The hypoxia-inducible transcription factors (HIFs)\(^2\) play a central role in the regulation of oxygen homeostasis. Their target genes have important roles in many physiological and pathological events such as angiogenesis, vascular remodeling, erythropoiesis, glucose utilization, iron transport, cell proliferation, survival and apoptosis, and tumor progression (1–3). HIFs are \(a\beta\) heterodimers, the human \(\alpha\) subunit having three isoforms, HIF-1\(\alpha\) to HIF-3\(\alpha\) (1–3). HIF-1\(\alpha\) and HIF-2\(\alpha\) are synthesized constitutively, and hydroxylation of at least one of two critical proline residues mediates their interaction with the von Hippel-Lindau (VHL) E3 ubiquitin-ligase complex, which targets them for rapid proteasomal degradation under normoxic conditions (4–6). This hydroxylation is catalyzed in humans by three cytoplasmic and nuclear HIF prolyl 4-hydroxylases (HIF-P4Hs, also known as PHDs 1–3, HPHs 3-1, and EGLNs 2, 1, and 3, respectively) (7–9), which are distinct from the well-characterized endoplasmic reticulum luminal collagen P4Hs (C-P4Hs) (10, 11). All P4Hs are 2-oxoglutarate dioxygenases and require Fe\(^2+\), 2-oxoglutarate, \(O_2\), and ascorbate (1, 10). Even small decreases in the \(O_2\) concentration (12, 13) will inhibit the activities of the HIF-P4Hs so that HIF-1\(\alpha\) and HIF-2\(\alpha\) escape degradation and dimerize with HIF-\(\beta\), this dimer then recognizing the HIF-responsive elements in numerous hypoxia-regulated target genes such as those for vascular endothelial growth factor (VEGF), erythropoietin (Epo), glucose transporters, and glycolytic enzymes (1–5).

Transcriptional activation in an oxygen-dependent manner is another key event that regulates HIF activity. Hydroxylation of a critical asparagine residue in the C-terminal transactivation domain of HIF-\(\alpha\) blocks its interaction with the transcriptional coactivator p300 (14). The asparaginyl hydroxylase catalyzing this modification is identical to a protein known as factor inhibiting HIF (FIH), this dimer then recognizing the HIF-responsive elements in numerous hypoxia-regulated target genes such as those for vascular endothelial growth factor (VEGF), erythropoietin (Epo), glucose transporters, and glycolytic enzymes (1–5).

Inhibition of hypoxia-inducible Factor (HIF) Hydroxylases by Citric Acid Cycle Intermediates

POSSIBLE LINKS BETWEEN CELL METABOLISM AND STABILIZATION OF HIF

Peppi Koivunen\(^5\), Maija Hirsilä\(^5\), Anne M. Remes\(\|\), Ilmo E. Hassinen\(\&\), Kari I. Kivirikko\(^5\)\(\&\), and Johanna Myllyharju\(\&\)

From the \(\&\)Collagen Research Unit, \(\&\)Biocenter Oulu, Departments of \(\&\)Medical Biochemistry and Molecular Biology and \(\|\)Neurology, University of Oulu, FIN-90014 Oulu, Finland

The stability and transcriptional activity of the hypoxia-inducible factors (HIFs) are regulated by two oxygen-dependent events that are catalyzed by three HIF prolyl 4-hydroxylases (HIF-P4Hs) and one HIF asparaginyl hydroxylase (FIH). We have studied possible links between metabolic pathways and HIF hydroxylases by analyzing the abilities of citric acid cycle intermediates to inhibit purified human HIF-P4Hs and FIH. Fumarate and succinate were analyzed as inhibitors of all three HIF-P4Hs, fumarate having \(K_i\) values of 50–80 \(\mu\)M and succinate 350–460 \(\mu\)M, whereas neither inhibited FIH. Oxaloacetate was an additional inhibitor of all three HIF-P4Hs with \(K_i\) values of 400–1000 \(\mu\)M and citrate of HIF-P4H-3, citrate being the most effective inhibitor of FIH with a \(K_i\) of 110 \(\mu\)M. Culturing of cells with fumarate diethyl or dimethyl ester, or a high concentration of monoethyl ester, stabilized HIF-1\(\alpha\) and increased production of vascular endothelial growth factor and erythropoietin. Similar, although much smaller, changes were found in cultured fibroblasts from a patient with fumarate hydratase (FH) deficiency and upon silencing FH using small interfering RNA. No such effects were seen upon culturing of cells with succinate diethyl or dimethyl ester. As FIH was not inhibited by fumarate, our data indicate that the transcriptional activity of HIF is quite high even when binding of the coactivator p300 is prevented. Our data also support recent suggestions that the increased fumarate and succinate levels present in the FH and succinate dehydrogenase-deficient tumors, respectively, can inhibit the HIF-P4Hs with consequent stabilization of HIF-\(\alpha\)s and effects on tumor pathology.

\(^2\) The abbreviations used are: HIF, hypoxia-inducible transcription factor; VHL, von Hippel-Lindau; HIF-P4H, HIF prolyl 4-hydroxylase; C-P4H, collagen prolyl 4-hydroxylase; VEGF, vascular endothelial growth factor; Epo, erythropoietin; FIH, HIF asparaginyl hydroxylase; SDH, succinate dehydrogenase; FH, fumarate hydratase; siRNA, small interfering RNA; HEK293, human embryonic kidney 293.

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\(^1\) To whom correspondence should be addressed: P. O. Box 5000, University of Oulu, FIN-90014 Oulu, Finland. Tel.: 358-8-537-5740; Fax: 358-8-537-5811; E-mail: Johanna.myllyharju@oulu.fi.

\(^2\) The abbreviations used are: HIF, hypoxia-inducible transcription factor; VHL, von Hippel-Lindau; HIF-P4H, HIF prolyl 4-hydroxylase; C-P4H, collagen prolyl 4-hydroxylase; VEGF, vascular endothelial growth factor; Epo, erythropoietin; FIH, HIF asparaginyl hydroxylase; SDH, succinate dehydrogenase; FH, fumarate hydratase; siRNA, small interfering RNA; HEK293, human embryonic kidney 293.
ing of cancer cells with 20 mM succinate dimethyl ester for 48 h (18) or with 2.5 mM succinate diethyl ester or 0.5 mM fumarate monoethyl ester together with 3-nitropropionic acid (19), a dual inhibitor of succinate dehydrogenase (SDH) and fumarate hydratase (FH), led to stabilization of HIF-1α, and a similar effect was seen upon silencing of SDH or FH by the corresponding siRNA (18, 19). Oxaloacetate and pyruvate have likewise been reported to stabilize HIF-1α in cultured cancer cell lines and inactivate HIF-P4Hs in a manner reversible by ascorbate (20, 21).

We have studied possible links between metabolic pathways and HIF hydroxylases by systematically analyzing the abilities of citric acid cycle intermediates and other aliphatic 2-oxoglutarate analogues to inhibit purified human HIF-P4Hs and FIH under initial velocity conditions and in the presence of saturating peptide substrate concentrations in vitro. Fumarate and succinate were identified as inhibitors of all three HIF-P4Hs, fumarate having 

\[ K_i = 50 - 80 \mu M \]

and succinate 350–460 

\[ \mu M, \]

whereas neither inhibited FIH to any significant extent even at a 10 mM concentration. Oxaloacetate was an additional inhibitor of all three HIF-P4Hs with 

\[ K_i = 400 - 1000 \mu M \]

and citrate was an inhibitor of HIF-P4H-3 with a 

\[ K_i = 180 \mu M, \]

whereas pyruvate and many other 2-oxoglutarate analogues showed no inhibition of any of the HIF-P4Hs even at a 10 mM concentration, variations in ascorbate levels having no effect on inhibition by oxaloacetate or pyruvate. FIH was inhibited most effectively by citrate and oxaloacetate with 

\[ K_i = 110 \] and 400 

\[ \mu M, \]

respectively. Our further experiments using culturing of cells with fumarate diethyl ester or dimethyl ester, which pass through cell membranes, led to stabilization of HIF-1α and to increases in VEGF production in all the cell types studied and in Epo production in human hepatocellular carcinoma Hep3B cells. Similar, although much smaller, changes were found in cultured skin fibroblasts from a patient with autosomal recessive FH deficiency and in cells transfected with FH siRNA. No such effects were seen upon culturing of cells with succinate diethyl ester or dimethyl ester.

**MATERIALS AND METHODS**

**Expression, Purification, and Activity Assays of Recombinant HIF-P4Hs and FIH—**FLAG His-tagged HIF-P4Hs 1–3 and FIH (17, 22) were expressed using recombinant baculoviruses in H5 insect cells cultured in suspension or on plates in SF900II SFM serum-free medium (Invitrogen). The cells, seeded at a density of 

\[ 1 \times 10^6/\text{ml}, \]

were infected at a multiplicity of 5 and harvested 72 h after infection, washed with a solution of 0.15 M NaCl and 0.02 M phosphate, pH 7.4, and homogenized in a 0.1 M NaCl, 0.1 M glycine, 10 mM dithiothreitol, 0.1% Triton X-100, and 0.01 M Tris buffer, pH 7.8. The soluble fractions were subjected to purification with an anti-FLAG M2 affinity gel (Sigma) (17, 22). HIF-P4H and FIH activities were assayed by methods based on measurement of the hydroxylated-coupled stoichiometric release of 

\[ ^{14} \text{CO}_2 \]

from 2-oxo-[1-14C]glutarate with synthetic HIF-1α peptides as substrates and the 

\[ K_{i50} \]

and IC_{50} values of the purified enzymes were determined as described previously (12, 17, 22). This method makes it possible to measure HIF-P4H activities under true initial velocity conditions and using saturating peptide substrate concentrations, and therefore the values obtained are often somewhat different from those obtained in assays involving capture of hydroxylated HIF-α or a fragment of this by pVHL. (Pro-Pro-Gly)_{10} was used as the substrate for a purified recombinant human type 1 C-P4H (23). The 

\[ K_i \]

values were determined by adding the 2-oxoglutarate analogues at four constant concentrations while varying the concentration of 2-oxoglutarate or the peptide substrate.

**Stabilization of HIF-1α by Diethyl Fumarate and Dimethyl Fumarate in Cultured Cells—**Human embryonic kidney (HEK293), hepatocellular carcinoma (Hep3B), and neuroblastoma (Kelly) cells, and fibroblasts (CRL-2086) were obtained from the American Type Culture Collection and cultured in Dulbecco’s (Biochrom) or Eagle’s (Sigma) minimal essential medium or in RPMI 1640 (Sigma) medium with 10% fetal bovine serum (BioClear) and 50 µg/ml ascorbate. They were then subcultured at a density of 

\[ 1 \times 10^6 \]

or 

\[ 1.6 \times 10^6 \]

cells/cm² in the case of fibroblasts, grown overnight, and incubated for 8–20 h with increasing concentrations of diethyl fumarate, dimethyl fumarate, monoethyl fumarate, diethyl succinate, or dimethyl succinate (Sigma). In control experiments the cells were treated with 300–500 

\[ \mu M \]

cobalt or subjected to hypoxia by culturing in an airtight incubator (Billups-Rothenberg) under 1% O₂ balanced with N₂. The viability of cells was determined using trypan blue dye exclusion. Skin fibroblasts from a patient with a homozygous loss of function mutation in the FH gene (24) and fibroblasts from a healthy control were cultured in Dulbecco’s medium with 10% fetal bovine serum. Medium samples were collected for analysis of the production of VEGF and Epo (see below) and total cell extracts were obtained by lysing the cells in 150 mM NaCl, 0.1% SDS, and 20 mM Tris-HCl, pH 6.8. Nuclear extracts were prepared by the NE-PER nuclear extraction reagent (Pierce). The extracts were analyzed by 8% SDS-PAGE under reducing conditions followed by Western blotting with a monoclonal antibody against human HIF-1α (BD Biosciences) and ECL™ immunodetection. Antibodies to α tubulin (total extracts, Sigma) and β actin (nuclear extracts, Novus Biologicals) were used as loading controls.

**RNA Interference—**Four siRNAs of the FH coding sequence (set of 4 siGENOMETM duplexes, Dharmacon) were transfected at 75 nM concentration into HEK293 cells using Lipofectamine™ 2000 (Invitrogen) according to the manufacturer’s instructions. The Silencer™ GAPDH and Silencer negative control siRNAs (Ambion) were used as controls. Medium samples were collected 48 h after transfection, and total RNA and protein were isolated from the cells using the PARIS™ kit (Ambion). The ability of FH siRNAs to down-regulate FH mRNA was studied by semiquantitative reverse transcriptase-PCR using the SMART™ PCR cDNA synthesis kit (BD Biosciences). PCR was carried out under the following conditions: 1 min at 94 °C followed by 22 cycles of 1 min at 94 °C, 1 min at 60 °C, and 1 min at 72 °C. The primers used for amplification were: 5’-GGAGGTGTGACAAAGCCATGC-GCACC-3’ and 5’-GCTGCTTGTCAACCTATATGAGG-3’. Stabilization of HIF-1α was analyzed by Western blotting of the protein samples as described above.

**Analysis of the Production of VEGF and Epo—**The VEGF and Epo concentrations in the medium samples were measured using the Quantikine human VEGF and Epo immunoassays.
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TABLE 1

| Compound          | Constant | HIF-P4H-1 | HIF-P4H-2 | HIF-P4H-3 | C-P4H-1 | FIH |
|-------------------|----------|-----------|-----------|-----------|---------|-----|
| 2-Oxoglutarate    | IC<sub>50</sub> | 120 ± 10  | 80 ± 30  | 50 ± 15  | ND      | ND  |
| Fumarate          | IC<sub>50</sub> | 830 ± 540 | 510 ± 310| 570 ± 170| ND      | ND  |
| Succinate         | IC<sub>50</sub> | 350 ± 20  | 460 ± 70 | 430 ± 200| 400<sup>b</sup> | ND  |
| Oxaloacetate      | IC<sub>50</sub> | 1000 ± 420| 3800 ± 1400 | 1200 ± 350 | ND  | 400 ± 40 |
| Citrate           | IC<sub>50</sub> | 400 ± 130 | 1000 ± 50 | 590 ± 120 | 100<sup>b</sup> | ND  |

* Values are mean ± S.D. from three to ten independent experiments.

<sup>a</sup> The IC<sub>50</sub> values of HIF-P4Hs 1, 2, and 3 for pyruvate, malate, isocitrate, 2-oxoadipinate, 2-oxovalerate, 2-oxobutyrate, adipinate, glutarate, malonate, 3-oxoglutarate, levulinate, and lactate were at least 10 mM, except those of HIF-P4H-3 for glutarate and HIF-P4H-2 for 3-oxoglutarate, which were 6 and 5 mM, respectively. Similar values were obtained from at least two independent experiments.

<sup>b</sup> The IC<sub>50</sub> value of FIH for isocitrate was about 5 mM, those for all the other compounds being at least 10 mM. Similar values were obtained from at least two independent experiments.

<sup>c</sup> Ref. 27.

<sup>d</sup> Ref. 17.

<sup>e</sup> Ref. 43.

<sup>f</sup> Ref. 26.

<sup>g</sup> Ref. 27.

(R&D Systems) according to the manufacturer’s instructions. Statistical analyses were performed with SPSS 14.0 for Windows.

Determination of Cellular Fumarate Concentrations—Equal numbers of HEK293 cells were incubated in the absence and presence of 40 μM diethyl fumarate for 20 h, the culture medium was carefully removed, and the cell pellets were immediately cooled in liquid N<sub>2</sub>. The frozen pellets were lysed in 8% cold perchloric acid and centrifuged. The supernatant was collected and the remaining pellet was washed and extracted with 6% perchloric acid and centrifuged. The supernatants were pooled and neutralized by K<sub>2</sub>CO<sub>3</sub> and used to determine the amount of fumarate with fumarase, malate dehydrogenase, and aspartate aminotransferase (Sigma) with a Fluoromax-2 spectrophuorometer (HORIBA Jobin Yvon, Inc., Edison, NJ) as described previously (25) with the exception that glycyl glycine, pH 9, supplemented with glutamic acid was used as a buffer. The maleate present in the commercial fumarase preparation was removed by gel filtration in a PD-10 column (GE Healthcare). The excitation wavelength was 340 nm and emission was recorded at 460 nm. Samples of skin fibroblasts from a patient with a homozygous FH mutation and from a healthy control were prepared and analyzed as above.

RESULTS

Inhibition of HIF-P4Hs and FIH by Citric Acid Cycle Intermediates and Certain Other Aliphatic 2-Oxoglutarate Analogues—FLAG His-tagged human HIF-P4Hs 1–3 and FIH were expressed in insect cells, which were harvested 72 h after infection, homogenized in a buffer containing Triton X-100, and centrifuged. The recombinant enzymes were purified from the soluble fraction by anti-FLAG affinity chromatography and their activities were assayed by methods based on measurement of the hydroxylation-coupled stoichiometric release of 14<sup>C</sup>CO<sub>2</sub> from 2-oxo-[1-14C]glutarate (12, 17, 22). The K<sub>m</sub> values of the purified HIF-P4Hs for 2-oxoglutarate were found to be 1–2 μM for isoenzymes 1 and 2, and 12 μM for isoenzyme 3 (Table 1), these values being distinctly lower than those previously reported for the HIF-P4Hs in crude insect cell extracts (12).

Inhibition of the purified recombinant HIF-P4Hs and FIH was studied using six citric acid cycle intermediates and 10 other aliphatic 2-oxoglutarate analogues (Table 1). The IC<sub>50</sub> values of the HIF-P4Hs and FIH were first determined in the presence of 10 and 50–100 μM 2-oxoglutarate, respectively. Fumarate was found to be the most efficient inhibitor of the three HIF-P4Hs, with IC<sub>50</sub> values of 120, 80, and 60 μM for HIF-P4Hs 1, 2, and 3, respectively, whereas succinate had IC<sub>50</sub> values of 830, 510, and 570 μM (Table 1). The IC<sub>50</sub> values of HIF-P4Hs 1, 2, and 3 for oxaloacetate were 1, 3.8, and 1.2 mM, respectively, and those for citrate 6.3, 4.8, and 0.55 mM, whereas the IC<sub>50</sub> values for pyruvate, malate, isocitrate, 2-oxoadipinate, 2-oxovalerate, 2-oxobutyrate, adipinate, glutarate, malonate, 3-oxoglutarate, levulinate, and lactate were at least 10 mM for all three isoenzymes, except that glutarate had an IC<sub>50</sub> of about 6 mM for HIF-P4H-3 and 3-oxoglutarate had an IC<sub>50</sub> of about 5 mM for HIF-P4H-2 (Table 1).

FIH was inhibited most effectively by citrate, with an IC<sub>50</sub> of 850 μM (Table 1). The IC<sub>50</sub> values of FIH for oxaloacetate and isocitrate were about 1.4 and 5 mM, respectively, whereas those of all the other compounds tested, including fumarate, succinate, and pyruvate were at least 10 mM (Table 1).

Inhibition of the HIF-P4Hs by fumarate, succinate, oxaloacetate, and citrate, the compounds with the lowest IC<sub>50</sub> values among those tested, and of FIH by citrate and oxaloacetate, its most effective inhibitors, was analyzed in more detail. The mode of inhibition and K<sub>i</sub> values were determined by adding fumarate, succinate, oxaloacetate, or citrate at four constant concentrations while varying the concentration of 2-oxoglutarate or the peptide substrate. The inhibition was found to be competitive with respect to 2-oxoglutarate (as shown for fumarate with HIF-P4Hs 2 and 3, succinate with HIF-P4H-1, and citrate with FIH in Fig. 1, A–D) and noncompetitive with respect to the peptide substrate (data not shown). The K<sub>i</sub> values for HIF-P4Hs 1, 2, and 3 for fumarate were 80, 60, and 50 μM, respectively, and those for succinate 350, 460, and 430 μM, whereas the values for oxaloacetate ranged from 400 to 1000 μM and the K<sub>i</sub> values of HIF-P4H-2 and HIF-P4H-3 for citrate...
were 1800 and 180 μM, respectively (Table 1). The $K_i$ values of FIH for citrate and oxaloacetate were 110 and 400 μM, respectively, whereas those for fumarate and succinate were more than 10 mM (Table 1).

Succinate, oxaloacetate, and citrate have also been reported (26, 27) to inhibit the C-P4Hs, their $K_i$ values for the C-P4H isoenzyme I being 400, 100, and 450 μM, respectively (Table 1). Our current data indicate that fumarate is an additional C-P4H-I inhibitor, with a $K_i$ of 190 μM (Table 1).

As oxaloacetate and pyruvate have been reported to inhibit the HIF-P4Hs in an in vitro VHL capture assay in the presence of nonsaturating ascorbate levels (30–100 μM) (20, 21), we also studied inhibition of the HIF-P4Hs by these two compounds in the presence of varying ascorbate concentrations. However, the IC$_{50}$ values obtained under these conditions were identical to those determined in the presence of 2 mM ascorbate (details not shown).

Stabilization of HIF-1α by Fumarate Diethyl and Dimethyl Esters in Cultured Cells—To study whether fumarate and succinate stabilize HIF-1α, we cultured human embryonic kidney (HEK293), hepatocellular carcinoma (Hep3B), and neuroblastoma (Kelly) cells and fibroblasts (CRL-2086) for 20 h in the presence of increasing concentrations of the membrane-permeable fumarate diethyl, dimethyl or monoethyl ester, or succinate diethyl or dimethyl ester, all of which are converted to fumarate or succinate by the action of cellular esterases (28, 29). These compounds gave no inhibition of purified HIF-P4Hs in vitro even at 10 mM concentrations (details not shown). In control experiments, cells were treated with cobalt or cultured under hypoxia. Total cell extracts and nuclear extracts were prepared and analyzed by 8% SDS-PAGE under reducing conditions followed by Western blotting with a HIF-1α antibody and an α tubulin antibody as a loading control. Stabilization of HIF-1α was seen in total extracts from the HEK293, Hep3B, and Kelly cells and fibroblasts with 20–100, 10–100, 40–120, and 100–150 μM diethyl fumarate, respectively (Fig. 2, A–D), higher concentrations leading to cell detachment (as shown by decreased staining for α tubulin). Similar results were obtained with dimethyl fumarate (as shown for HEK293 cells in Fig. 2E), whereas no stabilization of HIF-1α was seen with succinate diethyl or dimethyl ester in a concentration range of 50 μM to 5 mM (as shown for succinate dimethyl ester in HEK293 cells in Fig. 2F). Stabilization of HIF-1α by diethyl fumarate was also seen in nuclear extracts (as shown for the HEK293, Hep3B, and Kelly cells in Fig. 3, A–C). Fumarate monoethyl ester also stabilized HIF-1α (as shown for nuclear extracts from HEK293 cells in Fig. 3D), but the concentration required for a detectable stabilization was 5 mM, being thus about 2 orders of magnitude lower than that required for fumarate diethyl.
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Stabilization of HIF-1α by fumarate diethyl and monoethyl esters in cultured cells. HEK293 (A), Hep3B (B) and Kelly cells (C), and fibroblasts (D) were incubated for 20 h with increasing concentrations (µM, indicated below the lanes) of membrane-permeable fumarate diethyl ester. HEK293 cells were also incubated with increasing concentrations of fumarate dimethyl ester (E) and succinate dimethyl ester (F). Stabilization of HIF-1α in cells cultured under hypoxic conditions (lanes H, 1% O2) is also shown. Total cell extracts were analyzed by 8% SDS-PAGE under reducing conditions followed by Western blotting with HIF-1α and α-tubulin antibodies and ECL immunodetection. The exposure times of lanes H are 10, 4, 12, and 20 times shorter than those of other lanes in A–D, respectively. Similar results were obtained from at least three independent experiments.

Stabilization of HIF-1α in cultured fibroblasts from a patient with autosomal recessive FH deficiency. Skin fibroblasts from a patient with a homozygous Q376P mutation in the FH gene (FH+/−) and control fibroblasts (FH−/−) were cultured under normoxia (N) and hypoxia (H, 1% O2). Total cell extracts were analyzed by 8% SDS-PAGE under reducing conditions followed by Western blotting with HIF-1α and α-tubulin antibodies and ECL immunodetection. The exposure times of lanes H detected by the HIF-1α antibody are 10 times shorter than those of lanes N. Similar results were obtained from at least three independent experiments.

In further experiments endogenous FH was silenced by transfecting HEK293 cells with FH-specific siRNAs. FH mRNA level, analyzed by reverse transcriptase-PCR, was found to be lower in the FH siRNA-transfected cells than control-transfected ones (Fig. 5A), and a very weak but reproducible stabilization of the HIF-1α was seen in the FH siRNA-transfected cells (Fig. 5B).

Increased VEGF Production by Diethyl Fumarate, Dimethyl Fumarate, and FH Deficiency in Cultured Cells—To study whether the stabilized HIF-1α caused increased expression of a HIF target gene, we assayed the amount of VEGF in medium samples collected from cells cultured in the presence of increasing diethyl fumarate concentrations in 3 to 5 independent experiments. Dose-dependent increases were found in VEGF production, the highest mean values being 640% with 80 µM diethyl fumarate in HEK293 cells, 250% with 100 µM in Hep3B higher than the concentration of fumarate diethyl ester required for a similar extent of stabilization (compare with Fig. 3A).

Stabilization of HIF-1α in Cultured Fibroblasts from a Patient with Autosomal Recessive FH Deficiency and in HEK293 Cells Transfected with FH siRNA—To study whether lack of endogenous FH leads to stabilization of HIF-1α, skin fibroblasts from a patient with a homozygous Q376P mutation in the FH gene (24) were cultured and analyzed as above. The level of FH activity in the mitochondrial and cytosolic fractions of cultured fibroblasts from this patient is only 0.5% of the control mean (24). A very weak but distinct stabilization of HIF-1α was seen in extracts from these cells cultured under normoxia in three independent experiments, whereas no HIF-1α was detected in control fibroblasts (Fig. 4). HIF-1α was stabilized to an equal extent in the FH mutant cells and in control fibroblasts cultured under hypoxia (Fig. 4).
cells, 240% with 20 μM in Kelly cells, and 210% with 100 μM in fibroblasts as compared with that in nontreated cells (as shown for HEK293, Hep3B, and Kelly cells in Fig. 6, A–C). The highest mean values seen in various cells range from about 30 to 100% of those obtained under 1% O2 in the same cells, the increases in VEGF production by diethyl fumarate thus being much larger than those seen in the extent of stabilization of HIF-1α (Fig. 2). A similar effect was seen with dimethyl fumarate, although the magnitude of the increase was smaller (as shown for HEK293 cells in Fig. 6D). The different levels obtained are probably due in part to differences in the uptake of diethyl fumarate and dimethyl fumarate and their conversion to fumarate in various cell types, in part to differences in the detachment of cells at high diethyl and dimethyl fumarate concentrations (see the decreased staining for α tubulin in Fig. 2) and in part to differences in the abilities of various cell types to produce VEGF (22).

No increase in VEGF production was found with diethyl succinate or dimethyl succinate when studied in a concentration range of 50 μM to 200 μM (details not shown).

To study the temporal relation of cell viability, HIF-1α stabilization, and VEGF production in the presence of increasing concentrations of diethyl fumarate, a time course analysis was performed with HEK293 cells. The viability of cells treated with 40 μM diethyl fumarate was 93–95% at 12 and 20 h, no difference in viability being seen between the two time points (data not shown). The viability of cells treated with 100 μM diethyl fumarate was likewise 94% at 12 h, but decreased to 83% at 20 h. Stabilization of HIF-1α was seen in total extracts from cells cultured in the presence of 10–100 μM diethyl fumarate for 8 h, the amount of stabilized HIF-1α increasing at 12 and 20 h (Fig. 7, A–C). The amount of VEGF secreted into the culture medium increased in a time- and dose-dependent manner (Fig. 7D).

Additional experiments demonstrated that the stabilized HIF-1α also caused increased VEGF production in the fibroblasts from the patient with autosomal recessive FH deficiency and a small increase in HEK293 cells transfected with FH siRNA (above). The amount of VEGF in the medium of FH−/− fibroblasts was 170% (p < 0.05) of that in the medium of control fibroblasts (Fig. 6F), and the amount in the medium of the siRNA-transfected HEK293 cells was 140% of that in the medium of the non-transfected cells, although the last mentioned increase was not statistically significant (data not shown).

Increased Fumarate Concentrations in HEK293 Cells Cultured with Diethyl Fumarate and FH Mutant Skin Fibroblasts—Cellular fumarate concentration was measured in HEK293 cells cultured with 40 μM diethyl fumarate for 20 h and in cultured skin fibroblasts from a patient with autosomal recessive FH deficiency. Control experiments indicated that the
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**FIGURE 7.** Time course analysis of HIF-1α stabilization and VEGF production in HEK293 cells at increasing diethyl fumarate concentrations. Total extracts from HEK293 cells incubated with increasing concentrations (μM, indicated below the lanes) of diethyl fumarate for 8 (A), 12 (B), and 20 h (C) were analyzed by 8% SDS-PAGE under reducing conditions followed by Western blotting with HIF-1α and α-tubulin antibodies and ECL immunodetection. Similar results were obtained in at least three independent experiments. The amount of VEGF was measured in the corresponding medium samples by immunoassay (D). In control experiments the cells were subjected to hypoxia (H, 1% O2). The values are mean ± S.D. from at least three independent experiments.

enzyemtic assay used here did not utilize diethyl fumarate as a substrate, and thus the increased concentration measured in the HEK293 cells incubated with this compound represents that of intracellular fumarate produced from the membrane-permeable ester. The fumarate concentrations, when expressed per 10⁶ cells, were highly similar in the non-treated HEK293 cells and control fibroblasts and increased to almost 2-fold by the incubation of the former cells with 40 μM diethyl fumarate and in the latter cells by FH deficiency (Table 2). The cellular fumarate levels were converted to micromolar concentrations by using reported (30, 31) values for the volumes of the two cell types. Such calculations suggested that the concentration in cultured HEK293 cells was about 40 μM and increased to about 70 μM by incubation with 40 μM diethyl fumarate, whereas the fumarate concentrations in cultured control fibroblasts was about 20 μM, and that in the FH-deficient fibroblasts about 40 μM (Table 2). All these values are within concentrations that inhibited the activities of the three purified HIF-P4Hs, the concentrations in the diethyl fumarate-treated cells even exceeding the Ki values of HIF-P4Hs 2 and 3 (Table 1 and Fig. 1).

**TABLE 2**

Fumarate concentrations in HEK293 cells cultured in the presence of diethyl fumarate for 20 h and in fibroblasts from a patient with autosomal recessive FH deficiency

| Cells                      | Cellular fumarate concentrationa pmol/10⁶ cells | % Control | Micromolarb |
|---------------------------|-----------------------------------------------|-----------|-------------|
| HEK293                    | 123 ± 21                                      | 100       | 37          |
| HEK293 + 40 μM diethyl fumarate | 219 ± 83                                      | 178       | 66          |
| FH−/− fibroblasts         | 108 ± 28                                      | 100       | 22          |
| FH−/− fibroblasts         | 205 ± 6                                      | 190       | 42          |

a Values are from three to six measurements.

b The approximate μM cellular concentrations were calculated using the reported volumes of 3.3 × 10⁻¹² and 4.9 × 10⁻¹² liters for HEK293 cells (30) and fibroblasts (31), respectively.

c Statistical significance versus corresponding control cells (Student’s t test) was p < 0.02.

d Statistical significance versus corresponding control cells (Student’s t test) was p < 0.02.

and in vitro, fumarate being markedly more effective than the two others, its Ki for the most abundant and hence most important HIF-P4H, isoenzyme 2 (32, 33), being 60 μM, i.e. about 13% of that of succinate, whereas oxaloacetate was the weakest inhibitor, its Ki for isoenzyme 2 being 1 mm. Citrate was an effective inhibitor of HIF-P4H-3 but not of the two others. The Ki values determined here for fumarate and succinate under initial velocity conditions and using four inhibitor concentrations are in reasonable agreement with the IC₅₀ of about 0.5 mM reported for succinate in assays of nonspecified total HIF-P4H activity in crude cell extracts in vitro (18) but the Ki for fumarate is about 20-fold and succinate 50-fold when compared with the apparent Ki values for these two compounds using purified HIF-P4H-2 and an in vitro VHL capture assay performed under nonsaturating substrate concentrations and in the presence of only one inhibitor concentration (19). Although oxaloacetate and pyruvate have been reported to stabilize HIF-1α in cultured cancer cells and to inhibit the HIF-

**DISCUSSION**

Our data demonstrate that three citric acid cycle intermediates, fumarate, succinate, and oxaloacetate, inhibit all three human HIF-P4Hs in vitro, fumarate being markedly more effective than the two others, its Ki for the most abundant and hence most important HIF-P4H, isoenzyme 2 (32, 33), being 60 μM, i.e. about 13% of that of succinate, whereas oxaloacetate was the weakest inhibitor, its Ki for isoenzyme 2 being 1 mm. Citrate was an effective inhibitor of HIF-P4H-3 but not of the two others. The Ki values determined here for fumarate and succinate under initial velocity conditions and using four inhibitor concentrations are in reasonable agreement with the IC₅₀ of about 0.5 mM reported for succinate in assays of nonspecified total HIF-P4H activity in crude cell extracts in vitro (18) but the Ki for fumarate is about 20-fold and succinate 50-fold when compared with the apparent Ki values for these two compounds using purified HIF-P4H-2 and an in vitro VHL capture assay performed under nonsaturating substrate concentrations and in the presence of only one inhibitor concentration (19). Although oxaloacetate and pyruvate have been reported to stabilize HIF-1α in cultured cancer cells and to inhibit the HIF-

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P4Hs in an in vitro VHL capture assay in the presence of non-saturating ascorbate levels (20, 21), we found only a weak inhibition by oxaloacetate, especially in the case of HIF-P4H-2, and no inhibition by pyruvate in the presence of 2 mM ascorbate and no increase in the level of inhibition in the presence of lower ascorbate concentrations. Membrane-permeable diethyl and dimethyl ester derivatives of fumarate produced stabilization of HIF-1α in all the cell types studied, and a very low extent of HIF-1α stabilization was additionally demonstrated in cultured fibroblasts from a patient with autosomal recessive FH deficiency and in cells transfected with FH siRNA. Interestingly, by contrast with our findings, culturing with monoethyl fumarate stabilized HIF-1α in human A549 lung carcinoma cells only when added in combination with 3-nitropropionic acid, a dual SDH and FH inhibitor (19), and the concentrations of monoethyl fumarate (0.5 mM) needed to obtain this effect in the presence of the inhibitor were much higher than those of diethyl and dimethyl fumarate needed in our study. This difference agrees with findings indicating that monoethyl esters of dicarboxylic acids are much less efficient in passing through cell membranes than diethyl and dimethyl esters, and our finding that monoethyl fumarate gave a detectable stabilization of HIF-1α only at a 5 mM concentration. In agreement with our data, a very weak stabilization of HIF-1α was seen in the A549 cells upon transfection with FH siRNA, whereas a much stronger stimulation was obtained in the presence of 3-nitropropionic acid (19).

The stabilized HIF-1α produced in our study by incubation with diethyl or dimethyl fumarate or by the lack of FH activity was transcriptionally active, as an increased production of a HIF-1α target protein VEGF was found in all these cases, a small increase in the VEGF production having also been reported recently in FH siRNA-transfected cells even in the absence of 3-nitropropionic acid (19). Time course experiments with diethyl fumarate indicated that stabilization of HIF-1α was detectable already at 8 h and that the VEGF production increased between 8 and 20 h in a time- and concentration-dependent manner indicating that the two events were coupled. Although transfection of HEK293 cells with SDH siRNA or incubation with 20 mM succinate dimethyl ester for 48 h has been reported to cause stabilization of HIF-1α (18), we found no stabilization in cells treated with 5 mM succinate dimethyl or diethyl ester for 20 h or in the VEGF production even with 20 mM concentrations of either of these compounds. Others have likewise seen no stabilization of HIF-1α with succinate dimethyl ester in the absence of 3-nitropropionic acid (19). These findings are explained at least in part by our data indicating that the IC_{50} and K_i values of the three HIF-P4Hs for succinate are much higher than those for fumarate and possibly additionally in part by a less efficient cellular uptake of diethyl and dimethyl succinate and their cleavage by cellular esterases than those of diethyl and dimethyl fumarate.

Diethyl fumarate also increased Epo production by cultured Hep3B cells. Interestingly, this effect was seen at much lower diethyl fumarate concentrations than the increases in VEGF production, and the Epo levels returned to baseline values or even below them at concentrations that gave the highest increases in VEGF production. This finding agrees with data indicating that hypoxic conditions can show selectivity with respect to stimulation of Epo versus VEGF (34). One possible explanation for such differences is that Epo has been found to be regulated in Hep3B cells unequivocally by HIF-2α, whereas VEGF is regulated by HIF-1α (35).

Fumarate levels assayed in cultured HEK293 cells and human skin fibroblasts were about 40 and 20 μM, respectively, being about 70 and 30% of the K_i of the purified HIF-P4H-2 for fumarate. The concentration in HEK293 cells incubated with 40 μM diethyl fumarate increased to about 70 μM, i.e. slightly above the K_i of purified HIF-P4H-2, whereas the value in the FH-deficient fibroblasts was about 70% of this K_i. These concentrations could thus be expected to lead to HIF-P4H inhibition, as suggested by our data.

Distinct VHL mutations that prevent the binding of HIF-α, and thus their subsequent pVHL-mediated degradation, can lead to HIF-dependent Epo production and a hereditary polycythemia known as Chuvash polycythemia (36), or a cancer syndrome (2, 37) that is characterized by highly vascular tumors that overproduce hypoxia-inducible mRNAs such as those for VEGF. SDH and FH mutations likewise predispose to dominantly inherited highly vascular tumors, findings in SDH mutations including pheochromocytoma, paraganglioma, renal cell carcinoma, and papillary thyroid cancer (18, 38–40) and those in FH mutations including uterine fibroids, skin leiomyomata, and papillary renal cell cancer (19, 38, 41). The fumarate levels in FH-deficient uterine fibroids were 200-fold relative to the level in normal tissue, whereas the succinate levels in normal tissue were about 3-fold relative to the fumarate levels and increased in tumors with germline SDH mutations up to about 13-fold relative to the level in corresponding tumors without SDH mutations (19). It is very clear from our data that the fumarate levels in FH-deficient tumors, and probably also the succinate levels in SDH-deficient tumors, would strongly inhibit the HIF-P4Hs. Our data thus support recent suggestions that such tumors accumulate fumarate or succinate to levels that inhibit the HIF-P4Hs and stabilize HIF-1α, leading to expression of HIF target proteins such as VEGF (18, 19, 38). In agreement with this hypothesis, both FH- and SDH-deficient tumors, and also tumors arising from pVHL mutations associated with the VHL syndrome, have been shown to overproduce HIF-1α and VEGF and to have a high microvessel density (18, 19, 37, 38, 42). It should be noted, however, that although the increased HIF-1α levels are likely to play an important role in the pathogenesis of these tumors, such as the high microvessel density, there is no evidence that HIF is sufficient to induce tumorigenesis, and there is indeed some evidence that it is not (37). The very high cellular fumarate or succinate levels are likely to have various additional effects on cellular metabolism, some of which may play a crucial role in tumor formation. Although overproduction of reactive oxygen species has been speculated as a mechanism by which FH and SDH mutations may mediate the induction of HIF-1α, two very recent studies

3. V. Günzler, personal communication.
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found no evidence for the generation of these species in FH- or SDH-deficient cells (18, 38).

The IC<sub>50</sub> and K<sub>i</sub> values of FIH for fumarate and succinate were found to be more than 10 mM, our data thus indicating that inhibition of FIH is not likely to contribute to the expression of HIF target genes in tumors from patients with FH or SDH mutations. Despite the lack of FIH inhibition, we found increases in VEGF production in response to diethyl and dimethyl fumarate in all the cell types studied and in the FH<sup>-/-</sup> fibroblasts and the HEK293 cells transfected with FH siRNA as well as in Epo production in Hep3B cells. These findings agree with previous data demonstrating that silencing of HIF-P4Hs alone with siRNA is sufficient to lead to marked increases in the expression of HIF target genes in cultured cells under normoxic conditions (33). The transcriptional activity of HIF is thus likely to be quite high even when FIH is not inhibited and binding of the transcriptional coactivator p300 to HIF-α is prevented.

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