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Desferrioxamine, an iron chelator, enhances HIF-1α accumulation via cyclooxygenase-2 signaling pathway

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Abstract

Cyclooxygenase-2 (COX-2) is an important inducible enzyme in inflammation and is overexpressed in a variety of cancers. Evidence is rapidly accumulating that chronic inflammation may contribute to carcinogenesis through increase of cell proliferation, angiogenesis, and metastasis in a number of neoplasms, including colorectal carcinoma. In the present study, we investigated some mechanistic aspects of DFX-induced hypoxia-driven COX-2 expression. Desferrioxamine (DFX), an iron chelator, is known to upregulate inflammatory mediators. DFX induced the expression of COX-2 and accumulation of HIF-1α protein in dose-dependent manners, but hypoxia mimetic agent cobalt chloride ($CoCl_2$) induced accumulation of HIF-1α protein but not increase of COX-2 expression. DFX-induced increase of COX-2 expression and HIF-1α protein level was attenuated by addition of ferric citrate. This result suggested that the iron chelating function of DFX was important to induce the increase of COX-2 and HIF-1α protein. PD98059 significantly inhibited the induction of COX-2 protein and accumulation of HIF-1α, suggesting that DFX-induced increase of HIF-1α and COX-2 protein was mediated, at least in part, through the ERK signaling pathway. In addition, pretreatment with NS-398 to inhibit COX-2 activity also effectively suppressed DFX-induced HIF-1α accumulation in human colon cancer cells, providing the evidence that COX-2 plays as a regulator of HIF-1α accumulation in DFX-treated colon cancer cells. Together, our findings suggest that iron metabolism may regulate stabilization of HIF-1α protein by modulating cyclooxygenase-2 signaling pathway.

Keywords: COX-2; Desferrioxamine; HIF-1α; ERK; Colon cancer

It is well established that prostaglandins (PGs) are known to participate in multiple physiologic and pathologic processes, including maintaining homeostasis of bone tissue, wound healing, cardiovascular disease, inflammation, and development and growth of malignant tumors [1]. Cyclooxygenase (COX) is a key enzyme that catalyzes the conversion of PGs and has two subtypes, COX-1 and COX-2 [2]. COX-1 is ubiquitously expressed on cell membranes in normal tissue, while COX-2 is cytoplasmic in location and its expression is low in most tissues and is inducible during inflammatory and carcinogenic processes. COX-2 seems to be involved in the processes of malignant transformation and tumor progression by affecting cell proliferation, cell cycle progression, cell adhesion, apoptosis, immune surveillance, and angiogenesis [3]. An elevated COX-2 level has been shown to correlate with a worse prognosis for patients with some types of tumors such as breast cancer [4–6]. Chronic inflammation is a recognized as a risk factor for epithelial carcinogenesis [7]. Inflammation is associated with increase synthesis of prostaglandins due to cytokine-mediated induction of COX-2. Therefore, what a cause and effect link between chronic inflammation and carcinogenesis via overexpression of COX-2 is established might provide a rational mechanism by which chronic inflammation increases the risk of cancer.

Most solid human tumors have focal hypoxic areas that cause low oxygen tension. And low oxygen tension is known to play a critical role in embryonic development,

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cause the emergence of drug-resistant tumor cells, enhance mutagenesis of neoplastic lesions, and elevate metastatic potential of the tumor [8–11]. Under hypoxia conditions, an oxygen-sensing mechanism activates a transcription factor known as hypoxia-inducible factor-1 (HIF-1), which in turn upregulates a broad array of genes that activate mitogenic, pro-invasive, pro-angiogenic, and pro-metastatic genes [12,13]. Interestingly, recent studies discovered that HIF-1α is also regulated by oxygen-independent mechanisms, mainly activation of oncogenes and mutation of tumor suppressor genes [14]. Recently, COX-2 was also found to play a role in up-regulating of HIF-1α expression in gastric cancer cells [15].

Iron chelators can also strongly induce transcription of NO synthase (NOS) and increase the release of interleukin-1β (IL-1β) in human alveolar macrophages [16,17]. These observations suggest that iron chelators may modulate certain inflammatory mediators and regulate inflammatory processes. Desferrioxamine (DFX), an iron chelator, is known to reduce free radical-mediated cell injury and has the ability of hypoxia mimetics to upregulate certain inflammatory mediators. It was reported that hypoxia increases COX-2 protein levels in prostate cancer cells and DFX upregulates COX-2 expression and prostaglandin production in a human macrophage cell line [18,19]. Since DFX upregulated inflammatory mediator and COX-2 expression, they have been used as a model system to examine the factors involved in cross-talking inflammation and carcinogenesis induced by COX-2 expression. Hypoxia leads to the expression of a number of gene products that are involved in tumor progression, invasion, and metastasis formation. The most important of these proteins is thought to be hypoxia-inducible factor-1α (HIF-1α), which appears to be a master regulator of the cellular response to hypoxia [20]. However, there have been only a few studies dealing with the correlation of COX-2 and HIF-1α expression in DFX-induced hypoxia condition. In present study, we hypothesized that DFX-induced hypoxia could induce COX-2 up-regulation, which in turn upregulates HIF-1α expression. To prove our hypothesis, mouse macrophage cells and colon cancer cells were treated with DFX and analyzed correlation of COX-2 and HIF-1α expression.

Materials and methods

Cells and materials. All reagents were purchased from Sigma–Aldrich unless otherwise stated. The macrophage cell line Raw 264.7 and human colon cancer cell line HT29 and HCT116 were obtained from the American Type Culture Collection (Rockville, MD) and cultured in RPMI 1640 supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% fetal calf serum. Human colon cancer cell lines SNUC4 were obtained from the Korean cell line bank (KCLB, Seoul, Korea). The cells were subcultured twice weekly and grown on 6-well plates at 1×10^6 cells per well, at 37 °C in fully humidified 5% CO_2 air. Anti-iNOS, anti-COX-2, and anti-HIF-1α were purchased from Santa Cruz Biotechnology. Anti-phospho-ERK, anti-phospho-JNK, and anti-phospho-p38 mitogen-activated protein kinase (MAPK) were purchased from New England Biolabs.

Western blotting. Cell lysates were prepared by suspending 1.5×106 cells in 100 μl lysis buffer (137 mM NaCl, 15 mM EGTA, 0.1 mM sodium orthovanadate, 15 mM MgCl2, 0.1% Triton X-100, 25 mM Mops (4-morpholinepropane sulfonic acid), 100 μM phenylmethylsulfonyl fluoride, and 20 μM leupeptin, adjusted to pH 7.2), disrupted by sonication, and extracted at 4 °C for 30 min. The proteins were electrotransferred to Immobilon-P membranes and detection of specific proteins was carried out with an ECL Western blotting kit according to the manufacturer's instructions.

Nitrite quantification. NO accumulation in the medium was used as an indicator of NO production as previously described [21]. Raw 264.7 cells were plated at 1.5×10^6 cells/ml and stimulated with DFX and LPS for 18 h. The isolated supernatants were mixed with an equal volume of Griess reagent (1% sulfanilamide, 0.1% naphthylethylenediamine dihydrochloride, and 2% phosphoric acid) and incubated at room temperature for 10 min. $NaNO_2$ was used to generate a standard curve, and nitrite production was determined by measuring the optical density at 550 nm.

RNA isolation and reverse transcriptase-polymerase chain reaction (RT-PCR). Total RNA was isolated according to Chomczynski and Sacchi [22]. Single-strand cDNA was synthesized from 2 μg of total RNA using M-MLV (Moloney Murine leukemia virus) reverse transcriptase. The cDNA for iNOS, COX-2, HIF-1α, and actin was PCR amplified using the following specific primers: iNOS (sense) 5′-ATG GCT TGC CCC TGG AAG TTT CTC-3′ and (antisense) 5′-CCT CTG ATG GTG CCA TCG GGC ATC TG-3′; HIF-1α (sense) 5′-TATGACCTGCTTGGTGC TGA-3 and (antisense) 5′-GGGAGAAAATCAAGTCGTGC-3′; COX-2 (sense) 5′-CCGTGGTGAATGTATGAGCA-3′ and (antisense) 5′-CCTC GCTTCTGATCTGTCTT-3′. PCR amplification was carried out as follows: 1 × (94 °C, 3 min); 30 × (94 °C, 45 s; 59 °C, 45 s; 72 °C, 1 min); and 1 × (72 °C, 10 min). PCR products were analyzed by agarose gel electrophoresis and visualized by ethidium bromide.

Protein half-life determination. U937 cells were treated with 200 μM quercetin for 18 h and cycloheximide (2 μg/ml) was added to inhibit new protein synthesis. Extracts were harvested at the indicated times and protein half-life was determined. Equal amounts of cell lysates (40 μg) were subjected to electrophoresis and analyzed by Western blot for HIF-1α and ERK.

Results

Effect of DFX on COX-2 expression in cancer cells

To investigate the effect of DFX on the expression levels of COX-2, iNOS proteins, and NO production, Raw 264.7 cells were treated with various concentrations of DFX for 18 h. As shown in Fig. 1A, treatment with DFX-induced the expression of COX-2 protein in a dose-dependent manner. However, there is no induction in the iNOS protein and NO production. LPS (200 ng/ml) is used as positive control for iNOS induction and NO production. It was reported that DFX and cobalt appear to stabilize HIF-1α via different mechanisms [23]. DFX most likely inhibits hydroxylation of HIF-1α by chelating the iron required for activity of the HIF-1α specific proline hydroxylases. In contrast, cobalt prevents VHL binding when added at either the translation or the binding step. We have also performed a parallel analysis for HIF-1α and COX-2 in various cancer cells including HT29, HCT116, and SNUC4 throughout the hypoxic mimetic condition induced by DFX treatment. As shown in Fig. 1B, DFX treatment increased the expression of COX-2 protein and HIF-1α protein accumulation in a dose-dependent manner in all of the cancer cell lines. Next, we treated $CoCl_2$, known as

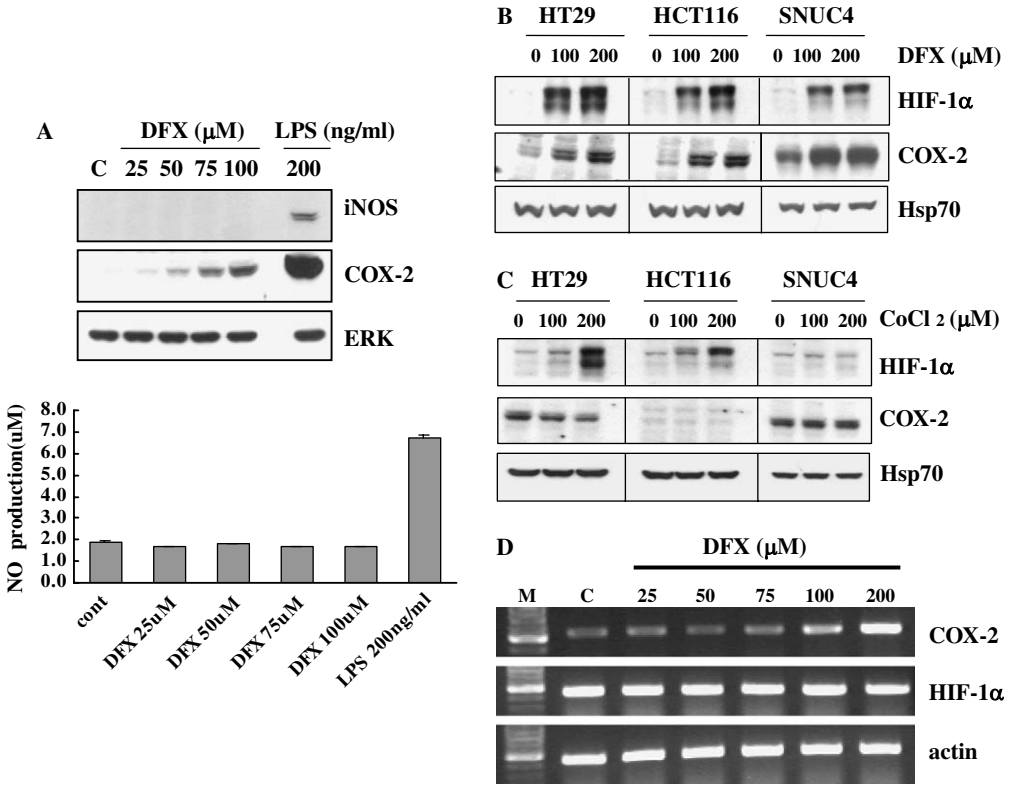

Fig. 1. Up-Regulation of COX-2 in Several Human Tumor Cell Lines under Hypoxic Treatments. (A) Raw 264.7 cells were treated with indicated concentrations of DFX and LPS for 18 h. The cells were lysed, and the lysates were analyzed by immunoblotting using anti-iNOS and anti-COX-2. The blot was stripped of the bound antibody and reprobed with anti-ERK antibody to confirm equal loading. The culture supernatants were subsequently isolated and analyzed for nitrite levels. The values for nitrite are averages ± SD from three independent experiments. (B) Human colon cancer cells were treated with indicated concentrations of DFX for 18 h and the lysates were analyzed by immunoblotting using anti-HIF-1α and anti-COX-2. (C) Human colon cancer cells were treated with indicated concentrations of $CoCl_2$ for 18 h and the lysates were analyzed by immunoblotting using anti-HIF-1α and anti-COX-2. (D) HCT116 cells were treated with indicated concentrations of DFX for 18 h, the total RNAs were isolated, and COX-2 and HIF-1α mRNA levels were analyzed by RT-PCR method. A representative study is shown; two additional experiments yielded similar results.

HIF-1α inducer, in colon cancer cell lines. Exposure to $CoCl_2$ induced increase of HIF-1α protein accumulation but not COX-2 expression (Fig. 1C). We also examine the COX-2 and HIF-1α mRNA expression levels in DFX-treated HCT116 cells. As shown in Fig. 1D, DFX treatment increased the COX-2 mRNA expression but not HIF-1α mRNA, suggesting that DFX-induced increase of COX-2 protein was caused by up-regulation of COX-2 mRNA expression while DFX-induced increase of HIF-1α protein was regulated by posttranscriptional levels. Taken together, these results suggest that exposure of cells to DFX under normoxia stimulates COX-2 expression and HIF-1α accumulation.

Inhibitory effect of ferrous iron on DFX-induced COX-2 expression and HIF-1α accumulation

To examine the direct inhibitory functions of iron on increase of HIF-1α and COX-2 protein, we analyzed HIF-1α and COX-2 protein expression by Western blotting. As shown in Figs. 2A and B, 200 μM DFX obviously induced COX-2 expression and HIF-1α protein accumula-

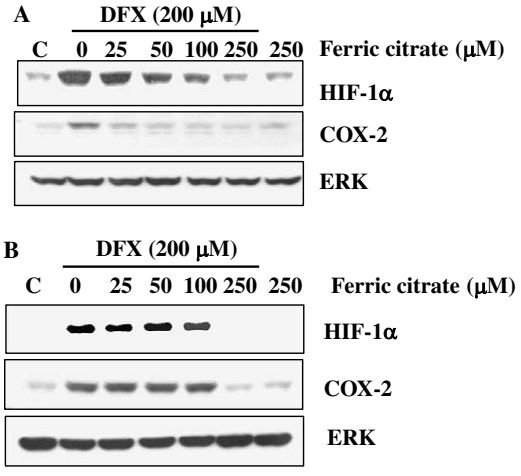

Fig. 2. Ferric citrate inhibits DFX-induced COX-2 expression and HIF-1α accumulation. Raw 264.7 (A) and HCT116 (B) cells were co-treated with 25–250 μM ferric citrate and 200 μM DFX for 18 h. The cells were lysed, and the lysates were analyzed by immunoblotting using anti-iNOS and anti-COX-2. The equal loading in each lane was demonstrated by the similar intensities of ERK. A representative study is shown; two additional experiments yielded similar results.

tion in both Raw 264.7 and HCT116 cells. COX-2 expression and HIF-1α protein accumulation were gradually reduced when coincubated with 25–250 μM ferric citrate in Raw 264.7 cells. However, HCT 116 cells were drastically reduced at 250 μM ferric citrate. These data clearly indicate that prevention of DFX-induced COX-2 expression and HIF-1α protein accumulation is associated with the iron chelating.

Mitogen-activated protein kinase signal pathway after DFX treatment

To examine the role of the mitogen-activated protein kinase (MAPK) signaling pathway in DFX-mediated COX-2 protein expression, we examined the activation of the three MAPKs in DFX-treated cells by Western blotting using phosphorylation specific MAPK antibodies. Treatment with DFX-induced a significant increase in phosphorylated ERK and JNK levels. However, treatment of Raw 264.7 cells with DFX slightly increased the levels of phospho-p38 MAPK without affecting the total amount of p38 MAPK protein. ERK and JNK were activated after 10 min, reached maximum activity at 90 min, and sustained to 2 h (Fig. 3A). To determine whether the MAPK signaling pathways are involved in the DFX-induced increase of HIF-1α and COX-2 protein, Raw 264.7 and HCT116 cells were pretreated with or without MAPK specific inhibitors

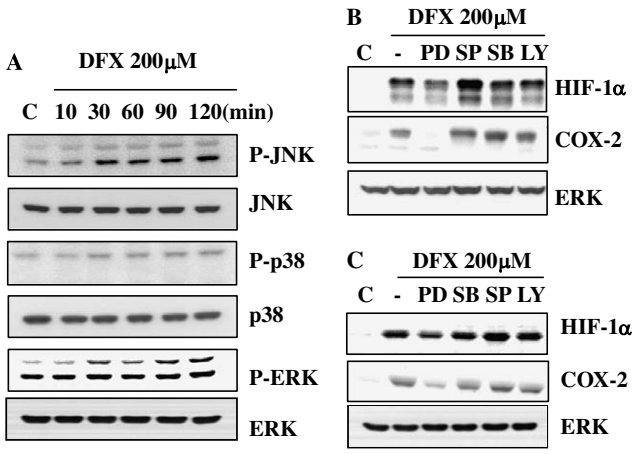

Fig. 3. DFX-induced HIF-1α and COX-2 expression was mediated through the ERK signaling pathway. (A) Effect of DFX on phosphorylation of MAPKs in Raw 264.7 cells. Raw 264.7 cells were stimulated with 200 μM DFX for the indicated time points. The whole-cell lysates were analyzed by immunoblot analysis using various antibodies against the activated MAPKs (dually phosphorylated on Tyr/Thr). To ascertain that the total level of each MAPK did not change, blots were stripped and reprobed with the antibodies raised against the corresponding phosphorylation-independent MAPK. A representative result is shown; two additional experiments yielded similar results. Raw 264.7 cells (B) or HCT116 cells (C) were pretreated with various inhibitors (50 μM PD98059, 25 μM SP600125, 10 μM SB203580, and 50 μM LY294002) for 60 min, and then treated with DFX (200 μM) for 18 h. Equal amounts of soluble lysates (50 μg) were subjected to electrophoresis. The blots were analyzed using a specific antibody against HIF-1α and COX-2. The equal loading in each lane was demonstrated by the similar intensities of ERK.

in the presence of DFX. Pretreatment of cells with PD98059, a selective ERK pathway inhibitor, significantly inhibited DFX-induced COX-2 expression and HIF-1α protein accumulation (Figs. 3B and C). These results suggest that DFX-induced COX-2 expression and HIF-1α protein accumulation was, at least in part, dependent on ERK-mediated signaling pathway.

The role of p53 in DFX-treated cells

There has been evidence to suggest that COX-2 expression is normally suppressed by wild-type p53, suggesting that loss of p53 function may result in the induction of COX-2 expression. It was reported that wild-type but not mutant p53 inhibited the binding of TATA-binding protein (TBP) to the promoter region of COX-2 gene [24]. To confirm the relationship between p53 expression status and HIF-1α expression, we treated with 100 or 200 μM DFX for 18 h in p53 wild-type HCT116 and HCT116 (p53−/−) cell lines. As shown in Fig. 4A, DFX-induced HIF-1α and COX-2 protein expression levels in p53 null HCT116 cells are greater than those of p53 wild-type cells. These findings are consistent with previous results, showing that the p53 represses HIF-1 stimulated transcription [25]. After treating with $CoCl_2$ to both cell lines, we analyzed protein expression levels of HIF-1α and COX-2. As shown in Fig. 4B, induction of HIF-1α was detected in both cells but not COX-2. Together, the status of p53 is associated with the HIF-1α and COX-2 protein expression in DFX- and $CoCl_2$-treated cells.

COX-2 increases HIF-1α protein stability in DFX-treated cells

To determine whether the DFX-induced increase of HIF-1α protein level was driven by COX-2, we assessed the HIF-1α expression levels in HCT116 and SNUC4 cells treated NS398, a specific COX-2 inhibitor, in addition to 200 μM DFX. As shown in Figs. 5A and B, HIF-1α protein accumulation by DFX was inhibited by NS-398, but HIF-1α mRNA expression was not affected by both DFX and NS-398. These results demonstrated that DFX-induced HIF-1α protein accumulation was regulated by COX-2 activity. However, NS-398 did not affect DFX-induced COX-2 protein expression levels in both cells (Figs. 5A and B). No significant cell death was seen using these concentrations of COX-2 inhibitors compared with control (data not shown). To investigate the mechanism by which DFX treatment increased HIF-1α protein levels, we investigated the effect of DFX on the half-life of HIF-1α protein. After HCT116 cells were treated with 200 μM DFX for 18 h, cycloheximide (2 μg/ml) was added to inhibit new protein synthesis. Extracts were harvested at the indicated times and protein half-life was determined. As shown in Fig. 5C, treatment with DFX increased the stability of HIF-1α protein. These data clearly indicate that DFX-induced increase of HIF-1α protein level is associated with

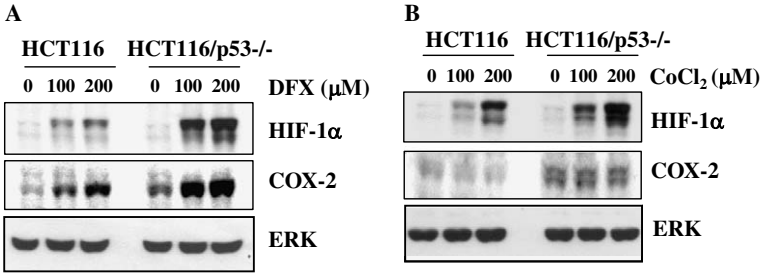

Fig. 4. The effect of the p53 status on DFX- and $CoCl_2$-induced HIF-1α and COX-2 protein expression. HCT116 (p53 wild-type) and HCT116 (p53−/−) cell lines were treated with 100 or 200 μM DFX (A) or $CoCl_2$ (B) for 18 h. The cells were lysed and the lysates were analyzed by immunoblotting using anti-HIF-1α and anti-COX-2. The equal loading in each lane was demonstrated by the similar intensities of ERK.

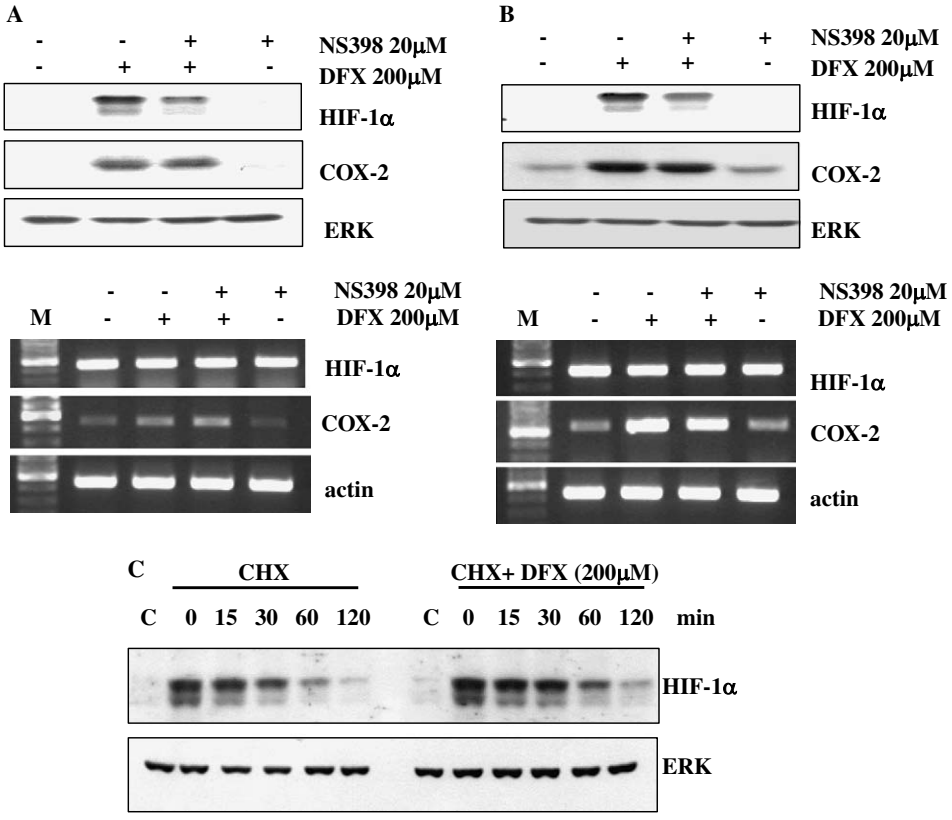

Fig. 5. COX-2 increase HIF-1α protein stability in DFX-treated cells. HCT116 (A) or SNUC4 (B) cells were pretreated with NS-398 and incubated with 200 μM DFX for 18 h. The cells were lysed and the lysates were analyzed by immunoblotting using anti-HIF-1α and anti-COX-2. Western blotting and RT-PCR analysis were performed. The total RNAs were isolated, and COX-2 and HIF-1α mRNA levels were analyzed by RT-PCR method. A representative study is shown; two additional experiments yielded similar results. (C) HCT116 cells were treated with 200 μM DFX for 18 h, washed out DFX and incubated with cycloheximide (2 μg/ml) alone or DFX plus cycloheximide for the indicated times. Extracts were harvested at the indicated times and protein half-life was determined.

accumulation of HIF-1α protein caused by enhance of HIF-1α protein stability.

Discussion

In the present study, we reported that DFX upregulates COX-2 protein expression and increases HIF-1α protein level in a dose-dependent manner in mouse macrophage cells and human colon cancer cells. In addition, the HIF-1α accumulation was regulated by COX-2 in DFX-treated cells.

Hypoxia is a major pathophysiological condition for the induction of angiogenesis, which is a crucial aspect of growth in solid tumors. Moreover, hypoxia is a dominant factor in the angiogenic phenotype through HIF-1 and other transcription factors combined with tumor-specific genetic alterations. Cellular adaptation to hypoxia is an important requirement of tumor progression independent of angiogenesis.

Desferrioxamine was obtained from *Streptomyces pilosus* and has the ability of hypoxia mimetics to upregulate several inflammatory mediators. DFX is also capable of

inducing HIF-1 and NF-κB activation, and hypoxia dependent gene expression, probably by replacing or removing the central iron of the putative heme oxygen sensor [12,26]. HIF-1 represents the most important transcription factor regulating gene expression under hypoxic conditions [27]. Expression of HIF-1α was induced by hypoxic conditions, transition metals including cobalt, nitric oxide, insulin, insulin like growth factor, lipopolysaccharide (LPS), TNF-α, and dibenzoylmethane [28–31]. The availability of HIF-1 is mainly determined by HIF-1α that is regulated in an oxygen-sensitive manner. HIF-1β may also be increased in response to hypoxic stress, but its abundance is more constitutive than that of HIF-1α. Under normoxia, HIF-1α is continuously expressed in the cell but immediately degraded the proteasomal system, thus keeping its protein level extremely low [32–34]. Up-regulation of gene expression induced by hypoxia can be mediated both by de novo synthesis of mRNA and by stabilization of the normally labile mRNAs under hypoxic conditions. In response to hypoxic stimulation, the mRNA of HIF-1α appears unchanged, but HIF-1α protein accumulates, interacts with HIF-1β, translocates to the nucleus, and binds to the hypoxia-responsive element that was found in the promoter region of target genes [34]. In our system, we found that DFX-induced hypoxia increased HIF-1α protein level through promoting the protein stability without any change of mRNA expression. This result suggested that DFX treatment caused accumulation of HIF-1α protein through the increase of protein stability.

COX-2 is an important inducible enzyme mediating inflammatory processes and is highly expressed in a diversity of cancers [35]. The known mechanisms by which COX-2 promotes carcinogenesis include evasion from apoptosis, suppression of immunity, and promotion of invasiveness [36]. However, the mechanism governing COX-2-induced angiogenesis caused by hypoxia remains largely unclear. Based on growing evidence implicating COX-2 and its derived prostanoids in angiogenesis [37], we explored the possibility that this enzyme may be an important mediator of hypoxic induction of HIF-1α caused by DFX. DFX could be blocked dosage dependently by adding ferrous iron salts [38]. Ferrous iron is rapidly oxidized by ambient oxygen yielding the DFX-chelatable ferric iron. We demonstrated that an increase of intracellular free iron correlated with HIF-1α and COX-2 disappearance, and suggested that multiple variables that modulate COX-2 expression to explain how DFX increased HIF-1α protein amount under hypoxia.

Growing evidence indicates that inflammation is a contributing factor leading to cancer development. However, pathways involved in this progression are not well understood. Several independent lines of research established the ability of DFX to enhance the expression of COX-2 [19,39]. Therefore, we postulate that the accumulation of HIF-1α and COX-2 expression functionally cooperates in DFX-stimulated cells. To examine the relationship between HIF-1α accumulation and COX-2 expression in DFX-treated cells, HCT116 cells were treated with DFX and analyzed protein and mRNA levels of HIF-1α and COX-2. As shown in Figs. 1D and 4, DFX resulted in a great increase in protein and mRNA levels of COX-2, but it did not affect HIF-1α mRNA expression. Also, treatment with the selective COX-2 inhibitor, NS398, had no effect on COX-2 expression but partially decreased HIF-1α accumulation in colon cancer cells. In consistent with our results, Huang et al. [15] reported that HIF-1α protein were increased by COX-2 overexpression and were reduced in a dose-dependent response to NS-398 in gastric carcinoma. NS398 decreases the expression of HIF-1α mRNA and reduces HIF-1α synthesis in a COX-2/PGE2 dependent way, which can be restored by addition of exogenous PGE2 in PC-3 cells under hypoxia condition [40]. Taken together, HIF-1α accumulation seems to be under COX-2 regulation in the DFX-induced hypoxic conditions on several cell types. Further studies will be required to define which COX-2 metabolite participates in the regulation of HIF-1α in the DFX-treated cells and the signaling pathways by which COX-2 regulates HIF-1α expression. Nevertheless, since decreasing COX-2 activity did not abolish completely DFX-induced HIF-1α expression, it is likely that additional COX-2 independent pathways are operative in regulating HIF-1α accumulation.

It is well established that MAPK signaling pathways mediate COX-2 induction in a number of cell types [41,42]. Tanji et al. [19] reported that DFX treatment induced COX-2 up-regulation that was inhibited by U0126, a specific inhibitor ERK signaling pathway. Our present study also showed that PD98059, a specific inhibitor of activation of ERK signaling pathway, inhibited COX-2 and HIF-1α protein induction by DFX, suggesting that activation of ERK is critical for COX-2 and HIF-1α expression after DFX exposure.

In conclusion, the present study demonstrated that DFX increases the HIF-1α protein stability through induction of the COX-2 under hypoxic conditions in human colon cancer cells although mechanistic details remained unclear.

Acknowledgments

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