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Deferoxamine synergistically enhances iron-mediated AP-1 activation: A showcase of the interplay between extracellular-signal-regulated kinase and tyrosine phosphatase

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Abstract
Deferoxamine (DFO) is a drug widely used for iron overload treatment to reduce body iron burden. In the present study, it was shown in mouse epidermal JB6 cells that all iron compounds transiently induced extracellular signal-regulated kinases (ERK) phosphorylation, whereas DFO further enhanced ERK phosphorylation over long periods. The ERK phosphorylation by DFO treatment appears to be due to the inhibition of MAPK phosphatases (MKP) by DFO. The combined effects of iron-initiated MAPK activation and DFO-mediated MKP inhibition resulted in a synergistic enhancement on AP-1 activities. The results indicate that the interplay between MAPK and MKP is important in regulating the extent of AP-1 activation. It is known that administration of DFO in iron overload patients often results in allergic responses at the injection sites. The results suggest that this synergistic AP-1 activation might play a role in DFO-induced skin immune responses of iron overload patients.

Keywords: *Iron overload, deferoxamine, activator protein-1, phosphatase, kinase.*

Abbreviations: *AP-1: activator protein-1; DFO: deferoxamine; ERK: extracellular signal-regulated kinases; FBS: foetal bovine serum; JNK: c-jun NH2 terminal kinases; MAPK: mitogen-activated protein kinases; MKP: MAPK phosphatases; MEM: minimum essential medium; NFAT: nuclear factor of activated T-cells; NTBI: non-transferrin-bound iron.*

Introduction

Iron is a critical nutritional element, essential for a great variety of important biological processes including cell growth and differentiation, electron transfer reactions and oxygen transport [1,2]. Iron can exert adverse health effects when the amount of iron entering the body exceeds the amount lost over a sustained period of time. Increasing evidence demonstrates that increased iron in the body may contribute to oxidative stress, inflammation and cancer development [3]. The mitogen-activated protein kinases (MAPK) family members of extracellular-signal-regulated kinases (ERK), c-jun NH_2 terminal kinases (JNK) and p38 MAPK are key players in antioxidant defenses, pro- and anti-inflammatory responses and cancer development [4–6]. Activator protein-1 (AP-1) is one of the early genes activated by a cascade of phosphorylation events in the MAPK signalling pathway. Previous studies have shown that iron induced phosphorylation of MAPK/ERK, p38, but not JNK pathways in mouse epidermal cells,

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hepatocytes and neuroblastoma cells, resulting in AP-1 activation and subsequently formation of pro-inflammatory cytokine interleukin 6 (IL-6) [7,8].

Deferoxamine (DFO), a siderophore originally isolated from *Streptomyces pylosus*, is a clinically approved drug for iron overload treatment to reduce body iron burden [9,10]. Patients with severe anaemia, such as β-thalassemia, which is due to haemolytic anaemia and ineffective erythropoiesis and requires regular blood transfusions causing iron overload, must be treated with iron chelating agents [11]. DFO forms a hexadentate complex with Fe^{3+} ions in a molar ratio of 1:1. Non-transferrin bound iron (NTBI) are more labile than transferrin-bound iron and, therefore, could constitute a potential source of catalytically redox active iron for adverse health effects [12]. Studies have demonstrated the ability of DFO to remove NTBI [13]. DFO was previously shown to activate MAPK/ERK and p38 but not JNK pathways in keratinocytes and intestinal epithelial cells, resulting in apoptosis and formation of chemokine IL-8 [14,15].

DFO is poorly absorbed from the gut. Therefore, parenteral routes of administration are the only realistic methods for DFO delivery. Administration of DFO at the injection site is known to result in allergic responses, causing discomfort and leading to frequent non-compliance during DFO treatment [16–18]. The exact mechanisms of how DFO causes allergic responses on the skin are not known. In the present study, we have used mouse epidermal JB6 cells, a cell line sensitive to oxidative stress [19], to test the effects of DFO and iron on AP-1 activation. When DFO was used to block iron-mediated MAPK signalling pathway in the cells, a synergistic increase on AP-1 activation was observed. This synergism on AP-1 appeared as a result of the combined effects of MAPK phosphorylation by iron and MAPK phosphatases (MKP) inhibition by DFO. Our study suggests that synergistic effects of iron and DFO on AP-1 might play a role in DFO-induced skin immune responses of iron overload patients.

Material and methods

Chemical reagents

Ferric sulphate [$Fe_2(SO_4)_3$], ferrous sulphate [$FeSO_4$], ferrous sulphide (FeS), iron oxide hydrated, [Fe_2O_3 as revealed by X-ray (data not shown)], deferoxamine mesylate (DFO) and Eagle's minimum essential medium (MEM) were obtained from Sigma (St. Louis, MO). Water-insoluble iron particles (FeS and Fe_2O_3) were used for comparison with water soluble iron sulphates to see whether insoluble iron plays a role in MAPK signalling. FeS and Fe_2O_3 were separately ground in a ball mill and size-classified by a Mercer Impactor as previously described [20]. Phospho- and non-phospho-MAPK antibody kits were from Cell Signaling Technology (Danvers, MA). Luciferase assay substrate was from Promega (Madison, WI).

Cell culture and iron treatment

Mouse epidermal JB6 cells and AP-1-luciferase reporter stably transfected JB6 P^+1-1 cells were prepared and cultured in monolayers in Eagle's MEM containing 5% foetal bovine serum (FBS), 2 mM L-glutamine and 25 μg of gentamicin/ml as previously described [21,22]. Nuclear factor of activated T-cells (NFAT) luciferase stable transfectants from mouse embryo fibroblasts, PW NFAT mass1 cells, were cultured in MEM with 10% FBS, 2 mM L-glutamine and 25 μg gentamicin/ml [23]. Cells were grown in a humidified atmosphere of 95% air and 5% CO_2 at 37°C.

All iron treatments for phosphorylation and phosphatase activities were carried out in a 6-well microplate. After JB6 cells reaching 80–90% confluency, cells were starved by culturing them in 0.1% FBS for 18 h and then exposed to ferrous sulphate in the presence or absence of DFO. 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 incubation times, cells were lysed for phosphorylation and phosphatase assays.

Protein kinase phosphorylation assay

Immunoblotting for the phosphorylation of ERK, p38 and JNK was carried out as described in the protocol provided by Cell Signaling Technology™, using phospho-specific antibodies against phosphorylated sites of ERK, JNK and p38 kinase. Non-phospho-specific antibodies against ERK, JNK and p38 kinase proteins provided in each assay kit were used to normalize the phosphorylation assay using the same transferred membrane blots.

Tyrosine and serine/threonine phosphatase assay

To measure tyrosine and serine/threonine phosphatase activities in JB6 cells, two methods were used. First, standard phospho-ERK (p-ERK) and phospho-p38 (p-p38) proteins, obtained from the Cell Signaling Technology™, were used. In brief, after various treatments, cells were collected using a rubber policeman. After washing and centrifugation, cells were suspended in 100 μl phosphate-buffered solution (10 mm, pH 7.4) and then lysed by repeatedly freezing at −80°C and thawing on ice. Cell extracts (15 μl) were incubated with standard p-ERK (1 μl) or p-p38 (2 μl) for 6 h or overnight at room temperature. This was to ensure that tyrosine and serine/threonine phosphatases in the cell extracts were able to remove the phosphate groups of phosphorylated

ERK or p38. Immunoblotting for the remaining phospho-ERK and phospho-p38 was carried out as described above.

To further confirm phosphatase activities in cell extracts, an IQ® phosphatase assay for serine/threonine and tyrosine phosphatases were used (Pierce, Rockford, IL). In brief, JB6 cell extracts from various iron and/or DFO treatments were incubated with phosphoserine peptide substrate (LRRApSLG) specific for serine/threonine phosphatase or phosphotyrosine peptide substrate (KVEKIGEGTpYGVVYK) specific for tyrosine phosphatase. At the end of the phosphatase reaction, a fluorescent compound was added to specifically bind the remaining phosphorylated peptide and its fluorescence was quenched. Therefore, the observed relative fluorescence units (RFU) are proportional to the extent of dephosphorylation. Data presented here were as percentage inhibition of phosphatase normalized to the control cells without any treatments.

AP-1 luciferase activity assay

Viable AP-1 luciferase reporter stably transfected JB6 P^+ 1-1 cells (8×10^3) cells were added to each well of a 96-well microtiter plate. After 18 h incubation in 100 μl MEM containing 5% FBS, cells were starved by culturing them in 0.1% FBS for 24 h and then exposed to water soluble ferrous and ferric sulphates and water insoluble iron sulphide and oxide in the presence or absence of DFO for AP-1 induction. 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. With a surface area of 0.36 cm^2 at the bottom of the well and 100 μl medium in the well, 40 $\mu g/cm^2$ of FeS and Fe_2O_3 equal to 2.1 mM and 1.8 mM iron, respectively, assuming that iron is completely solubilized. When DFO was used, cells were pre-treated with DFO for 0.5 h before adding iron. After different time periods, cells were extracted with lysis buffer and luciferase activity was measured using a luminometer (Monolight 2010; Monolight, Gaithersburg, MD). The results were expressed as fold increase on AP-1 activities relative to those of the controls.

NFAT luciferase activity assay

To study whether the synergistic effect of DFO and iron is AP-1 specific, viable PW NFAT mass1 cells (5×10^3) suspended in 100 μl medium were added to each well of a 96-well microtiter plate. After exposing these cells to Fe or DFO or DFO + Fe, cells were extracted with lysis buffer. Luciferase activity was measured and results were expressed as relative NFAT activity to the control cells.

Statistical analysis

The experimental results were analysed for their statistical significance by the paired, two-tailed Student's *t*-test. Confidence levels of $p < 0.05$ were taken to represent a significant difference between the two means.

Results

Variation of ERKs phosphorylation as a function of time

Figure 1 shows that 200 μM iron significantly induces ERK phosphorylation (lane 2 in Figure 1A) and slightly p38 MAPK phosphorylation (Figure 1C) but not JNK phosphorylation (Figure 1D) after 1 h treatment. These results confirm our previous finding on activation of both ERK and p38 but not JNK pathways by high concentration of iron (e.g. 800 μM) [7]. DFO itself had no effect on ERK phosphorylation at 1 h treatment (lane 3 at the top) but temporarily prevented iron-induced ERK phosphorylation (lane 4). Interestingly, 6 h treatment of cells with DFO or DFO followed by iron (DFO + Fe) showed increased ERK phosphorylation (Figure 1A, lanes 3 and 4 at the 3rd line of the bands). Quantitation of ERK phosphorylation as a function of time is shown in Figure 1B, indicating a delayed ERK activation in the presence of DFO.

Inhibition of phosphatase activity by DFO

Equal amounts of standard p-ERK or p-p38 as substrates for MKP were incubated with extracts from cells treated with iron, DFO or DFO + Fe. Figure 2A shows that extracts obtained from control and iron-treated cells can dephosphorylate, leading to decreased levels of p-ERK as well as p-p38 after 6 h and 18 h incubation. Interestingly, extracts from cells treated with DFO or DFO + Fe cannot readily dephosphorylate the standard p-ERK or p-p38 proteins.

To further confirm the ability of DFO or DFO + Fe to inhibit tyrosine- and serine/threonine phosphatase activities, substrates specific for each phosphatase were used. Figure 2B and C show that cell extracts from DFO- or DFO + Fe-treated cells were less active in removing phosphate from their phosphopeptide substrates. DFO or DFO + Fe had more inhibitory effects on tyrosine phosphatase than serine/threonine phosphatase (Figure 2B vs C).

Synergistic enhancement of AP-1 but not NFAT luciferase activities by DFO + Fe

AP-1 and NFAT transcriptional activities induced by various iron compounds were measured in stable AP-1 and NFAT luciferase reporter-transfected cells, respectively. Results obtained from these two transfectants show that both water soluble and water

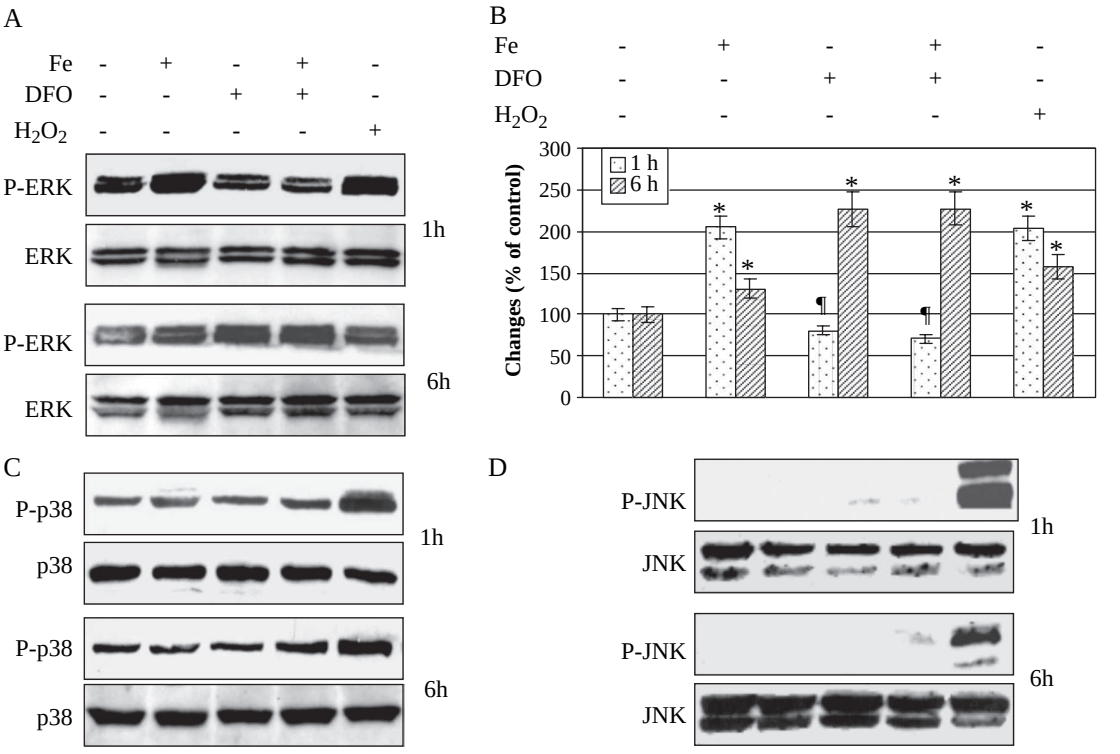

Figure 1. Effects of iron, DFO and DFO+Fe on the phosphorylation of ERK, JNK and p38 as a function of time. Mouse epidermal JB6 cells were seeded, cultured and processed in a 6-well microplate. Cells were then exposed to iron, DFO or DFO followed by iron (DFO+Fe) at a concentration of 200 μM for different incubation time points as indicated. After lysis, cell extracts were subjected to Western blot analysis with antibodies specific for phosphorylated and total ERK (A), p38 (C) and JNK (D). Data are representative of three independent experiments and the quantification of ERK phosphorylation at 1 h and 6 h treatments is shown in panel (B). H_2O_2 (200 μM) was used as a positive control. *Significantly different from control cells ($p < 0.05$); ¶ significantly different from iron-treated cells ($p < 0.05$).

insoluble iron compounds can markedly induce AP-1 as well as NFAT activities (Figure 3A and B). Without treatment, background readings of AP-1 luciferase activities in the control cells were 2100 arbitrary units. After treatment with the four iron compounds tested, AP-1 luciferase activities were all significantly increased, varying from 2–4-folds of the control levels (Figure 3A). Water soluble iron

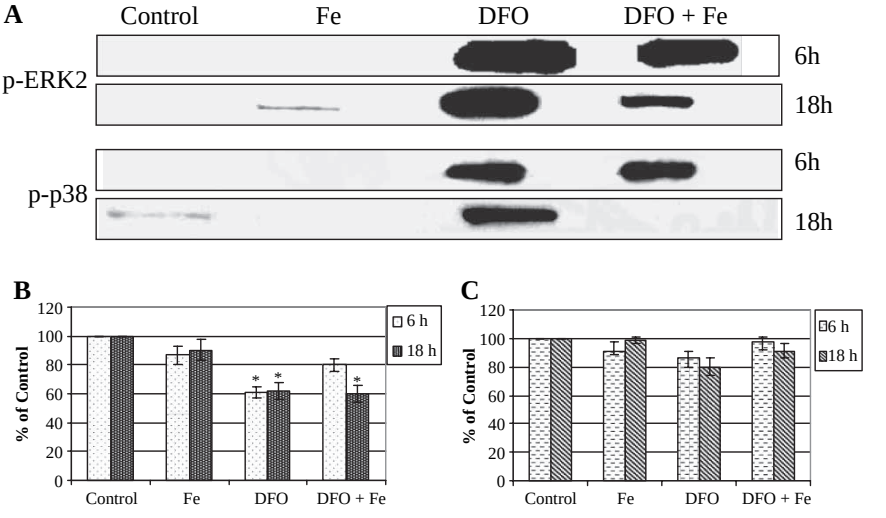

Figure 2. Inhibitory effects of DFO or DFO+Fe on tyrosine or serine/threonine phosphatases activities. JB6 cells were treated with iron, DFO or DFO followed by iron as described in the Figure 1 legend. Cell extracts were incubated with standard p-ERK or p-p38. Immunoblotting for the remaining p-ERK or p-p38 was then carried out. A representative of three independent experiments is shown (A). Cell extracts were also incubated with phospho-peptide substrates specific for tyrosine phosphatase (B) and serine/threonine phosphatase (C). Relative fluorescence units (RFU)/mg were measured and were normalized to the control levels. *Significantly different from the controls ($p < 0.05$, $n = 3$).

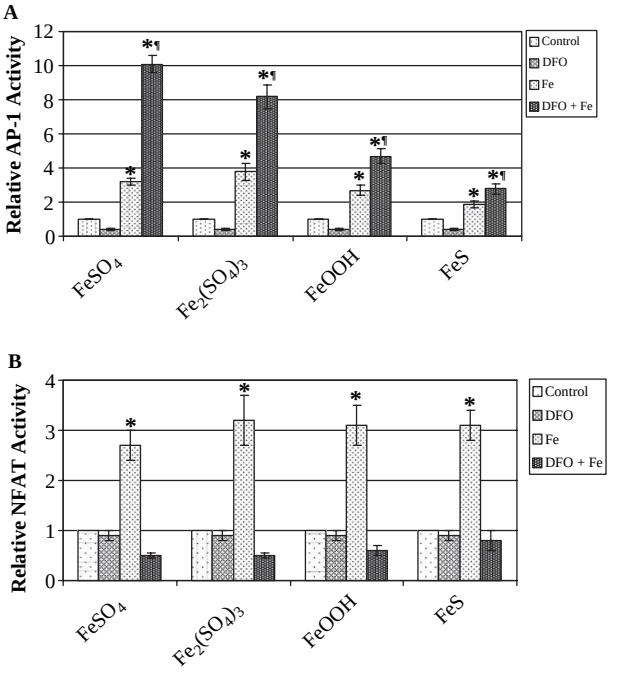

Figure 3. Effects of DFO on iron-induced AP-1 and NFAT activation. AP-1- luciferase reporter stably transfected mouse epidermal JB6 cells and NFAT-luciferase reporter stably transfected mouse embryo fibroblast PW cells were treated separately with $FeSO_4$ (200 μM), $Fe_2(SO_4)_3$ (50 μM), Fe_2O_3 (40 μg/cm^2) and FeS (40 μg/cm^2) in the presence or absence of DFO. After 36 h treatment, the luciferase activities were measured and the results were presented as fold increase on AP-1 (A) and NFAT (B) activities relative to the controls. Each bar indicates the mean and standard deviation of four identically treated assay wells. *Significantly different from control cells ($p < 0.05$); ¶ significantly different from iron-treated cells ($p < 0.05$).

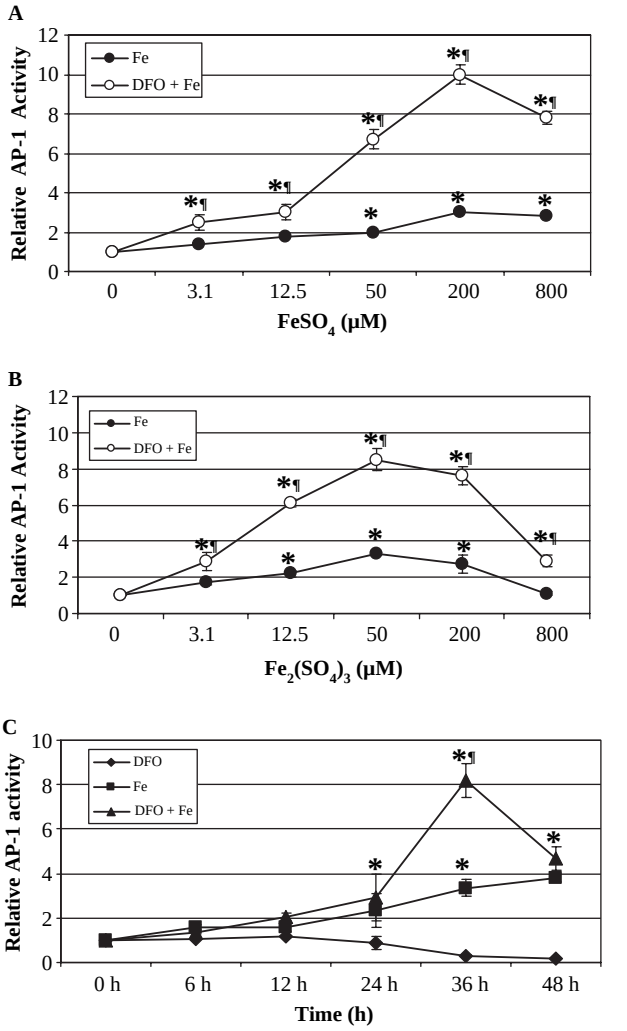

Figure 4. Dose- and time-dependence of AP-1 luciferase activities induced by iron and DFO. JB6 cells carrying AP-1 luciferase reporter were first treated with 200 μM DFO for 0.5 h, followed by various concentrations of $FeSO_4$ (A) and $Fe_2(SO_4)_3$ (B) for 36 h or as a function of time in the presence of DFO and/or $FeSO_4$ (C). AP-1 luciferase activities were measured and data were presented as described in Figure 3 legend. *Significantly different from control cells ($p < 0.05$); ¶significantly different from iron-treated cells ($p < 0.05$).

sulphates (0.1 mM range) were apparently the most active forms of iron in inducing AP-1 and NFAT. Water insoluble FeS and Fe_2O_3, which were less readily bioavailable due to limited solubility, induced the same activation but at much higher doses (2 mM range if iron is completely solubilized). Interestingly, pre-treatment of cells with DFO (0.2 mM), followed by iron treatment (e.g. $FeSO_4$, 0.2 mM), synergistically increased AP-1 activity 10-fold over the control as compared to $FeSO_4$ alone, which had a 3-fold increase (Figure 3A). In contrast, DFO prevented iron-induced NFAT activation (Figure 3B).

Figure 4A and B show that, at 0.2 mM DFO, synergistic activations of AP-1 luciferase activities at 36 h treatment were iron-concentration dependent for $FeSO_4$ and $Fe_2(SO_4)_3$, respectively. Treatment of cells with higher concentrations of Fe^{3+} ions resulted in lower luciferase activities, which may be due to the cytotoxicities caused by Fe^{3+} ions as measured by the Trypan blue exclusion test. Dose-dependent AP-1 activations were also observed for water-insoluble iron compounds Fe_2O_3 and FeS (data not shown). Figure 4C shows that DFO itself had no effect on AP-1 activity up to 24 h treatment. Longer periods of incubation (> 36 h) inhibited AP-1 luciferase activity. Interestingly, the maximal synergistic effect of DFO and Fe on AP-1 was at 36 h treatment.

Discussion

The mechanisms that lead to activation of the MAPK signalling pathway have been studied intensively. Nearly all growth factors and cytokines, as well as various environmental stimuli, such as arsenic, UV and reactive oxygen species, can activate and translocate ERK to the nucleus and promote activation of downstream early genes and their protein products [5,24,25]. Yet, these diverse extracellular stimuli can evoke markedly different biological responses, leading to MAPK signal specificity [5,26]. Factors such as receptor density on the cell membrane, the rate and

extent of receptor internalization, expression of scaffolding proteins and the surrounding extracellular matrix can modulate the strength and duration of MAPK signalling and, thus, its specificity in downstream gene expression [5]. For example, it was shown that sustained but not transient activation of ERK signalling preceded the differentiation of rat PC12 pheochromocytoma cells into sympathetic-like neuron [27]. Sustained ERK activation resulting from upstream Ras mutation also correlates with carcinogenesis [5,28].

In the present study, we have shown that iron and H_2O_2 can transiently lead to ERK activation after 1 h treatment (Figure 1). As the incubation time increased (e.g. 6 h), ERK phosphorylation in cells treated with iron and H_2O_2 started to decrease. It is noteworthy that DFO also stimulated ERK phosphorylation at 6 h but not at 1 h treatment (Figure 1B). These results are in agreement with those previously reported showing ERK phosphorylation in keratinocytes, breast cancer MCF-7 cells and monocytes after 18 h treatment with DFO [15,29,30]. Because DFO itself had no significant effect on AP-1 luciferase activity (Figure 4C), these results suggest that DFO-mediated ERK phosphorylation may be due to MKP inhibition by DFO. Indeed, by incubating cell extracts with standard phospho-ERKs or phospho-p38, extracts from the control cells, as well as the cells treated with iron readily removed phosphate from the substrates, resulting in weak or no bands (Figure 2A). In contrast, extracts from cells treated with DFO or DFO + Fe had little activities in the removal of phosphate from the substrates. Using specific phosphotyrosine or phosphoserine peptide substrates, it was further confirmed that DFO or DFO + Fe significantly inhibited tyrosine phosphatase activities and slightly inhibited serine/threonine phosphatase activities.

MKP are considered to be important negative-feedback regulators of MAPK signalling [6,28,31]. In general, activation of MAPK signalling is accompanied by induction of MKP-mediated dephosphorylation and transient over-expression of MKP. DFO was previously shown as effective in inhibiting iron-mediated lipid peroxidation and ferritin synthesis [32]. DFO or iron were separately shown to activate ERK and p38 MAPK activation but not JNK pathways, with iron leading to IL-6 formation and DFO contributing to increased IL-8 levels [7,15]. In the present study, it was shown that iron stimulated AP-1 by 3-fold and DFO + Fe resulted in 10-fold increases over the controls. DFO alone had no significant effect on AP-1 at early treatment (6 h) and an inhibitory effect at long period of treatment ($\geq$ 36 h). These results suggest that passive accumulation of ERK phosphorylation due to DFO-mediated MKP inhibition may not be sufficient to trigger AP-1 activities. Our previous study has shown that iron-mediated AP-1 activation is mainly through the ERK pathway [7]. PD98059, a specific MEK1 inhibitor (ERK pathway), was shown to block iron-mediated AP-1 luciferase activities. Although ERK phosphorylation is not evident, as shown by the semi-quantitative Western blot, in the presence of DFO and iron, it is reasonable to assume that ERK phosphorylation initiated by iron combined with phosphatase inhibition by DFO is responsible for the synergistic increase in AP-1 activities. Our results indicate that interplay between MAPK and MKP is important in regulating the extent and timing of AP-1 activation [33–35].

To investigate whether this synergistic effect of iron and DFO is AP-1 specific, NFAT transfectants were used. In contrast to AP-1, dephosphorylation activates NFAT protein. This is achieved through the activation of signalling pathways that produce a rise in the intracellular free calcium levels. An increase in intracellular free calcium induces activation of the phosphatase, calcineurin, which dephosphorylates NFAT proteins [34,36]. It was shown that iron stimulated NFAT and that DFO completely prevented iron-mediated NFAT activation (Figure 3B). These results suggest that the observed synergistic effect is AP-1 specific.

AP-1 is an important transcription factor controlling the regulation of many genes such as pro-inflammatory cytokines [37], which may play an important role in skin immune responses [38,39]. At present, DFO is the most widely used iron chelator for treatment of β-thalassemia patients. Besides its high cost, other disadvantages include its requirement for long subcutaneous infusion and its poor absorption from the gut [13]. Administration of DFO often results in allergic responses, which may cause discomfort to the patients at the injection site [16–18]. In clinical studies, DFO has recently been shown to increase cardiac morbidity and mortality in patients with thalassemia major as compared to another iron chelator deferiprone [40]. Therefore, this synergistic effect of DFO and iron on AP-1 may be relevant to DFO treatment in iron overload patients and the clinical implication may warrant further investigation.

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