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The inhibitory effect of sodium nitroprusside on HIF-1 activation is not dependent on nitric oxide-soluble guanylyl cyclase pathway

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Abstract

Adaptation to hypoxia and maintenance of O_2 homeostasis involve a wide range of responses that occur at different organizational levels in the body. One of the most important transcription factors that activate the expression of O_2-regulated genes is hypoxia-inducible factor 1 (HIF-1). Nitric oxide (NO) mediates a variety of biological effects including relaxation of blood vessels and cytotoxicity of activated macrophages. We investigated the effect of the clinically used nitrates nitroglycerin (NTG), isosorbide dinitrate (ISDN), and sodium nitroprusside (SNP) on HIF-1-mediated transcriptional responses to hypoxia. We demonstrate that among the three nitrates, only SNP inhibits HIF-1 activation in response to hypoxia. In contrast, NTG or ISDN does not affect HIF-1 activity. SNP inhibits the accumulation of HIF-1α, the regulatory subunit of HIF-1, and the transcriptional activation of HIF-1α via a mechanism that is not dependent on either NO or soluble guanylate cyclase.

Keywords: Nitroglycerin; Isosorbide dinitrate; Sodium nitroprusside; Nitric oxide; Hypoxia; Hypoxia-inducible factor 1; Hydroxylase; Von Hippel–Lindau

Hypoxia (reduced O_2 availability) induces a series of adaptive physiological responses [1]. At the cellular level, the adaptation includes a switch of energy metabolism from oxidative phosphorylation to anaerobic glycolysis, increased glucose uptake, and the expression of stress proteins related to cell survival. At the molecular level, the adaptation involves changes in gene expression.

One of the most important transcription factors that activate the expression of O_2-regulated genes is hypoxia-inducible factor 1 (HIF-1) [2,3]. HIF-1 is a heterodimer composed of a constitutively expressed HIF-1β subunit and an inducibly expressed HIF-1α subunit [2]. The regulation of HIF-1 activity occurs at multiple levels in vivo [4]. Among those, the mechanisms regulating HIF-1α protein expression and transcriptional activity have been most extensively analyzed. The von Hippel–Lindau tumor-suppressor protein (VHL) has been identified as the HIF-1α-binding component of a ubiquitin–protein ligase that targets HIF-1α for proteasomal degradation in non-hypoxic cells [5]. Under hypoxic conditions, the hydroxylation of specific proline and asparagine residues in HIF-1α is inhibited due to substrate (O_2)

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limitation, resulting in HIF-1α protein stabilization and transcriptional activation [5–7]. The iron chelator desferrioxamine (DFX) inhibits the prolyl and asparaginyl hydroxylases, which contain Fe^{2+} at their catalytic sites, causing HIF-1α stabilization and transactivation under normoxic conditions [5,7].

Nitric oxide (NO) is known to mediate many physiological and pathological functions including vascular dilatation, cytotoxicity mediated by activated macrophages, and cGMP formation following glutamate receptor activation in neurons [8]. There are several reports demonstrating that exposure of cells to certain NO donors or gaseous NO modulates HIF-1 activity [9–12]. NO donors including *S*-nitrosoglutathione (GSNO) and NOC18 induce HIF-1 activity under non-hypoxic conditions [11,13]. Nitrate reagents including nitroglycerin (NTG), isosorbide dinitrate (ISDN), and sodium nitroprusside (SNP), which are supposed to act by releasing NO, have important clinical applications because of their vasoactive properties. There are several reports that describe the effect of SNP on cellular hypoxic gene responses mediated by HIF-1 [14,15]. However, there is no comprehensive study on the effect of the clinically used nitrates on HIF-1 activation. In this study we demonstrate that nitrates differentially regulate HIF-1 activity and hypoxia-induced gene expression and that selective inhibitory effect of SNP is not dependent on NO-soluble guanylyl cyclase (sGC) pathway.

Materials and methods

Cell culture and reagents. Hep3B cells were maintained in MEM with Earl's salts supplemented with 10% fetal bovine serum (FBS), essential amino acids and pyruvate, 100 U/mL penicillin, and 0.1 mg/mL streptomycin. HEK293 cells were maintained in DMEM supplemented with 10% FBS, 100 U/mL penicillin, and 0.1 mg/mL streptomycin. DFX was obtained from Sigma (St. Louis, MO). The spontaneous NO releaser NOC18, the NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide (carboxyl-PTIO), and the soluble guanylyl cyclase (sGC) inhibitor *1H*-[1,2,4-]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) were obtained from Dojindo (Kumamoto, Japan). NTG was from Mitsubishi Pharma (Osaka, Japan). ISDN was from Eisai (Tokyo, Japan). SNP was from Calbiochem (San Diego, CA).

Plasmid constructs. Reporter plasmid p2.1, harboring a 68-bp hypoxia response element (HRE) from the human enolase 1 (ENO1) gene inserted upstream of an SV40 promoter and *Photinus pyralis* (firefly) luciferase coding sequences, was described previously [16,17]. The *Renilla* luciferase expression plasmid pRL-SV40 was from Promega (Madison, WI). The expression vector pGAL4/HIF-1α(531–826) and the reporter G5E1bLuc, which contains five copies of a GAL4-binding site upstream of a TATA sequence and firefly luciferase coding sequences, were described previously [17]. Mutagenesis of HIF-1α was performed based on a protocol [18] using pCMV-3XFLAG-HIF-1α [13] or pGAL4/HIF-1α(531–826) as template. The sequences of the primer used for the mutagenesis will be provided upon request.

Immunoblot assays. Whole cell lysates were prepared following a protocol described previously [13,19]. One hundred micrograms aliquots were fractionated by 7.5% SDS–PAGE and subjected to immunoblot assay using mouse monoclonal antibody against HIF-1α (BD Biosciences, San Jose, CA) or HIF-1β (H1β234; Novus Biologicals, Littleton, CO) at 1:1000 dilution and HRP-conjugated mouse monoclonal antibody for mouse IgG (Amersham Bioscience, Piscataway, NJ, 1:1000 dilution). Anti-ERK3 antibody was from Santa Cruz and used at 1:1000 dilution (San Diego, CA). Signal was developed using ECL reagent (Amersham Biosciences).

Hypoxic treatment. Tissue culture dishes were transferred to a modular incubator chamber (Billups-Rothenberg, Del Mar, CA) which was flushed with 1% O_2–5% CO_2–94% N_2, sealed, and placed at 37 °C [13,16].

NO scavenger or sGC inhibitor treatment. The NO scavenger carboxyl-PTIO [13,20] or the sGC inhibitor ODQ [21] was added before exposure to 1% O_2, the nitrates or DFX.

Reverse transcription-PCR assay. The reverse transcription (RT)-PCR protocol is described elsewhere [13,22]. Cells were harvested and RNA was isolated with TRIzol (Invitrogen). One microgram of total RNA was subjected to first strand cDNA synthesis using random hexamers (SuperScript II RT kit, Invitrogen). cDNAs were amplified with TaqGold polymerase (Roche, Mannheim, Germany) in a thermal cycler with the gene-specific primers (sequences of the primers can be provided by request). For each primer pair, PCR was optimized for cycle number to obtain linearity between the amount of input RT product and output PCR product. Thermocycling conditions were 30 s at 94 °C, 60 s at 57 °C, and 30 s at 72 °C for 25 (VEGF), 25 (HIF1A), or 20 (18S rRNA) cycles preceded by 10 min at 94 °C. PCR products were fractionated by 3% Nusieve agarose gel electrophoresis, stained with ethidium bromide, and visualized with UV.

HRE-dependent gene expression assay and HIF-1α transactivation assay. Reporter gene assays were performed in Hep3B cells or HEK293 cells with p2.1 and pRL-SV40 for HRE-dependent gene assay, pGAL4/HIF-1α(531–826), G5E1bLuc, and pRL-SV40 for HIF-1α transactivation assay [13,19,23]. For each experiment, at least two independent transfections were performed in triplicate and representative data are shown.

In vitro HIF-1α–VHL interaction assay. Glutathione *S*-transferase (GST)-HIF-1α(429–608) fusion protein was expressed in *Escherichia coli* and the assay was performed as described [6,13,22].

NO assay in conditioned medium. 1×10^5 cells were plated in complete culture media and treated by nitrates or NOC18. Eighty microliters of the condition media was transferred to an assay system, NO_2/NO_3 assay kit-C II (Greiss Reagent Kit) (Dojindo, Kumamoto, Japan) to measure nitrite (NO_2^-) and nitrate (NO_3^-) content for estimation of the amount of generated NO. The principle is based on the Greiss reaction.

Results

Differential effect of nitrates on hypoxia-induced HIF-1α accumulation

To study the effect of nitrates on HIF-1 activation induced by hypoxia, we tested three different types of nitrates: NTG, ISDN, and SNP. Dosages of the nitrates were determined based on the precedent reports [10,13,15]. Hep3B human hepatoma cells were exposed to 1% O_2 with or without the treatment with one of the nitrates for 4 h (lanes 3–8), harvested, and subjected to immunoblot analysis using anti-HIF-1α or -HIF-1β antibody (Fig. 1A). Expression of HIF-1α protein was barely detected in non-hypoxic Hep3B cells (lane 1), and was induced markedly in cells exposed to 1% O_2 for 4 h (lane 2). Neither NTG (10–100 μM) (lanes 3 and 4) nor ISDN (10–100 μM) (lanes 5 and 6) had an effect

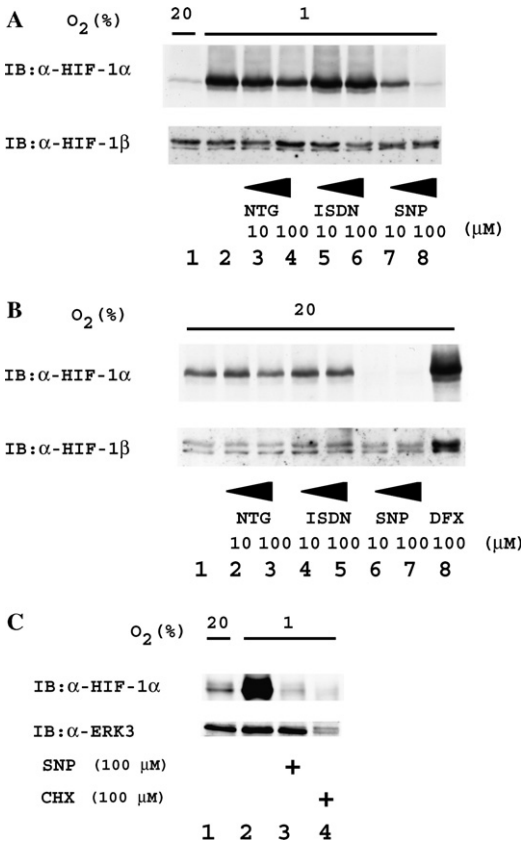

Fig. 1. SNP inhibits HIF-1α protein accumulation. Hep3B cells were treated with NTG, ISDN, or SNP in the indicated dosages at 1% (A) or 20% (B) O_2 for 4 h and harvested for immunoblot assays using anti-HIF-1α and -HIF-1β Abs. (C) Hep3B cells were treated with 100 μM SNP or 100μM CHX for 4 h and harvested for immunoblot assay using anti-HIF-1α Ab or anti-ERK3 Ab.

on the accumulation of HIF-1α. In contrast, SNP (10–100 μM) markedly inhibited the accumulation under hypoxia (lanes 7 and 8). Expression of HIF-1β protein was not affected by hypoxia or nitrates. We next examined HIF-1α expression in cells exposed to 20% O_2 using longer image exposures (Fig. 1B). The iron chelator DFX induced HIF-1α accumulation under non-hypoxic conditions (lane 8) as previously described [17]. SNP (10–100 μM) reduced the basal level of HIF-1α protein (lanes 6 and 7) whereas neither NTG (10–100 μM) nor ISDN (10–100 μM) had any effect (lanes 2–5). ERK3 is a protein with rapid turnover and mainly degradated by ubiquitin–proteasome system [24]. Notably, SNP selectively suppresses HIF-1α accumulation induced by hypoxia (Fig. 1C, lane 3). In contrast, CHX, which is general translational inhibitors, suppresses both HIF-1α and ERK3 protein accumulation (lane 4).

SNP preferentially suppresses HIF-1α expression in HEK293 cells and HUVECs

In addition to in Hep3B cells, SNP suppressed HIF-1α expression in HEK293 cells (Fig. 2A) and HUVEC

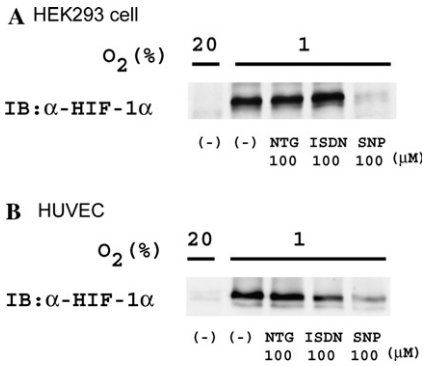

Fig. 2. SNP inhibit HIF-1α protein accumulation in HEK293 cells and HUVECs HEK293 cells (A) or HUVECs (B) were treated with 100 μM of NTG, ISDN, or SNP under 1% O_2 conditions for 4 h and harvested for immunoblot assays using anti-HIF-1α.

(Fig. 2B) under 1% O_2 conditions. In HEK293 cells and HUVECs HIF-1α protein was barely detected under 20% O_2 conditions. As well as in the case of Hep3B cells, SNP preferentially suppressed hypoxia-induced HIF-1α accumulation.

SNP preferentially inhibits HIF-1-dependent gene expression

We investigated the effect of the nitrates on hypoxia-induced *VEGF* gene expression. VEGF mRNA expression was induced in response to hypoxia (Fig. 3A, lane 2). Treatment with SNP (100 μM) inhibited hypoxia-induced VEGF mRNA expression (lane 5). Neither NTG (100 μM) nor ISDN (100 μM) affected the expression of VEGF mRNA (lanes 3 and 4), which is consistent with the analysis of HIF-1α protein (Fig. 1). HIF-1α mRNA expression was not affected by nitrates (Fig. 3A, middle panel), indicating that the effect of SNP occurs at the level of HIF-1α protein expression. No significant difference in the expression of 18S rRNA was detected (bottom panel), indicating that the nitrates did affect the reverse transcriptase reaction or PCR process.

Hep3B cells were transfected with the reporter p2.1, which contains an HIF-1-dependent HRE driving the expression of firefly luciferase, and pRL-SV40, which encodes *Renilla* luciferase, exposed to 20% or 1% O_2, and harvested for luciferase assays. SNP (100 μM) inhibited hypoxia-induced HRE-dependent gene expression whereas neither NTG (100 μM) nor ISDN (100 μM) had any effect (Fig. 3B). Finally, we investigated the impact of the nitrates on HIF-1α transcriptional activity using GAL4-HIF-1α(531–826) fusion protein construct. Because expression of GAL4-HIF-1α(531–826) fusion protein is not affected by O_2 tension, the activity of the transcriptional activity of HIF-1α can be assayed with this system [6,17]. Only SNP suppressed the hypoxia-induced HIF-1α transcriptional activation (Fig. 3C). Expression of GAL4-HIF-1α(531–826) fusion

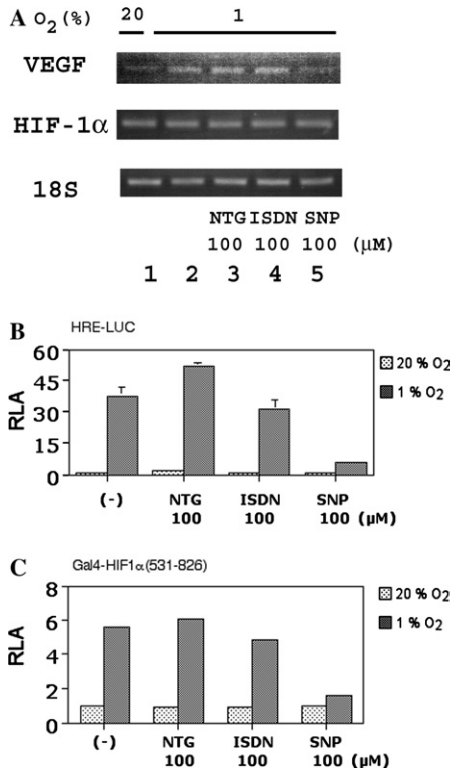

Fig. 3. SNP inhibits hypoxia-induced gene expression. (A) Hep3B cells were exposed to 20% or 1% O_2 in the presence or absence of the nitrates for 24 h and total RNA was isolated. Expression of VEGF mRNA, HIF-1α mRNA, and 18S rRNA was analyzed by RT-PCR. (B,C) Hep3B were transfected with pRL-SV40 (control reporter encoding *Renilla* luciferase) and p2.1 (HRE-driven reporter encoding firefly luciferase). (B) The ratio of firefly:*Renilla* luciferase activity was determined and normalized to the value obtained from non-hypoxic untreated (−) cells to obtain the relative luciferase activity (RLA). Results shown represent mean ± SD of three independent transfections.

protein was not affected by either hypoxic treatment or SNP.

Impact of an NO scavenger and a sGC inhibitor on the effect of SNP

NTG, ISDN, and SNP are considered to be NO donors. To examine signal transduction pathways mediating effects of nitrates on HIF-1α protein levels, the NO scavenger carboxyl-PTIO was tested (Fig. 4A, top panel). Under non-hypoxic conditions, the spontaneous NO donor NOC18 (500 μM) induced HIF-1α accumulation (lane 2) as previously reported [13] and the accumulation was suppressed almost completely by treatment with the NO scavenger carboxyl-PTIO (100 μM) (lane 3). In contrast, the effect of SNP (100 μM), which inhibited HIF-1α accumulation induced by hypoxia, was not neutralized completely by carboxyl-PTIO (compare lanes 7 and 10). Neither NTG (100 μM) nor ISDN (100 μM) had an effect in the presence or absence of carboxyl-PTIO. HIF-1β expression was not affected by carboxyl-PTIO (Fig. 4A, bottom panel). Next, we examined whether sGC activity was required for the inhibitory effects of SNP on HIF-1α accumulation (Fig. 4B, top panel). In Hep3B cells, the specific sGC inhibitor ODQ did not block the inhibitory effect of SNP on hypoxia-induced HIF-1α accumulation (lanes 12–14). NOC18-induced HIF-1α accumulation also was not suppressed by ODQ (1–20 μM). ODQ treatment did not affect the expression of HIF-1β protein (Fig. 4B, bottom panel). The three nitrates tested are not spontaneous NO donors. They require enzymatic activity and intracellular free thiols for NO generation. We investigated NO generation in culture media by Greiss method (Fig. 4C). NOC18 is a spontaneous NO donor and generates NO_2^- and NO_3^- efficiently. SNP induced NO_2^- and NO_3^- accumulation in cell-free condition but the amount of NO_2^- and NO_3^- was far less than NO released from NOC18. ISDN did not promote the accumulation of NO_2^- and NO_3^- in Hep3B cells or HUVECs. NTG induced the accumulation of NO_2^- and NO_3^- only in HUVECs.

Effects of the nitrates on the interaction between HIF-1α and VHL in vitro

Intracellular protein expression of HIF-1α is regulated by ubiquitin–proteasome system. VHL is the component of the E3 ubiquitin ligase for HIF-1α. The interaction between HIF-1α and VHL is governed by prolyl hydroxylation status of HIF-1α. Incubation of a GST-HIF-1α(429–608) fusion protein with HEK293 cell lysate resulted in prolyl hydroxylation of HIF-1α and interaction with VHL (Fig. 4D). Lysate treated with DFX (100 μM) did not promote the interaction of GST-HIF-1α(429–608) with VHL (lane 6). None of the nitrates (100 μM) affected the interaction, demonstrating that hydroxylase activity is not directly inhibited by nitrates in vitro. On the other hand, lysate from cells treated with SNP promoted the interaction (Fig. 4E, lane 4).

Effects of SNP on the expression and transactivity of wild or mutant HIF-1α

To elucidate the molecular mechanism of the inhibitory effect of SNP on HIF-1 activity, we adopted the assays using mutant forms of HIF-1α. HEK293 cells were transfected with 1 μg of wild or mutant (P402A/P564G) FLAG-tagged HIF-1α and then underwent hypoxic or SNP treatment. Cells were harvested and then subjected to Western blotting using anti-FLAG antibody. Expression of FLAG-HIF-1α-wt was sensitive to SNP treatment under 20% and 1% O_2 conditions (Fig. 5A, lanes 1 and 2). In contrast, expression of FLAG-HIF-1α-P402A/P564G was sensitive to neither SNP treatment nor change of O_2 tension (lanes 3 and 4). As next wild

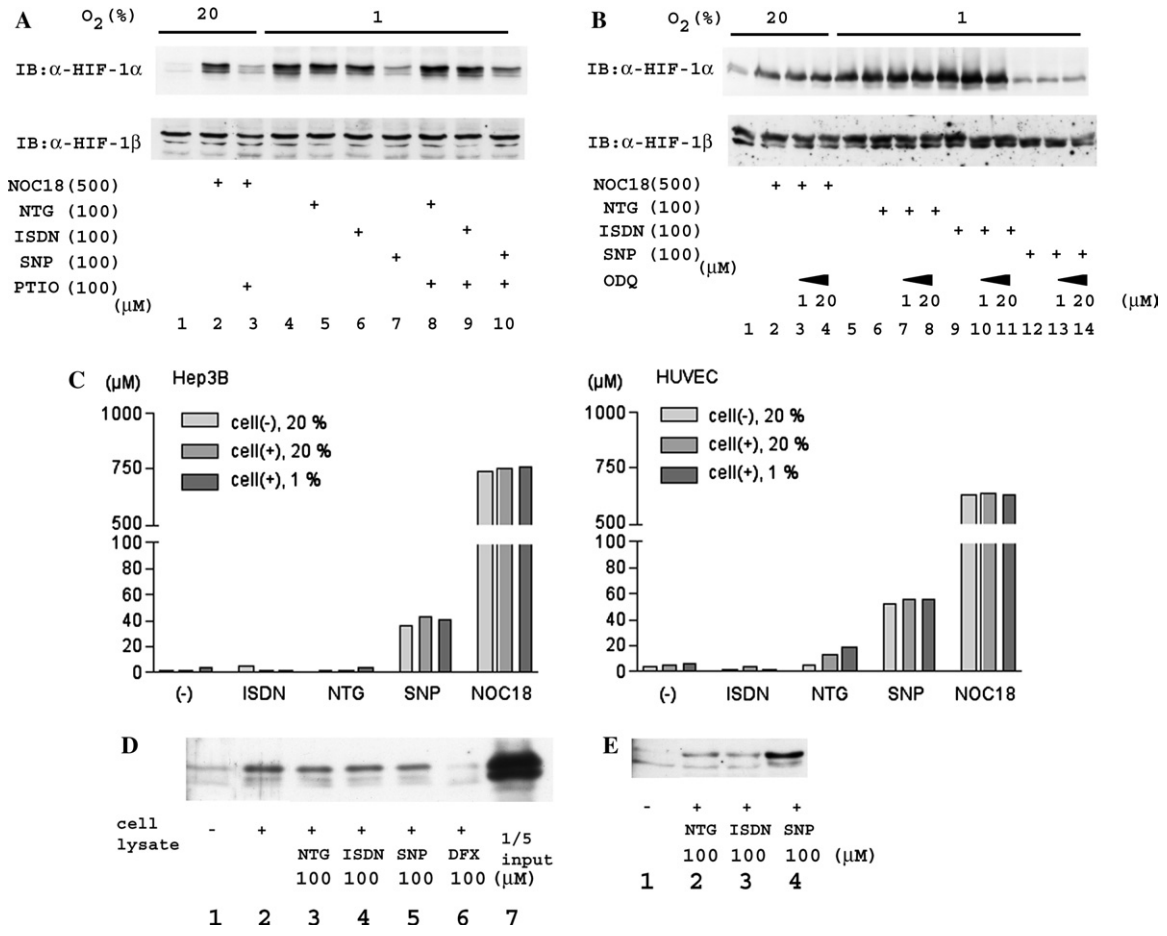

Fig. 4. Impact of NO scavenger and sGC inhibitor on HIF-1α accumulation. (A,B) Hep3B cells were exposed to 20% or 1% O_2, were treated with the reagents indicated for 4 h with or without PTIO (A) or ODQ (B) and then harvested for immunoblot assays using anti-HIF-1α and -HIF-1β Abs. (C) NO assay was performed as described in Materials and methods using Hep3B cells (upper) or HUVECs (lower). Cell(−) indicated that the assay was done without cells plated. (D,E) GST-HIF-1α(429–608) fusion protein was incubated with in vitro translated VHL in the presence of PBS or HEK293-lysates untreated or treated with the indicated reagents. Glutathione–Sepharose beads were used to capture GST-HIF-1α and the presence of bound VHL was determined by PAGE. One-fifth of the input VHL protein was also analyzed.

or mutant (N803A) GAL4-HIF-1α(531–826) transactivation activity was assayed by the reporter assay. As shown in Fig. 3C, SNP suppressed hypoxia-induced luciferase expression. In contrast, SNP failed to suppress the luciferase activity from N803A mutant.

Discussion

In this study, we demonstrate that the clinically used nitrates NTG, ISDN, and SNP differentially modulate hypoxia-induced gene expression mediated by HIF-1. SNP treatment reduced HIF-1α protein levels and HIF-1 transcriptional activity in Hep3B cells that were untreated or exposed to hypoxia. Similar results were observed in human embryonic kidney HEK293 cells and HUVECs, suggesting that the phenomenon is not dependent on the cell or tissue type.

The nitrates investigated in this study are generally regarded as NO donors. NO stimulates the activity of sGC, which catalyzes the production of cGMP, an important second messenger for NO-mediated signal transduction. As previously reported [11,13] and shown in Fig. 4A, the spontaneous NO releaser NOC-18 induces HIF-1α accumulation in a NO-dependent manner, because the NO scavenger carboxyl-PTIO completely suppresses the accumulation induced by NOC-18. In contrast, SNP suppressed hypoxia-induced accumulation of HIF-1α and the suppression was not sensitive to either carboxyl-PTIO or the sGC inhibitor ODQ. Moreover, neither NTG nor ISDN suppressed hypoxia-induced HIF-1α accumulation. These three nitrates are not spontaneous NO donors. They require enzymatic activity and intracellular free thiols for NO generation [25]. There is a report that NTG is metabolized within mitochondria by aldehyde dehydrogenase [25], although the molecular mechanisms are not well understood. We tested Hep3B, HEK293, and HUVECs, the latter of which has been shown to metabolize NTG [25]. Notably the result of NO assay (Fig. 4C) demonstrates ISND

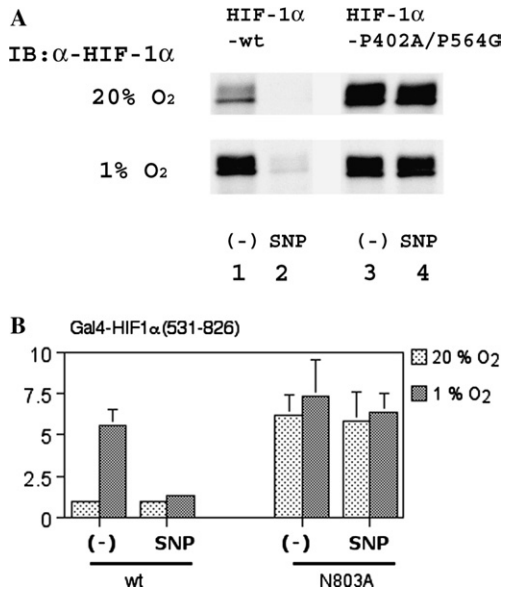

Fig. 5. Effects of SNP on the expression or transactivity of wild or mutant type of HIF-1α. (A) HEK293 cells were transfected with 1 μg of pCMV-FLAGX3-HIF-1a-wt or pCMV-FLAGX3-HIF-1a-P402A/P564G. Cells were exposed to 20% or 1% O_2 conditions with or without SNP-treatment and harvested and the whole cell lysates were subjected to immunoblot assay using anti-FLAG Ab. (B) Constructs encoding the GAL4 DNA-binding domain (amino acids 1–147) fused to the transactivation domain of HIF-1α (531–826) with or without mutation (N803A) were analyzed for their ability to transactivate reporter gene G5E1bLuc containing five GAL4-binding sites. Hep3B cells were co-transfected with pRL-SV40 (50 ng), GAL4E1bLuc (100 ng), and GAL4-HIF-1α fusion protein expression plasmids (100 ng). After 6 h incubation, cells were treated with the nitrates under 20% (stippled bars) and 1% (shaded bars) O_2 for 18 h and harvested for luciferase assays. The ratio of firefly:*Renilla* luciferase activity was determined and normalized to the value obtained from non-hypoxic untreated (−) cells to obtain the relative luciferase activity (RLA). Results shown represent mean ± SD of three independent transfections.

and NTG barely induce the accumulation of NO_2^-/NO_3^-. In contrast, 100 μM of SNP induces the accumulation comparably with 50 μM NOC18, which does not induce the activation of HIF-1. These results strongly suggest that the effects of SNP on HIF-1 are not dependent on NO or its metabolites NO_2^- and NO_3^-.

Expression level and transactivation activity of HIF-1α are regulated by prolyl hydroxylases and asparagine hydroxylase, respectively. The results in Figs. 5A and B together indicate that prolyl hydroxylation and asparagine hydroxylation are critically involved in the expression and transactivity of HIF-1α and that SNP inhibited both HIF-1α protein expression and HIF-1α transactivation domain function, suggesting that it may be acting to maintain hydroxylase activity in hypoxic cells. Taking account of the evidence that SNP suppresses both HIF-1α accumulation and transcriptional activity under both hypoxic and non-hypoxic conditions, SNP may hyperactivate HIF-1α hydroxylases. Our in vitro experimental result (Figs. 4D and E) provides very interesting evidence. In Fig. 4D, cell lysate treated with SNP does not affect the interaction between HIF-1α and VHL. In contrast, lysate from cell treated with SNP facilitates the interaction as we previously reported (Fig. 4E) [13]. In fact, recently Hagen et al. [26] reported that inhibition of mitochondrial respiration promotes O_2 to redistribute toward non-respiratory O_2 targets such as HIF-1α hydroxylases, thus inhibiting HIF-1 activation under hypoxic conditions. In this case, SNP might not affect HIF-1α hydroxylase activity directly but affects the activity by redirecting intracellular O_2 availability. This is consistent with our in vitro result. SNP is known to disintegrate into ferricyanide, ferrocyanide, and cyanide, and produces hydrogen peroxide during the degradation process. The by-products of SNP may modulate cellular redox conditions, which in turn may affect HIF-1 activation.

We conclude that SNP selectively inhibits HIF-1 activation and HIF-1-mediated gene expression and that this inhibition is not dependent either on NO or sGC activity.

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References

[1] P.W. Hochachka, L.T. Buck, C.J. Doll, S.C. Land, Unifying theory of hypoxia tolerance: molecular/metabolic defense and rescue mechanisms for surviving oxygen lack, Proc. Natl. Acad. Sci. USA 93 (1996) 9493–9498.
[2] G.L. Wang, B.H. Jiang, E.A. Rue, G.L. Semenza, Hypoxia-inducible factor 1 is a basic-helix–loop–helix-PAS heterodimer regulated by cellular O_2 tension, Proc. Natl. Acad. Sci. USA 92 (1995) 5510–5514.
[3] K. Hirota, Hypoxia-inducible factor 1, a master transcription factor of cellular hypoxic gene expression, J. Anesthesiol. 16 (2002) 150–159.
[4] G.L. Semenza, Hypoxia-inducible factor 1: master regulator of O_2 homeostasis, Curr. Opin. Genet. Dev. 8 (1998) 588–594.
[5] G.L. Semenza, HIF-1, O_2, and the 3 PHDs: how animal cells signal hypoxia to the nucleus, Cell 107 (2001) 1–3.
[6] P.C. Mahon, K. Hirota, G.L. Semenza, FIH-1: a novel protein that interacts with HIF-1alpha and VHL to mediate repression of HIF-1 transcriptional activity, Genes Dev. 15 (2001) 2675–2686.
[7] D. Lando, D.J. Peet, J.J. Gorman, D.A. Whelan, M.L. Whitelaw, R.K. Bruick, FIH-1 is an asparaginyl hydroxylase enzyme that regulates the transcriptional activity of hypoxia-inducible factor, Genes Dev. 16 (2002) 1466–1471.
[8] L.J. Ignarro, G. Cirino, A. Casini, C. Napoli, Nitric oxide as a signaling molecule in the vascular system: an overview, J. Cardiovasc. Pharmacol. 34 (1999) 879–886.
[9] Y. Liu, H. Christou, T. Morita, E. Laughner, G.L. Semenza, S. Kourembanas, Carbon monoxide and nitric oxide suppress the

hypoxic induction of vascular endothelial growth factor gene via the 5′ enhancer, J. Biol. Chem. 273 (1998) 15257–15262.
[10] K. Sogawa, K. Numayama-Tsuruta, M. Ema, M. Abe, H. Abe, Y. Fujii-Kuriyama, Inhibition of hypoxia-inducible factor 1 activity by nitric oxide donors in hypoxia, Proc. Natl. Acad. Sci. USA 95 (1998) 7368–7373.
[11] L.A. Palmer, B. Gaston, R.A. Johns, Normoxic stabilization of hypoxia-inducible factor-1 expression and activity: redox-dependent effect of nitrogen oxides, Mol. Pharmacol. 58 (2000) 1197–1203.
[12] H. Kimura, A. Weisz, Y. Kurashima, K. Hashimoto, T. Ogura, F. D'Acquisto, R. Addeo, M. Makuuchi, H. Esumi, Hypoxia response element of the human vascular endothelial growth factor gene mediates transcriptional regulation by nitric oxide: control of hypoxia-inducible factor-1 activity by nitric oxide, Blood 95 (2000) 189–197.
[13] K. Kasuno, S. Takabuchi, K. Fukuda, S. Kizaka-Kondoh, J. Yodoi, T. Adachi, G.L. Semenza, K. Hirota, Nitric oxide induces hypoxia-inducible factor 1 activation that is dependent on MAP kinase and phosphatidylinositol 3-kinase signaling, J. Biol. Chem. 279 (2003) 2550–2558.
[14] F. Wang, H. Sekine, Y. Kikuchi, C. Takasaki, C. Miura, O. Heiwa, T. Shuin, Y. Fujii-Kuriyama, K. Sogawa, HIF-1α-prolyl hydroxylase: molecular target of nitric oxide in the hypoxic signal transduction pathway, Biochem. Biophys. Res. Commun. 295 (2002) 657–662.
[15] H. Kimura, T. Ogura, Y. Kurashima, A. Weisz, H. Esumi, Effects of nitric oxide donors on vascular endothelial growth factor gene induction, Biochem. Biophys. Res. Commun. 296 (2002) 976–982.
[16] G.L. Semenza, B.H. Jiang, S.W. Leung, R. Passantino, J.P. Concordet, P. Maire, A. Giallongo, Hypoxia response elements in the aldolase A, enolase 1, and lactate dehydrogenase A gene promoters contain essential binding sites for hypoxia-inducible factor 1, J. Biol. Chem. 271 (1996) 32529–32537.
[17] B.H. Jiang, J.Z. Zheng, S.W. Leung, R. Roe, G.L. Semenza, Transactivation and inhibitory domains of hypoxia-inducible factor 1α. Modulation of transcriptional activity by oxygen tension, J. Biol. Chem. 272 (1997) 19253–19260.
[18] A. Sawano, A. Miyawaki, Directed evolution of green fluorescent protein by a new versatile PCR strategy for site-directed and semi-random mutagenesis, Nucleic Acids Res. 28 (2000) E78.
[19] K. Hirota, G.L. Semenza, Rac1 activity is required for the activation of hypoxia-inducible factor 1, J. Biol. Chem. 276 (2001) 21166–21172.
[20] T. Akaike, H. Maeda, Quantitation of nitric oxide using 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide (PTIO), Methods Enzymol. 268 (1996) 211–221.
[21] M.A. Moro, R.J. Russel, S. Cellek, I. Lizasoain, Y. Su, V.M. Darley-Usmar, M.W. Radomski, S. Moncada, cGMP mediates the vascular and platelet actions of nitric oxide: confirmation using an inhibitor of the soluble guanylyl cyclase, Proc. Natl. Acad. Sci. USA 93 (1996) 1480–1485.
[22] S.O. Ang, H. Chen, K. Hirota, V.R. Gordeuk, J. Jelinek, Y. Guan, E. Liu, A.I. Sergueeva, G.Y. Miasnikova, D. Mole, P.H. Maxwell, D.W. Stockton, G.L. Semenza, J.T. Prchal, Disruption of oxygen homeostasis underlies congenital Chuvash polycythemia, Nat. Genet. 32 (2002) 614–621.
[23] T. Itoh, T. Namba, K. Fukuda, G.L. Semenza, K. Hirota, Reversible inhibition of hypoxia-inducible factor 1 activation by exposure of hypoxic cells to the volatile anesthetic halothane, FEBS Lett. 509 (2001) 225–229.
[24] P. Coulombe, G. Rodier, S. Pelletier, J. Pellerin, S. Meloche, Rapid turnover of extracellular signal-regulated kinase 3 by the ubiquitin–proteasome pathway defines a novel paradigm of mitogen-activated protein kinase regulation during cellular differentiation, Mol. Biol. Cell 23 (2003) 4542–4558.
[25] Z. Chen, J. Zhang, J.S. Stamler, Identification of the enzymatic mechanism of nitroglycerin bioactivation, Proc. Natl. Acad. Sci. USA 99 (2002) 8306–8311.
[26] T. Hagen, C.T. Taylor, F. Lam, S. Moncada, Redistribution of intracellular oxygen in hypoxia by nitric oxide: effect on HIF1α, Science 302 (2003) 1975–1978.