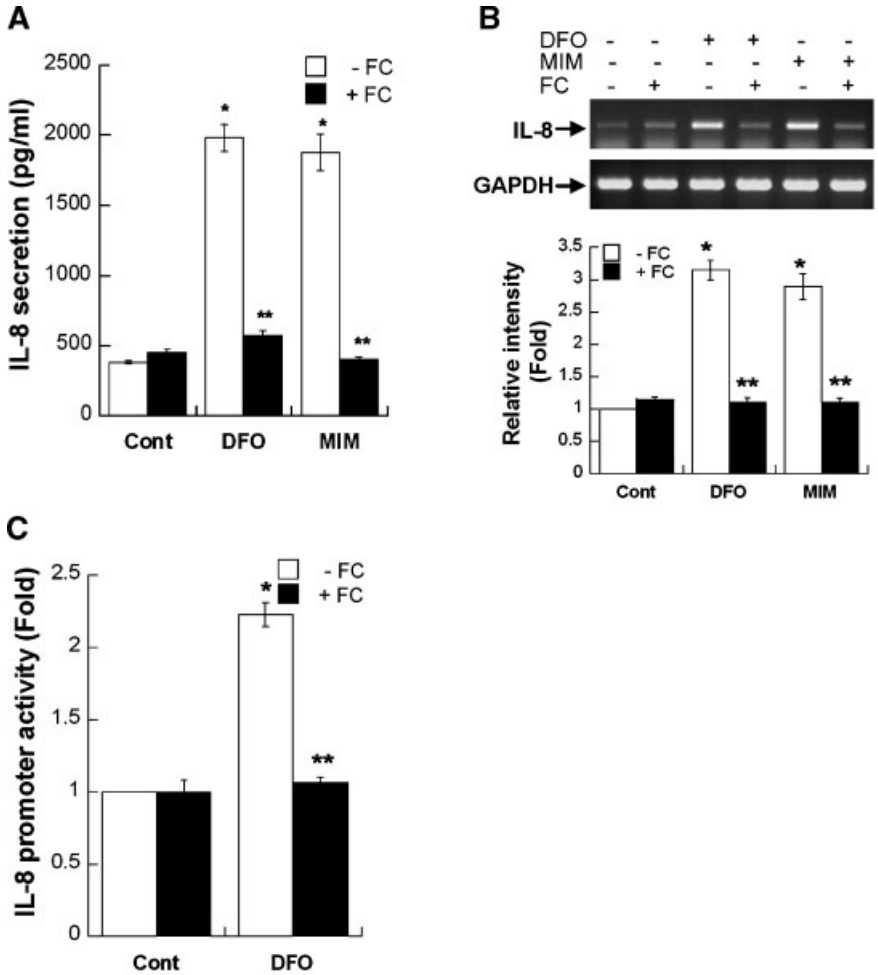

Fig. 1. Iron chelator induces IL-8 promoter activity in HT-29 cells. **A**,**B**: Cells (2×10^5/well) were treated for 16 h with DFO (0.2 mM) or MIM (0.2 mM), in the presence or absence of FC (Fe^{3+}, 0.5 mM). A: Levels of IL-8 secretion were determined by ELISA. B: Levels of mRNA were determined by semiquantitative RT-PCR. The data were represented as the relative intensity of control IL-8 mRNA as normalized to GAPDH. The bar graphs represent the combined densitometry data from these experiments. **C**: Cells were transfected with IL-8 promoter reporter construct, and then treated for 24 h with DFO (0.2 mM) in the presence or absence of FC (0.5 mM). IL-8 promoter activity was determined by luciferase activity assay. Normalized luciferase activity was expressed as the fold difference relative to the control activity (medium only). No statistical difference was found between untreated cells and cells treated with medium (vehicle carriers). Results are expressed as means ± SD of four independent experiments. $^*P < 0.01$ versus control (−FC); $^{**}P < 0.01$ versus DFO- or MIM-treated cells.

involvement of one or more transcription factors in iron chelator-induced IL-8 expression.

AP-1 Involvement in Iron Chelator-Induced IL-8 Promoter Activity

We have reported that the effect of DFO is independent of NF-κB pathways as determined by I-κBα degradation, NF-κB DNA binding, and NF-κB-dependent transcriptional activity [Choi et al., 2004]. In the present study, we further examined whether pharmacological inhibitors that affect NF-κB pathways could alter DFO-induced IL-8 release as well as NF-κB activity. As shown in Figure 2A, treatment of HT-29 cells with inhibitors of NF-κB activation such as MG132 and caffeic acid phenethyl ester (CAPE) failed to abrogate the DFO-induced IL-8 secretion. Similarly, neither NF-κB inhibitor had an effect on NF-κB luciferase activity under conditions with or without DFO (Fig. 2B). However, as expected, both inhibitors significantly diminished TNF-α-induced NF-κB luciferase activity in HT-29 cells (Fig. 2B), thereby confirming that the NF-κB system is not involved in iron chelator-induced IL-8 production. An interesting result is that MG312 (5 μM) alone could also produce a high level of IL-8

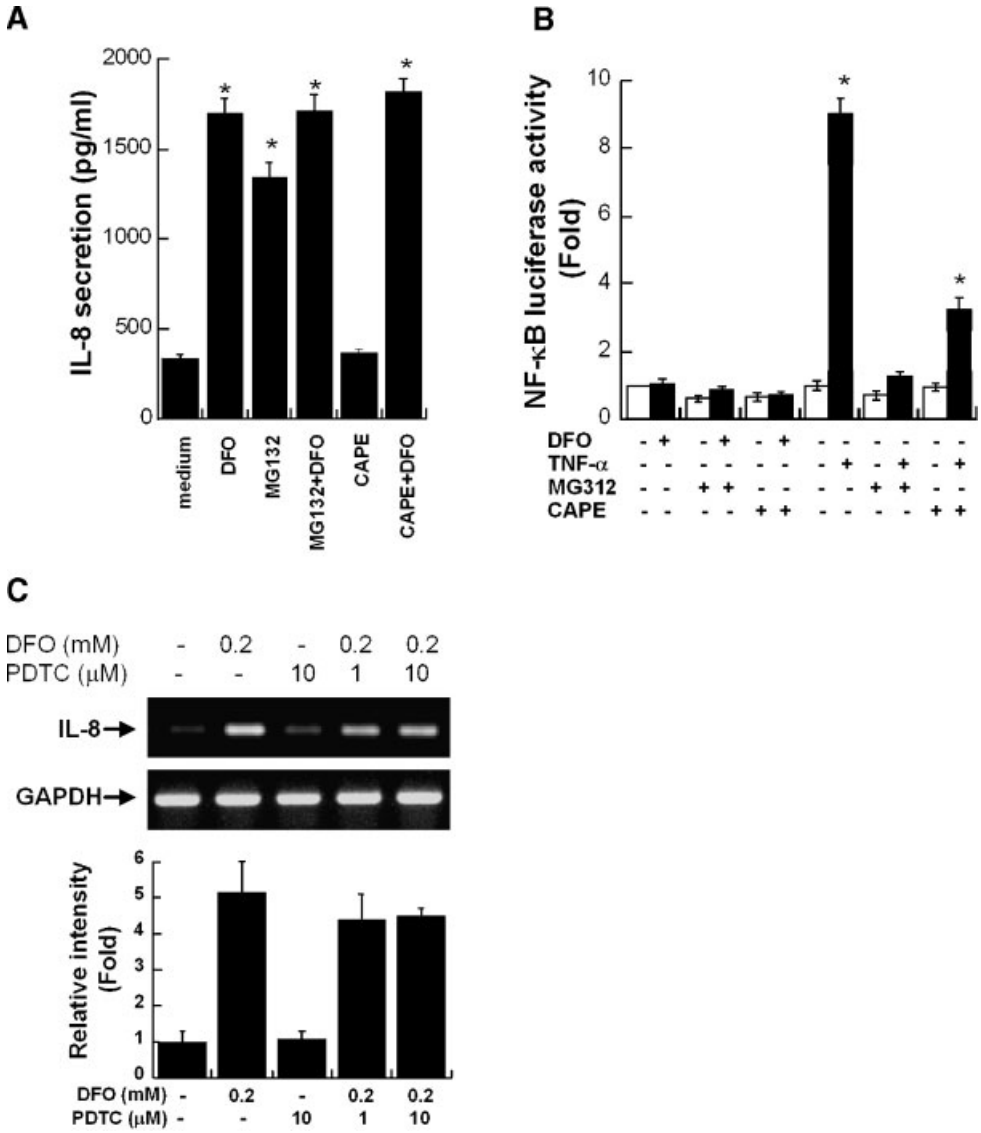

Fig. 2. Effect of NF-κB inhibitors on iron chelator-induced IL-8 production and NF-κB-dependent transcriptional activity. **A**: HT-29 cells (2×10^5/well) were treated with NF-κB inhibitors MG132 (5 μM) or CAPE (20 μM) for 30 min. The cells were then further incubated for 24 h with DFO (0.2 mM). Levels of IL-8 protein were determined by ELISA. **B**: HT-29 cells were transiently transfected with NF-κB luciferase reporter vector. Then, the cells were treated as in (A). NF-κB-dependent transcriptional activity was determined by luciferase activity assay. As a positive control, TNF-α (1 ng/ml) was used. Normalized luciferase activity was expressed as the fold difference relative to the control activity (medium only). Results are expressed as means ± SD of five independent experiments. *$P < 0.01$ versus control (medium only). **C**: HT-29 cells (2×10^5/well) were treated with NF-κB inhibitor PDTC (1–10 μM) for 30 min. The cells were then further incubated for 24 h with DFO (0.2 mM). Levels of mRNA were determined by semiquantitative RT-PCR. The data were represented as the relative intensity of control IL-8 mRNA as normalized to GAPDH. Results are expressed as means ± SD of three independent experiments. *$P < 0.01$ versus DFO-treated cells.

protein (Fig. 2A). Since MG132 is also known to inhibit proteasome function, we think the effect of MG132 in IL-8 secretion may be related with the inhibition of proteasome function. Accordingly, a previous report demonstrated that MG132 induces expression of IL-8, both at the protein and the mRNA levels, in hepatocytes and hepatoma cells [Joshi-Barve et al., 2003]. We therefore evaluated another NF-κB inhibitor, PDTC, to confirm the relationship between NF-κB activation and DFO-induced IL-8 expression. As shown in Figure 2C, treatment with PDTC (1–10 μM) also failed to abrogate the DFO-induced IL-8 secretion in HT-29 cells.

As an IL-8 promoter contains AP-1 binding site as well, we next carefully examined

whether DFO induces AP-1-dependent transcriptional activity by measuring the AP-1-dependent luciferase activity. To test this, a vector containing the AP-1 consensus binding site was transfected into HT-29 cells, after which the cells were treated with DFO (0.2 mM, 24 h). Luciferase activity was increased 3.1-fold in cells treated with DFO, suggesting that DFO-mediated signals are coupled with the AP-1 pathways (Fig. 3A). Moreover, transfection with DN c-Jun showed decrease of AP-1-dependent luciferase activity induced by DFO compared to the empty vector control ($^{*}P < 0.01$ vs. DFO-treated cells), while WT-c-Jun revealed increased AP-1-dependent luciferase activity ($^{**}P < 0.05$ vs. DFO-treated cells) (Fig. 3A). Similar results were also obtained when the cells were transfected with DN-c-Jun or WT-c-Jun in wild-type IL-8 promoter transfected cells (Fig. 3B). These data further suggest that AP-1, rather than NF-κB, functions in HT-29 cells when stimulated with DFO.

As transcription factors may be activated at both transcriptional and post-transcriptional levels, we further checked whether DFO can change the expression levels of the two transcription factors in HT-29 cells. To this end, several subunits of transcription factors NF-κB and AP-1 were tested. As shown in Figure 4A, we observed a significant up-regulation of c-Fos, junB, and c-Jun, and to a less extent, c-Rel, whereas little changes in the mRNA levels of p65 (RelA) was seen. To further quantify the levels of gene expression, the mRNAs of each subunit were assessed by real-time PCR using a SYBR green dye. As shown in Figure 4B, relative values of each subunit as normalized to internal control GAPDH were well matched with the results of RT-PCR. These data indicate that DFO may also activate transcription factors at a transcriptional level, thereby resulting in pro-inflammatory chemokine production.

Next, we also used EMSA to examine whether the AP-1 binds to the IL-8 promoter after DFO treatment. To this end, nuclear extracts from DFO-treated cells were incubated with specific oligonucleotides containing the NF-κB or AP-1 binding sites present in the human IL-8 promoter. The specificity of NF-κB- or AP-1-binding complexes was shown by adding excess unlabeled (cold) probes to the binding reaction. We observed that DFO treatment led to the binding of specific oligonucleotides to AP-1/IL-8 but not to NF-κB/IL-8 (Fig. 5A,B). Taken together, these results strongly demonstrate that AP-1 but not NF-κB is involved in DFO-induced IL-8 production in transcriptional levels.

Inhibition of ERK1/2 and p38 Kinase Pathways Reduces the AP-1 Binding Activity

AP-1 is known as a target transcription factor of activated MAPKs [Parhar et al., 2003; Hoffmann et al., 2005]. As we have shown that DFO induces phosphorylation of ERK1/2 and

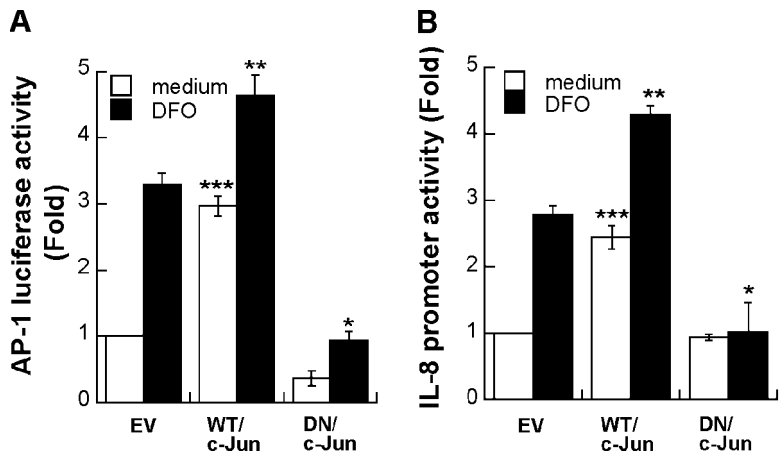

Fig. 3. AP-1 involves in iron chelator-induced IL-8 promoter activity. **A**: HT-29 cells (2×10^5/well) were transiently transfected with empty vector (EV), wild-type (WT) c-Jun, or dominant-negative (DN) c-Jun vector, together with AP-1 luciferase reporter construct. Then, the cells were treated for 24 h with DFO (0.2 mM). AP-1-dependent transcriptional activity was determined by luciferase activity assay. **B**: Cells were transiently transfected as in (A), together with IL-8 promoter luciferase reporter construct. Then, the cells were incubated for 24 h with DFO (0.2 mM). IL-8 promoter activity was determined by luciferase activity assay. Normalized luciferase activity was expressed as the fold difference relative to the control activity (medium only). Results are expressed as means ± SD of five independent experiments. $^{*}P < 0.01$ versus DFO-treated cells; $^{**}P < 0.05$ versus DFO-treated cells; $^{***}P < 0.01$ versus empty vector (EV) controls.

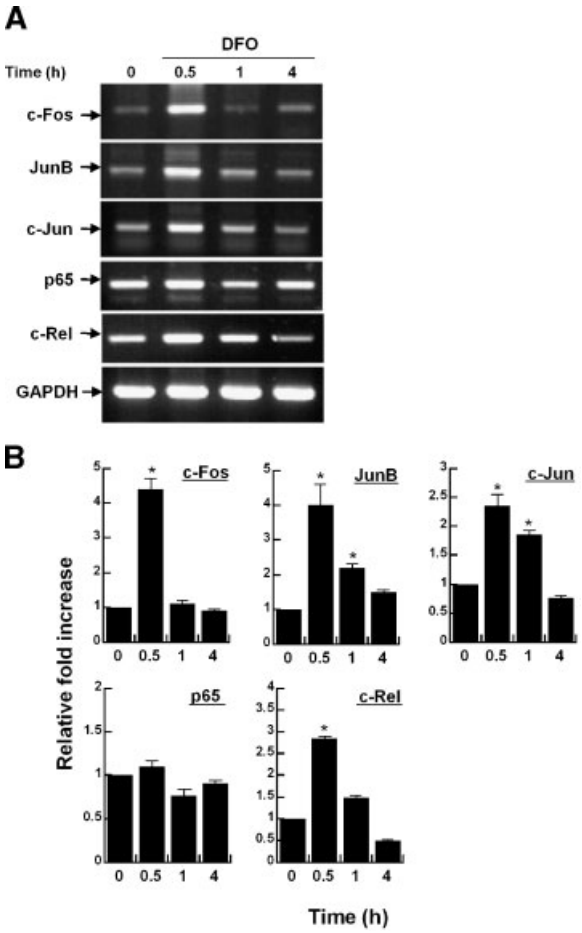

Fig. 4. Iron chelator changes mRNA levels of NF-κB and AP-1 subunits. HT-29 cells (2×10^5/well) were treated with DFO (0.2 mM). At the indicated time points, total RNA was purified and the levels of mRNA were determined by semiquantitative RT-PCR (**A**) and quantitative real-time PCR (**B**). Normalized gene induction was expressed as the fold increases relative to the gene expression of untreated cells (=0 h). Results are expressed as means ± SD of four independent experiments. $^*P < 0.05$ versus control (untreated cells).

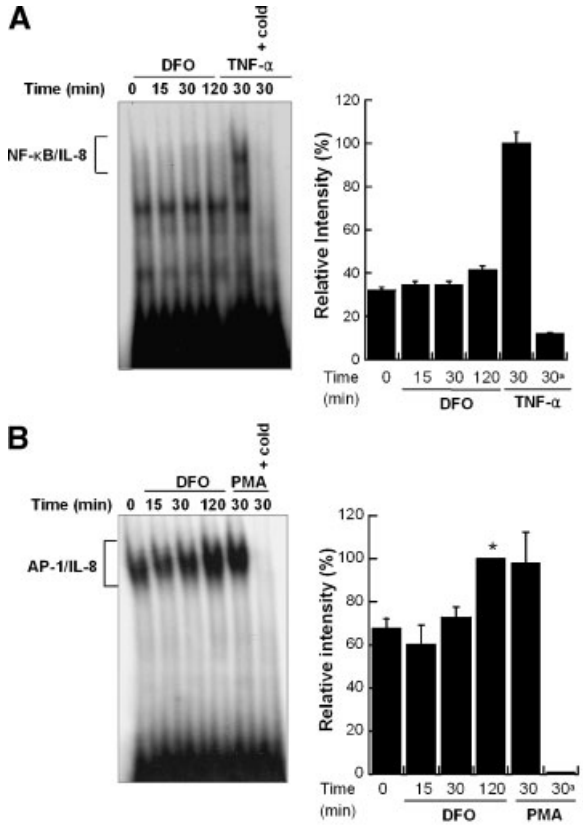

Fig. 5. Iron chelator induces specific binding of AP-1, but not NF-κB, to the IL-8 promoter. HT-29 cells (1×10^7/dish) were treated with DFO (0.2 mM) for the indicated time periods. The nuclear extracts were incubated with either a radiolabeled NF-κB/IL-8 probe (**A**) or AP-1/IL-8 probe (**B**). As positive controls, the cells were treated for 30 min with TNF-α (10 ng/ml) and PMA (100 nM), respectively. The unradiolabeled (i.e., cold) probes were added to the reactions to detect the specific bands. These data are representative of four independent experiments. Relative intensities of the NF-κB-DNA and AP-1-DNA complexes are shown in the **right panels**, with the intensities for the complex of the highest lane as 100%. Results are expressed as means ± SD. $^*P < 0.05$ versus control (medium only). [a] represents a cold probe control.

p38 kinase [Choi et al., 2004], we further examined whether the AP-1 activation may be regulated by ERK1/2 and p38 kinase pathways. As shown in Figure 6, DFO induced phosphorylation of MEK1/2, ERK1/2, and p38 kinase. MEK1/2 activation was seen at 5–10 min treatment with DFO, whereas activations of both ERK1/2 and p38 kinase were seen at relatively later time (>∼10 min after DFO treatment). Interestingly, treatment with PD98059 (20 μM) or SB202190 (10 μM) significantly inhibited IL-8 promoter activity as well as AP-1-mediated transcriptional activity (Fig. 7A,B). In addition, both PD98059 and SB202190 attenuated the binding of specific oligonucleotides to AP-1/IL-8. These results suggest that ERK1/2 and p38 kinase are functionally upstream of the transcription factor AP-1 for the IL-8 production and potentially for other chemokine productions by iron chelator. The concentrations of MAPK inhibitors used in this study showed no significant effect on cell viability, as determined by MTT assay (data not shown).

Iron Chelator Increases Specific Binding of CREB and Egr-1, but not NF-IL6

In addition to evaluating the potential role of AP-1, we investigated possible implications of

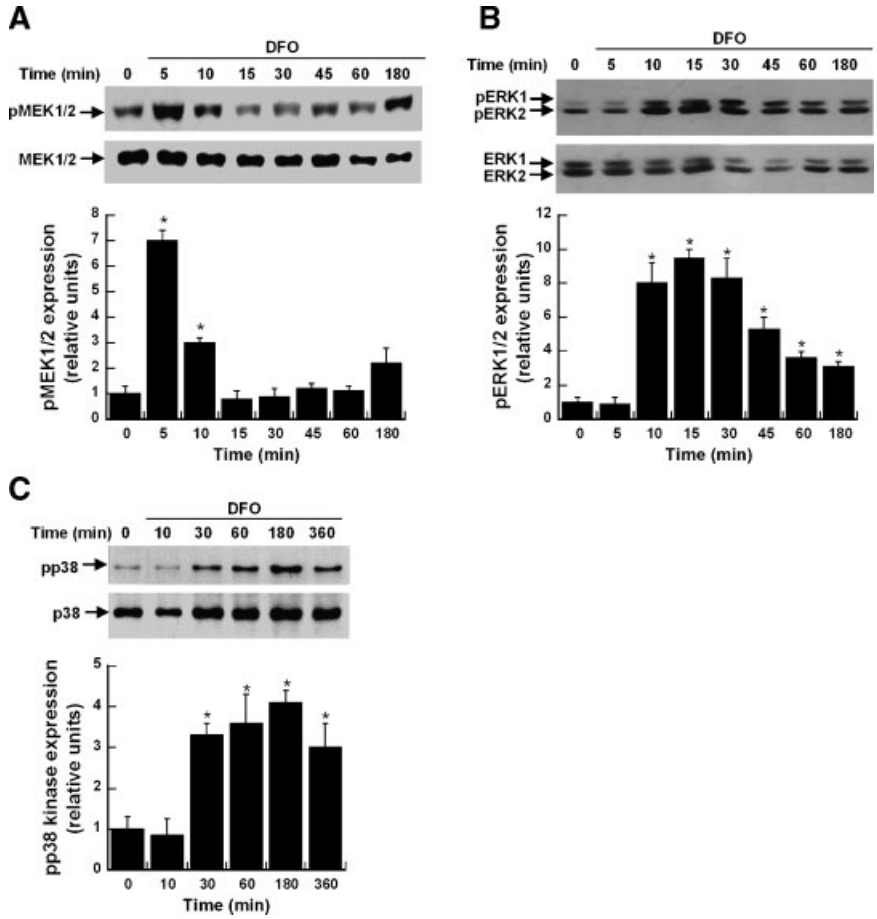

Fig. 6. Iron chelator induces ERK1/2, its immediate upstream mediator MEK1/2, and p38 kinase. HT-29 cells (5×10^5/well) were treated with DFO (0.2 mM) for the indicated time periods, after which the cells were harvested, and the cell lysates were blotted with Abs specific for the phosphorylated form of MEK1/2 (**A**), ERK1/2 (**B**) or p38 kinase (**C**). After striping with 0.1 M glycine (pH 2.5), the membranes were further blotted for the total forms. These data are representative of three independent experiments. The bar graphs represent the combined densitometry data from these experiments. Results are expressed as means ± SD. $^*P < 0.05$ versus control (0 h).

other transcription factors such as CREB, NF-IL6 and Egr-1 on DFO-induced IL-8 production. CREB is a known transcription factor involved in inflammation and chemokine production or a coactivator which may interact with subunits of transcription factors NF-κB or AP-1 [Grassl et al., 1999; Shenkar et al., 2001; Jaramillo and Olivier, 2002]. Based on this knowledge, we examined whether DFO activates the transcriptional factor CREB in HT-29 cells. As shown in Figure 8A, we observed a significant CREB binding activity after DFO stimulation. NF-IL6 binding site is also contained in the IL-8 promoter. We therefore used a specific oligonucleotide containing the NF-IL6 binding site in the IL-8 promoter. However, NF-IL6 probe binding to the nuclear extract from DFO-treated HT-29 cells was not significantly changed during the indicated time periods (Fig. 8B). On the contrary, the involvement of Egr-1 is likely, because time-dependent experiment revealed Egr-1 DNA binding activity in the nuclear extracts from DFO-treated HT-29 cells (Fig. 8C). The specificity of each probe was determined by adding excess unlabeled probe to the binding reaction (Fig. 8A–C). As the 5′-flanking region of the human IL-8 promoter does not contain a putative Egr-1 binding site, we do not believe that this strong DNA binding activity of Egr-1 in DFO-treated cells is directly involved in IL-8 production. However, as Egr-1 is a target transcription factor of activated ERK1/2 and of Elk1/2 and putative Egr-1-binding site resides in some chemokine promoters [Silverman and Collins, 1999; Yan et al., 2000; Gousseva et al., 2001; Giri et al., 2003],

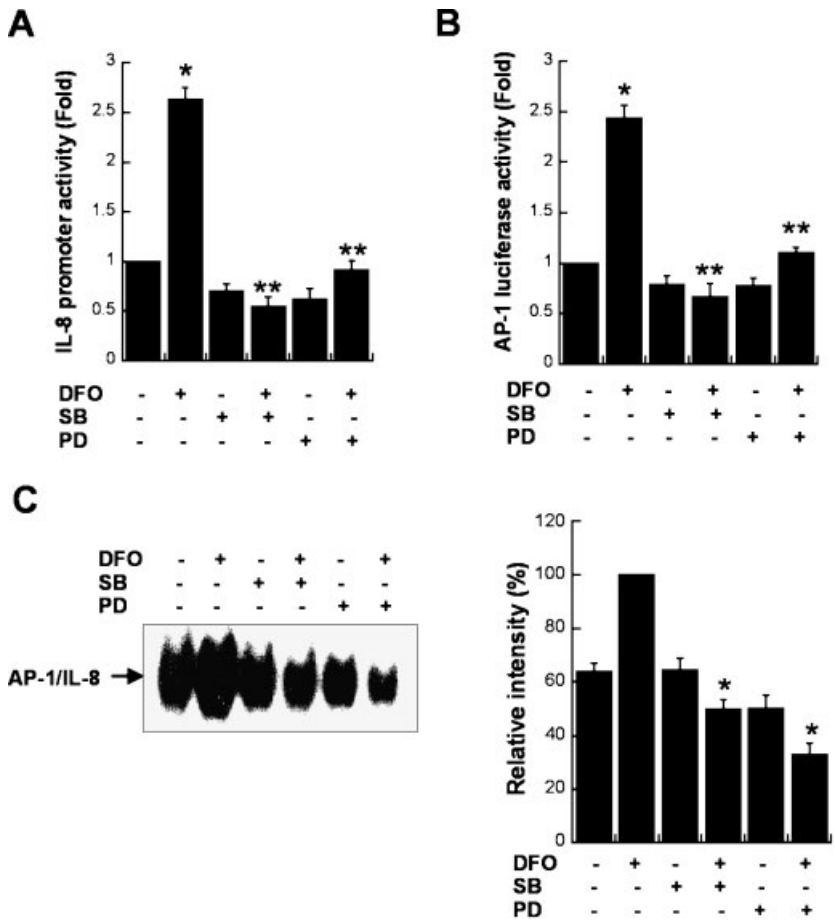

Fig. 7. Inhibition of ERK1/2 and p38 kinase pathways reduces DFO-induced IL-8 promoter activity, AP-1-dependent transcriptional activity, and binding of AP-1 to IL-8 promoter. HT-29 cells (2×10^5/well) were transiently transfected with either IL-8 promoter luciferase reporter construct (**A**) or AP-1 reporter construct (**B**). The cells were then treated with SB202190 (10 μM) or PD98059 (20 μM) for 30 min. The cells were further incubated for 24 h with DFO (0.2 mM). The transcriptional activity was determined by luciferase activity assay. Normalized luciferase activity was expressed as the fold difference relative to the control activity (medium only). Results are expressed as means ± SD of three independent experiments. *$P < 0.01$ versus control (medium only); **$P < 0.01$ versus DFO-treated cells. **C**: HT-29 cells (1×10^7/dish) were treated with SB202190 (10 μM) or PD98059 (20 μM) for 30 min and further incubated with DFO (0.2 mM) for 2 h. The nuclear extracts were incubated with the radiolabeled AP-1 probe. These data are representative of three independent experiments. Relative intensities of the AP-1-DNA complexes are shown in the **right panel**, with the intensities for the complex of the highest lane as 100%. Results are expressed as means ± SD. *$P < 0.05$ versus DFO-treated cells.

this result suggests that Egr-1 may be indirectly involved in DFO-induced IL-8 expression.

Based on the results observed in this study, we suggest a putative sequence of signaling events initiated by the cellular iron chelation (Fig. 9). The schematic representation of the signaling events shows that the iron chelator causes the activation of p38 kinase and ERK1/2, which then modulates the activity of AP-1. The activation of AP-1 leads to the expression of IL-8 mRNA. A possible link between iron status and two additional transcription factors, that is, CREB, and Egr-1, rather than NF-κB also could be suggested in IL-8 production in IECs (Fig. 9).

DISCUSSION

Iron is vital for microorganisms [Weinberg, 1978; Neilands, 1981]. To obtain iron from the iron-restricted environment, micro-organisms have developed many strategies. Molecules involved in iron uptake and transport may be receptors or channels (localized in the outer and inner membrane of the microbe) and siderophores (Greek for 'iron bearers'). In the present study, we have demonstrated the possibility that direct chelation of Fe by infected bacteria could be an alternative way to induce host inflammatory responses, presumably via a

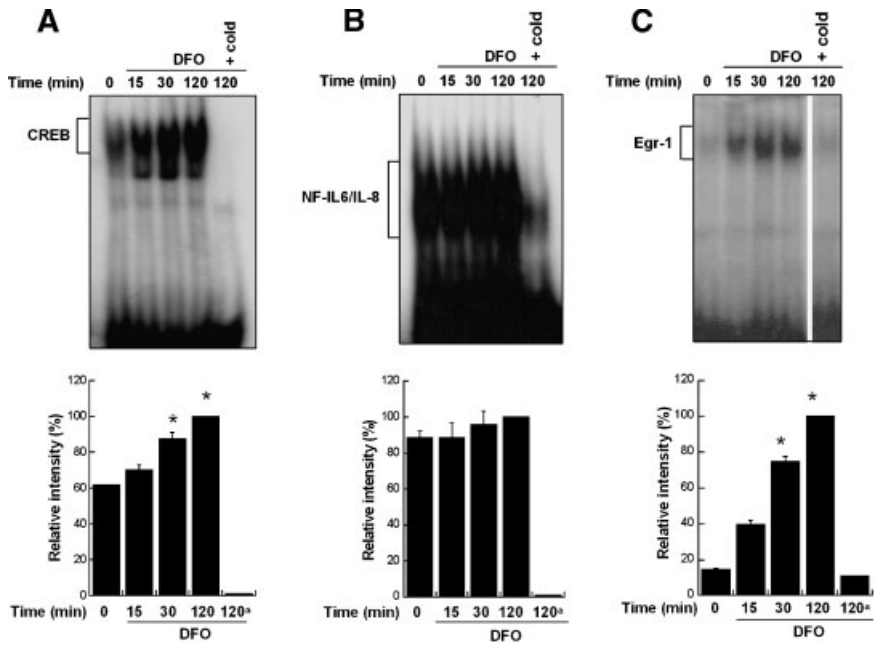

Fig. 8. Iron chelator activates transcription factors CREB and Egr-1, but not NF-IL6. HT-29 cells (1×10^7/dish) were treated with DFO (0.2 mM) for the indicated time periods. The nuclear extracts were incubated with the radiolabeled CREB (**A**), NF-IL6 (**B**), and Egr-1 (**C**) probes, respectively. The unradiolabeled (i.e., cold) probes were added to the reactions to test whether they compete with the radiolabeled probes. These data are representative of three independent experiments. Relative intensities of the complexes are shown in **lower panels**, with the intensities for the complex of the highest lane as 100%. Results are expressed as means ± SD. $^*P < 0.05$ versus untreated control cells. [a] represents a cold probe control.

distinct mechanism from that induced by inflammatory mediators such as TNF-α, IL-1β, or LPS. We identified that transcriptional regulation of IL-8 by iron chelator is independent from NF-κB, a central regulator of inflammatory responses, but is dependent on AP-1 system. Central to this iron chelator-induced activation of transcription factors in IECs is the early activation of ERK1/2 and p38 kinase systems. These early pathways may have a pivotal role in mediating later events of the IEC inflammatory response by regulating such transcription factor as AP-1. We also identified that CREB and Egr-1 are activated in response to DFO stimulation, while binding of NF-IL6 to IL-8 promoter is not altered.

NF-κB signaling is an accepted regulator of IL-8 synthesis [Jung et al., 2002], and immune cells exposed with various stimuli do mobilize NF-κB [Baeuerle and Baltimore, 1996; Ghosh et al., 1998]. However, in contrast to these publications, others do not require NF-κB, but instead they require AP-1 and/or some other transcription factors [Hipp et al., 2002; Akhtar et al., 2003; Joshi-Barve et al., 2003; Kumar et al., 2003]. The mechanism by which iron chelator induces IL-8 secretion in IECs may be similar to the later case as our previous results showed no significant degradation of I-κBα and no p65 DNA-binding in response to DFO [Choi et al., 2004]. However, as there is also a possibility of I-κBβ-mediated NF-κB activation or IκB-independent NF-κB activation [Antonsson et al., 2003; Jiang et al., 2004], we further measured the DFO-induced NF-κB binding with a specific oligonucleotide probe containing a NF-κB binding site of the IL-8 promoter by EMSA. No significant binding of olionucleotide probe to the NF-κB binding site corroborates that iron chelator-induced IL-8 production is independent from NF-κB activation. In contrast, the DNA binding activity of AP-1 could be essential for the expression and production of IL-8 as inhibition of the activity of AP-1 by DN-c-Jun significantly reduced the DFO-induced IL-8 promoter activity as well as the AP-1-dependent transcriptional activity. Taken together, our present results support the contention that the regulatory mechanism of IL-8 by iron chelator is quite different from that occurring in the case of proinflammatory cytokines.

We also observed that iron chelator induces the activity of MEK1/2, ERK1/2, and p38 kinase

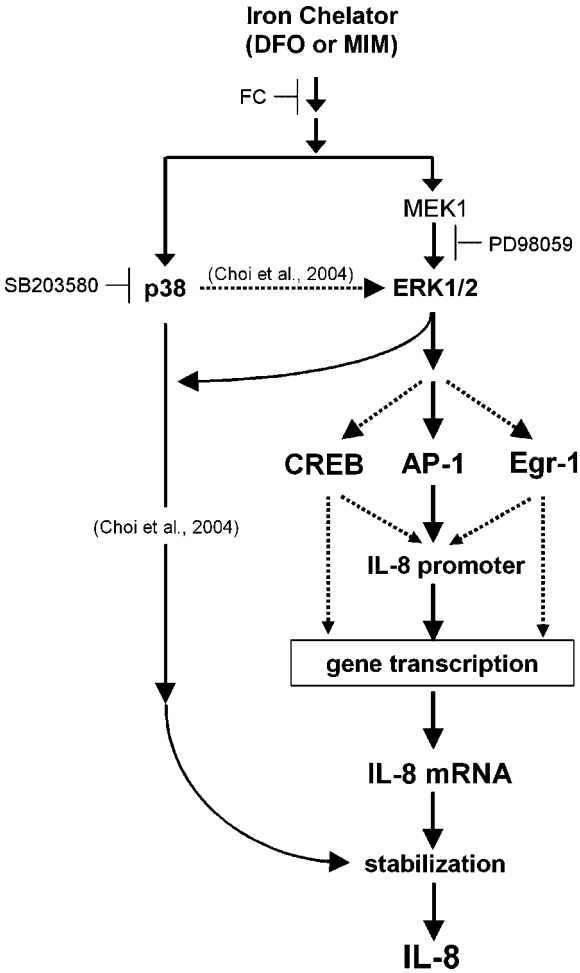

Fig. 9. A putative mechanism of iron chelator-induced IL-8 production in HT-29 cells. The schematic representation of the signaling events shows that the iron chelator causes the activation of p38 kinase and ERK1/2 by iron-specific manner, which then modulates the activity of AP-1 transcription factor. The activation of AP-1 leads to the expression of IL-8 mRNA followed by its translation into protein and subsequent release in the culture supernatants. A possible link between iron status and two additional transcription factors, that is, CREB, and Egr-1, rather than NF-κB also could be suggested in IL-8 production in IECs. Dotted lines represent a putative position of transcription factors in the signaling pathway while it is not directly confirmed in the present study.

in HT-29 cells. The role of various MAPKs in the expression and production of IL-8 is not well understood. As we previously reported, however, iron chelator increases the IL-8 mRNA stability, presumably through the activation of p38 and ERK1/2 pathways. In the present study, we also observed that the inhibition of p38 kinase or MEK1/2 with SB203580 or PD098059 significantly reduces the IL-8 mRNA half-lives compared with un-treated samples [Choi et al., 2004], suggesting that MAPKs contribute to IL-8 secretion by IECs via a post-transcriptional mechanism, as described by other researchers [Jijon et al., 2002]. However, promoter study of IL-8 suggested that iron chelator can also act at the transcriptional level. Therefore, we further investigated whether there is any link in the ERK1/2 and p38 kinase signaling with the downstream transcription factors that influence the expression of IL-8 in response to iron chelator. Interestingly, our results revealed that the inhibition of ERK1/2 or p38 kinase activities significantly reduced the DNA binding activity of the AP-1 transcription factor (Fig. 6).

As transcription factors can be activated transcriptionally and post-transcriptionally, we also tested whether the essential components of some transcription factors are induced in response to DFO. As for NF-κB/Rel proteins, among 5 distinct subunits RelA (p65), RelB, c-Rel, NF-κB1 (p50/p105) and NF-κB2 (p52/p100), RelA, RelB and c-Rel are transcriptionally active members, while p50 and p52 may serve as DNA binding subunits [Baeuerle and Baltimore, 1996; Ghosh et al., 1998]. Although c-Rel was slightly induced in response to DFO in HT-29 cells, the fact that both PDTC and CAPE failed to reduce DFO-induced IL-8 production (Fig. 2A,C) corroborates the previous results in which we demonstrated that NF-κB is not involved in DFO-induced IL-8 production in human IECs [Choi et al., 2004]. On the contrary, it will be interesting to test whether induction of c-Rel may have a role in DFO-induced toxicity, as c-Rel has been implicated in pro-apoptotic function [Chen et al., 2003]. As for AP-1 proteins, we tested specifically c-Fos, JunB, and c-Jun. DFO treatment significantly changed the expression levels of all three AP-1 subunits tested (Fig. 4A,B). These results strongly suggest that transcriptional regulation of the AP-1 components is also important for the iron chelator-induced IL-8 production in IECs.

The promoter region of IL-8 contains potential binding sites for a variety of transcriptional factors including NF-κB, AP-1, and NF-IL-6 (C/EBPβ) [Kikuchi et al., 2002; Zhu et al., 2003]. In addition, the transcriptional regulation in the chemokine promoter involves interactions of these transcription factors with other transcription factors such as CREB and Egr-1, depending on the cell types and stimuli used [Jaramillo and Olivier, 2002; Wu et al., 2002;

Giri et al., 2003; Li et al., 2003; Zhu et al., 2003]. For this reason, we were also interested in Egr-1 activation in response to DFO. As Egr-1 plays a critical role in differentiation and chemokine production [Grimbacher et al., 1997; Giri et al., 2003], DFO-induced Egr-1 activation may imply that iron imbalance is important for the cellular differentiation or activation of IECs. Of interest, therefore, this Egr-1 activation may offer an important direction for future studies, in terms of a possible link between iron status and IEC differentiation.

There are multiple lines of evidence suggesting that both MAPKs can directly activate CREB transcription factor [Iordanov et al., 1997; Xing et al., 1998]. Consistent with these reports our unpublished data revealed that both ERK1/2 and p38 kinase are involved in the phosphorylation of CREB. Moreover, we found that the DNA binding activity of CREB was decreased in the presence of either MAPK inhibitor (data not shown). These results may suggest that although AP-1 is an important transcriptional activator in inducing IL-8 transcription in HT-29 cells, CREB is also likely to be in another alternative pathway, finally resulting in chemokine production. The cooperative action of AP-1 and CREB for maximized chemokine production should be considered.

In summary, our study shows that iron chelator induces increased expression of chemokines such as IL-8 and CCL20 in human IECs [Choi et al., 2004; Lee et al., 2005]. We show that iron chelator-mediated cellular signaling cascades involve downstream activation of p38 kinase and ERK1/2. We have provided evidence that ERK1/2 and p38 kinase pathways are linked with the activation of downstream transcription factor AP-1, and are involved in the regulation of IL-8 expression and production. A possible link between iron status and other transcription factors such as CREB and Egr-1 are also suggested for the chemokine production or cellular differentiation of IECs. We also suggest that the regulatory mechanism of IL-8 secretion by iron chelator is quite different from that involved in either cytokine or growth factor-induced production of IL-8.

ACKNOWLEDGMENTS

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