NADPH-Cytochrome P-450 Reductase in the Plasma Membrane Modulates the Activation of Hypoxia-inducible Factor 1*

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Hypoxia induces a group of physiologically important genes that include erythropoietin (EPO) and vascular endothelial growth factor (VEGF). Hypoxia-inducible factor 1 (HIF-1) was identified as a hypoxia-activated transcription factor; however, the molecular mechanisms that underlie hypoxia signal transduction in mammalian cells remain undefined. In this study, we found that a flavoprotein, NADPH-P450 reductase (NPR), could regulate the induction of EPO mRNA under hypoxic conditions. Hypoxic EPO mRNA induction in Hep3B cells was inhibited by diphenylethyleniodionium chloride, which is an inhibitor of NADPH-dependent enzymes. NPR antisense cDNA was transfected into Hep3B cells, and NPR-deficient hepatocyte cells (NPR− cells) were established. NPR− cells lacked EPO induction under hypoxia, and HIF-1α in NPR− cells did not respond to either transcriptional activation or translocation to the nucleus based on electrophoretic mobility shift assays and reporter gene assay including hypoxia response element. In contrast, NPR overexpression in Hep3B cells enhanced the DNA binding activity of HIF-1α by luciferase reporter gene assay. A study with HeLa S3 cells produced the same results. Furthermore, anti-NPR IgG inhibited EPO induction. EPO induction inhibited by diphenylethyleniodionium chloride was recovered by bovine serum albumin-NADPH (a covalent binding complex of bovine serum albumin and NADPH) as well as NADPH. These results suggested that NPR located at the plasma membrane regulates EPO expression in hypoxia, including HIF-1 activation and translocation. We further studied the expression of NPR and VEGF mRNAs in human tumor tissues and found that the NPR mRNA levels were correlated with the VEGF mRNA levels, suggesting that NPR might be an important factor in the hypoxic induction of genes such as VEGF in vivo.

Decreased cellular oxygen tension, that is hypoxia, occurs under physiological conditions such as high altitudes and physiological exercise and under pathological conditions including ischemia, inflammation, and neoplast. The cells adapt to hypoxia mainly via propagating energy metabolism, ventilation of blood flow, and erythropoiesis. Hypoxia stimulates a group of physiologically important genes such as erythropoietin (EPO)1

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¶ The abbreviations used are: EPO, erythropoietin; HIF, hypoxia-inducible factor; VEGF, vascular endothelial growth factor; NPR, and vascular endothelial growth factor (VEGF). Transcriptional activation of the EPO gene is controlled via an enhancer element located in the 3′-flanking region of the gene, hypoxia response element (HRE), and requires binding of a specific transcription factor termed hypoxia-inducible factor 1 (HIF-1) (1). Identification and cloning of HIF-1 revealed a heterodimeric protein consisting of two subunits: HIF-1α and HIF-1β (2). HIF-1α protein undergoes rapid degradation by proteasomes under normoxic conditions (3, 4). Targeting for the protein degradation is determined by binding to the von Hippel-Lindau protein (VHL) to form a ubiquitin-ligating complex (5). In hypoxia, degradation of HIF-1α is blocked, and then HIF-1α is transferred into the nucleus to bind to HRE.

The mechanisms of sensing low oxygen and transduction of this signal to HIF-1 are not well understood. Recently, researchers have hypothesized that the mechanisms involve the generation of reactive oxygen species by NADPH-oxidase. Cytochrome b568 is the redox core of the NADPH-oxidase complex in phagocytes (6) and B lymphocytes (7, 8) and is a membrane-bound protein consisting of two subunits, p22phox and gp91phox (9). Although the presence of cytochrome b568 was previously believed to be restricted to these cell types, its presence has also been demonstrated in other cells, such as rat carotid body cells (10) and HepG2 cells (11, 12). In the carotid body, reactive oxygen species produced by NADPH-oxidase opens a K+ channel whose signal is transferred to HIF-1. In support of this model, exogenous H2O2 and diphenylethyleniodionium chloride (DPI), an inhibitor of NADPH-dependent enzymes, are found to inhibit hypoxic stabilization of HIF-1α (13, 14). However, Archer et al. (15) showed that NADPH-oxidase is not an oxygen sensor by using knockout mice lacking the gp91phox gene. They found that the K+ channel of the knockout mouse was opened by hypoxia, although DPI inhibited it efficiently.

A different model based on the role of the mitochondrial electron transport chain has been suggested as a mechanism of hypoxic response. Inhibition of the mitochondrial respiratory chain blocks HIF-1 DNA binding activity by electrophoretic mobility shift assays (EMSA) and also blocks the induction of EPO, VEGF, aldolase, and phosphoglycerate kinase mRNA, which are HIF-1 target genes in Hep3B cells under hypoxic conditions (16). Whereas, Srinivas et al. (17) found that mitochondrial DNA-less (ρ−) cells that lack a respiratory chain protein have a normal response to hypoxia.

Cytochrome P-450 (P450) monoxygenase has been proposed as a microvascular oxygen-sensing protein. P450 enzymes produce a series of vasoactive metabolites from arachidonic acid

NADPH-P450 reductase; DPI, diphenylethyleniodionium chloride; NPR− cell, NPR-deficient hepatocyte cell; EMSA, electrophoretic mobility shift assay; HRE, hypoxia response element; P450, cytochrome P-450; pcDNA-nNPR, anti-sense NPR mRNA expression plasmid; pcDNA-sNPR, NPR expression plasmid; RT, reverse transcriptase; EDTA, ethylenediamine-N,N,N′,N′-tetraacetic acid; BSA, bovine serum albumin.
(18, 19). Harder et al. (20) demonstrated that the production of 20-hydroxyeicosatetraenoic acid by P450 in rat vascular tissue is directly dependent on the concentration of oxygen. P450 enzymes require molecular oxygen for their activity, and the majority of them require only very low PO2 levels for normal activity. The unique characteristic of the extraplatik P450 enzymes responsible for 20-hydroxyeicosatetraenoic acid formation is that a high level of PO2 is required for the catalytic activity of these enzymes. Furthermore, CO, which efficiently binds to heme, inhibits chloramphenicol acetyltransferase activity of these enzymes. Furthermore, CO, which efficiently binds to heme, inhibits chloramphenicol acetyltransferase activity of these enzymes. Furthermore, CO, which efficiently binds to heme, inhibits chloramphenicol acetyltransferase activity of these enzymes. Furthermore, CO, which efficiently binds to heme, inhibits chloramphenicol acetyltransferase activity of these enzymes.

**EXPERIMENTAL PROCEDURES**

**Materials**—Dulbecco’s modified Eagle’s medium, fetal calf serum, and DPIC were purchased from Sigma. LipofectAMINE PLUS reagent, antibiotic G418, pBluescript II, and pcDNA 3.1 were purchased from Invitrogen. DH5α-competent cells were purchased from Toyobo (Osaka, Japan). pGL3-SV40 and pRL-TK vector were purchased from Promega (Madison, WI). [γ-32P]ATP was obtained from PerkinElmer Life Sciences. Isogen and restriction enzymes were purchased from Nippon Gene (Toyama, Japan). RNA PCR Kit (AMV) version 2.1 and Pyrobest DNA polymerase were purchased from Takara (Shiga, Japan). Nitrilecelllose membrane, horseradish peroxidase conjugated to goat anti-rabbit IgG, and 4-chloro-1-naphthol were purchased from Bio-Rad. Proteinase inhibitor (Complete, Mini: inhibition of serine and cysteine proteases) was purchased from Roche Molecular Biochemicals.

**Isolation of cDNA and Construction of Plasmid**—The entire coding region of human NPR was isolated from a human liver cDNA library (CLONTECH, Palo Alto, CA) by PCR. PCR was performed with Pyrobest DNA polymerase as follows: 35 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 1.5 min. The PCR was performed under hypoxia for normal activity. The unique characteristic of the extraplatik P450 enzymes responsible for 20-hydroxyeicosatetraenoic acid formation is that a high level of PO2 is required for the catalytic activity of these enzymes. Furthermore, CO, which efficiently binds to heme, inhibits chloramphenicol acetyltransferase activity including HRE element in rat aortic smooth muscle cells (21) or HIF-1 binding activity by EMSA into Hep3B cells (22). There is no definitive evidence that the P450 system contributes to gene regulation under hypoxic conditions.

P450s require NADPH-P450 reductase (NPR) for electron transfer as well as molecular oxygen. NPR is known as a flavoprotein and requires NADPH. In this study, we observed that DPIC inhibited hypoxic EPO induction in Hep3B cells that did not express gp91phox mRNA. This result suggested that DPIC did not inhibit gp91phox (a component of NADPH-oxidase) in the cells, suggesting the presence of another flavoprotein that regulates hypoxic response of the cells. We found that NPR plays an important role in the regulation of HIF-1 activation, stabilization, and HRE binding under hypoxic conditions. We also propose that NPR is present in the plasma membrane, although it was previously believed to exist only in the endoplasmic reticulum. We further studied the correlation of NPR expression with VEGF and HIF-1 expression in human tumor tissues.

**Preparation of Antibodies**—Human NPR expressed in E. coli was a generous gift of Dr. S. Asahi (Department of Biology, Graduate School of Science, Osaka University). Human NPR was purified as described previously (25). Human HIF-1α, which has a His tag, was expressed in E. coli as DH5α cells according to the manufacturer’s instructions.
CoCl\(_2\) was added. The ratio (EPO mRNA/0.25 M imidazole. Purified HIF-1 with a nickel-nitrilotriacetic acid-Sepharose gel (Qiagen) according to the manufacturer’s instructions. The absorbed protein was eluted with 0.25 \(\text{M}\) imidazole. Purified HIF-1 with His tag in its N terminus was purified by affinity chromatography.

1% O\(_2\), 5% CO\(_2\) and nitrogen-balanced with a modulator incubator chamber (Napco 7101, Winchester, VA).

**RNA Isolation and Reverse Transcriptase-PCR**—Total RNA was extracted from both Hep3B cells and HeLa S3 cells with Isogen and transferred to cDNA using a RNA PCR kit according to the manufacturer’s protocol. PCR containing 10 pmol of each primer, 1.5 units of AmpliTaq, and 100 ng of cDNA was performed with the following cycles: 10 min at 94°C and then 1 min at 94°C, 1 min at 56°C, and 2.5 min at 72°C for 35 cycles. The PCR primers of p22\(^{phox}\) (antisense), and the PCR primers of p91\(^{phox}\) (antisense), and the PCR primers of VEGF (30) were 5’-GGCTTCTTGCGGGC-3’ (antisense), and the PCR primers of p91\(^{phox}\) (antisense), and the PCR primers of VEGF (30) were 5’-GGATTTCAAGATGCCTGGGAAACTA-3’ (sense) and 5’-ACGTA-CAATTGTCGGGACTC-3’ (antisense). The PCR primers of NPR (23) were 5’-GGCGGAGCTGTACCTGAGCTG-3’ (sense) and 5’-GCCAGACT-TTCTTCAG-3’ (antisense), and the PCR primers of p91\(^{phox}\) (antisense), and the PCR primers of VEGF (30) were 5’-TTCCAGTCTCATCCTATCC-3’ (antisense), and the PCR primers of EPO (29) were 5’-TACTGGTACGATGATCC-3’ (antisense), and the PCR primers of VEGF (30) were 5’-TACTGGTACGATGATCC-3’ (antisense), and the PCR primers of VEGF (30) were 5’-TACTGGTACGATGATCC-3’ (antisense), and the PCR primers of VEGF (30) were 5’-ACGTA-CAATTGTCGGGACTC-3’ (antisense). The oligonucleotide sequences of PCR reaction products were confirmed by sequencing.

**Electrophoretic Mobility Shift Assay**—The Hep3B cells were washed with phosphate-buffered saline and collected by centrifugation in ice-cold phosphate-buffered saline. The nuclear extracts for the cells were prepared by the method of Wang et al. (2). The oligonucleotides for

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**FIG. 3. Effect of NPR expression on EPO induction.** A, Western blot analysis from whole cell lysates of Hep3B cells lipofected with empty vector (Control cell) or pcDNA-aNPR (NPR\(^{-}\) cell). We cloned Hep3B cells lipofected with empty vector or pcDNA-aNPR. Purified human NPR was used as a standard. B, induction of EPO in control or NPR\(^{-}\) cells. The cells were incubated for 3 h in hypoxia or 6 h after CoCl\(_2\) was added. The ratio (EPO mRNA/\(\beta\)-actin mRNA) under hypoxia was set at 100%. The values are given as the means \(\pm\) S.D. of six separate experiments. Std., standard.

**FIG. 4. Effect of DPIC on HIF-1\(\alpha\) stabilization and translocation to the nucleus.** A, Western blot analysis with an anti-HIF-1\(\alpha\) antibody. The cells were incubated for 3 h under hypoxia in the presence or absence of DPIC (100 \(\mu\)M). B, HIF-1\(\alpha\) binding activity to HRE in the presence of DPIC. Hep3B cells were exposed to normoxia or hypoxia (1%) in the presence of DPIC. The nuclear extracts were prepared from Hep3B cells and analyzed by electrophoretic mobility shift assay.
Western Blot Analysis—Cells at 80% confluence were lysed in 50 mM Tris-HCl buffer (pH 7.5) containing 0.1% Triton X-100, 100 mM NaCl, 5 mM EDTA, and protease inhibitor. Immunoblotting with antibodies against HIF-1α and NPR was done by a method described previously (26). Each protein was visualized using horseradish peroxidase conjugated to goat anti-rabbit IgG and by 4-chloro-1-naphthol or ECL (Amersham Biosciences).

Preparation of Neutrophils—Human neutrophils were isolated from the peripheral blood of healthy adult volunteers by sedimentation through mono-poly-resolving medium (Dainippon Pharmaceutical Company, Osaka, Japan).

RESULTS

Involvement of NADPH-dependent Enzymes in EPO Induction—We examined the effect of DPIC upon the induction of the EPO gene by hypoxia in human hepatoma cells (Hep3B cells). DPIC is an inhibitor of NADPH-dependent enzymes such as NADPH-oxidase and NPR. The Hep3B cells were incubated under hypoxia (1% oxygen) for 3 h in the presence of DPIC (100 μM). Exposure to DPIC markedly reduced the hypoxic induction of EPO (Fig. 1). The presence of NADPH (1 mM) together with DPIC recovered the induction of EPO mRNA in hypoxia, but the presence of NADH did not. These findings suggest that the NADPH-dependent enzyme NADPH-oxidase, a candidate oxygen sensor, plays a role in the hypoxic EPO mRNA induction. For that reason, we investigated the expression of

![Graph](image-url)

**Fig. 5.** Dual luciferase reporter assay in Hep3B cells. A, effect of DPIC on the luciferase activity. B, effect of NPR antisense cDNA and NPR sense cDNA on the luciferase activity. Hep3B cells were co-transfected with a reporter plasmid (pGL3-SV40HRE vector) containing the HIF-1 binding domain of the human EPO gene (HRE) and a control plasmid (pRL-TK vector), containing a basal T4 promoter. At 48 h post-transfection, DPIC was added to the transfected Hep3B cells, and the cells were incubated for 2 h under hypoxia or normoxia (A). Either pcDNA-aNPR or pcDNA-sNPR (B) was transfected together with pGL3-SV40HRE and pRL-TK vector, and the cells were incubated for 2 h under hypoxia or normoxia. The amount of luciferase activity was quantitated with the cell lysates. The ratio (reporter/control luciferase activity) obtained from the cell lysates under hypoxia was set at 100%. Luciferase activity is given as the mean ± S.D. of six separate experiments.

![Graph](image-url)

**Fig. 6.** Dual luciferase reporter assay in HeLa S3 cells. A, RT-PCR using RNA from HeLa S3 cells with specific primers for gp91phox. B, effect of NPR antisense cDNA and NPR sense cDNA on the luciferase activity. HeLa S3 cells were co-transfected with a reporter plasmid (pGL3-SV40HRE vector) containing the HIF-1 binding domain of the human EPO gene (HRE) and a control plasmid (pRL-TK vector), containing a basal T4 promoter. Either pcDNA-aNPR or pcDNA-sNPR was transfected together with pGL3-SV40HRE and pRL-TK vector, and the cells were incubated for 2 h under hypoxia or normoxia. The amount of luciferase activity was quantitated with the cell lysates. The ratio (reporter/control luciferase activity) obtained from the cell lysates under hypoxia was set at 100%. Luciferase activity is given as the mean ± S.D. of six separate experiments.
NADPH-oxidase component proteins, p22phox and gp91phox, using reverse transcriptase (RT)-PCR (Fig. 2A). However, the two proteins were not detected in Hep3B cells. On the contrary, NPR, which is also an NADPH-dependent enzyme, was detected by RT-PCR. NPR protein was abundantly present in Hep3B cells (Fig. 2B).

Study of NPR Function Using NPR− Cells—We examined whether NPR regulates EPO mRNA induction by hypoxia. NPR− cells were prepared by transfection of pcDNA-nNPR plasmid. The cell lysates were subjected to immunoblotting with anti-human NPR antibody (Fig. 3A) or to cytochrome c enzyme assay. The NPR in NPR− cells was much less than that in control cells transfected with vector only. The NPR activity assayed by cytochrome c reduction in NPR− cells was also extensively reduced (data not shown). Hypoxic EPO mRNA induction using NPR− cells was investigated (Fig. 3B). The expression of EPO mRNA was suppressed by NPR knockdown, whereas it was induced by hypoxia in control cells. NPR− cells were next treated with CoCl2, which mimics hypoxia (32). The induction of EPO mRNA by CoCl2 in NPR− cells occurred to the same degree as in control cells, indicating that these cells maintain the capacity of EPO induction. This study suggests that NPR is an important factor in hypoxic induction of EPO mRNA.

Regulation of HIF-1α Stabilization and HRE Binding by NPR—Next, we investigated whether NPR regulates the EPO expression at the transcriptional level in Hep3B cells. Hypoxia stabilized HIF-1α and increased the HIF-1α protein level, but the level was dramatically decreased in the presence of DPIC (Fig. 4A), indicating that DPIC inhibited HIF-1α stabilization (the HIF-1α mRNA level was not changed). We examined whether NPR influences the activation or stabilization of HIF-1 and the interaction of HIF-1 with HRE of the EPO gene (Fig. 4B). Hep3B cells were incubated under normoxia or hypoxia for 3 h, in the presence or absence of DPIC. The nuclear protein extracts were prepared from the cells, and the HIF-1 binding activity was examined with EMSA. The HRE oligonucleotide probe produced one major DNA-protein complex under hypoxia, and the protein complex disappeared when DPIC was added to the cells. We also investigated the binding of HIF-1 with HRE with a reporter gene assay including the plasmid constituted with HRE and the luciferase gene (Fig. 5). Under hypoxic conditions, the cells lipofected by the reporter gene and empty vector to Hep3B cells (Fig. 5B). The induction of luciferase activity was also...
inhibited by the reduction in NPR. In contrast, NPR overexpression increased the luciferase activity under hypoxic conditions, although there was no effect under normoxic conditions. These results indicate that the expression levels of NPR in Hep3B cells are important for hypoxic response. We further studied the hypoxic response in another cell line, HeLa S3, which expresses NADPH-oxidase (Fig. 6A), although Hep3B did not express NADPH-oxidase. Either pcDNA-aNPR or pcDNA-sNPR was transfected into HeLa S3 cells, and the hypoxic response was investigated (Fig. 6B). As in Hep3B cells, the induction of luciferase activity in hypoxia was inhibited by the reduction in NPR and was increased by the overexpression of NPR. These results indicate that NPR is important for the activation of HIF-1 and the interaction of HIF-1 with HRE.

**Elucidation of NPR Localization**—To elucidate the function of NPR, NPR IgG was added to Hep3B cells under hypoxic conditions (Fig. 7A). DNA-protein complex under hypoxia by EMSA was reduced by anti-NPR IgG, although control IgG had no effect. We next expressed transient fusion luciferase proteins containing the HRE and added anti-NPR IgG to the cells (Fig. 7B). The addition of control IgG did not affect the luciferase activity induced by hypoxia, whereas anti-NRP IgG reduced it by 50% in comparison. Because the IgG protein cannot pass through the plasma membrane, NPR IgG may bind with NPR located on the surface of the plasma membrane. This antibody against NPR did not cross-react with gp91phox, which was expressed abundantly in human neutrophils, indicating that the specificity of an anti-NPR antibody is high (Fig. 7, C and D). We have supplied evidence that BSA-NADPH (a covalent complex of NADPH and BSA) recovered the EPO induction inhibited by DPIC as well as NADPH (Fig. 8). We concluded that NADPH acted on the plasma membrane, because neither BSA-NADPH nor NPR IgG can pass through the membrane.

**DISCUSSION**

NPR is a major NADPH-dependent enzyme transferring electrons to microsomal P450 and heme oxygenase (33). NPR is distinct from many other flavoproteins in that it contains one molecule each of FAD and FMN per polypeptide chain. NPR reduces NADPH in FMN. We have shown here a new function of NPR, as a regulator of HIF-1 activation and gene induction by hypoxia.

Despite various studies, thus far the molecular mechanisms responsible for oxygen sensing and downstream pathways utilized by the hypoxic signal are still poorly understood. Recent studies of oxygen sensing have explored the role of oxygen as an electron acceptor in a variety of nonmitochondrial redox systems involving electron transport and have focused on an NADPH-oxidase from phagocytes that catalyzes the production of hydrogen peroxide (12). We utilized hypoxic EPO induction to test the hypothesis that NADPH-oxidase is an oxygen sensor. Although we observed that DPIC as an NADPH-oxidase inhibitor inhibited EPO induction in Hep3B cells by hypoxia, NADPH-oxidase mRNA was not detected in Hep3B cells by RT-PCR. These results indicate that DPIC is probably not an inhibitor of NADPH-oxidase in Hep3B cells. Our data provide evidence that NPR is the target of DPIC.

We cloned cells that had low NPR expression (NPR− cells) to elucidate whether the target of DPIC is NPR. The EPO gene was not induced in the NPR− cells under hypoxic conditions, whereas the hypoxic EPO induction in the NPR+ cells was normally induced by cobalt, which is known to induce EPO mRNA, as happens with hypoxia (32). These findings were consistent with our results, in which DPIC had little or no effect on EPO induction by cobalt. Therefore, we studied whether NPR influences either transportation of HIF-1 to the nucleus or its HRE binding. As expected, binding of HIF-1 to HRE of the EPO gene examined by EMSA and reporter gene assay was extensively reduced in Hep3B cells lipofected with pcDNA-aNPR. The same experiments with HeLa cells that expressed NADPH-oxidase produced the same results. These results suggest that NPR but not the HIF-1/HRE complex is important for the activation of HIF-1 under hypoxic conditions in general.

Moreover, we found novel evidence that NPR is located on the plasma membrane. This was demonstrated by both the anti-NPR antibody, which did not cross-react with NADPH-oxidase, and BSA-NADPH. Thus far it has been understood that NPR and P450 are located in the endoplasmic reticulum; however, in this study, anti-NPR IgG also inhibited the binding of HIF-1 to HRE of the EPO gene. An important function of
NPR in the plasma membrane is the regulation of EPO induction and HIF-1 activation.

The activation of HIF-1 by hypoxia appears to be complex and to involve changes in protein stability, nuclear localization, DNA binding capability, and transcriptional activation function (34, 35). In the presence of oxygen, HIF-1 is targeted for inhibition of EPO induction by CO cannot be explained by this consistent with tissues that had a high expression of NPR. These findings are relevant for HIF-1 inhibition and HIF-1 activation.

We have presented evidence for the relation of NPR to HIF-1/2. Wang, G. L., Jiang, B.-H., Rue, E. A., and Semenza, G. L. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 275–280.

In conclusion, NPR regulates gene expression under hypoxic conditions by modulating the HIF-1α activation and its HRE binding. We also found that NPR was present in the plasma membrane and had the function of regulating gene expression. We have presented evidence for the relation of NPR to HIF-1α expression in human bladder tissues and have suggested that NPR is an important factor in hypoxic expression of genes such as VEGF in vivo.

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REFERENCES
1. Semenza, G. L., and Wang, G. L. (1992) Mol. Cell. Biol. 12, 5447–5454
2. Wang, G. L., Jiang, B.-H., Rue, E. A., and Semenza, G. L. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 5510–5514
3. Huang, L. E., Gu, J., Schau, M., and Bunn, H. P. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 7987–7992
4. Saldana, S., and Caro, J. (1997) J. Biol. Chem. 272, 23642–23647
5. Maxwell, P. H., Wiesener, M. S., Chang, G. W., Clifford, S. C., Vaux, E. C., Cockman, M. E., Wykoff, C. C., Pugh, C. W., Maher, E. R., and Ratcliffe, P. J. (1999) Nature 399, 271–275
6. Babier, B. M. (1992) Adv. Enzymol. Relat. Areas Mol. Biol. 65, 49–95
7. Maly, F. E., Cross, A. R., Jones, O. T., Wolf-Verbeck, G., Walker, C., Dahinden, C. A., and De Weck, A. L. (1988) J. Immunol. 140, 2334–2339
8. Vonman, D. J., Buescher, E. S., Gallin, J. I., and Fauci, A. S. (1984) J. Immunol. 133, 3006–3009
9. Parkos, C. A., Allen, R. A., Cochrane, C. G., and Jessitai, A. J. (1987) J. Clin. Invest. 80, 732–742
10. Caro, J., and Jones, O. T. (1986) Biochem. J. 237, 111–116
11. Ackerman, H. (1994) Respir Physiol 95, 1–10
12. Gorlach, A., Holtermann, G., Jelkmann, W., Hancock, J. T., Jones, A. S., Jones, O. T., and Ackerman, H. (1992) Biochem. J. 282, 771–776
13. Gleadle, J. M., Ebert, B. L., and Ratcliffe, P. J. (1995) Eur. J. Biochem. 234, 92–99
14. Wiesener, M. S., Turley, H., Allen, W. E., William, C., Eckardt, K. U., Talks, K. L., Wood, S. M., Gatter, K. C., Harris, A. L., Pugh, C. W., Ratcliffe, P. J., and Maxwell, P. H. (1998) Blood 92, 2260–2268
15. Archer, S. L., Reeve, H. L., Michelakis, E., Puttagunta, L., Waite, R., Nelson, D. P., Dinazer, M. C., and Weir, E. K. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 7844–7849
16. Chandell, N. S., Maltepe, E., Goldwasser, E., Mathieu, C. E., Simon, M. C., and Schumacker, P. T. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 11715–11720
17. Sinovius, V., Leschinsky, I., Sang, N. K., Minchenko, A., and Caro, J. (2001) J. Biol. Chem. 276, 21995–21998
18. Harder, D. R., Gembrededin, D., Narayan, J., Jefcoat, C., Falck, J. R., Campbell, W. B., and Roman, R. (1994) Am. J. Physiol. 266, H2098–H2107
19. Zou, A. P., Imig, J. D., Kaldunkov, M., Orixi de Mentallione, P. R., Sui, Z., and Roman, R. J. (1994) Am. J. Physiol. 266, F275–F282
20. Harder, D. R., Narayan, J., Birks, E. K., Liard, J. F., Imig, J. D., Lombard, R. J., Lange, A. R., and Brennan, B. J. (1996) Cell 85, 54–61
21. Liu, Y., Christou, H., Morita, T., Laughner, E., Semenza, G. L., and Ratcliffe, P. J. (1996) Cell 85, 54–61
22. Huang, L. E., Willmore, W. G., Gu, J., Goldberg, M. A., and Bunn, H. F. (1999) J. Biol. Chem. 274, 9038–9044
23. Jacobs, K. E., Shoemaker, C., Hudson, S. D., Kaufman, R. J., Mufson, A., Sehra, J. H., Jones, S. S., Hewick, R., Fritsch, E. F., Wakawaki, M., Shimizu, T., and Miyake, T. (1998) Nature 393, 806–810
24. Leung, D. W., Cachianes, G., Kuang, W. J., Goeddel, D. V., and Ferrara, N. (1989) Science 246, 1306–1309
25. Imaoka, S., Yoneo, Y., Matsuda, T., Degawa, M., Fukushima, S., and Funae, Y. (1987) Biochem. Biophys. Res. Commun. 166, 349–358
26. Wang, G. L., and Semenza, G. L. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 4304–4308
27. Tenhunen, R., Marver, H. S., and Schmid, R. (1969) J. Biol. Chem. 244, 6388–6394
28. Wengar, R. C. (2000) J. Exp. Biol. 203, 1253–1263
29. Semenza, G. L. (1999) Annu. Rev. Cell Biol. 15, 551–578
30. Cockman, M. E., Masson, N., Mole, D. R., Jakobola, P., Chang, G. W., Clifford, S. C., Maher, E. R., Pugh, C. W., Ratcliffe, P. J., and Maxwell, P. H. (2000) J. Biol. Chem. 275, 25737–25741
31. Kamura, T., Sato, S., Iwai, K., Czyzyk-Krzeska, M., Conway, R. C., and Conway, J. W. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 10430–10435
32. Ohm, M., Park, C. W., Ivan, M., Hoffman, M. A., Kim, T. Y., Huang, L. E., Pavletich, N., Chau, V., and Kaelin, W. G. (1999) J. Biol. Chem. 274, 4225–427
33. Tanimoto, K., Makino, Y., Pereira, T., and Poelinger, L. (2000) EMBO J. 19, 4288–4306
34. Ivan, M., Kondo, K., Yang, H., Kim, W., Valiando, J., Ohm, M., Salie, A., Asara, J. M., Lane, W. S., and Kaelin, W. G. Jr. (2001) Science 292, 464–468
35. Jakobola, P., Mole, D. R., Tian, Y. M., Wilson, M. I., Gliedt, J., Gaskell, S. J., Kriegsmann, A., Hebestreit, H. F., Mukherji, M., Schofield, C. J., Maxwell, P. H., Pugh, C. W., and Ratcliffe, P. J. (2001) Science 292, 468–472
36. Yu, F., White, S. H., Zhao, Q., and Lee, F. S. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 9044–9049
37. Epstein, A. C., Gleadle, J. M., McNeill, L. A., Hewison, K. C., O’Rearke, J., Mole, D. R., Mukherji, M., Metzen, E., Wilson, M. I., Dhand, A., Tian, Y. M., Masson, N., Hamilton, B. L., Jakobola, P., Barstead, R., Hodgkin, J., Maxwell, P. H., Pugh, C. W., Schofield, C. J., and Ratcliffe, P. J. (2001) Cell 107, 43–54
38. Li, D., Gan, Y., Wijntjes, M. G., Bashamal, R. A., and Au, J. L. (2001) J. Urol. 166, 2500–2505