Hypoxia Stimulates Degradation of 3-Hydroxy-3-methylglutaryl-coenzyme A Reductase through Accumulation of Lanosterol and Hypoxia-Inducible Factor-mediated Induction of Insigs*§

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The enzyme 3-hydroxy-3-methylglutaryl (HMG)5-CoA reductase catalyzes reduction of HMG-CoA to mevalonate, a rate-determining step in synthesis of cholesterol and nonsterol isoprenoids that are indispensable for cell function (1). HMG-CoA reductase is embedded in membranes of the endoplasmic reticulum (ER) through an N-terminal domain that contains eight membrane-spanning helices separated by short loops (2). The catalytic domain of reductase projects into the cytosol (3, 4). Sterol and nonsterol end-products of mevalonate metabolism exert stringent feedback control on reductase through multiple mechanisms (5). This complex feedback control system permits continuous production of essential nonsterol isoprenoids while avoiding overproduction of cholesterol and potentially toxic cholesterol precursors. One mechanism for feedback control involves rapid degradation of reductase, which is mediated by a pair of ER membrane proteins called Insig-1 and Insig-2 (6). Accumulation of sterols in ER membranes triggers binding of the membrane domain of reductase to a subset of Insigs that carry a membrane-anchored ubiquitin ligase called gp78, which initiates ubiquitination of reductase (7). Ubiquitination marks reductase for proteasomal degradation, reducing the half-life of the protein from 12 h in sterol-depleted cells to less than 1 h under sterol-replete conditions. Sterol regulatory element-binding proteins (SREBPs), a family of membrane-bound transcription factors, constitute another mechanism for feedback regulation of reductase (8). In sterol-deprived cells, SREBPs are transported from the ER to the Golgi, whereupon they encounter proteases that release soluble fragments from membranes into the cytosol. Processed forms of SREBPs migrate to the nucleus and enhance transcription of genes encoding reductase and other enzymes known to be required for cholesterol synthesis (9). Translocation of SREBPs from the ER to Golgi requires the sterol-responsive escort protein Scap (10–12). Scap, like reductase, contains a hydrophobic N-terminal domain that spans the membrane eight times and a C-terminal domain located in the cytosol that mediates association with SREBPs (13, 14). Sterols trigger binding of Insigs to a region in the membrane domain of Scap that comprises membrane-spanning helices 2–6 and resembles the Insig binding site in reductase (15–17). Insig binding prevents incorporation of Scap and its bound SREBPs into COPII-coated vesicles that are destined for fusion with the Golgi. In the absence of this transport, SREBPs remain tethered to membranes and are not cleaved; consequently, expression of target genes falls, and cholesterol synthesis declines.
With regard to sterol specificity, recent studies have revealed a crucial difference in Insig-mediated regulation of Scap and reductase (8). Cholesterol directly binds to the membrane domain of Scap (18), inducing a conformational change that triggers Insig binding, thereby blocking exit of Scap-SREBP from the ER (19, 20). Scap recognizes the sterol nucleus and the 3β-hydroxy group of cholesterol; the iso-octyl side chain is dispensable for binding. Lanosterol, the first sterol intermediate in cholesterol synthesis (Fig. 1), neither binds Scap nor blocks proteolytic activation of SREBPs (21). Instead, lanosterol potently stimulates Insig-dependent ubiquitination and degradation of reductase. Methyl groups present in the 4α, 4β, and 14α positions are key determinants for the action of lanosterol in stimulating reductase degradation. These methyl groups are removed in the conversion of lanosterol to cholesterol; the initial reaction is catalyzed by the cytochrome P450 enzyme lanosterol 14α-demethylase (22). Lanosterol demethylation has been implicated as a rate-limiting step in the posttranslational portion of the cholesterol synthetic pathway (23, 24), thereby situating the reaction as a potential focal point in sterol regulation.

Synthesis of cholesterol is an oxygen-intensive process; 11 molecules of dioxygen are consumed by four enzymes during production of one molecule of cholesterol from acetyl-CoA (25, 26) (Fig. 1). A single molecule of dioxygen is required for epoxidation of squalene, which is catalyzed by the enzyme squalene monooxygenase. Nine molecules of dioxygen are utilized in removal of the 4α, 4β, and 14α methyl groups in lanosterol through successive actions of lanosterol 14α-demethylase and C4-methyl sterol oxidase. The final oxygen-requiring step in cholesterol synthesis is catalyzed by sterol 5-desaturase and consumes one molecule of dioxygen, resulting in reduction of lathosterol to 7-dehydrocholesterol.

In the fission yeast Schizosaccharomyces pombe, orthologs of mammalian SREBP and Scap called Sre1 and Scp1, respectively, function in a mechanism that uses sterol synthesis as an indicator of oxygen availability (27). Oxygen deprivation (hypoxia) inhibits synthesis of the fungal sterol ergosterol, which triggers Scp1-dependent proteolytic activation of Sre1. In the nucleus, processed forms of Sre1 modulate transcription of genes encoding enzymes that catalyze oxygen-dependent steps in synthesis of ergosterol to maintain sterol homeostasis. Other Sre1 target genes include those that encode oxygen-requiring enzymes in heme, sphingolipid, and ubiquinone synthesis (28). Hypoxia has been reported to inhibit cholesterol synthesis in mammalian cells (29), but a role for SREBP and Scap has not been demonstrated. However, an intriguing possibility exists that degradation of reductase is modulated by low oxygen in mammalian cells, considering that demethylation of lanosterol consumes the majority of molecular oxygen required for cholesterol synthesis.

In the current study, we explored the consequences of hypoxia on regulation of reductase in cultured Chinese hamster ovary (CHO) cells. Our results show that hypoxia triggers accumulation of lanosterol and its metabolite 24,25-dihydrolanosterol, due to inhibition of lanosterol 14α-demethylase. The accumulation of methylated sterols serves as one signal for rapid degradation of reductase, which ultimately reduces flow through early steps of cholesterol synthesis when oxygen is limiting. The other signal is provided by hypoxic induction of Insig-1 and Insig-2. Pharmacologic and genetic data presented in this paper indicate that the hypoxic induction of Insigs requires the oxygen-sensitive transcription factor hypoxia-inducible factor (HIF)-1α (30, 31). Importantly, these effects were specific for reductase inasmuch as hypoxia did not block SREBP processing. Considered together, these results not only highlight the importance of lanosterol as a physiologic regulator of reductase degradation, but they also establish a new connection between cholesterol synthesis and oxygen sensing in mammalian cells.

**EXPERIMENTAL PROCEDURES**

**Materials**—We obtained hydroxypropyl-β-cyclodextrin from Cyclodextrin Technologies Development; bovine serum albumin and the squalene monooxygenase inhibitor NB-598 were from Sigma; [14C]pyruvate, [14C]acetate, and [3H]cholesterol were from American Radiolabeled Chemicals; silica gel TLC plates were from Macherey-Nagel. Dimethylxylglycine (DMOG) was obtained from Frontier Scientific. The lanosterol 14α-demethylase inhibitor RS-21607 (32, 33) was synthesized by the Core Medicinal Chemistry Laboratory, University of Texas Southwestern Medical Center. Other reagents, including lipoprotein-deficient serum (d > 1.215 g/ml), sodium compactin, and sodium mevalonate, were prepared or obtained from sources as described previously (11).

**Cell Culture**—All cell lines used in this study were grown in monolayer at 37 °C in 8–9% CO2. CHO-7 cells were maintained in medium A (1:1 mixture of Ham’s F-12 medium and Dulbecco’s modified Eagle’s medium containing 100 units/ml penicillin and 100 µg/ml streptomycin sulfate) supplemented with 5% (v/v) lipoprotein-deficient serum. SRD-15 and SRD-17 cells are mutant cell lines derived from CHO-7 cells. SRD-15 cells were maintained in medium A containing 5% lipoprotein-deficient serum and 2.5 µM 25-hydroxycholesterol (34). SRD-17 cells were grown in medium A containing 5% lipoprotein-deficient serum and 10 µM SR-12813 (35). C4.5 cells are CHO-K1 cells that stably express the cell surface marker E-selectin under transcriptional control of hypoxia response elements. Mutant Ka13 cells were derived from C4.5 cells and lack HIF-1α (36). C4.5 and Ka13 cells were cultured in medium A supplemented with 5% fetal calf serum. Hypoxia treatments were performed at 37 °C in a humidified hypoxic chamber (Coy Laboratory Products) filled with 1% O2 and 5% CO2 and balanced with N2. Control cells were incubated at 37 °C under normoxic conditions in 21% O2 and 5% CO2. RS-21607, NB-598, and DMOG were added to the culture medium in Me2SO at a final concentration of 0.1% (v/v).

**Metabolic Labeling Studies**—Incorporation of [14C]pyruvate and [14C]acetate into sterols and fatty acids was determined as described (37) with minor modifications. CHO-7 cells were set up for experiments on day 0 and treated as described in the figure legends. On day 1, cells were switched to 2 ml of medium A containing 5% lipoprotein-deficient serum and 6.25 µCi of [14C]pyruvate or 10 µCi of [14C]acetate; cold pyruvate and acetate were added to achieve final concentrations of 2 and 0.5 mM, respectively. After incubation at 37 °C for the times indicated in the figure legend, cell monolayers were washed twice with 2 ml
of cold buffer containing 5 mM Tris-Cl, pH 7.4, 0.15 M NaCl, and 0.2% (w/v) bovine serum albumin and then twice with 2 ml of cold buffer containing 5 mM Tris-Cl, pH 7.4, and 0.15 M NaCl. Cells were harvested and dissolved in 1 ml of 0.1 N NaOH at room temperature for at least 30 min. Cholesterol, lanosterol, and squalene dissolved in chloroform/methanol (2:1) were added to each sample as carrier sterols, and ∼40 µCi of [3H]cholesterol was added as an internal standard. Lipids were saponified in 15% KOH, 40% ethanol at 80 °C for 1 h. Lipids were extracted twice with 4 ml of petroleum ether, and the organic phase was evaporated under nitrogen gas with heat. Lipids were resuspended in heptane and separated by thin layer chromatography on plastic-backed silica gel TLC plates developed in 100% chloroform. For fatty acids, 2 ml of H2O was added to the remaining aqueous phase before acidifying with 2 ml of concentrated HCl. After adding 2 ml of ethanol, fatty acids were extracted twice with 4 ml of petroleum ether, and the amount of radioactivity in each was measured by scintillation counting. Values were corrected for recovery and background from parallel dishes incubated at 4 °C. An aliquot of each sample was taken prior to saponification for protein determination using the BCA protein assay reagent (Pierce). The mean values from triplicate dishes are presented as nmol of [14C]pyruvate incorporation/mg of protein.

Analysis of Cellular Cholesterol Intermediates—Cells were set up for experiments on day 0 as described in the figure legends. Conditions prior to harvest of the cells are also described in the figure legends. Following treatments, cells were harvested, washed twice with PBS, and stored at −80 °C until analyzed. Lipids were extracted with chloroform/methanol (38), and relative amounts of sterols were determined by HPLC-MS. Values were normalized for protein amount, and data are presented as -fold changes relative to the amount of sterols at 0 h. These values are given in the figure legends.

Cell Fractionation and Immunoblot Analysis—Pools of 3–5 dishes were used to isolate membrane and/or nuclear extract fractions as described previously (6). Conditions prior to harvest of the cells are described in the figure legends. Primary antibodies used for immunoblotting were as follows: IgG-A9, a monoclonal antibody against the catalytic domain (amino acids 450–887) of hamster reductase (39); IgG-R139, a rabbit polyclonal antibody against hamster Scap (amino acids 54–277 and 540–707) (13); IgG-7D4, a monoclonal antibody against the NH2 terminus of hamster SREBP-2 (40); a monoclonal antibody against human HIF-1α (amino acids 432–528; UpstateBio-technology); and a rabbit polyclonal antibody against human calnexin (amino acids 575–593; Novus Biologicals). Following incubations with primary antibodies and horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch) as indicated in the figure legends, bound antibodies were visualized with Supersignal Chemiluminescent Substrate (Pierce). As a loading control, membranes used to detect reductase were stripped and subsequently immunoblotted for calnexin.

RNA Isolation and Quantitative Real Time PCR Analysis—The protocol for quantitative real time PCR was identical to that described previously (41). Total RNA was isolated using the RNeasy kit (Qiagen) and treated with DNase to remove contaminating DNA (Qiagen). Two micrograms of resulting RNA were subjected to reverse transcription reactions using TaqMan Reverse Transcription Reagents (Applied Biosystems) and
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The next experiment was designed to determine whether accumulation of lanosterol 14α-demethylase causes accumulation of lanosterol and triggers degradation of HMG-CoA reductase in CHO cells. A, CHO-7 cells were set up for experiments on day 0 at 5 x 10⁵ cells/60-mm dish in medium A supplemented with 5% lipoprotein-deficient serum. On day 1, the cells were reseeded 0.5 ml of the identical medium containing 10 μCi of [14C]acetate; cold acetate was added to achieve a final concentration of 0.5 mM. Some of the dishes also received 10 μM sodium compactin, 30 μM NB-598, or 0.1 μM RS-21607. Following incubation at 37°C for the indicated periods of time, cell lysates were prepared, and lipids were extracted. Aliquots of the resulting lipid extracts were then subjected to thin layer chromatography in parallel with authentic standards for squalene, lanosterol, and cholesterol. Incorporation of [14C]acetate into squalene, lanosterol, and cholesterol was determined by scintillation counting. Data are presented as mean values from triplicate dishes. B, CHO-7 cells were set up on day 0 at 5 x 10⁵ cells/100-mm dish in medium A with 5% lipoprotein-deficient serum. On day 3, cells were subjected to treatment for 1 h at 37°C in medium A containing 5% lipoprotein-deficient serum and incubated at 37°C for an additional 5-6 h. The cells were then subjected to treatments in the identical medium containing various combinations of 0.1 μM RS-21607 and 10 μM compactin. After incubation at 37°C for the indicated times, cells were harvested, lysed, and subjected to cell fractionation. Aliquots of protein from the membrane (12 μg of protein/lane) and nuclear extract (40 μg of protein/lane) fractions were separated by 8% SDS-PAGE and transferred to nitrocellulose membranes. Immunoblot analysis was carried out with 1 μg/ml monoclonal IgG-A9 (against reductase) or 5 μg/ml monoclonal IgG-7D4 (against SREBP-2). Filters were exposed to film for 30–40 s at room temperature. Experiments in A and B have been replicated at least two times with similar results.

RESULTS

Accumulation of Endogenous Lanosterol Stimulates Degradation of HMG-CoA Reductase—The experiment of Fig. 2A compares effects of the reductase inhibitor compactin (37), the squalene monooxygenase inhibitor NB-598 (44), and the lanosterol 14α-demethylase inhibitor RS-21607 (33) (see Fig. 1) on lipid synthesis in CHO-7 cells. CHO-7 cells are a subline of CHO-K1 cells selected for growth in lipoprotein-deficient serum (45). The cells were grown in the absence of exogenous cholesterol and then metabolically labeled with [14C]acetate. Lipids extracted from the labeled cells were separated by thin layer chromatography, and incorporation of the radiolabel into squalene, lanosterol, and cholesterol was determined. In untreated cells, time-dependent incorporation of [14C]acetate into cholesterol was observed; small amounts of radioactivity (<10% of that incorporated into cholesterol) were found in squalene and lanosterol. As expected, compactin prevented incorporation of [14C]acetate into squalene, lanosterol, and cholesterol. NB-598 abolished incorporation of [14C]acetate into lanosterol and cholesterol; the majority of the radiolabel was found in squalene. RS-21607 treatment led to complete inhibition of [14C]cholesterol synthesis with concomitant accumulation of [14C]lanosterol, a result attributable to inhibition of lanosterol 14α-demethylase activity.

The next experiment was designed to determine whether accumulation of lanosterol that results from RS-21607 treatment stimulates degradation of reductase (Fig. 2B). Activity through the cholesterol biosynthetic pathway was stimulated by first subjecting cells to treatment with hydroxypropyl-β-cyclodextrin, a reagent that removes cholesterol from the plasma membrane (46). The cells were then incubated in medium containing lipopro-
tein-deficient serum to allow for maximal expression of SREBP target genes. Following this recovery period, cells were incubated in the absence or presence of RS-21607, after which they were harvested and separated into membrane and nuclear extract fractions. Immunoblotting revealed the complete disappearance of reductase from the membrane fraction of cells treated for 6 h with RS-21607 (top panel, lane g); this effect was blocked by compactin (lanes k–m). In contrast, RS-21607 treatment did not lead to suppression of SREBP processing, as indicated by equivalent amounts of nuclear SREBP-2 in treated and untreated cells (bottom panel; compare lanes b–d with lanes e–g).

Hypoxia Triggers Accumulation of Lanosterol and 24,25-Dihydrolanosterol and Insig-mediated Degradation of HMG-CoA Reductase—Noting that demethylation of lanosterol consumes the majority of molecular oxygen required for cholesterol synthesis (Fig. 1), we next sought to determine whether the reaction becomes inhibited when oxygen is limiting, leading to accumulation of lanosterol in cells. Cells were first subjected to cyclodextrin treatment and subsequently cultured in lipoprotein-deficient serum under hypoxic (1% oxygen) or normoxic (21% oxygen) conditions. Following incubation at 37°C for the indicated periods of time, incorporation of [14C]pyruvate into squalene, lanosterol, and cholesterol was determined as described in the legend to Fig. 2A. These results are from a representative experiment that has been repeated two times with similar results.

FIGURE 3. Hypoxia triggers accumulation of lanosterol and 24,25-dihydrolanosterol in CHO cells. A, CHO-7 cells were set up for experiments on day 0 and subjected to cyclodextrin treatment on day 3 as described in the legend to Fig. 2A. The cells were then incubated at 37°C with medium A containing 5% lipoprotein-deficient serum in the absence or presence of 10 μM compactin under normoxic (21% oxygen) or hypoxic (1% oxygen) conditions. After the indicated periods of time, cells were harvested, cell lysates were prepared, and lipids were extracted with chloroform/methanol. The amounts of cholesterol and sterol precursors in the resulting lipid extracts were determined by HPLC-MS and normalized for protein content. The data are presented as fold changes relative to the amount of the indicated sterol prior to hypoxia treatment (t = 0 h). These values per mg of protein were 5.1 μg of lanosterol, 2.0 μg of 7-dehydrocholesterol, 2.3 μg of desmosterol, and 41.9 μg of cholesterol. 24,25-Dihydrolanosterol and zymosterol were not quantified; thus, relative fold changes were calculated based on peak areas. B, CHO-7 cells were set up on day 0 as described in the legend to Fig. 2A. On day 1, cells were subjected to cyclodextrin treatment at 37°C for 1 h. Cells were subsequently incubated for 6 h under normoxic or hypoxic conditions, after which they were labeled with 6.25 μCi of [14C]pyruvate as described under “Experimental Procedures.” Following incubation at 37°C for the indicated periods of time, incorporation of [14C]pyruvate into squalene, lanosterol, and cholesterol was determined as described in the legend to Fig. 2A. These results are from a representative experiment that has been repeated two times with similar results.
24,25-dihydroxycholesterol is low compared with lanosterol. Indeed, based on peak areas, we estimate the amount of lanosterol exceeded that of 24,25-dihydroxycholesterol by 26-fold under normoxic conditions. Importantly, accumulation of lanosterol and 24,25-dihydroxycholesterol upon hypoxia was blocked by compactin, demonstrating that it required de novo sterol synthesis. Intermediates distal to lanosterol (zymosterol, desmossterol, and 7-dehydrocholesterol) tended to decrease in hypoxic cells. The amount of cholesterol was quite high at zero time (42 μg/mg protein) and did not change during the 6 h of hypoxia. Similar results were obtained in two other independent experiments. When the cells were incubated with [14C]pyruvate, the incorporation of [14C] into cholesterol fell, and incorporation into [14C]lanosterol rose during hypoxia (Fig. 3A). There was no buildup of [14C]squalene, although its conversion to 2,3-epoxysqualene requires a single molecule of dioxygen (Fig. 1).

An experiment was next designed to determine whether reductase is degraded when lanosterol accumulates in oxygen-deprived cells. Fig. 4A shows that reductase protein declined markedly after 3 h of hypoxia (top panel, lane i) and even further at 6 h (lane j). This disappearance was prevented by compactin (lanes k–m), consistent with the hypothesis that lanosterol accumulation mediates the effect. The amount of processed SREBP-2 in the nuclear extract actually rose after 6 h of hypoxia (second panel, lane j), and it rose further when compactin was present without or with hypoxia (lanes g and m). As expected, oxygen depletion stabilized HIF-1α, one of the three HIF-α subunits that mediate transcriptional responses to hypoxia (30) (fourth panel, lanes h–m). HIF-1α is known to be rapidly degraded during normoxia and stabilized upon hypoxia due to an inhibition of its ubiquitination (47, 48). Levels of a control membrane protein, calnexin, were unchanged regardless of culture under normoxic or hypoxic conditions (fifth panel). Like compactin, NB-598 prevented oxygen-regulated degradation of reductase (Fig. 4B, top, compare lanes b–d with lanes e–j), but the enzyme continued to become degraded when the hypoxic cells were treated with RS-21607 (lanes k–m).

To address the Insig requirement for oxygen-regulated degradation of reductase, experiments were conducted in two lines of mutant CHO cells (Fig. 4C). SRD-15 cells lack both Insig-1 and Insig-2; thus, neither reductase degradation nor inhibition of SREBP processing is stimulated when these cells are treated with sterols (34). SRD-17 cells harbor a point mutation in the membrane domain of reductase (G87R) that prevents its sterol-induced binding to Insigs, thereby abrogating sterol-accelerated degradation of the enzyme (35). The results show that, as expected, reductase was degraded in wild type cells exposed to hypoxia for 6 h (top panels, lanes e–g). In contrast, reductase was refractory to hypoxia-induced degradation in mutant SRD-15 and SRD-17 cells (top panels, lanes l–n), although HIF-1α was stabilized in the mutant cells to a level comparable with that in wild type cells (bottom panels, lanes l–n).

Degradation of HMG-CoA Reductase in Oxygen-depleted Cells Requires the Presence of HIF-1α—Ubiquitination and subsequent degradation of HIF-α subunits requires hydroxylation of specific proline residues, which enhances their binding to von Hippel-Lindau tumor suppressor protein (pVHL) (49). pVHL is the recognition component of a ubiquitin ligase complex containing 5% lipoprotein-deficient serum in the absence or presence of either 10 μM compactin, 30 μM NB-598, or 0.1 μM RS-21607. The following indicated times of incubation at 37 °C under normoxic or hypoxic conditions, cells were harvested, lysed, and subjected to cell fractionation. Aliquots of the membrane (12 μg of protein/lane) and nuclear extract (40 μg of protein/lane for SREBP-2, 10 μg of protein/lane for HIF-1α) fractions were subjected to 8% SDS-PAGE, and immunoblot analysis was performed with 1 μg/ml IgG-A9 (against reductase), 5 μg/ml IgG-7D4 (against SREBP-2), 2 μg/ml monoclonal anti-HIF-1α IgG, 5 μg/ml polyclonal IgG-R139 (against Scap), or polyclonal antibody against calnexin (1:2000 dilution). Filters were exposed to film for 2–40 s at room temperature. Similar results have been obtained in at least two replicate experiments of A–C.

FIGURE 4. Hypoxia increases degradation of HMG-CoA reductase through an Insig-dependent mechanism. CHO-7, SRD-15, and SRD-17 cells were set up for experiments on day 0 and subjected to cycloheximide treatment on day 3 as described in the legend to Fig. 28. The cells were subsequently refed medium A containing 5% lipoprotein-deficient serum in the absence or presence of either 10 μM compactin, 30 μM NB-598, or 0.1 μM RS-21607. Following the indicated times of incubation at 37 °C under normoxic or hypoxic conditions, cells were harvested, lysed, and subjected to cell fractionation. Aliquots of the membrane (12 μg of protein/lane) and nuclear extract (40 μg of protein/lane for SREBP-2, 10 μg of protein/lane for HIF-1α) fractions were subjected to 8% SDS-PAGE, and immunoblot analysis was performed with 1 μg/ml IgG-A9 (against reductase), 5 μg/ml IgG-7D4 (against SREBP-2), 2 μg/ml monoclonal anti-HIF-1α IgG, 5 μg/ml polyclonal IgG-R139 (against Scap), or polyclonal antibody against calnexin (1:2000 dilution). Filters were exposed to film for 2–40 s at room temperature. Similar results have been obtained in at least two replicate experiments of A–C.


**Oxygen-mediated Regulation of HMG-CoA Reductase**

**A**

| DMOG (mM) | 0   | 0.3 | 1.0 |
|-----------|-----|-----|-----|
| Time (h)  | 0 1 5 3 6 | 0 1 5 3 6 | 0 1 5 3 6 |
| HMG CoA Reductase | abc def ghi jkl | abc def ghi jkl | abc def ghi jkl |
| HIF-1α    |     |     |     |
| Nuclear SREBP-2 |      |      |      |
| Calnexin  |     |     |     |

**B**

| Time (h) | 0 1 5 3 6 | 0 1 5 3 6 | 0 1 5 3 6 |
|----------|-----------|-----------|-----------|
| HMG CoA Reductase | abc def ghi jkl | abc def ghi jkl | abc def ghi jkl |
| HIF-1α    |     |     |     |
| Calnexin  |     |     |     |

**C**

| DMOG | 0   | 0.3 | 1.0 |
|------|-----|-----|-----|
| Time (h) | 0 1 5 3 6 | 0 1 5 3 6 | 0 1 5 3 6 |
| HMG CoA Reductase | abc def ghi jkl | abc def ghi jkl | abc def ghi jkl |
| HIF-1α    |     |     |     |

**FIGURE 5.** 2-Oxoglutarate-dependent dioxygenase inhibition stimulates Insig-mediated degradation of HMG-CoA reductase. CHO-7, SRD-15, and SRD-17 cells were set up for experiments on day 0 and treated with cyclodextrin on day 3 as described in the legend to Fig. 2A. The cells were subsequently incubated at 37 °C under normoxic conditions for various periods of time in medium A containing 5% lipoprotein-deficient serum with or without DMOG (0.3 and 1.0 mM in normoxic conditions for various periods of time in medium A containing 5% lipoprotein-deficient serum with or without DMOG (0.3 and 1.0 mM in A; 1 mM in B and C) in the absence or presence of 10 μM compactin. After the indicated times of incubation, cells were harvested, lysed, and subjected to cell fractionation. Aliquots of membrane and nuclear extract (40 μg of protein/lane for SREBP-2, 10 μg of protein/lane for HIF-1α) fractions were subjected to 8% SDS-PAGE, and immunoblot analysis was performed with 1 μg/ml IgG-A9 (against reductase), 5 μg/ml IgG-7D4 (against SREBP-2), 2 μg/ml monoclonal anti-HIF-1α IgG, 5 μg/ml polyclonal IgG-R139 (against Scap), or polyclonal antibody against calnexin (1:2000 dilution). Filters were exposed to film for 5 to 8 min at room temperature. Experiments of A–C have been replicated at least two times with similar results.

Complex that targets HIF-α for degradation. Prolyl hydroxylation of HIF-α is catalyzed by a class of dioxygenases that use 2-oxoglutarate as a co-substrate and exhibit an absolute requirement for molecular oxygen (50–52). When cells are deprived of oxygen, prolyl hydroxylation is suppressed, allowing HIF-α to escape degradation and accumulate to high levels. Stabilized HIF-α subunits associate with the constitutive HIF-β subunit, forming the heterodimeric transcription factor HIF. The HIF-α/β heterodimer binds to hypoxia response elements present in oxygen-regulated elements of more than 70 genes that play a central role in both systemic and cellular responses to hypoxia (30).

Rapid degradation of reductase correlated with stabilization of HIF-1α in oxygen-deprived cells (see Figs. 4 and 5), which prompted us to next explore a possible role for HIF activation in reductase degradation. To this end, we employed DMOG, a cell-permeable analog of 2-oxoglutarate that inhibits 2-oxoglutarate-dependent dioxygenases (53). In the experiment of Fig. 5A, DMOG treatment of oxygenated cells stimulated degradation of reductase in a fashion that paralleled enhanced stability of HIF-1α (top two panels, compare lanes a–d with lanes e–h and i–l). The response to DMOG was remarkably similar to the hypoxic response shown in Fig. 4. First, levels of nuclear SREBP-2 were not reduced by DMOG treatment (Fig. 5A, third panel). Second, compactin and NB-598, but not RS-21607, blocked DMOG-induced degradation of reductase (Fig. 5B, top panel). Finally, DMOG failed to accelerate reductase degradation in mutant SRD-15 and SRD-17 cells (Fig. 5C, top panel, lanes h–n) but not in mutant Ka13 cells (top panels, lanes a–g) but not in mutant Ka13 cells (top panels, lanes h–n). The anti-HIF-1α immunoblot revealed the presence of the protein in parental cells and its absence in mutant cells regardless of hypoxia or DMOG treatment (Fig. 5A, A and B, respectively) for 6 h triggered degradation of reductase in parental CHO-7 cells (top panels, lanes a–g) but not in mutant Ka13 cells (top panels, lanes h–n). Despite their HIF-1α deficiency, the cholesterol synthetic pathway appears intact in Ka13 cells, as was indicated by the accumulation of lanosterol and 24,25-dihydrolanosterol when the cells were depleted of oxygen (Fig. 5C).

**Oxygen-sensitive Degradation of HMG-CoA Reductase Mediated by HIF-dependent Induction of Insigs—**A clue to the identities of HIF-1α target genes required for oxygen-regulated degradation of reductase is provided by studies focused on the transcriptional profile of oxygen-depleted cells. In several DNA microarray analyses, transcripts for Insig-1 and/or Insig-2 have been identified among those induced by hypoxia or DMOG treatment (54–57). Importantly, induction of Insig-1 in hypoxic cells was verified in one study by Northern blot analysis (54). The quantitative
responsed requires the presence of HIF-1α. We could not determine protein levels for Insig-1 and Insig-2 in these experiments, due to the lack of an antibody capable of detecting the protein in cells that either lack Insigs (SRD-15 cells) or express a mutant form of reductase that cannot bind Insigs in the presence of lanosterol (SRD-17 cells). These results indicate that the disappearance of reductase upon hypoxia is a result of accelerated degradation of the enzyme in response to accumulation of lanosterol and 24,25-dihydrolanosterol. The Insig requirement for oxygen-regulated enzyme in response to accumulation of lanosterol and 24,25-dihydrolanosterol is accompanied by accumulation of lanosterol and its metabolite 24,25-dihydrolanosterol by successive actions of lanosterol 14α-demethylase and C4-methyl sterol oxidase (Fig. 1).

Results of the current study show that when cells are deprived of oxygen, demethylation of lanosterol and 24,25-dihydrolanosterol becomes rate-limiting, and both sterols accumulate (Fig. 3). Accumulation of lanosterol and 24,25-dihydrolanosterol is accompanied by the disappearance of reductase from membranes of oxygen-deprived cells (Fig. 4, A and B). This disappearance was blocked by the reductase inhibitor compactin and the squalene monooxygenase inhibitor NB-598 but not by the lanosterol 14α-demethylase inhibitor RS-21607 (Fig. 4C), which allows for production of lanosterol (Fig. 2A) and presumably 24,25-dihydrolanosterol. These results indicate that the disappearance of reductase upon hypoxia is a result of accelerated degradation of the enzyme in response to accumulation of lanosterol and 24,25-dihydrolanosterol. The Insig requirement for oxygen-regulated degradation of reductase is revealed by results of Fig. 4D. Reductase was not subject to hypoxia-induced degradation in cells that either lack Insigs (SRD-15 cells) or express a mutant form of reductase that cannot bind Insigs in the presence of lanosterol (SRD-17 cells).
Oxygen-mediated Regulation of HMG-CoA Reductase

Although hypoxia (and subsequent accumulation of lanosterol and 24,25-dihydrolanosterol) accelerated reductase degradation, these treatments failed to block processing of SREBPs (Fig. 4, A and B). This finding is consistent with our previous observation that exogenous 4,4-dimethylated sterols stimulate reductase degradation but do not block SREBP processing (21). The lack of SREBP inhibition can be rationalized through knowledge of the fact that genes encoding all known cholesterol synthetic enzymes, including those that convert lanosterol and 24,25-dihydrolanosterol to cholesterol, are targets of SREBPs. Under conditions in which lanosterol accumulates, a reduction in SREBP processing would slow conversion of lanosterol to cholesterol. On the other hand, a selective decrease in reductase degradation while maintaining high levels of the postlanosterol enzymes.

A surprising finding of the current study is the hitherto unappreciated role of HIF-1α in oxygen-regulated degradation of reductase. Remarkably, treatment of cells with the 2-oxoglutarate-dependent dioxygenase inhibitor DMOG not only stabilized HIF-1α as previously reported, but also led to Insig-dependent degradation of reductase in oxygen-deprived cells (Fig. 5). Although DMOG treatment did not result in the accumulation of lanosterol and 24,25-dihydrolanosterol, the effect of the compound on reductase degradation was blocked by compactin and NB-598. Given that DMOG nonselectively inhibits 2-oxoglutarate-dependent dioxygenases, the results of Fig. 6, A and B, are important in that they provide a direct link between HIF-1α and reductase degradation. The results show that reductase was refractory to hypoxia- and DMOG-induced degradation in HIF-1α-deficient Ka13 cells. Importantly, lanosterol and 24,25-dihydrolanosterol continued to accumulate in hypoxic Ka13 cells (Fig. 6C), indicating the mutant cells are not defective in sterol synthesis. Moreover, Ka13 cells responded to exogenous sterol treatment by stimulating rapid degradation of reductase (supplemental Fig. 1A). Thus, HIF-1α-dependent gene transcription appears to be a second requirement for rapid degradation of reductase in oxygen-deprived cells.

Fig. 7 provides evidence that the HIF-1α target genes required for oxygen-dependent regulation of reductase encode Insig-1 and Insig-2. Expression of mRNAs for Insig-1 and Insig-2 is enhanced by hypoxia and DMOG treatment through a mechanism that requires the presence of HIF-1α. HIF-1α-mediated induction of Insigs helps to explain why reductase became degraded upon DMOG treatment without an accumulation of lanosterol (Fig. 5). This is consistent with unpublished studies in which we observed that increased expression of Insigs through transfection enhances sensitivity of reductase degradation to sterols. Moreover, extended treatment of cells with DMOG rendered reductase more sensitive to the regulatory oxysterol 25-hydroxycholesterol (supplemental Fig. 1B). Collectively, the results of the current study indicate that oxygen-regulated degradation of reductase in CHO cells is the combined result of two actions: 1) inhibition of lanosterol 14α-demethylase and subsequent accumulation of lanosterol and 24,25-dihydrolanosterol and 2) HIF-1α-dependent induction of Insig-1 and Insig-2. Sequences conforming to consensus hypoxia response elements are found in 5′-flanking and intronic regions of genes encoding mouse Insig-1 and Insig-2. However, it remains to be determined whether these potential hypoxia response elements are responsible for induction of Insigs observed in oxygen-deprived cells.

Recently, DNA microarray analysis was used to compare transcriptional profiles in livers of mice overexpressing nondegradable forms of HIF-1α or HIF-2α and of mice harboring a liver-specific deletion of pVHL (59). Consistent with results of the current study, expression of Insig-2 mRNA was found to be enhanced in livers of all three groups of mice. Further analysis revealed a significant down-regulation of several SREBP target genes, including those encoding reductase, lanosterol synthase, lanosterol 14α-demethylase, and C4-methyl sterol oxidase. A similar reduction in SREBP target genes was recently observed in livers of mice exposed to hypoxia (60); however, an induction of Insig-2 was not observed in that study.

Significant changes in SREBP processing or in the levels of SREBP target genes were not observed upon hypoxia in the

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6 A. D. Nguyen and R. A. DeBose-Boyd, unpublished observations.
A link has been established between cholesterol metabolism and oxygen sensing in animal cells. The link is provided by hypoxia-induced accumulation of the cholesterol biosynthetic intermediate lanosterol and 24,25-dihydrolanosterol and HIF-1α-mediated induction of Insigs. Convergence of these signals leads to rapid degradation of HMG-CoA reductase, thereby limiting synthesis of cholesterol. Considering that cholesterol (an important component of cell membranes) and nonsterol isoprenoids (including the prenyl groups that become attached to many signaling proteins) are essential for cell growth and viability, we envision accelerated degradation of reductase as part of a protective mechanism that guards against wasting of oxygen and inappropriate cell growth in the face of hypoxia. Further insight into oxygen-regulated degradation of reductase will be provided by future studies focused on determining the consequences of hypoxia on cholesterol metabolism in the liver (the major site of cholesterol synthesis in vivo) as well as other tissues of whole animals.

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