Chelation of Cellular Calcium Modulates Hypoxia-inducible Gene Expression through Activation of Hypoxia-inducible Factor-1α*

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Hypoxia-Inducible Factor-1 (HIF-1) is the key transcription factor in control of the expression of hypoxia-inducible genes needed by cells to adapt to decreased oxygen availability. Herein, we investigated the HIF-1α-mediated gene expression of carbonic anhydrase 9 (CA9) in response to hypoxia and changes of intracellular calcium levels in the neuroblastoma cell line SH-SYSY. Decreasing the intracellular calcium level by BAPTA (1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid) induced HIF-1α nuclear accumulation and enhanced HIF-1 DNA binding within 1 h of incubation. Like hypoxia, BAPTA stimulated HIF-1-dependent transcription by increasing the activity of the C-terminal transactivation domain of HIF-1α and greatly enhanced expression of the HIF-1 target gene CA9. Detailed analysis of HIF-1α accumulation revealed that BAPTA attenuated the interaction of HIF-1α with von-Hippel-Lindau protein thus decreasing proteasomal degradation of HIF-1α. Knock down of HIF-1α mRNA and protein by small interference RNA for HIF-1α revealed that both hypoxia and the BAPTA-induced gene expression of CA9 were strictly dependent on HIF-1α. In contrast, elevation of cytosolic calcium level by thapsigargin reduced the BAPTA-mediated effects. Measurements of intracellular calcium under hypoxia revealed a change in the cellular calcium distribution. BAPTA-dependent induction of HIF-1 activity was not caused by its in vitro capability to chelate iron. Instead, effective chelation of cellular calcium caused the accumulation of HIF-1α protein through inhibition of HIF-prolyl hydroxylases and activated HIF-1α-dependent gene expression under normoxic conditions.

Hypoxia induces a variety of genes that act in concert to facilitate the supply of oxygen and nutrients and to promote cell growth and survival. Hypoxia-inducible factor-1 (HIF-1) is of central importance for control of the expression of most of these genes (1). HIF-1 is a heterodimer consisting of one of three α-subunits (HIF-1α and isoforms HIF-2α or HIF-3α) and the β-subunit (HIF-1β), also called aryl hydrocarbon receptor nuclear translocator or ARNT) (2–5). HIF-1α is constitutively found in the nucleus, whereas stabilization of HIF-1α and nuclear accumulation are induced by hypoxia. HIF-1α is constitutively synthesized but sent to destruction by the ubiquitin-proteasome pathway in normoxia (6–8). This process is mediated by binding of the von-Hippel-Lindau protein (pVHL) (9), which is the substrate-recognizing component of an E3 ubiquitin ligase complex (10–12). Oxygen dependence of this process depends on the post-translational hydroxylation of HIF-1α at proline residues 564 and 402 by prolyl hydroxylases (13, 14). Activity of these prolyl hydroxylases (PHDs) requires oxygen. Thus, under hypoxic conditions when prolyl hydroxylation ceases, HIF-1α is not recognized by pVHL and evades degradation. In addition, trans-activity of HIF-1α is regulated by oxygen-sensitive hydroxylation of the asparagine residue 803 by a related asparagine hydroxylase called FIH-1 (15). Under hypoxic conditions, HIF-1α accumulates, translocates into the nucleus, and forms an active HIF-1 complex that turns on expression of target genes such as vascular endothelial growth factor (VEGF), erythropoietin, glucose transporter-1, and carbonic anhydrase (CA9) (16, 17).

In addition to this well established O2-dependent activation mechanisms of HIF-1α, a role for changes in intracellular calcium levels in hypoxia-induced gene expression has been postulated. After prolonged hypoxic incubation, an increase in cytosolic calcium concentration was observed in some cell lines (18–20) that was due to the release of calcium from intracellular stores (21, 22). Increased calcium concentrations have been suggested to be involved in hypoxia-induced expression of tyrosine hydroxylase gene (23–25), VEGF, and NDRG-1/Cap43 (26, 27). But unlike hypoxia or hypoxia-mimicking agents, the elevation of intracellular calcium concentration neither induced HIF-1α protein nor stimulated HIF-1-dependent transcription (27). Instead, increased intracellular calcium activated VEGF expression through an AP-1-dependent pathway during hypoxia (26, 27). Recently Mottet et al. (20) reported that elevated calcium levels after prolonged hypoxia increased extracellular signal-regulated kinase 2/1 (ERK 2/1) activation and increased HIF-1 transcriptional activity but did not induce HIF-1α accumulation. However, HIF-1α accumulation and activation is instantaneous upon the onset of hypoxia (28), and it has not yet been clarified whether changes in intracellular calcium level play a role in this early phase of hypoxia. Herein,
Calmodulin Modulates HIF-1α Activity

We studied the effect of decreased intracellular calcium level in SH-SY5Y neuroblastoma cells on HIF-1 activation and expression of a typical HIF-1 target gene, carbonic anhydrase 9, under normoxic and hypoxic conditions.

**EXPERIMENTAL PROCEDURES**

Reagents—Me2SO and citric acid trisodium salt (dihydrate) were obtained from Merck (Darmstadt, Germany) and desferoxamine mesylate (Desfera®) was from Novartis Pharma (Nuremberg, Germany). 2,2'-Dipyridyl, cholera toxin (inhibiting diacytic acid), ferrous ammonium sulfate, 8-hydroxyquinoline, and propidium iodide were obtained from Sigma. BAPTA-AM (1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid-acetoxymethyl ester), BAPTA hexapotassium salt, thapsigargin, and the fluorescent dyes Oregon Green® 488 BAPTA-1-AM (OG-BAPTA-AM), Oregon Green® 488 BAPTA-1, hexapotassium salt, Fura-2-AM, and PhenGreen® SK (PG SK, diacetate) were purchased from Molecular Probes Europe BV (Leiden, Netherlands). 1,10-Phenanthroline was obtained from Aldrich (Sigma-Aldrich).

Cell Culture Experiments and Hypoxic Induction—SH-SY5Y neuroblastoma cells (American Type Culture Collection, Manassas, VA) were kept in RPMI 1640 medium (BioWhittaker, Cambrex, Verviers, Belgium) supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 100 units/ml streptomycin in a normoxic atmosphere of 5% CO2 and 95% air.

DNA, RNA, and protein assay reagents (Bio-Rad). 50 whole cell extract, and the protein concentration was determined using a bicinchoninic acid assay (Pierce, Rockford, IL). The pH265 plasmid, containing six HIF-1α binding sites, was generously provided by Peter Ratcliffe (31).

Plasmid Constructs—The pH3SVL plasmid, containing six HIF-1α binding sites, was generously provided by Roland Wenger (30). The empty vector without HIF-1α-binding sites (pGL3 vector, Promega, Heidelberg, Germany) was generously provided by Peter Ratcliffe (31).

**In Vitro Protein Interaction Assay**—The impact of BAPTA on recombining HIF-1α prolyl hydroxylase 2 (PHD2) was studied by the in vitro protein interaction assays as described previously (32). The assay makes use of the interaction between the C-terminal oxygen degradation domain of HIF-1α (ODD, amino acids 549–582) and pVHL, which requires the presence of hydroxyproline in the ODD. To test hydroxylation-dependent effects of BAPTA on HIF-1α-pVHL interaction in normoxia, we produced GalDBD-HIF-1α-pVHL, HD2, and 12-labeled pVHL in T7-coupled rabbit reticulocyte lysate in vitro transcription/translation system (Promega). GalDBD-HIF-1α-pVHL was purified with GalDBD antibodies conjugated to agarose beads (Santa Cruz Biotechnology) and suspended in a reaction buffer (5 mM KCl, 1.5 mM MgCl2, 20 mM Tris, pH 7.5) supplemented with cofactors (1 mM ascorbate, 1 mM α-ketoglutarate, 20 μM FeCl3). GalDBD-HIF-1α-pVHL was then incubated for 30 min with BAPTA hexapotassium salt (final concentration, 10–50 μM) before loading into a nitrocellulose membrane. After incubation with BAPTA-pVHL or thapsigargin under normoxic or hypoxic atmosphere for 4 or 6 h, cells were lysed in a buffer made of 2 mM dithiothreitol, 2 mM EDTA, 10% glycerol, 1% Triton X-100, 25 mM Tris, pH 7.8. Luciferase activity was determined with the luciferase assay system (Promega) and normalized to total cellular protein. Luciferase activity of the control vector without HIF-1α-binding sites was not inhibited by hypoxia, BAPTA-AM, or thapsigargin treatment (data not shown). All transfections were at least done in three separate culture dishes; data are expressed as means ± S.D.

**Reverse Transcription and Quantitative Real Time PCR**—Total RNA was extracted with the guanidinium isothiocyanate method as described previously (32). 1 μg of total RNA was reverse transcribed with M-MLV reverse transcriptase (Promega). The empty vector without HIF-1α-binding sites (pGL3 vector, Promega) was generously provided by Peter Ratcliffe (31).

**Site-Directed Mutagenesis**—The pH265 plasmid, containing six HIF-1α binding sites, was generously provided by Peter Ratcliffe (31).

**Cell Transfection and Reporter Gene Assays**—1 × 10⁶ SH-SY5Y cells were transfected with 10 μg of pH3SVL plasmids or control plasmid (Promega) or with 5 μg of Gal4-C-TAD and 15 μg of GALA-Luc plasmids together by electroporation method at 975 microfarads, 250 V, in 0.4-mm thick cuvettes using a Gene Pulser and Capacitance Extender apparatus (Bio-Rad). After recovering, the cells were split in 18 aliquots and grown in 6-well dishes overnight. After treatment with BAPTA-AM or thapsigargin under normoxic or hypoxic atmosphere for 4 or 6 h, cells were lysed in a buffer made of 2 mM dithiothreitol, 2 mM EDTA, 10% glycerol, 1% Triton X-100, 25 mM Tris, pH 7.8. Luciferase activity was determined with the luciferase assay system (Promega) and normalized to total cellular protein. Luciferase activity of the control vector without HIF-1α-binding sites was not inhibited by hypoxia, BAPTA-AM, or thapsigargin treatment (data not shown). All transfections were done in at least three separate culture dishes; data are expressed as means ± S.D.
Cell Viability—For the fluorescence microscopic studies, the uptake of the vital dye propidium iodide (5 μg/ml) was routinely determined either during or at the end of the experiments to detect loss of cell viability. The red fluorescence of propidium iodide excited at 543 nm was collected through a 560-nm long-pass filter when laser scanning microscopy was used; using digital fluorescence microscopy, propidium iodide was detected at λ_{exc} = 535 ± 17.5 nm and λ_{em} = 590 nm. Toxicity of BAPTA-AM and thapsigargin was excluded as judged from the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (39).

Statistics—All experiments with SH-SY5Y cells were repeated at least three times with cells from different subcultures; experiments in a cell-free system were repeated at least twice. Cellular microfluorographs and traces shown in the figures are representative for all the corresponding experiments carried out. The results are expressed as means ± S.D.

RESULTS

Effects of BAPTA and Thapsigargin on the HIF-1α Accumulation—In normoxic SH-SY5Y neuroblastoma cells HIF-1α protein was absent. Hypoxia-induced HIF-1α accumulation was detectable after 30 min and was maximal after 4 h. Application of the calcium chelator BAPTA induced HIF-1α protein levels in normoxia and enhanced HIF-1α protein accumulation under hypoxic conditions. Maximum HIF-1α protein induction was observed after 1 h of incubation with BAPTA-AM (Fig. 1). In contrast, elevation of intracellular calcium using thapsigargin diminished hypoxia-induced HIF-1α protein levels after incubation from 0.5 to 4 h (Fig. 1).

Because BAPTA-induced HIF-1α accumulation was maximal after 1 h, we next investigated the effects of BAPTA-AM, thapsigargin, or both under normoxic and hypoxic conditions at this time point (Fig. 2). Western analysis of whole cell lysate showed that the HIF-1α protein expression was maximally stimulated under hypoxia plus BAPTA. Additional treatment with thapsigargin reduced the BAPTA-induced HIF-1α protein accumulation under normoxia and hypoxia (Fig. 2A). Immunofluorescence was performed to study nuclear translocation of HIF-1α by BAPTA-AM treatment. As shown in Fig. 2B, BAPTA-induced HIF-1α nuclear accumulation under normoxic conditions and increased the hypoxic effect, which as expected was decreased by treatment with thapsigargin. These experiments clearly indicate that chelation of calcium induced HIF-1α protein accumulation and nuclear localization, whereas elevation of cytosolic calcium by inhibition of the Ca^{2+}-ATPase attenuated this effect.

BAPTA Increases HIF-1α Levels by Inhibiting Its Degradation—Under normoxia BAPTA induced HIF-1α accumulation despite the presence of cycloheximide, an inhibitor of translation that prevents the de novo synthesis of HIF-1α (Fig. 3A) and was added as FeCl₃, or as ferrous ammonium sulfate from a freshly prepared stock solution (1 mM) in distilled water supplemented with 20 mM ascorbate (35). Fe²⁺, Fe³⁺ was added as FeCl₃, or as ferrous ammonium sulfate from a freshly prepared stock solution (1 mM) in distilled water supplemented with 20 mM ascorbate (35).
stabilized HIF-1α during re-oxygenation (Fig. 3B). To test whether BAPTA affected proteasomal degradation of HIF-1α, we compared the effect of BAPTA on HIF-1α with the one of the membrane-permeable proteasomal inhibitors, MG132. Inhibition of the proteasome by MG132 resulted in accumulation of high molecular bands that represent polyubiquitinated HIF-1α (33). Neither BAPTA nor hypoxia induced a similar accumulation of polyubiquitinated HIF-1α (Fig. 3C). In addition, MG132 inhibited proteasomal degradation of the short-lived p53, whereas BAPTA and hypoxia hardly affected p53 (Fig. 3C). This effect is most likely due to the stabilizing interaction of p53 with HIF-1α (40). Finally, BAPTA did not further increase HIF-1α in renal clear carcinoma cells lacking functionally active pVHL (Fig. 3D). Therefore, the accumulation of HIF-1α

FIG. 1. Effect of BAPTA and thapsigargin on HIF-1α protein accumulation. SH-SY5Y cells were exposed to normoxic (21% O2) or hypoxic (3% O2) conditions in the presence or absence of the Ca2+ chelator BAPTA-AM (5 μM) or the inhibitor of the endoplasmatic Ca2+-ATPase thapsigargin (10 nM) for the indicated periods (0.5–4 h). Whole cell extracts were used for Western blots for HIF-1α and α-tubulin as a loading control as described under “Experimental Procedures.” Data are representative for comparable results from at least three separate experiments each.

FIG. 2. BAPTA-induced nuclear accumulation of HIF-1α. A, SH-SY5Y cells were incubated with 5 μM BAPTA-AM, and/or 10 nM thapsigargin in normoxic (21% O2) and hypoxic (3% O2) atmosphere for 1 h. Western blot analyses of whole cell extracts (50 μg per lane) for HIF-1α and α-tubulin as a loading control were performed as described under “Experimental Procedures.” Numbers below the bands indicate the fold induction of HIF-1α protein accumulation normalized to α-tubulin compared with control cells under normoxia. B, HIF-1α nuclear accumulation was detected by immunofluorescence analysis. Cells were grown on coverslips, exposed to 21% or 3% O2 for 1 h, fixed, and stained with anti-HIF-1α antibodies as described under “Experimental Procedures.” Fluorescence emission as recorded using fluorescence microscopy is shown in the gray levels. Scale bar, 50 μm. Data are representative for comparable results from at least three separate experiments each.

FIG. 3. BAPTA inhibits the HIF-1α degradation pathway. HIF-1α accumulation was characterized by Western analysis of whole cell protein extracts (70 μg per lane). A, protein synthesis of SH-SY5Y cells was inhibited by addition of cycloheximide (CHX, 10 μg/ml) after incubation with BAPTA-AM (5 μM, 1 h) or CoCl2 (100 μM, 4 h) under normoxia. B, SH-SY5Y cells were preincubated under severe hypoxia (0.1% O2, 1 h) and then re-oxygenated in the absence or presence of BAPTA (50 μg of whole cell protein extracts were loaded per lane). C, SH-SY5Y cells were incubated with BAPTA-AM (5 μM, 1 h), hypoxia (3% O2 or 0.1% O2, 4 h) or with proteasome inhibitor MG132 (10 μM, 2 h) to study the effects of this treatment on proteasomal degradation of HIF-1α and p53. Polyubiquitinated HIF-1α was only detected after treatment with MG132 but not in cells incubated with BAPTA or under hypoxia. D, renal clear carcinoma cells deficient for pVHL (RCC4/VHL) and RCC4 cells reconstituted with pVHL (RCC4/VHL) were incubated with BAPTA-AM (5 μM) under normoxic and hypoxic conditions (3% O2) for 1 h. BAPTA did not further increase HIF-1α in pVHL-deficient cells. Data are representative for results from at least two separate experiments each.
induced by BAPTA under normoxic conditions suggests an inhibition of the HIF-1α degradation pathway mediated by PHDs/pVHL.

**BAPTA Decreases HIF-1α Prolyl Hydroxylase Activity in Vitro**—To study the hypothesis, that the very rapid accumulation of HIF-1α induced by BAPTA under normoxic conditions results from an inhibition of prolyl hydroxylation and subsequent pVHL-dependent proteasomal degradation, as it is known from hypoxic stabilization of HIF-1α protein (14, 41), recombinant PHD2 was incubated with GalDBD-HIF-1α549–582 in the presence or absence of BAPTA. Fe2+ did not attenuate the inhibition of PHD2 by BAPTA (Fig. 4C) indicating that the effect of BAPTA did not result from iron chelation (see also below). To exclude direct effects of BAPTA on the interaction of HIF-1α with pVHL, a synthetic 19-mer hydroxyproline564-ODD was incubated with pVHL. BAPTA, however, had no effect on this interaction (Fig. 4D).

**BAPTA Increases the HIF-1 Binding to a Hypoxia Response Element**—To test for activation of HIF-1α, DNA-binding activity of the HIF-1 complex was studied using nuclear extracts from BAPTA-AM-treated cells (Fig. 5). BAPTA induced HIF-1 DNA-binding activity under normoxic conditions and increased DNA binding under hypoxia (complex i in Fig. 5). In contrast, thapsigargin failed to induce HIF-1-binding activity under normoxia and decreased BAPTA-induced DNA binding. To confirm the presence of HIF-1α in the DNA-bound complex, supershift experiments were performed with monoclonal HIF-1α antibodies that shifted the complete DNA-protein complex to a higher molecular mass (complex ss in Fig. 5).

**BAPTA Enhances the Transcriptional Activity of HIF-1α**—Transcriptional activity of the BAPTA-AM-induced HIF-1 complex was determined in reporter gene assays using the luciferase gene under the control of six HIF-1-binding sites from the erythropoietin 3′ enhancer. To test whether the increase was solely dependent on HIF-1α accumulation (Fig. 1) or whether the trans-activity of HIF-1α was also influenced by BAPTA, the effect of BAPTA on the C-TAD of HIF-1α was tested. BAPTA stimulated HIF-1α transactivation under both for 4 h (Fig. 6A). BAPTA induced a 2.6-fold increase in luciferase activity compared with normoxic controls. Hypoxia alone increased luciferase activity 3.1-fold, whereas BAPTA plus hypoxia showed a 27-fold increase over normoxic controls. Thapsigargin greatly attenuated the stimulatory effect of BAPTA-AM treatment and reduced hypoxic activation. To dissect whether the increase was solely dependent on HIF-1α accumulation (Fig. 1) or whether the trans-activity of HIF-1α was also influenced by BAPTA, the effect of BAPTA on the C-TAD of HIF-1α was tested. BAPTA stimulated HIF-1α transactivation under both...
Calcium Modulates HIF-1α Activity

To prove the critical role of HIF-1α in the up-regulation of CA9 gene expression by hypoxia or BAPTA in SH-SY5Y cells, HIF-1α mRNA and protein were reduced by siRNA treatment against HIF-1α (Fig. 8A). siRNA treatment greatly decreased the hypoxic and the BAPTA-dependent induction of CA9 mRNA levels to values close to those in normoxia (Fig. 8B). Scrambled oligonucleotides confirmed the specificity of the treatment, because they affected neither HIF-1α mRNA levels (Fig. 8A) nor CA9 expression.

Effects of Alterations of the Intracellular Chelatable Iron Concentration on the Fluorescence of OG-BAPTA and PhenGreen SK—Several other chemicals, most prominently iron chelators, can mimic the BAPTA-mediated effects on hypoxic response. Although BAPTA has the highest specificity for Ca^{2+} of all Ca^{2+} chelators, one has to consider that BAPTA might bind Fe^{2+}/Fe^{3+}. To exclude that the effects of BAPTA on HIF-1α action are due to the chelation of intracellular iron, BAPTA was studied in SH-SY5Y cells as well as in a cell-free system for its iron-chelating properties. For these experiments, the fluorescent BAPTA-AM analogue OG-BAPTA-AM (Oregon Green 488-BAPTA-AM) was used and compared with the known iron indicator PhenGreen SK (35, 36, 45). The fluorescence of both indicators was homogenously and comparably distributed inside the cells. The chelation of iron by a fluorescent indicator always leads to a dynamic fluorescence quenching, because both the quantum yield of the indicator as well as the life span of fluorescence are decreased (for review see Ref. 35). Therefore, strong non-fluorescent iron chelators (which remove indicator-bound iron and thus “dequenche” the indicator’s fluorescence (35)) were added to OG-BAPTA-AM-loaded SH-SY5Y cells to study whether the indicator is capable of chelating intracellular labile iron. The addition of the Fe^{2+}-chelator 2,2’-dipyridyl (5 mM, Fig. 9, A and B) and of other iron chelators (2.0 mM 1,10-phenanthroline or 10 mM desferoxamine mesylate; data not shown) to OG-BAPTA-AM-loaded SH-SY5Y cells had no effect on the fluorescence intensity of the indicator, indicating that no chelatable iron was bound to OG-BAPTA within these cells. Because the concentration of chelatable iron in SH-SY5Y cells may be too low to significantly quench OG-BAPTA fluorescence, we increased the concentration of the lipophilic and membrane-permeable Fe^{3+} (1): 8-hydroxyquinoline (2) complex (FeCl_{3}/8-HQ; 10 μM) in the incubation medium (2). The addition of the ferric iron ions, which undergo rapid intracellular reduction to the lipophilic and membrane-permeable Fe^{3+} (1): 8-hydroxyquinoline (2) complex (FeCl_{3}/8-HQ; 10 μM) in the incubation medium (2). The addition of the ferric iron ions, which undergo rapid intracellular reduction to the lipophilic and membrane-permeable Fe^{3+} (1): 8-hydroxyquinoline (2) complex (FeCl_{3}/8-HQ; 10 μM) in the incubation medium (2). The addition of the ferric iron ions, which undergo rapid intracellular reduction to the

Fig. 6. Induction of HIF-1α transcriptional activity by hypoxia and BAPTA. A, SH-SY5Y cells were transfected with pHSV1-Luciferase reporter plasmids containing the luciferase gene under control of six HIF-1 binding sites from the transferrin 3’ enhancer (HRE-luc). Cells were treated with 5 μM BAPTA-AM and/or 10 μM thapsigargin, or solvents for the substances (control) under normoxia (21% O_2) or hypoxia (3% O_2) for 4 h. Luciferase activities were expressed as fold induction compared with untreated control cells (B). Cells were cotransfected with the activator plasmid Gal4-CTAD encoding a fusion protein of the C-terminal transactivation domain of HIF-1α fused to the GAL4 DNA-binding domain and with the GAL4-driven luciferase reporter plasmid Gal4-luc as described (31). Transfected cells were treated as described under A but for 6 h. Luciferase activities were expressed as fold induction compared with untreated control cells. Data are the means ± S.D. of at least three separate culture dishes from one representative experiment. Experiments were repeated at least three times.

Fig. 7. Activation of carbonic anhydrase 9 expression by hypoxia and BAPTA. SH-SY5Y cells were incubated under normoxic (21% O_2) or hypoxic (3% O_2) conditions in the presence of 5 μM BAPTA-AM, 10 μM thapsigargin, or both for 6 h. Real time transcription-PCR was performed for carbonic anhydrase 9 (CA9) cDNA and normalized to 60 S acidic ribosomal protein cDNA as an internal control. Numbers above the bars indicate the fold induction compared with untreated cells kept under normoxia. Data are the means ± S.D. of at least three separate culture dishes from one representative experiment. Experiments were repeated at least three times.
ferrous state, did not decrease the intracellular fluorescence of OG-BAPTA (Fig. 9E). In contrast, and to our surprise FeCl₃/8-HQ led to a strong fluorescence increase (Fig. 9F), which most likely reflected an iron-mediated release of intracellular Ca²⁺/H⁺ (46, 47). These data clearly demonstrate that OG-BAPTA is not able to complex labile iron ions but chelates Ca²⁺/H⁺ ions in SH-SY5Y cells.

In contrast to OG-BAPTA, intracellular PhenGreen SK fluorescence readily increased upon the addition of 2,2'-dipyridyl (5 mM) within 20–30 min (Fig. 9, compare A and B with C and D) indicating that intracellular chelatable iron was bound to the indicator (35, 36, 45). Likewise, addition of the Fe²⁺ chelator 1,10-phenanthroline (2 mM) and the Fe³⁺ chelator desferoxamine mesylate (10 mM) increased PG SK fluorescence (data not shown). As expected and in contrast to OG-BAPTA, the intracellular fluorescence of PG SK was strongly quenched (~80% within 1 min) when the intracellular chelatable iron pool was increased by addition of FeCl₃/8-HQ (10 µM; Fig. 9, G and H). Neither loading of the cells with OG-BAPTA-AM or PG SK, the scanning procedures, nor the addition of the iron chelators and of FeCl₃/8-HQ, respectively, was cytotoxic to SH-SY5Y cells.

![Fig. 8. Specific knock down of HIF-1α prevents CA9 gene induction by hypoxia or BAPTA-AM. A, SH-SY5Y cells were exposed to HIF-1α siRNA duplexes for specific knock down of HIF-1α mRNA or to scrambled siRNA duplexes as controls. Cells were subjected to normoxic (21% O₂) or hypoxic (3% O₂) atmosphere for 6 h. 5 µM BAPTA-AM was added to cells incubated under hypoxia. Real time reverse transcription-PCR was performed as described above. B, the PCR products of HIF-1α or 60 S acidic ribosomal protein cDNAs were separated on agarose gels and stained with ethidium bromide (upper four panels). A representative Western blot illustrates the parallel decrease of HIF-1α protein after 1 h of normoxic or hypoxic incubation (panel 5). C, statistical evaluation of the quantified CA9 cDNA normalized to the cDNA of the 60 S acidic ribosomal protein. siRNA treatment was repeated twice. Data are the means ± S.D. of at least three separate culture dishes.](image8)

![Fig. 9. Effects of alterations in the intracellular chelatable iron concentration on the fluorescence of OG-BAPTA and PG SK in SH-SY5Y cells. Cells were cultured on glass coverslips and loaded either with 5 µM Oregon Green 488 BAPTA-1-AM (OG-BAPTA-AM; A, B, E, and F) or with 20 µM fluorescent iron indicator PhenGreen SK (PG SK; C, D, G, and H) in HBSS (37 °C) as described under “Experimental Procedures.” The intracellular fluorescence of OG-BAPTA and PG SK, respectively, was imaged using laser-scanning microscopy (λ_ex = 488 nm; λ_em ≈ 505 nm). After establishing the baseline fluorescence (10 min), images were collected (A and C) before addition of the iron chelator 2,2'-dipyridyl (2,2'-DPD, 5 mM) and (B and D) after complete equilibration (20 min later) following addition of 2,2'-DPD, or (E and G) before and (F and H) 1 min after the addition of 10 µM of the highly membrane-permeable Fe³⁺-8-hydroxyquinoline complex. Bar in G indicates 50 µm. Pictures are representative for three series of experiments.](image9)
Using the interaction assay, further experiments were performed with cofactors (1 mM ascorbate, 1 mM fluorescent intensity of OG-BAPTA (5 μM) in the cytosolic medium). This indicates that the iron-binding site of OG-BAPTA cannot compete with the chelating ligands in the "cytosolic" medium (e.g. citrate, phosphate, ATP, and ascorbate), i.e. those that have been reported to form iron chelates within cells (35). In addition, we studied the chelating properties of OG-BAPTA under the conditions of the in vitro interaction assay (see Fig. 4). In contrast to the quenching effect in a simple buffered solution, the addition of chelatable iron ions had no effect on the fluorescence of OG-BAPTA when the indicator was dissolved in a medium composed to simulate the cytosol (Fig. 10A). This indicates that the iron-binding site of OG-BAPTA cannot compete with the chelating ligands in the "cytosolic" medium (e.g. citrate, phosphate, ATP, and ascorbate), i.e. those that have been reported to form iron chelates within cells (35). In addition, we studied the chelating properties of OG-BAPTA under the conditions of the in vitro interaction assay (see Fig. 4). In contrast to the quenching effect in a simple buffered solution, the addition of chelatable iron ions had no effect on the fluorescence of OG-BAPTA in the "interaction medium" of the PHD assay containing reaction buffer supplemented with cofactors (1 mM ascorbate, 1 mM α-ketoglutarate, 20 μM FeCl₂) and reticulocyte lysate. Moreover, addition of the Fe³⁺ chelator 1,10-phenanthroline to the "interaction medium" did not increase OG-BAPTA fluorescence indicating that no intracellular chelatable iron was bound to the indicator under these conditions (Fig. 10B).

**Effects of Acute Hypoxia on Intracellular Calcium Levels in SH-SY5Y Cells**—Because Ca²⁺ chelation by BAPTA mimicked hypoxic HIF-1 activation, we raised the question whether decreased O₂ concentrations affect the intracellular Ca²⁺ level in SH-SY5Y cells. SH-SY5Y cells loaded with the fluorescent Ca²⁺ indicator OG-BAPTA-AM were exposed to hypoxia while the intracellular indicator fluorescence and the pO₂ in the medium were simultaneously recorded. Acute exposure to hypoxia up to 120 min (3% to 0.2% O₂) did not change the basal overall Ca²⁺ level of the cells under conditions (Fig. 11, A and B) when HIF-1α protein accumulation was already induced. The addition of BAPTA-AM readily decreased the intracellular OG-BAPTA fluorescence, which was increased by thapsigargin, indicating that the indicator well responded to changes in intracellular Ca²⁺ levels under the present experimental conditions (Fig. 11C). In further experiments, Ca²⁺ measurements using the indicator OG-BAPTA or Fura-2 confirmed that total cellular Ca²⁺ levels were not altered during 4 h of prolonged hypoxic superfusion (data not shown). However, microfluorographs with subcellular resolution revealed changes in the intracellular distribution of Ca²⁺ during hypoxia Ca²⁺ levels gradually increased within the nuclei, whereas they slightly decreased within the cytosol (Fig. 11D).

**DISCUSSION**

Calcium-dependent signaling is known to affect the expression of hypoxia-inducible genes (26, 27, 49). However, very little is known to what extent induction of hypoxia-inducible factor-1 (HIF-1), the master regulator of oxygen homeostasis, is affected by changes in intracellular Ca²⁺ concentrations. Herein, for the first time we provide evidence that lowering intracellular Ca²⁺ levels can induce HIF-1α accumulation, activation and HIF-1 target gene expression.

In contrast, however, and in contrast to the quenching effect in a simple buffered solution, the addition of chelatable iron ions had no effect on the fluorescence of OG-BAPTA when the indicator was dissolved in a medium composed to simulate the cytosol (Fig. 10A). This indicates that the iron-binding site of OG-BAPTA cannot compete with the chelating ligands in the "cytosolic" medium (e.g. citrate, phosphate, ATP, and ascorbate), i.e. those that have been reported to form iron chelates within cells (35). In addition, we studied the chelating properties of OG-BAPTA under the conditions of the in vitro interaction assay (see Fig. 4). In contrast to the quenching effect in a simple buffered solution, the addition of chelatable iron ions had no effect on the fluorescence of OG-BAPTA in the "interaction medium" of the PHD assay containing reaction buffer supplemented with cofactors (1 mM ascorbate, 1 mM α-ketoglutarate, 20 μM FeCl₂) and reticulocyte lysate. Moreover, addition of the Fe³⁺ chelator 1,10-phenanthroline to the "interaction medium" did not increase OG-BAPTA fluorescence indicating that no intracellular chelatable iron was bound to the indicator under these conditions (Fig. 10B).
Interestingly, Oehme et al. (51) have described a putative PHD-4 that possesses an EF-hand motif indicating Ca\(^{2+}\)-dependent regulatory functions. However, further experiments are necessary to determine whether active Ca\(^{2+}\)-binding sites exist in PHDs and whether these binding sites influence the activity of prolyl hydroxylases. Because BAPTA also increased the activity of the C-TAD of HIF-1\(\alpha\), it is very likely that FIH-1 was inhibited like the PHDs, although this was not explicitly tested in this study.

Nuclear accumulation of HIF-1\(\alpha\) by BAPTA was accompanied by increased binding of the HIF-1 complex to DNA (Fig. 5) and activation of the HRE-driven reporter gene (Fig. 6). Again, this effect of BAPTA is most likely related to its Ca\(^{2+}\)-chelating capacity, because in both assays co-treatment with thapsigargin...
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From the in vivo-like conditions fluorescence of OG-BAPTA became insensitive to iron ions (Fig. 10A). Further support of a specific Ca$^{2+}$-effecting role is provided by the experiments using the “interaction medium” of the PHD assay (Fig. 10B). Collectively, HIF-1α induction by BAPTA most likely results from chelation of intracellular free Ca$^{2+}$ but not iron ions and is thus clearly different from the hypoxia mimicking effects of desferoxamine or 2,2′-dipyridyl.

Herein, we provide evidence that lowering intracellular Ca$^{2+}$ concentration activates HIF-1 through inhibition of hydroxyla-
tion of HIF-1α. In that way, BAPTA acts like iron chelators (13) or NO donors (33) that cause accumulation of HIF-1α protein through inhibition of prolyl hydroxylases and thus mimic hypoxic signaling. Interestingly, our data indicate that total cellular Ca$^{2+}$ levels were not affected during 120 min of acute or up to 4 h of prolonged hypoxic exposure, although HIF-1α levels were already increased (Fig. 11). Instead we found an intracellular change in the distribution of Ca$^{2+}$ between the cytosolic and the nuclear compartment. Ca$^{2+}$ levels increased within the nuclei most prominently after 4 h of hypoxia resulting in decreased cytosolic levels, whereas the overall Ca$^{2+}$ did not change. Because PHD2 is mainly localized within the cytosol (56), a decrease in Ca$^{2+}$ availability may be a physiological factor, that modulates PHD activity. In this context, the effect of BAPTA could mimic this change in cellular Ca$^{2+}$ distribution by chelating Ca$^{2+}$ within the cytosolic compartment. On the other hand, however, PHD1 and -3 are localized within the nucleus where Ca$^{2+}$ may be essential for their activity during reoxygenation subsequent to hypoxia. Therefore, the Ca$^{2+}$ dependence of the regulation of PHDs and, consequently, gene expression needs to be studied in future projects.

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