Stabilization of Hypoxia-inducible Factor-1α by Prostacyclin under Prolonged Hypoxia via Reducing Reactive Oxygen Species Level in Endothelial Cells

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Hypoxia-inducible factor-1 (HIF-1) takes part in the transcriptional activation of hypoxia-responsive genes. HIF-1α, a subunit of HIF-1, is rapidly degraded under normoxic conditions by the ubiquitin-proteasome system. Hypoxia up-regulates HIF-1α by inhibiting its degradation, thereby allowing it to accumulate to high levels with 3–6 h of hypoxia treatment and decreasing thereafter. In vascular tissues, prostacyclin (prostaglandin I2 (PGI2)) is a potent vasodilator and inhibitor of platelet aggregation and is known as a vasoprotective molecule. However, the role of PGI2 in HIF-1 activation has not been studied. In the present study, we investigated the effect of PGI2 on HIF-1 regulation in human umbilical vein endothelial cells under prolonged hypoxia (12 h). Augmentation of PGI2 via adenovirus-mediated gene transfer of both cyclooxygenase-1 and PGI2 synthase activated HIF-1 by stabilizing HIF-1α in cells under prolonged hypoxia or the hypoxia-normoxia transition but not under normoxia. Exogenous H2O2 abolished PGI2- and catalase-induced HIF-1α up-regulation, which suggests that degradation of HIF-1α under prolonged hypoxia is through a reactive oxygen species-dependent pathway. Moreover, PGI2 attenuated NADPH oxidase activity by suppressing Rac1 and p47phox expression under hypoxia. These data demonstrate a novel function of PGI2 in down-regulating reactive oxygen species production by attenuating NADPH oxidase activity, which stabilizes HIF-1α in human umbilical vein endothelial cells exposed to prolonged hypoxia.

Hypoxia induces a number of cellular responses, such as angiogenesis, erythropoiesis, and glycolysis, through both gene regulation and post-translational modification of proteins. Hypoxia-inducible factor-1 (HIF-1)2 takes part in the transcriptional activation of hypoxia-responsive genes through binding to the hypoxia-responsive element (HRE) in the promoter or enhancer regions and activating a number of genes (1–4). HIF-1 is a heterodimer composed of HIF-1α and HIF-1β. HIF-1α is rapidly degraded under normoxic conditions by the ubiquitin-proteasome system, whereas HIF-1β is constitutively expressed (5). Under hypoxia, HIF-1α has been shown to be up-regulated to high levels at 3–6 h and decrease thereafter (6, 7). A number of studies have focused on the mechanism of HIF-1α stabilization under hypoxia for 4–6 h. Recently, a natural antisense HIF-1α was suggested to down-regulate HIF-1α in A549 cells under prolonged hypoxia (6). However, regulation of HIF-1α under prolonged hypoxia (12 h) in endothelial cells remains largely unknown.

Under normoxic conditions, HIF-1α is regulated through hydroxylation of proline residues by prolyl hydroxylase enzymes (8, 9). The von Hippel-Lindau tumor suppressor protein (pVHL) associates with hydroxylated HIF-1α and targets it for ubiquitination and rapid degradation (10). Under hypoxia, prolyl hydroxylase is inactivated through oxygen-sensing mechanisms, and the unmodified HIF-1α accumulates, permitting dimerization with HIF-1β (3, 11). Various redox-dependent signaling pathways for oxygen sensing and HIF-1α stabilization have been proposed. Under hypoxia, the level of reactive oxygen species (ROS) generated by NADPH oxidase is attenuated because of low pO2, which leads to stabilization of HIF-1α (11, 12). However, contradictory results suggest that the NADPH oxidase inhibitor has differential inhibitory effects on HIF-1α regulation under hypoxia (13, 14). Moreover, overexpression of NADPH oxidase 1 up-regulates HIF-1α (15), whereas stabilization of HIF-1α is not altered in gp91phox knock-out mice (16). These results demonstrate controversial HIF-1α regulatory mechanisms via ROS generated by NADPH oxidase under hypoxia. Another model proposes that increased ROS production in mitochondria results in HIF-1α stabilization under hypoxia (17, 18). However, paradoxical results have been reported in cells lacking mitochondria (19) or a functional mitochondrial respiratory chain (20). Although results are conflicting, they suggest that redox-dependent signaling is involved in the HIF-1 activation pathway. Nevertheless, whether ROS signaling is involved in HIF-1α regulation under prolonged hypoxia remains elusive.

Prostacyclin (prostaglandin I2 (PGI2)) is the major eicosanoid produced in vascular endothelial and smooth muscle cells. PGI2 is a potent vasodilator and inhibitor of platelet aggregation and monocyte attachment and known as a vasoprotective molecule. It is synthesized by a series of enzymatic reactions whereby cytosolic phospholipase A2 cleaves arachidonic acid from phospholipids, cyclooxygenase (COX) converts arachidonic acid to PGH2, and PGI2 synthase (PGIS) catalyzes PGH2 to PGI2 (21, 22). Two COX isoforms (COX-1 and COX-2) have been identified. COX-1 is constitutively expressed, whereas COX-2 is inducible (22, 23) and is the primary source of PGI2 biosynthesis, espe...
**PGI₂ Reduces ROS and Stabilizes HIF-1α**

PGI₂ reduces ROS and stabilizes HIF-1α, which is crucial in the cardiovascular system (24). Previously, we have shown that overexpression of COX-1 and PGIS by adenovirus-mediated gene transfer can selectively increase PGI₂ production in endothelial cells without a concurrent overproduction of other prostanooids (25, 26).

In the present study, we investigated the effect of PGI₂ on HIF-1α regulation in human umbilical vein endothelial cells (HUVECs) under prolonged hypoxia. PGI₂ up-regulated HIF-1α under prolonged hypoxia via down-regulating ROS production. This finding demonstrates a novel function of PGI₂ and sheds light on the regulatory mechanism of HIF-1α under prolonged hypoxia.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—HUVECs in passages 3–5 were isolated from freshly obtained umbilical veins and cultured in 95% air and 5% CO₂ at 37 °C as described previously (25). Hypoxia culture was in a gas-controlled chamber (Forma Scientific) maintained at 1% oxygen, 94% N₂, and 5% CO₂ described previously (25). Hypoxia culture was in a gas-controlled system under the regimen of 40 cycles of 95 °C for 20 s, 52 °C for 10 s, and 72 °C for 40 s followed by 20 cycles of 95 °C for 40 s, 55 °C for 40 s, and 72 °C for 40 s for VEGF. Quantitative real-time PCR was performed in a 10–ml reaction volume by the standard protocols of the Roche Applied Science LightCycler system under the regimen of 40 cycles of 95 °C for 40 s, 52 °C for 10 s, and 72 °C for 10 s for HIF-1α.

The relative gene expression was obtained by ΔC_T assay (ΔC_T = C_T (Target gene) - C_T (actin)). All reactions were performed in triplicates and normalized by reference gene expression.

**ROS Measurement**—Intracellular ROS generation was assessed with use of 2',7'-dichlorofluorescein diacetate (Molecular probes, Eugene, OR). ROS in cells causes oxidation of 2',7'-dichlorofluorescein diacetate, yielding the fluorescent product 2',7'-dichlorofluorescein. The cells were cultured in medium containing 2',7'-dichlorofluorescein diacetate (10 µmol/liter) for 30 min. The medium was then removed, cells were lysed and centrifuged, and the level of 2',7'-dichlorofluorescein in the supernatant was measured with the use of a spectrophotometer at an excitation/emission ratio of 488/525 nm.

**NADPH Oxidase Activity Assay**—Cells were suspended in lysis buffer (phosphate-buffered saline, pH 7.4) containing 1 mmol/liter EDTA, 5 µmol/ml aprotinin, 2 µmol/ml pepstatin, 2 µmol/ml leupeptin, pH 7.0. NADPH (100 µmol/liter) (Sigma) and cytochrome c (40 µmol/liter) (Sigma) were added into 400 µl of phosphate-buffered saline containing samples of 5 mg of protein with or without Cu/Zn superoxide dismutase (SOD) (200 units/ml) (Sigma) and incubated at room temperature for 20 min. The O₂⁻-dependent cytochrome c reduction was calculated from the difference of absorbance at 550 nm between samples incubated with and without SOD (650, reduced – oxidized = 21.1 nmol/liter⁻¹ cm⁻¹).

**Statistical Analysis**—Unpaired Student’s t test was used for comparisons between treatments, and data are expressed as mean ± S.E. A p value < 0.05 was considered significant.

**RESULTS**

**Effect of PGI₂ on HIF-1α Regulation in HUVECs Exposed to Hypoxia**—HUVECs were infected with an adenovirus carrying both COX-1 and PGIS, Ad-COP1, at a multiplicity of infection of 25 for 48 h. Western blot analysis showed both COX-1 and PGIS protein levels highly increased in Ad-COP1-infected cells compared with cells mock-infected with Ad-PGK under both hypoxia and normoxia (Fig. 1, A and B). The level of 6-keto-PGF₁α, a stable hydrolyzed product of PGI₂, in Ad-COP1-infected cells was augmented to ~10× that in mock-infected and control cells (Fig. 1C). To examine the effect of PGI₂ on HIF-1α regulation, HUVECs were infected with Ad-PGK or Ad-COP1 and exposed to hypoxia for 3–15 h. HIF-1α was up-regulated in cells exposed to hypoxia for 3–9 h but not in cells exposed to normoxia (Fig. 2A). In
control cells, HIF-1α levels were down-regulated to a very low level at 12 and 15 h. Surprisingly, HIF-1α was significantly up-regulated in cells infected with Ad-COP1 under hypoxia for 12 and 15 h (Fig. 2, A and B). Up-regulation of HIF-1α was also detected in cells treated with iloprost, a stable analogue of PGI2 (Fig. 2B), whereas exogenous PGE2 did not alter HIF-1α expression (Fig. 2C).

HIF-1 activity was measured by HRE reporter assay in HUVECs transfected with pHRE-Luc and infected with Ad-PGK or Ad-COP1. Ad-COP1 infection did not up-regulate HIF-1 activity in HUVECs under normoxia (Fig. 3A). However, the activity was increased in control cells exposed to hypoxia for 12 h and further up-regulated in cells infected with Ad-COP1. Because HIF-1 is the major mediator of VEGF up-regulation, augmented transactivation activity of HIF-1 was also supported by RT-PCR results showing the VEGF mRNA level up-regulated in Ad-COP1-infected cells as compared with Ad-PGK-infected cells (Fig. 3B). These data imply that augmentation of PGI2 up-regulates HIF-1 activity in HUVECs under prolonged hypoxia. To investigate whether HIF-1α up-regulation was through gene transactivation or protein stabilization, real-time PCR revealed that HIF-1α mRNA levels did not significantly differ between cells infected with Ad-PGK and those with Ad-COP1 (Fig. 3C). Thus, up-regulation of HIF-1α by PGI2 is through protein stabilization but not DNA transactivation.
PGI₂ Stabilizes HIF-1α in HUVECs Treated with Hypoxia and Then Normoxia—Because HIF-1α is rapidly degraded through a proteosome pathway in cells exposed to hypoxia and then normoxia (5), HUVECs were incubated under hypoxia for 6 h and then exposed to normoxia for 15–120 min to investigate whether PGI₂ stabilizes HIF-1α in such cells. Western blot analysis showed that HIF-1α in Ad-PGK-infected cells was degraded to a very low level in cells exposed to hypoxia and then normoxia for 15 and 30 min (Fig. 4A). However, a significant amount of HIF-1α was detected in cells infected with Ad-COP1 exposed to hypoxia and then normoxia for up to 60 min, markedly reduced at 90 min, and down-regulated to a very low level at 120 min (Fig. 4B). A similar effect was identified in Ad-COP1-infected cells treated with cyclohexamine, a translational inhibitor, that a high level of HIF-1α was detected at 45 min, gradually decreased at 60 and 90 min, and down-regulated to a very low level at 120 min (Fig. 4C). These results suggest that overexpression of both COX-1 and PGIS prolongs HIF-1α stability in the hypoxia-normoxia transition.

Interaction of pVHL with HIF-1α was investigated by co-immunoprecipitation of HIF-1α and pVHL. Ad-COP1 infection abolished the interaction of pVHL with HIF-1α in cells exposed to prolonged hypoxia and in control cells exposed to hypoxia for 6 h, whereas high levels of pVHL protein co-immunoprecipitated with HIF-1α in control and Ad-PGK-infected cells (Fig. 4D). Because pVHL mediated HIF-1α degradation, this result suggests that Ad-COP1 infection abolishes pVHL interaction with HIF-1α and, hence, inhibits its degradation.

Stabilization of HIF-1α by PGI₂ is ROS-dependent—To determine whether stabilization of HIF-1α is related to the ROS signal, we found...
that exogenous H$_2$O$_2$ abolished the PGI$_2$ effect on HIF-1$\alpha$ up-regulation (Fig. 5A), whereas HIF-1$\alpha$ was up-regulated in cells infected with Ad-catalase (Fig. 5B). Moreover, HIF-1$\alpha$ levels were not altered by H$_2$O$_2$ or catalase overexpression in cells under normoxia. Thus, under prolonged hypoxia, HIF-1$\alpha$ degradation is signaled by ROS and can be stabilized by reduction of endogenous H$_2$O$_2$ by catalase. To examine whether PGI$_2$ attenuates ROS production, as implied above, HUVECs infected with Ad-PGK or Ad-COP1 were exposed to hypoxia and their ROS levels measured. As shown in Fig. 5C, ROS levels were increased in Ad-PGK-infected cells exposed to hypoxia for 6 h, further elevated in control cells under hypoxia for 12 h, and markedly attenuated in Ad-COP1-infected and iloprost-treated cells, whereas Ad-COX-1 infection did not alter the ROS level. These results suggest an antioxidant effect of PGI$_2$ under hypoxia but not normoxia. To examine whether the antioxidant effect of PGI$_2$ was through up-regulation of some inhibitor, up-regulated HIF-1$\alpha$ in the hypoxia-normoxia transition (Figs. 4, B and C; and 6B), which confirms that HIF-1$\alpha$ down-regulation is through a proteosome degradation pathway.

**PGI$_2$ Attenuated NADPH Oxidase Activity**—Because NADPH oxidase is one of the major sources of ROS in HUVECs, we examined whether augmentation of PGI$_2$ suppressed NADPH oxidase activity in HUVECs under prolonged hypoxia. As shown in Fig. 7A, Ad-COP1 infection reduced NADPH oxidase activity in HUVECs as compared with cells infected with the control virus under normoxic conditions. Surprisingly, under prolonged hypoxia, NADPH oxidase activity was markedly attenuated to a very low level in cells infected with Ad-COP1, which supports the antioxidant effect of PGI$_2$ shown in Figs. 5C and 6A. For an unknown reason, NADPH oxidase activity was reduced in cells infected with the control virus under prolonged hypoxia. Moreover, HIF-1$\alpha$ expression was not affected in cells treated with exogenous diphenylene iodonium, an NADPH oxidase inhibitor, and exposed to normoxia or hypoxia for 6 h (Fig. 7B). However, under prolonged hypoxia, HIF-1$\alpha$ was significantly up-regulated in such cells and in those infected with Ad-COP1 (Fig. 7B). Thus, inhibition of NADPH oxidase activity up-regulates HIF-1$\alpha$ in HUVECs under prolonged hypoxia.

To investigate the molecular mechanism of NADPH oxidase attenuation by PGI$_2$, protein levels of NADPH oxidase subunits were analyzed by Western blotting in cells exposed to prolonged hypoxia. p67phox was...
PGI₂ up-regulates under hypoxia, and infection with Ad-COP1 significantly suppressed the up-regulation (Fig. 8A). The p67phox level in Ad-COP1-infected cells was similar to that in cells under normoxia, which implies that regulation of p67phox does not contribute to marked attenuation of NADPH oxidase activity (Fig. 7A). In addition, p47phox and Rac1 were markedly down-regulated in cells infected with Ad-COP1 as compared with those infected with Ad-PGK (Fig. 8, B and C). These data suggest that PGI₂ attenuates NADPH oxidase activity by down-regulating Rac1 and p47phox in HUVECs infected with Ad-COP1 under prolonged hypoxia.

**DISCUSSION**

In the cardiovascular system, the up-regulation of HIF-1 activates VEGF and many other genes, which may protect endothelial cells or surrounding organs by promoting angiogenesis, anti-apoptosis (29), and cell proliferation (30). In this study, we identified a novel function of PGI₂ in down-regulating ROS production and activating HIF-1 by stabilizing HIF-1α under prolonged hypoxia in HUVECs. Fig. 9 delineates the model of PGI₂ function and is supported by the major new findings of this study. 1) Overexpression of COX-1 and PGIS via adenovirus-mediated gene transfer augments PGI₂ production, which stabilizes HIF-1α in cells under prolonged hypoxia and hypoxia followed by normoxia. 2) Stabilization of HIF-1α by PGI₂ or Ad-catalase infection is mediated through reduction of ROS production under prolonged hypoxia. 3) PGI₂ attenuates NADPH oxidase activity by suppressing Rac1 and p47phox expression under prolonged hypoxia. Although antisense HIF-1α has been suggested to down-regulate HIF-1α in A549 cells exposed to prolonged hypoxia (6), our data suggest a different regulatory mechanism, because the mRNA level of HIF-1α was not altered (Fig. 3C).

Up-regulation of ROS by hypoxia or the hypoxia-normoxia transition was associated with HIF-1α degradation, which was attenuated by PGI₂ production, catalase overexpression, or exogenous diphenylene iodonium. These results indicate that up-regulation of ROS mediates HIF-1α degradation in such conditions, which explains why exogenous H₂O₂ pretreatment suppressed HIF-1α stabilization in cells exposed to hypoxia for 8 h (11). Although NADPH oxidase activity was not up-regulated under hypoxia, increased ROS levels might be generated by the complex III of mitochondria (17). In addition, diphenylene iodonium has no effect on HIF-1α expression in cells under normoxia or 6-h hypoxia (Fig. 7B), which implies that HIF-1α up-regulation in HUVECs exposed to hypoxia for 6 h is not mediated through suppression of NADPH oxidase activity. Conversely, up-regulation of HIF-1α by accumulated H₂O₂ has been reported in immortalized JunD⁻/⁻ cells via suppression of PGIS and other antioxidant gene expression, as well as transactivation of NADPH oxidase 4 (18). Moreover, HIF-1α stabilization by Ad-COP1 infection was not detected in human lung cancer (H1299) and hepatoma (HepG2) cells under prolonged hypoxia (data not shown).
PGF1α cytokines by attenuating NADPH oxidase activity through down-regulation of Rac1. Intriguingly, for unclear reasons, NADPH oxidase activity was greatly diminished by PGI2 in cells exposed to prolonged hypoxia but only partially reduced under normoxia. Moreover, both Ad-COP1 infection and iloprost treatment up-regulated HIF-1α but not PGE2, which suggests that stabilization of HIF-1α under hypoxia is through PGI2 but not PGE2. Although PGE2 has been suggested to induce VEGF expression via stabilization of HIF-1α under normoxic conditions in human prostate tumor and colon carcinoma cells (41, 42), PGI2 and PGE2 did not up-regulate HIF-1α under normoxia in HUVECs in our study. Because iloprost alone attenuated ROS production but Ad-COX1 did not alter the ROS level (Fig. 5C), the antioxidant effect in cells infected with Ad-COP1 is mediated through PGI2 but not the increased expression of COX-1.

In conclusion, as illustrated in Fig. 9, our results demonstrate a novel function of PGI2 in attenuating NADPH oxidase activity, which results in ROS reduction and activation of HIF-1 by stabilizing HIF-1α in HUVECs exposed to prolonged hypoxia or the hypoxia-normoxia transition but not normoxia. PGI2 inhibits NADPH oxidase activity by suppressing the protein expression of Rac1 and p47phox. Under prolonged hypoxia, HIF-1α degradation is signaled by H2O2, in that reduction of H2O2 stabilizes HIF-1α. These results reveal that PGI2 not only protects the cardiovascular system against thrombosis and atherosclerosis but also attenuates ROS production and extends HIF-1 activation under prolonged hypoxia.

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