Inhibitory Effect of 6-Formylpterin on HIF-1 α Protein Accumulation

Piyanart SOMMANI,^a Kouhei YAMASHITA,^{*,b} Takashi MIYOSHI,^b Hiroko TSUNEMINE,^b Tsutomu KODAKI,^a Hiroko MORI,^c Kiichi HIROTA,^d Toshiyuki ARAI,^d Masataka SASADA,^e and Keisuke MAKINO^a

^aInstitute of Advanced Energy, Kyoto University; Uji 611–0011, Japan; ^bDepartment of Hematology and Oncology, Kyoto University Hospital; ^dDepartment of Anesthesia, Kyoto University Hospital; Kyoto 606–8507, Japan; ^eSchool of Health Science, Faculty of Medicine, Kyoto University; Kyoto 606–8507, Japan; and ^cDepartment of Anesthesia, Higashiyama Takeda Hospital; Kyoto 605–0932, Japan. Received May 14, 2007; accepted July 31, 2007

Hypoxia-inducible factor-1 (HIF-1) is a main regulator of metabolic adaptation to hypoxia. HIF-1 α is induced by hypoxia, or by hypoxia-mimicking reagents, such as desferrioxamine (DFX), under a normoxic condition. A xanthine oxidase inhibitor, 6-formylpterin (6FP), is reported to exert its functions on reactive oxygen species (ROS) modulation. In this study, we investigated the effect of 6FP on HIF-1 α expression under a DFX-treated or hypoxic condition. 6FP decreased HIF-1 α expression at the protein level, but not at the mRNA level, in a dose-dependent manner, and this suppressive effect was reversed by the antioxidant, *N*-acetyl-L-cysteine (NAC). Furthermore, the ROS generated by 6FP was reversed with NAC coincubation. These findings suggest that intracellular ROS generated by 6FP decreased the HIF-1 α protein accumulation under a DFX-treated or hypoxic condition.

Key words 6-formylpterin; hypoxia-inducible factor 1; reactive oxygen species; desferrioxamine

Adaptation to hypoxia elicits both acute and chronic responses in a tissue-specific manner. The activation of hypoxia-inducible factor (HIF)-1, a master transcriptional regulator under low oxygen conditions, induces expression of many target genes, which play pivotal roles in various developmental and physiological processes, such as angiogenesis, energy metabolism, cell proliferation and survival.^{1–4)} HIF-1 is a heterodimer of a hypoxia-stabilized and -activated α -subunit and an oxygen-insensitive β -subunit. Therefore, the biological activity of HIF-1 is determined by the HIF-1 α expression.¹⁾ Under a normoxic condition, the O₂-dependent hydroxylation of proline residues 402 and/or 564 leads to the ubiquitination and proteasomal degradation of HIF-1 α .^{1,2)} Moreover, hydroxylation of the asparagine residue at amino acid 803 interferes with the interaction of HIF-1 α with the transcriptional coactivator CBP/p300, and thus suppresses the HIF-1 α transcriptional activity.⁵⁾ In a hypoxic condition, hydroxylation of the proline and asparagine residues is inhibited, leading to the stabilization of HIF-1 α protein and the upregulation of HIF-1 α transcriptional activity, respectively. Desferrioxamine (DFX), a well-known iron chelator, is widely used to mimic hypoxia.^{6,7)} Even under normoxia, DFX induces the expression of HIF-1 α by the removal of iron, thus leading to the inactivation of prolyl hydroxylases. In the last decade, reactive oxygen species (ROS) have been reported to be involved in HIF-1 modulation, although the mechanism is still a matter of debate.^{1,3)} One model revealed that hypoxia-induced mitochondrial ROS formation increases HIF-1 α stabilization, while another the reduction of ROS formation by hypoxia *via* an NADPH-oxidase pathway leads to HIF-1 α induction.

6-Formylpterin (6FP) is a conjugated pterin attached to an aldehyde residue⁸⁾ and is well known as a potent xanthine oxidase inhibitor with an inhibition constant (*K_i*) of about 0.6 nM.^{9,10)} We previously reported that 6FP generates ROS, mainly hydrogen peroxide, in T-lymphocytes,¹¹⁾ neutrophils,¹²⁾ and carcinoma cell lines such as HL-60, PanC-1 and Jurkat cells.¹³⁾ The application of 6FP is a reasonable approach with which to modulate HIF-1 activity because of its

noticeable actions on ROS generation.^{11–13)} Nevertheless, there have been no reports thus far on the effect of 6FP on HIF-1 α activity. In this study, using a cell line derived from a human hepatocellular carcinoma, Hep3B, we found that intracellular ROS generated by 6FP decreased HIF-1 α protein accumulation, but not gene expression, under a DFX-treated or hypoxic condition.

MATERIALS AND METHODS

Materials 6FP was obtained from Sankyo Kasei Kogyo (Hiratsuka, Japan). Dulbecco's modified Eagle's medium (DMEM), fetal calf serum (FCS), and penicillin–streptomycin liquid were purchased from Gibco (Grand Island, NY, U.S.A.). DFX and *N*-acetyl-L-cysteine (NAC) were obtained from Nacalai (Kyoto, Japan).

Cell Culture The human hepatocellular carcinoma Hep3B cells were cultured in DMEM medium supplemented with 10% FCS, 100 U/ml penicillin, and 100 μ g/ml streptomycin at 37 °C in a 5% CO₂ incubator or in a hypoxic incubator (1% O₂, 5% CO₂, and 94% N₂). To maintain the exponential growth, cells were seeded at 2 $\times$ 10⁵ cells/ml and passaged every 4 to 5 d.

Western Blot Analysis HIF-1 α expression was quantified by Western blot analysis. Briefly, scraped cells were lysed in lysis buffer, followed by centrifugation (20000 $\times$ *g*, 15 min). Protein was added to the same volume of 2 $\times$ sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer and boiled for 5 min. The lysate was quantified by the Bradford method. One hundred micrograms of protein was separated by 7.5% SDS-PAGE and transferred to polyvinylidene difluoride membranes (Immobilon-P; Millipore, Bedford, MA, U.S.A.). At 1 : 1000 dilution, mouse anti-human HIF-1 α monoclonal antibody (BD Bioscience, San Jose, CA, U.S.A.) and anti-mouse IgG HRP-linked antibody (Cell Signaling, Beverly, MA, U.S.A.) were used as the first and secondary antibodies, respectively. Finally, a signal was developed using ECL reagent (Amersham Biosciences, Zurich, Switzerland). β -Actin (Chimicon,

* To whom correspondence should be addressed. e-mail: kouhei@kuhp.kyoto-u.ac.jp

Temecula, CA, U.S.A.) was used as an internal control. The intensity of each band was quantified with the software Quantity One-4.6.1 (Bio-Rad, Hercules, CA, U.S.A.) and normalized by β -actin.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) and Real-Time PCR Total RNAs were isolated from the Hep3B cells using RNAiso reagent (Takara Bio, Shiga, Japan), according to the manufacturer's instructions. Extracted RNA was dissolved in diethyl pyrocarbonate-treated water and quantified by measuring the absorbance at 260 nm.

For RT-PCR, 1 μ g of total RNA was subjected to first strand cDNA synthesis using random primers (SuperScript III RT Kit; Invitrogen, Carlsbad, CA, U.S.A.). PCR was performed in a 20 μ l reaction volume and cDNAs were amplified with rTaq polymerase (Takara Bio, Shiga, Japan). For amplification of cDNA, primers of HIF-1 α (forward, 5'-GAAAGCGCAAGTCCTCAAA-3'; backward 5'-CTATATGGTGATGATGTGGCACTA-3'), and β -actin (forward 5'-GTGGGGCGCCCCAGGCACCA-3'; backward 5'-CTCCTTAATGTCACGCACGATTTC-3') (Sigma-Aldrich Japan, Kyoto, Japan) were used. Thermal cycling conditions were as follows: 1 min at 94 °C, and 30 (HIF-1 α) or 25 (β -actin) cycles of 30 s at 94 °C, 30 s at 56 °C (HIF-1 α) or 62 °C (β -actin), and 1 min at 72 °C, with a final extension step of 10 min at 72 °C. The resulting PCR fragments (10 μ l) were electrophoresed on a 1.5% agarose gel.

For quantitative real-time PCR, first-strand cDNA was synthesized from 500 ng of total RNA using random hexamer primers (ExScript RT reagent kit (Perfect Real Time); Takara Bio, Shiga, Japan). Real-time PCR was performed using SYBR Premix Ex Taq (Perfect Real Time) (Takara Bio, Shiga, Japan) and an ABI 7300 real-time instrument with Applied Biosystems Sequence Detection System, Version 1.2. (Applied Biosystems Japan, Tokyo, Japan). The primers of HIF-1 α (forward, 5'-TTTGCTGAAGACACAGAAGCAAAGA-3'; backward 5'-TTGAGGACTTGCGCTTTTCAGG-3'), and β -actin (forward 5'-ATTGCCGACAGGATGCAGA-3'; backward 5'-GAGTACTTGCGCTCAGGAGGAA-3') (Takara Bio, Shiga, Japan) were used. Thermal cycling conditions were as follows: 10 s at 95 °C, 40 cycles of 5 s at 95 °C, and 31 s at 60 °C. The relative expression of HIF-1 α mRNA was normalized by β -actin mRNA. Statistical analysis was performed on three experiments using the paired Student's *t* test. Statistical significance was designated at $p < 0.05$. Values are expressed as the mean $\pm$ S.D.

Flow Cytometry Intracellular ROS generation in Hep3B cells was examined by flow cytometry using 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA), an ROS sensitive dye, and an FACSCalibur flow cytometer (Becton Dickinson, Mountain View, CA, U.S.A.). The data were analyzed using CELLQuest software (Becton Dickinson).

RESULTS AND DISCUSSION

The effect of 6FP on HIF-1 α protein accumulation was studied using Western blot analysis (Fig. 1A). Hep3B cells were pretreated with 6FP at 0.5 to 2 mM for 1 h, and incubated with 100 μ M DFX for 4 h. HIF-1 α protein levels were very low under a normoxic condition, and increased markedly in response to DFX, which is a strong inducer of

Fig. 1. Effect of 6FP on HIF-1 α Accumulation in Hep3B Cells

(A—C) Hep3B cells were pretreated with 0.5 to 2 mM 6FP for 1 h and exposed to 100 μ M DFX under 21% O₂ for 4 h. The cells were then harvested and examined for HIF-1 α protein expression using Western blot analysis (A) and HIF-1 α mRNA expression using RT-PCR (B) and real-time PCR (C). β -Actin was used as an internal control. In (C), the HIF-1 α expression levels are shown relative to those for β -actin. Values are shown as the mean $\pm$ S.D. ($n=3$). No significant changes were observed in HIF-1 α mRNA expression.

Fig. 2. Effect of 6FP on Intracellular ROS Generation in Hep3B Cells and the Counteraction by the Antioxidant *N*-Acetyl-L-cysteine (NAC)

Hep3B cells were pretreated with 1.5 mM 6FP and 20 mM NAC for 1 h, and then the cells were incubated with 2 μ M DCFH-DA for 30 min under a DFX-treated condition. The cells were then harvested and analyzed for intracellular ROS generation by flow cytometry.

HIF-1 α .^{5,6)} The suppressive effect of 6FP on the protein expression of HIF-1 α was dependent on its concentration. At 2 mM 6FP, the accumulation of HIF-1 α protein had nearly disappeared. In contrast, the mRNA expression of HIF-1 α was not affected significantly by 6FP (Figs. 1B, C).

Next, intracellular ROS generation by 6FP in the Hep3B cells was examined using a flow cytometry technique (Fig. 2). Under a DFX-treated condition, an increase in the intensity of DCFH, a fluorescent probe for ROS, was observed, when the Hep3B cells were incubated with 1.5 mM 6FP for 4 h. This increase was reversed by *N*-acetyl-L-cysteine (NAC), which is a well-known antioxidant.

To study the molecular mechanism of the action of 6FP on HIF-1 α protein accumulation, the effect of the concomitant use of 6FP and NAC on HIF-1 α protein expression was eval-

Fig. 3. Reversible Effect of the Antioxidant NAC on HIF-1 α Accumulation Induced by 6FP in Hep3B Cells

(A—C) Hep3B cells were pretreated with 20 mM NAC and 1.5 mM 6FP for 1 h and exposed to 100 μ M DFX under 21% O₂ for 4 h. The cells were then harvested and examined for HIF-1 α protein expression using Western blot analysis (A) and HIF-1 α mRNA expression using RT-PCR (B) and real-time PCR (C). β -Actin was used as an internal control. In (A), from the left, the band densities of HIF-1 α relative to β -actin by densitometry were 1, 74.7, 31.8, and 53, when the intensity of the untreated cells was assigned to 1 (mean value, $n=3$). Significant changes were observed between the samples without NAC (the third lane) and with NAC (the fourth lane) ($p<0.01$). In (C), the HIF-1 α expression levels are shown relative to those for β -actin. Values are shown as the mean $\pm$ S.D. ($n=3$). No significant changes were observed in HIF-1 α mRNA expressions.

uated using Western blot analysis. As shown in Fig. 3A, the pretreatment of Hep3B cells with 1.5 mM 6FP under a DFX-treated condition revealed markedly reduced HIF-1 α expression. This reduction was then reversed by the addition of 20 mM NAC. This result suggests that the diminished level of HIF-1 α was caused by the ROS produced by the 6FP. No significant change in HIF-1 α mRNA expression was observed as shown in Figs. 3B, C.

We investigated the effect of 6FP on HIF-1 α accumulation in Hep3B cells under a hypoxic condition. The suppressive effect of 6FP on the protein expression of HIF-1 α was dependent on its concentration under 1% O₂ as well as in a DFX-treated condition (Fig. 4A). The mRNA expression of HIF-1 α was not affected significantly by 6FP (Fig. 4B). We also observed that the addition of 20 mM NAC abrogated the suppressive effect of 6FP on HIF-1 α protein expression without alteration of mRNA expression under 1% O₂ (Figs. 4C, D).

In this current study, we have revealed that 6FP attenuated the expression of HIF-1 α protein under a DFX-treated or hypoxic condition without the modulation of mRNA expression. This suppressive effect of 6FP on HIF-1 α expression is most likely mediated through the ROS produced by 6FP, based on the results with the antioxidant NAC. What is the mechanism of action of ROS generated by 6FP on the reduc-

Fig. 4. Effect of 6FP on HIF-1 α Accumulation in Hep3B Cells under Hypoxia

(A, B) Hep3B cells were pretreated with 0.5 to 2 mM 6FP for 1 h under 1% O₂ for 4 h. The cells were then harvested and examined for HIF-1 α protein expression using Western blot analysis (A) and HIF-1 α mRNA expression using RT-PCR (B). β -Actin was used as an internal control. (C, D) Reversible effect of the antioxidant NAC on HIF-1 α accumulation induced by 6FP, in Hep3B cells under a hypoxic (1% O₂) condition. Hep3B cells were pretreated with 20 mM NAC and 1.5 mM 6FP for 1 h under 1% O₂ for 4 h. The cells were then harvested and examined for HIF-1 α protein expression using Western blot analysis (C) and HIF-1 α mRNA expression using RT-PCR (D). β -Actin was used as an internal control. In (C), from the left, the band densities of HIF-1 α relative to β -actin by densitometry were 84.5, 36.6, and 47.8, when the intensity of the untreated cells in a normoxic condition was assigned to 1 (mean value, $n=3$). Significant changes were observed between the samples without NAC (the middle lane) and with NAC (the right lane) ($p<0.05$).

tion of HIF-1 α protein expression? Under a hypoxic condition, hydroxylation of the specific proline in HIF-1 α is inhibited due to substrate (O₂) limitation, resulting in HIF-1 α protein stabilization without induction of mRNA.¹⁴ Given the results in this study, ROS generated by 6FP probably hydroxylates Pro402 and/or Pro564 residues in the oxygen-dependent degradation domain of HIF-1 α , leading to the binding of von Hippel–Lindau protein to HIF-1 α and an increase in the degradation of HIF-1 α protein by the ubiquitin-proteasome system. Further study is required to elucidate the mechanism of action of 6FP in HIF-1 α degradation.

REFERENCES

- 1) Zagorska A., Dulak J., *Acta Biochim. Pol.*, **51**, 563–585 (2004).
- 2) Hirota K., Semenza G. L., *Biochem. Biophys. Res. Commun.*, **338**, 610–616 (2005).
- 3) Semenza G. L., *Biochem. Pharmacol.*, **59**, 47–53 (2000).
- 4) Semenza G. L., *Trends Mol. Med.*, **7**, 345–350 (2001).
- 5) Mahon P. C., Hirota K., Semenza G. L., *Genes Dev.*, **15**, 2675–2686 (2001).
- 6) Ho V. T., Bunn H. F., *Biochem. Biophys. Res. Commun.*, **223**, 175–180 (1996).
- 7) Wang G. L., Semenza G. L., *Blood*, **82**, 3610–3615 (1993).
- 8) Watanabe K., Arai T., Mori H., Nakao S., Suzuki T., Tajima K., Makino K., Mori K., *Biochem. Biophys. Res. Commun.*, **233**, 447–450 (1997).
- 9) Lowry O. H., Bessey O. A., Crawford E. J., *J. Biol. Chem.*, **180**, 399–410 (1949).

- 10) Kalckar H. M., Kjeldgaard N. O., Klenow H., *Biochim. Biophys. Acta*, **5**, 586—594 (1950).
- 11) Arai T., Yamada H., Namba T., Mori H., Ishii H., Yamashita K., Sasada M., Makino K., Fukuda K., *Biochem. Pharmacol.*, **67**, 1185—1193 (2004).
- 12) Yamashita K., Arai T., Fukuda K., Mori H., Ishii H., Nishioka M., Tajima K., Makino K., Sasada M., *Biochem. Biophys. Res. Commun.*, **289**, 85—90 (2001).
- 13) Arai T., Endo N., Yamashita K., Sasada M., Mori H., Ishii H., Hirota K., Makino K., Fukuda K., *Free Radic. Biol. Med.*, **30**, 248—259 (2001).
- 14) Semenza G. L., *Cell*, **107**, 1—3 (2001).