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Iron-induced interleukin-6 gene expression: possible mediation through the extracellular signal-regulated kinase and p38 mitogen-activated protein kinase pathways

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Abstract

Increased iron store in the body may increase the risk of many diseases such as cancer and inflammation. However, the precise pathogenic mechanism of iron has not yet been elucidated. In the present study, the early biological responses of cells to iron treatment were investigated in AP-1 luciferase reporter stably transfected mouse epidermal JB6 cells and primary rat hepatocytes. It was shown that water-soluble iron compounds, such as $FeSO_4$ and $Fe_2(SO_4)_3$, were more active in inducing AP-1 in JB6 cells than water-insoluble iron compounds, such as Fe_2O_3 and FeS. Iron stimulated mitogen-activated protein kinase (MAPK) family members of extracellular signal-regulated kinases (ERKs) and p38 MAPK but not c-jun NH_2 terminal kinases (JNKs), both in JB6 cells and in primary rat hepatocytes, as determined by the phosphorylation assay. Interestingly, the increase in AP-1 luciferase activity by iron was inhibited by the pretreatment of the cells with PD98059, a specific MEK1 inhibitor, and SB202190, a p38 kinase inhibitor. Levels of interleukin-6 (IL-6), a pro-inflammatory cytokine, were increased in JB6 cells by iron in a dose-dependent manner. The increase in IL-6 and its mRNA by iron was also eliminated by the pretreatment of the cells with PD98059 and SB202190. Since the IL-6 promoter contains an AP-1 binding site, our studies indicate that the iron-induced IL-6 gene expression may be mediated through ERKs and p38 MAPK pathways, possibly one of the important mechanisms for the pathogenesis of iron overload.

Keywords: Iron; Activator protein-1; Interleukin-6; Mitogen-activated protein kinase

1. Introduction

Iron deficiency anemia is a common condition known to the medical profession for several centuries, but iron overload is mistakenly believed to be rare. Iron overload may be primary, resulting from a deregulation of intestinal iron absorption as in genetic hemochromatosis, or secondary to other congenital or acquired conditions, such as β-thalassemia (Sheth

Abbreviations: AP-1, activator protein-1; IL-6, interleukin-6; LMW, low molecular weight; MAPK, mitogen-activated protein kinase; ERKs, extracellular signal-regulated kinases; JNKs, c-jun NH_2 terminal kinases

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and Brittenham, 2000). Tissue iron overload affects several organs, such as liver and kidney (Bonkovsky, 1991). For example, elevated hepatic concentration of iron can cause hepatocellular injury, fibrosis, and cirrhosis (Pietrangelo, 1998, 2002). If such a patient becomes cirrhotic, there is a 200-fold increase in relative risk of developing hepatoma (Bradbear et al., 1985; Niederau et al., 1985). Increasing evidence demonstrates that iron is an important risk factor in contributing to the development of these diseases (Huang, 2003), since removal of excess iron by phlebotomy or chelation results in clinical improvement (Jacobs, 1977; Link et al., 2001; Porter, 1997).

Iron-catalyzed oxidative stress is believed to be the main mechanism involved in the iron-induced pathogenic processes (Brown and Knudsen, 1998; Lesnefsky, 1994; Toyokuni, 1996). Iron bound to low molecular weight (LMW) chelators has been thought to be the most likely active species, producing oxidants through Haber-Weiss, Fenton, or autoxidation reactions (Huang et al., 2002; Qian and Buettner, 1999). Previously reported in vitro and in vivo studies, which mainly focused on the oxidative damage (e.g., lipid peroxidation) or antioxidant enzymes, support these points of view (Lesnefsky, 1994). However, early biological responses of cells to iron that initiate signaling cascades leading to pathogenic processes have not yet been elucidated.

The goal of the present study was to investigate iron-induced early signal pathways that modulate activity of activator protein-1 (AP-1) and to assess the effect of iron on interleukin-6 gene expression in mouse epidermal JB6 cells and primary rat hepatocytes. AP-1 is an important transcription factor controlling the up-regulation of pro-inflammatory cytokines, which may contribute to the development of liver fibrosis (Friedman, 2000; Poli and Parola, 1997). AP-1 consists of a family of Jun/Fos dimers that include different Jun proteins (c-Jun, JunB, and JunD) and Fos proteins (c-Fos, FosB, Fra-1, Fra-2, and FosB2) (Angel and Karin, 1991). AP-1 activation is regulated at multiple levels by activation of mitogen-activated protein kinases (MAPK), involving three major pathways of extracellular signal-regulated kinases (ERKs), stress-activated protein kinases/c-jun NH_2 terminal kinases (SAPK/JNK), and p38 MAPK (Kelly and Chu, 2000). Putative AP-1 binding sites are found in the promoters of many genes, such as IL-6, IL-8, as well as granulocyte-macrophage colony-stimulating factor (GM-CSF) (Hirano, 1998). IL-6 is a pro-inflammatory cytokine that can be both mitogenic and fibrogenic (Hirano, 1998). Therefore, induction of IL-6 by iron may contribute to the pathogenesis of iron overload. Although previous studies have shown that activation of AP-1 is essential for the transcriptional regulation of IL-6 (Beetz et al., 2000; Smart et al., 2001), there is little evidence that AP-1 plays a key role in iron-induced IL-6 gene expression. In the present study, we have shown that iron can activate AP-1 and induce IL-6 formation, and iron-induced IL-6 appears to be mediated through ERKs and p38 MAPK pathways.

2. Materials and methods

2.1. Chemical reagents

Ferric sulfate [$Fe_2(SO_4)_3$], ferrous sulfate [$FeSO_4$], ferrous sulfide (FeS), iron oxide hydrated, [Fe_2O_3 as revealed by X-ray (data not shown)], fetal bovine serum (FBS), and alpha-minimum essential medium (α-MEM) were from Sigma–Aldrich Chemical Co. (St. Louis, MO). Water-insoluble iron particles (FeS and Fe_2O_3) were ground in a ball mill and size-classified by a Mercer Impactor as previously described (Zhang et al., 2002). The diameters of over 80% of iron particles were less than 5 μm (data not shown). ERKs, p38, JNKs antibody were from Cell Signaling Technology (Bevery, MA). Rainbow Marker was from Amersham (Piscataway, NJ). Second antibody (goat anti-rabbit) was from Pierce (Rockford, IL). Twelve percent ready gel was from Bio-Rad (Hercules, CA). PD98059 and SB 202190 were from Calbiochem (San Diego, CA). Superscript II reverse transcriptase was from Gibcol BRL (Grand Island, NY). RNase inhibitor, DNA marker, and Blue/Orange loading dye were from Promega (Madison, WI). AmpliTaq Gold was from Perkin-Elmer (Shelton, CT). dNTPs and Oligo d(T) 18 primers were from New England Biolabs (Beverly, MA). Primers for IL-6 were from Invitrogen (Carlsbad, CA).

2.2. Isolation of primary rat hepatocytes and cell culture

Primary rat hepatocytes were isolated from adult Long Evans male rats (200–250 g, Taconic, New York,

NY) by a two-step collagenase perfusion in situ with slight modification (Neufeld, 1997). Briefly, adult male rats were anesthetized and the portal veins were cannulated. The liver was perfused for 15–20 min with calcium-free buffer A containing 118 mM NaCl, 4.7 mM KC1, 1.2 mM KH_2PO_4, and 25 mM $NaHCO_3$ to remove red blood cells before adding collagenase. Absence of calcium in this initial perfusion allows for optimal cell dispersion. Collagenase perfusate solution (buffer B) contained 118 mM NaCl, 6.7 mM KC1, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, 4.8 mM $CaCl_2$, 1.2 mM $MgSO_4$, and 0.5 mg/ml collagenase. The collagenase perfusate was circulated for 15–20 min. After removing the liver and lightly dispersing it with tweezers in a culture dish containing buffer C (140 mM NaCl, 6.7 mM KC1, 1 mM $CaCl_2$, and 2.4 mM Hepes), the dispersed liver tissue was incubated for 10 min at 37 °C with 5% CO_2. The mixed liver cell suspension was filtered through 100-mesh net to remove other tissue debris. The isolated hepatocytes were washed twice with buffer C and centrifuged (40 × *g* for 5 min at 4 °C) before plating cells for primary culture at a concentration of 10^6 cells/ml. Culture medium was iron-free α-MEM containing 10% FBS, 2 mM L-glutamine, 25 μg of gentamicin/ml, and 10 μg/ml insulin. The resulting hepatocytes had cell viability above 90%, as measured by Trypan blue exclusion test. Hepatocytes were maintained in culture at 37 °C in a humidified atmosphere of 5% CO_2–95% air overnight before iron treatment.

Mouse epidermal JB6 parent cells as well as AP-1 luciferase reporter stably transfected JB6 P^+1-1 cells were grown in α-MEM containing 5% FBS, 2 mM L-glutamine, and 25 μg of gentamicin/ml. Cells were cultured in a humidified atmosphere of 95% air–5% CO_2 at 37 °C. Previous studies have shown that dominant negative ERK or c-Jun can block AP-1 transactivation in stable transfectants of clone 41 cells with the same AP-1 luciferase reporter, suggesting that luciferase activity is under the control of AP-1 promoter (Dong et al., 1994; Watts et al., 1998).

2.3. AP-1 luciferase activity assay

Confluent monolayers of AP-1 luciferase reporter stably transfected JB6 P^+1-l cells were trypsinized, and 8 × 10^3 viable cells suspended in 100 μl of 5% FBS MEM were added to each well of a 96-well plate, and incubated at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air for 12–24 h. At this time point, the cells reached 80–90% confluency and cell numbers per well were approximately the same before iron exposure. Cells were starved by culturing them in 0.1% FBS MEM for an additional 18 h and then exposed to various iron compounds for AP-1 induction. Water-soluble ferrous and ferric sulfates and water insoluble iron sulfide and oxide were used for the study. Cells were treated with different doses of iron for various periods. Water-soluble iron doses were expressed as μM in the media and water-insoluble iron doses were expressed as $\mu g/cm^2$ surface area of the culture dish. The reason for using 0.1% FBS was to ensure that iron would not be chelated by transferrin present in serum. Controls consisted of cells grown in the same media with 0.1% FBS. After different periods of time cultures, cells were extracted with lysis buffer. Luciferase activity was measured using a luminometer and was normalized with protein concentration (Monolight, 2010). Total protein in the cell lysates was determined using bicinchoninic acid (Sigma). The results are expressed as relative AP-1 activity to the control without iron treatment and shown as fold change over control.

To confirm that specific ERKs and p38 MAPK signal transduction pathways are regulating iron-induced AP-1, specific inhibitors for each pathway were added to the tissue culture media 30 min before iron treatment. SB202190, a potent and cell permeable inhibitor of p38 MAPK with no effect on the activity of ERKs or JNK MAPK subgroups, and PD98059, a selective and cell permeable inhibitor of MAP kinase (MEK1, ERKs pathway) that acts by inhibiting the activation of MAP kinase and subsequent phosphorylation of MAP kinase substrates, were used.

2.4. Protein kinase phosphorylation assay

Immunoblotting for the phosphorylation of ERKs, JNKs, and p38 kinase was carried out as described in the protocol from Cell Signaling Technology™, using phospho-specific antibodies against phosphorylated sites of ERKs, JNKs, and p38 kinase, respectively. Non-phospho-specific antibodies against ERKs,

JNKs, and p38 kinase proteins were used to normalize the phosphorylation assay using the same transferred membrane blots. Mouse epidermal JB6 cells and primary rat hepatocytes were starved by culturing them in 0.1% FBS MEM for 24 and 2 h, respectively. Cells were then treated with various concentrations of ferric sulfate from 30 min to 24 h before performing the phosphorylation assay.

2.5. IL-6 determination

JB6 cells were treated with various concentrations of Fe^{3+} in α-MEM containing 0.1% FBS for 48 h, and culture media were collected for IL-6 determinations using ELISA kit, following the Manufacturer's instruction (R&D System). To assess the regulatory effects of AP-1 on IL-6, SB202190 and PD98059 were also added 30 min before iron treatment for IL-6 measurement.

2.6. RNA isolation and RT-PCR

Total RNA was extracted using Trizol solution. Reverse transcription reactions were carried out using Superscript RNase H^- reverse transcriptase according to the Manufacturer's instructions. Primers used were 5′-ATG AAG TTC CTC TCT GCA AG-3′ and 5′-CTG TAT CTC TCT GAA GGA CT-3′ for IL-6, 5′-CGT CTT CAC CAC CAT GGA GA-3′ and 5′-CGG CCA TCA CGC CAC AGT TT-3′ for GAPDH. Mouse IL-6 fragments were amplified by 30 cycles of PCR, each cycle consisting of 90 s at 94 °C, 45 s at 58 °C and 90 s at 72 °C. All PCR reactions were carried out in the linear range. The resultant RT-PCR products along with DNA markers were electrophoresed in 2% agarose gels, then stained with 1× SYBR and visualized under UV light.

2.7. Statistical analysis

The experimental results were analyzed for their statistical significance by the paired, two-tailed Student's *t*-test when comparing two groups. For comparisons among three or more groups, data were analyzed using a one-way analysis of variance, followed by Duncan's multiple range test. A confidence level of $P < 0.05$ was taken to represent a significant difference in all cases.

3. Results

3.1. Induction of AP-1 in JB6 cells by various iron compounds

AP-1 transcription activities induced by various iron compounds were measured in stable AP-1 luciferase reporter plasmid-transfected mouse epidermal JB6 P^+ cells. The results observed from this stable transfectant show that AP-1 activity was markedly induced by exposure of the cells to both water-soluble and water-insoluble iron compounds. For example, control cells without iron treatment yielded a luciferase activity reading of 2128 ± 51 (standard deviation, $n = 8$, in arbitrary units). At a concentration of 50 μM, Fe^{3+} ions increased by four-fold relative AP-1 luciferase activity as compared to the control cells (Fig. 1A). Cells transfected with vehicles only (no AP-1 luciferase reporter) gave activity less than 100. The activation of AP-1 by iron compounds appeared to be dose-dependent (Fig. 1A and B). In general, Fe^{3+} ions in the forms of sulfates were more potent than Fe^{2+} ions in inducing AP-1 (Fig. 1A). The maximal concentration for the induction of AP-1 by Fe^{2+} ions was at 200 μM. Treatment of cells with higher concentrations of iron, particularly Fe^{3+} ions, resulted in lower AP-1 luciferase activities, which may be due to the cytotoxicities caused by Fe^{3+} ions as measured by Trypan blue exclusion test. Water-insoluble iron compounds were also able to transactivate AP-1. At a dose of 40 μg/cm^2 (or 160 μg/ml culture media), relative luciferase activities were increased 174% by iron oxide and 79% by iron sulfide, respectively (Fig. 1B).

3.2. Effects of Fe^{3+} ions on phosphorylation of ERKs and p38 MPAK in JB6 cells

Because Fe^{3+} ions appeared the most active among the four iron compounds tested in inducing AP-1 activity, ferric sulfate was chosen for the rest of the study. Phosphorylation of ERKs, JNKs, and p38 MAPK by an upstream kinase results in AP-1 activation. We studied ERKs, JNKs, and p38 MAPK proteins and their phosphorylation using antibodies specific for the MAPKs and phospho-specific for the phosphorylated MAPKs. Fig. 2 shows that Fe^{3+} ions significantly stimulated the phosphorylation of ERKs in a dose- and time-dependent manner. Greater phosphorylation of

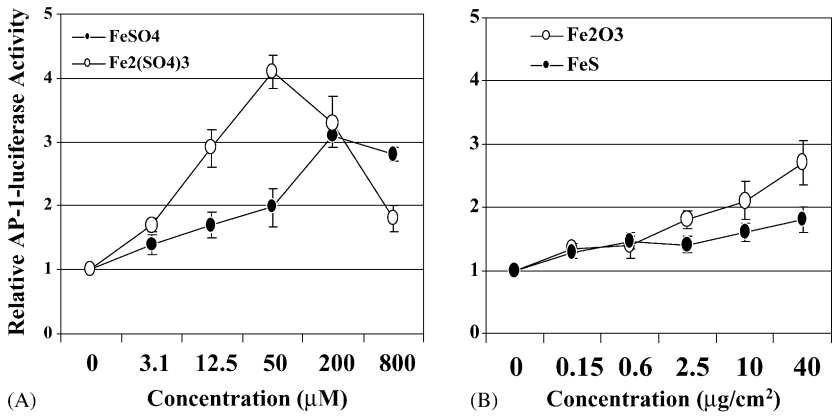

Fig. 1. Effects of various iron compounds on AP-1 activation in mouse epidermal JB6 cells. JB6 cells were seeded into each well of 96-well plates. After being cultured at 37 °C overnight, the cells were starved for 18 h by replacing medium with 0.1% FBS α-MEM. Then, the cells were treated separately with different concentrations of $FeSO_4$, $Fe_2(SO_4)_3$, Fe_2O_3, and FeS. After 36 h treatment, the luciferase activity was measured and normalized with protein concentration. The results are presented as fold change over control. Each bar indicates the mean and standard deviation of four identically treated assay wells.

ERKs induced by Fe^{3+} ions was observed at 200 μM (3.48 ± 0.42-fold of control, $n = 3$) for 30 min treatment (2.85 ± 0.26-fold of control, $n = 3$) (representative gels were shown in Fig. 2A and B). Fig. 3 shows that Fe^{3+} ions can also induce phosphorylation of p38 MPAK in a dose- and time-dependent manner. Interestingly, phosphorylation of p38 MAPK required higher concentrations of Fe^{3+} ions (1.98 ± 0.30-fold of control at 800 μM, $n = 3$) and longer treatment time (2.79 ± 0.36-fold of control at 200 μM for 18 h) for maximal response as compared to the phosphorylation of ERKs (representative gels were shown in Fig. 3A and B). Fig. 4 shows that Fe^{3+} ions did not induce phosphorylation of JNKs. The positive control hydro-

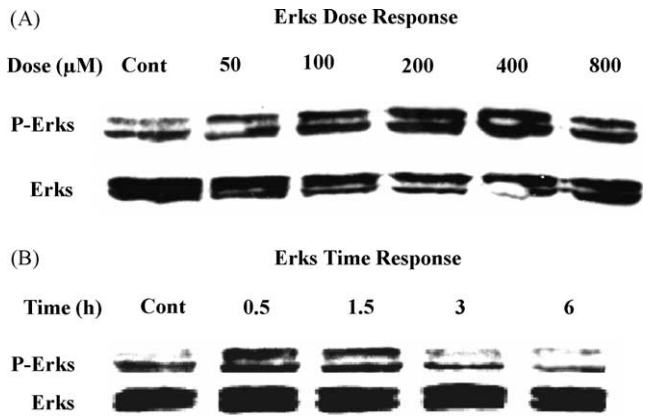

Fig. 2. Dose response and time course studies on the phosphorylation of ERKs by ferric sulfate in mouse epidermal JB6 cells. Cells were cultured in six-well plates to 90% confluence. (A) After starving them in 0.1% FBS α-MEM for 18 h, cells were treated with different concentrations of Fe^{3+} for 90 min. (B) 200 μM of Fe^{3+} were used for cell treatment from 30 min to 6 h. Control indicates time 0 without iron. After treatment, cells were lysed with SDS lysis buffer in six-well plates, and cell lysis solutions were collected for the phosphorylation assay. Phosphorylated and non-phosphorylated ERKs were assayed using the corresponding specific antibodies. The phosphorylated and non-phosphorylated proteins were analyzed using the same transferred membrane blots.

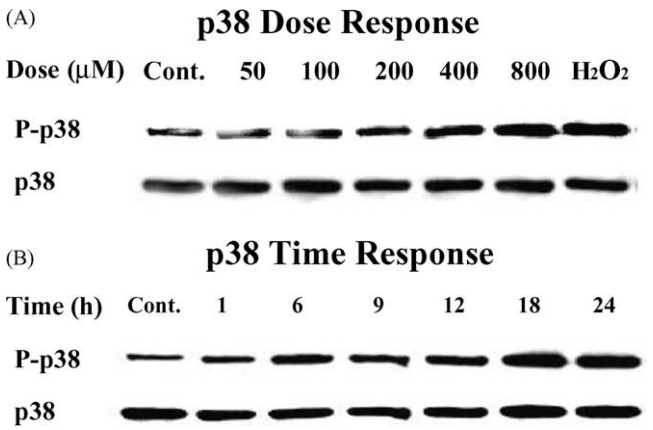

Fig. 3. Dose response and time course studies on the phosphorylation of p38 MAPK. Cells were seeded, cultured, and processed as described in the legend of Fig. 2. H_2O_2 was used as a positive control for p38 MAPK: (A) 90 min treatment; (B) 200 μM Fe^{3+}.

gen peroxide (H_2O_2, 200 μM) did activate the phosphorylation of JNKs, indicating that Fe^{3+} ions did not stimulate phosphorylation of JNKs under these experimental conditions.

3.3. Effects of iron on the phosphorylation of MAPKs in primary rat hepatocytes

Since liver is the main target organ of iron overload, we used primary rat hepatocytes to verify the pathways of AP-1 activation induced by iron. Fig. 5 shows an iron-induced phosphorylation of ERKs and p38 MPAK at 0.8 and 1.6 mM Fe^{3+}. Similar to the effects of iron on mouse epidermal JB6 cells, iron did not induce phosphorylation of JNKs. Although the concentrations of iron (above 0.8 mM) in the induction of phosphorylation seem high, it was reported that the liver iron concentration within cells of hemochromatosis patients could be as high as 160 mM (Bassett et al., 1986; Huang et al., 2002).

3.4. Inhibition of the iron-induced AP-1 activation by PD98059 and SB202190

To further confirm that iron-induced AP-1 activation is through ERKs and p38 MPAK pathways, specific in-

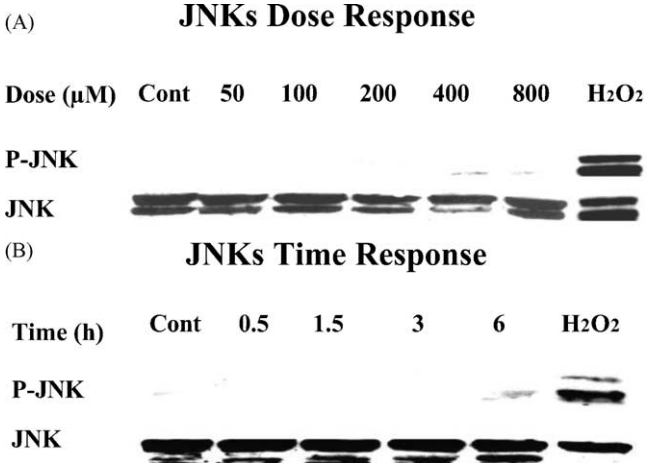

Fig. 4. Dose response and time course studies on the phosphorylation of JNKs. Cells were seeded, cultured, and processed as described in the legend of Fig. 2. H_2O_2 was used as a positive control for JNKs: (A) 90 min treatment; (B) 200 μM Fe^{3+}.

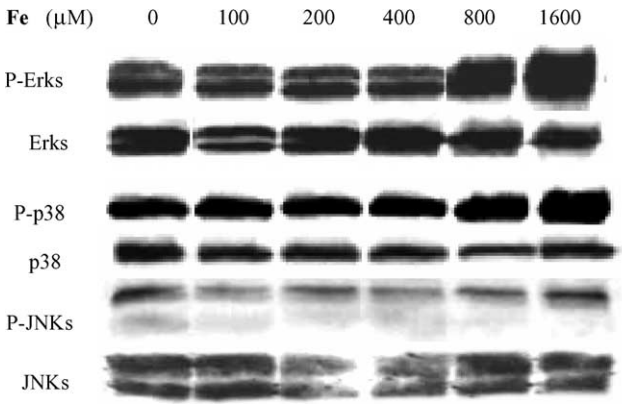

Fig. 5. Effects of ferric sulfate on the phosphorylation of ERKs, JNKs, and p38 MAPK in primary rat hepatocytes. Cells were exposed to various amounts of iron for 30 min. After treatment, cells were lysed and processed as described in Fig. 2.

hibitors were added to the tissue culture media 30 min before iron treatment. We have found that the pretreatment of JB6 cells with PD98059 and SB202190 resulted in the inhibition of the iron-induced AP-1 luciferase activity at different time points (Fig. 6A). Fig. 6B shows that the two inhibitors alone had no effects on AP-1 as compared to the control.

3.5. Induction of IL-6 by Fe^{3+} ions

IL-6 is a multifunctional cytokine that regulates many genes such as matrix metalloproteinases, which may contribute to iron-induced fibrosis and cancer (Hirano, 1998). Fig. 7 shows a dose-dependent increase in levels of IL-6 in the tissue culture media of mouse epidermal JB6 cells as a function of Fe^{3+} concentration. The control levels of IL-6 were 152.6 ± 14.0 pg/ml culture media ($n = 3$). At 400 μM Fe^{3+} treatment, levels of IL-6 reached 1747.3 ± 107.8 pg/ml ($n = 3$). We have observed that maximal induction of IL-6 by iron was approximately at 48 h treatment, while the greatest transactivation of AP-1 was at 36 h (Fig. 1).

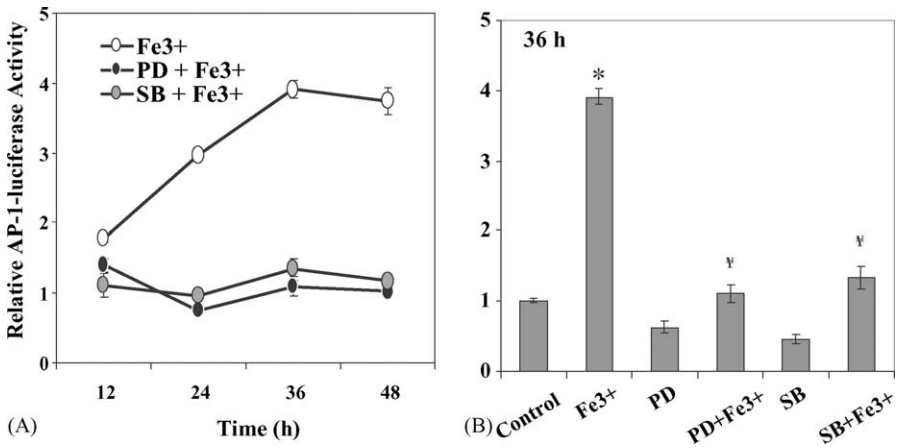

Fig. 6. Effects of PD98059 or SB202190 on iron-induced AP-1 luciferase activity. (A) Time-dependency. Inhibitors were added to the culture media 30 min before Fe^{3+} ion treatment (50 μM). Time periods showed on the X-axis were hrs after iron addition. (B) PD or SB alone showed no effect on AP-1. (*) Significantly different from the control ($P < 0.05$); (¥) significantly different from the iron-treated cells ($P < 0.05$).

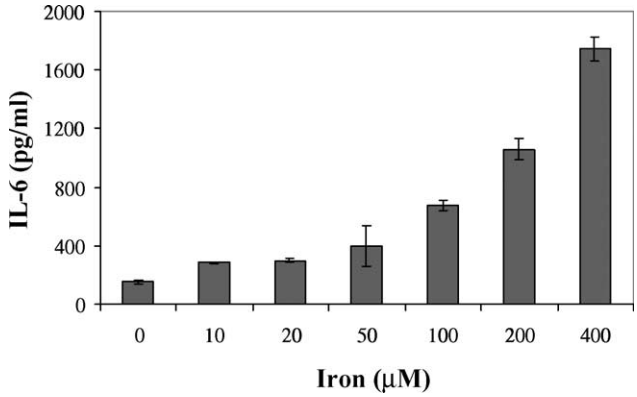

Fig. 7. Effect of iron on IL-6 formation. Mouse epidermal JB6 cells grown in six-well plates containing 0.1% FBS α-MEM were treated with various doses of Fe^{3+} for 48 h. The media were collected for IL-6 measurements.

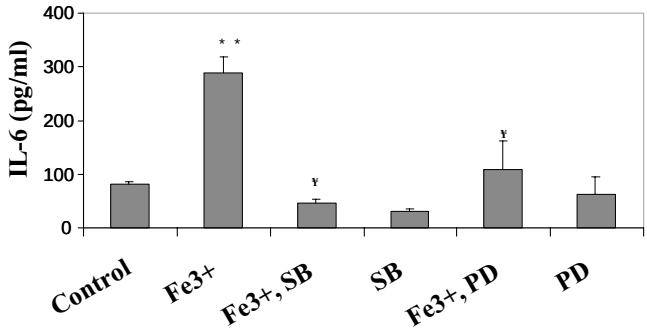

Fig. 8. Effects of various MAPK inhibitors on iron-induced IL-6. Mouse epidermal JB6 cells were pretreated with SB 202190 (2 μM), an inhibitor of p38 MAPK, and PD 98059 (100 μM), an inhibitor of MAP kinase (MEK), followed by iron treatment (100 μM) for 48 h. After treatment, the media were collected for IL-6 measurements. (**) Significantly different from the control ($P < 0.01$); (¥) significantly different from the iron-treated cells ($P < 0.05$).

3.6. Inhibition of iron-induced IL-6 as well as IL-6 mRNA by specific inhibitors of ERKs and p38 MAPK pathways

To determine whether iron-induced IL-6 is mediated through ERKs and p38 kinase pathways, specific inhibitors of each pathway were added to the tissue culture media 30 min before Fe^{3+} ions treatment. We have found that the pretreatment of cells with PD98059 (100 μM) and SB202190 (2 μM) resulted in the inhibition of the Fe^{3+} ions-induced IL-6 formation (Fig. 8). Fig. 9 shows that the increased level of IL-6 mRNA by

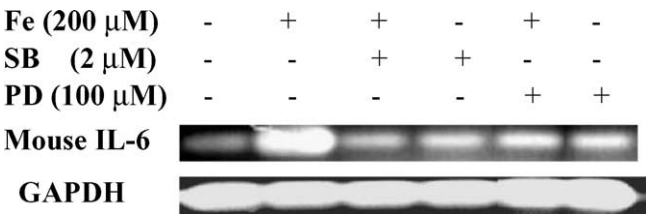

Fig. 9. Effects of MAPK inhibitors on iron-induced IL-6 mRNA. Mouse epidermal JB6 cells were grown in 72 cm^2 flask to 90% confluence, and changed to 0.1% FBS MEM for 4 h. Cells were pretreated with SB 202190 (2 μM) or PD 98059 (100 μM) for 30 min, followed by iron treatment (200 μM) for 24 h. Cells were collected for RNA isolation and RT-PCR assay as described in the Section 2.

Fe^{3+} ions was attenuated by the pretreatment of cells with PD 98059 and SB202190. The inhibitory effects of SB202190 on IL-6 and its mRNA appeared to be more pronounced than those of PD 98059, although the difference was statistically insignificant (Fig. 8). These two MAPK inhibitors alone had no significant effects on the levels of IL-6 protein or IL-6 mRNA as compared to the control levels.

4. Discussion

Iron can exert adverse health effect when the amount of iron entering the body exceeds the amount lost over a sustained period of time. However, the mechanism by which iron overload causes disease remains unknown, although redox cycling and oxidant formation through Haber-Weiss, Fenton, or autoxidation reactions were suggested (Comporti et al., 2002; Ryan and Aust, 1992; Welch et al., 2002). Because of the intrinsic properties of the mouse epidermal JB6 cells being sensitive to oxidative stress (Hsu et al., 2000), this cell line was chosen for testing our hypothesis. In the present study, we have shown that both water-soluble and water-insoluble iron compounds transactivated AP-1 in mouse epidermal JB6 cells, as measured by the luciferase activity assay. Water-soluble Fe^{3+} and Fe^{2+} sulfates induced more AP-1 activation than water-insoluble iron oxide or ferrous sulfide. The lesser activation of AP-1 by FeS and Fe_2O_3 may be due to the insolubility of these iron compounds. For example, at 40 μg/cm^2 or 160 μg/ml of FeS, it could represent a concentration of 550 μM free iron if FeS is completely solubilized in the media. Therefore, one would expect a higher AP-1 activation by FeS than that by the water-soluble $FeSO_4$. To the contrary, higher AP-1 luciferase activity was observed in cells treated with water-soluble iron. These observations suggest that greater accessibility by water-soluble iron compounds to oxygen in an aqueous solution may result in more oxidant formation and AP-1 activation than those by water-insoluble iron compounds.

AP-1 is an important oxidant-responsive transcription factor controlling expression of many genes. The signal transduction pathways leading to the AP-1 activation have been extensively studied in the last several years. The ERKs pathway is considered responsive to the growth/differentiation signals, while the JNKs and p38 pathways are responsive to environmental stresses such as heat shock. A variety of environmental pollutants, which include arsenic, vanadium, and quartz, activate all or part of the ERKs and JNKs/p38 pathways (Ding et al., 1999; Dong et al., 2001; Huang et al., 1999, 2001). In the present study, we have found that the phosphorylation of ERKs and p38 kinase, but not JNKs, is induced by iron, both in mouse epidermal JB6 cells and primary rat hepatocytes. Using H_2O_2 (200 μM) as a positive control, we found that H_2O_2 induced phosphorylation of JNKs under the same experimental conditions. Moreover, pretreatment of cells with the p38 and ERKs inhibitors SB202190 and PD98059 suppressed AP-1 transactivation induced by Fe^{3+} ions (Fig. 6). Thus, these results suggest that iron-induced AP-1 activation may occur through ERKs and p38 MAPK but not JNKs pathways. However, it is still unclear whether co-activation of ERKs and p38 is required for iron-induced AP-1 and which pathway may be rate-limiting. This awaits further investigation. It is also noteworthy that differences in incubation times and concentrations for obtaining maximal responses of phosphorylation and AP-1 activation were distinct. For example, greater ERKs phosphorylation occurred at 0.5 h treatment with 200 μM Fe^{3+}. Maximal AP-1 activation was at 36 h with 50 μM Fe^{3+}. As shown by Trypan blue exclusion assay, higher concentrations of Fe^{3+} for longer period of treatment caused cytotoxicity, and therefore, affected AP-1 activation.

Regulation of IL-6 production has been shown to occur both at the transcriptional and post-transcriptional level (Rola-Pleszczynski and Stankova, 1992). As shown here, mouse epidermal JB6 cells produced and released IL-6 in the culture media without any treatment (152.6 pg/ml). Exposure of the cells to iron resulted in a dose-dependent increase in IL-6 production. For example, at 50 and 400 μM Fe^{3+}, levels of IL-6 were increased 2.6- and 11.4-fold over the controls. To determine the mechanism responsible for the increase in IL-6 levels in iron-treated cells, we measured levels of IL-6 mRNA. Exposure of cells to iron caused an increase in IL-6 mRNA as shown in Fig. 9. Several potential transcriptional control elements, such as glucocorticoid-responsive elements, an active AP-1 binding, a c-fos serum-responsive element (c-fos SRE) homology, as well as a nuclear

factor-kB binding site have been identified within the conserved region of the IL-6 promoter (Hirano, 1998). Among them, c-fos SRE and AP-1-like elements appear to contain the major *cis*-acting regulatory elements that confer responsiveness to several reagents, such as serum and phorbol ester (Hirano, 1998). In monocytic cell lines, the NF-kB site is crucial for lipopolysaccharide-induced IL-6 gene expression (Sanceau et al., 1995). In HeLa cells, concomitant activation of NF-kB and AP-1 is required for ionizing radiation-induced IL-6 gene expression (Beetz et al., 2000). In the present study, we have shown that increased levels of IL-6 protein as well as IL-6 mRNA by iron can be abolished by the pretreatment of cells with PD98509 and SB203580, two specific inhibitors of MEK1 (ERKs pathway) and p38 MAPK, respectively. These data provide evidence that the activation of ERKs and p38 MAPK pathways may be crucial for the iron-induced IL-6 formation.

In conclusion, our studies have shown that iron can stimulate IL-6 release from mouse epidermal JB6 cells. The increased levels of IL-6 by iron may be an important mechanism for iron overload pathogenesis. Our results also indicate that the iron-induced IL-6 increase involves AP-1 through ERKs and p38 MAPK pathways.

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