Hypoxia Inhibits Myogenic Differentiation through Accelerated MyoD Degradation*

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Cells undergo a variety of biological responses when placed in hypoxic conditions, including alterations in metabolic state and growth rate. Here we investigated the effect of hypoxia on the ability of myogenic cells to differentiate in culture. Exposure of myoblasts to hypoxia strongly inhibited multinucleated myotube formation and the expression of differentiation markers. We showed that hypoxia reversibly inhibited MyoD, Myf5, and myogenin expression. One key step in skeletal muscle differentiation involves the up-regulation of the cell cycle-dependent kinase inhibitors p21 and p27 as well as the product of the retinoblastoma gene (pRb). Myoblasts cultured under hypoxic conditions in differentiation medium failed to up-regulate both p21 and pRb despite the G1 cell cycle arrest, as evidenced by p27 accumulation and pRb hypophosphorylation. Hypoxia-dependent inhibition of differentiation was associated with MyoD degradation by the ubiquitin-proteasome pathway. MyoD overexpression in C2C12 myoblasts overrode the differentiation block imposed by hypoxic conditions. Thus, hypoxia by inducing MyoD degradation blocked accumulation of early myogenic differentiation markers such as myogenin and p21 and pRb, preventing both permanent cell cycle withdrawal and terminal differentiation. Our study revealed a novel anti-differentiation effect exerted by hypoxia in myogenic cells and identified MyoD degradation as a relevant target of hypoxia.

Skeletal muscle differentiation is characterized by myoblast withdrawal from the cell cycle, induction of muscle specific gene expression, and cell fusion into multinucleated myotubes. The antagonism between proliferation and differentiation implies that signaling pathways driving proliferation must be suppressed to allow induction of differentiation. The muscle regulatory transcription factors (MRFs)1 MyoD, myogenin, MRF4, and Myf5 were initially identified as master regulators of cell fate because of their ability to confer a skeletal muscle phenotype to non-muscle cells (1, 2). These factors form heterodimers with ubiquitous basic helix-loop-helix (bHLH) proteins and, through their subsequent binding to specific sequences, termed E boxes, in the promoter-regulatory regions of muscle-restricted target genes, activate myogenic differentiation (1, 2). Proliferating myoblasts express MyoD and Myf5 before the onset of muscle differentiation (2, 3). Once activated, MyoD and Myf5 induce the withdrawal of myoblasts from the cell cycle together with the expression of myogenin. The link between myoblast cell cycle withdrawal and differentiation is established through regulation of cyclin-dependent kinases (CDKs) and cyclin-dependent kinase inhibitors (CDKIs). The CDKI p21 and the retinoblastoma protein (pRb) are critical in establishing the post-mitotic state during myogenesis (4, 5). In myogenic cells, p21 expression increases during the G1 phase of the cell cycle, and this up-regulation is associated with permanent cell cycle arrest of muscle cells (6). One key target of the CDKIs is pRb, which is regulated by its phosphorylation state. The CDKI p21, by blocking CDK activity, induces accumulation of pRb in the hypophosphorylated, active form, which is necessary for the maintenance of the permanent cell cycle withdrawal in myotubes (4, 7, 8). During myogenesis, the cell cycle regulatory pathways are modulated by muscle-specific regulators of the MyoD family (1, 9). Additionally, MyoD is a key transcription factor regulating both p21 and pRb gene expression during muscle differentiation. MyoD has been found to enhance activity of the p21 promoter in transient transfection experiments (6) and to stimulate p21 mRNA and protein accumulation in muscle cells and fibroblasts (6, 7). Attempts to characterize factors responsible for myoblast proliferation and differentiation have focused largely on growth factors and their receptors. However non-protein factors have been shown to regulate myogenic cell functions as well. Indeed nitric oxide has been involved in myoblast activation and fusion to generate multinucleated myotubes (10, 11).

Mammalian cells require a constant supply of oxygen to maintain adequate energy production, which is essential for ensuring normal function and cell survival. A decrease of oxygen levels, which occurs in pathophysiologic conditions, induces different metabolic and biological responses, including transition from oxidative phosphorylation to glycolysis. Depletion of adenosine triphosphate (ATP) results in either growth arrest or apoptosis, depending on the cell types (12–15). Many of these responses are mediated by the transcription factor HIF-1 (16), which activates the expression of genes involved in growth arrest, such as the tumor suppressor p53, as well as the CDKIs p21 and p27 (17). Several studies indicate that hypoxia-induced growth arrest is associated with a decrease cyclin-CDK complexes and an increase in CDKI p27, leading to hypophosphorylation of pRb (18, 19). Previous studies show that, al-

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‡ The abbreviations used are: MRF, muscle regulatory transcription factor; CDK, cyclin-dependent kinases; CDKI, CDK inhibitor; BrdUrd, bromodeoxyuridine; pRb, retinoblastoma protein; GM, growth medium; DM, differentiation medium; PBS, phosphate-buffered saline; mAb, monoclonal antibody; MyHC, myosin heavy chain mAb; GFP, green fluorescent protein.
though hyperoxia (from 20 to 40% oxygen) enhanced fusion of mononucleated myoblasts into myotubes (20), mild hyperoxia (6% oxygen) increased cell proliferation and survival (21). However, the response of myogenic cells to hyperoxia (1–2% oxygen) as well as the molecular mechanisms involved in oxygen-mediated myoblast functions have not been investigated.

In the present study we have examined the effect of hyperoxia on myogenic differentiation of C2C12 myoblasts. Hyperoxia negatively regulates myogenic differentiation by inhibiting MRP's expression and cell cycle withdrawal. In addition, hyperoxia accelerates MyoD degradation through the ubiquitin-proteasome pathway in a cell cycle-independent mechanism. Because MyoD overexpression in hypoxic myoblasts overrode the hyperoxia-mediated inhibition of differentiation, we identified MyoD as a novel target of hyperoxia.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfections—The murine myoblast C2C12 (22) and L6E9 cell lines (23) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% fetal bovine serum (Euroclone Inc., Milan, Italy), 20 mM glutamine, 100 units/ml penicillin, and 100 mg/ml streptomycin (growth medium (GM), Invitrogen). To induce differentiation cells at 20–80% confluence were shifted to Dulbecco's modified Eagle's medium supplemented with 2% horse serum (differentiation medium (DM)). Myoblasts were induced by culturing cells in a contractile medium. Myoblasts were then fixed in ice-cold 70% ethanol and stored at 4 °C. The fixed cells were then washed at high stringency with 0.2 M sodium phosphate, 1 M NaCl, 200 mM dATP, dCTP, dGTP, and dTTP, and 50 μg/ml leupeptin and pepstatin at 4 °C for 15 min at 4 °C followed by centrifugation at 14,000 rpm for 10 min. Equal amounts of total cellular proteins (100 μg/ml) were resolved by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane (Amersham Biosciences). Membranes were probed with MyoD rabbit polyclonal antibody (0.4 μg/ml BD Biosciences), myogenin monoclonal antibody (mAb; 0.4 μg/ml, Santa Cruz Biotechnology, Santa Cruz, CA), Myf-5 mAb (0.4 μg/ml, Santa Cruz), myosin heavy chain mAb (MyHC) (1:40, Sigma), g-actin mAb (0.3 μg/ml, Oncogene Science Inc., Cambridge, MA) followed by horseradish peroxidase-coupled secondary antibodies and developed with a chemiluminescence-based detection system (ECL, Amersham Biosciences).

Protease Inhibitors—Protease inhibitors MG132 (N-benzyloxycarbonyl-Leu-Leu-leucinal) and clastolactacystin β-lactone (Calbiochem) were diluted in dimethyl sulfoxide and added to the DM-cultured C2C12 cells for 6 h at 50 μg and 25 μg/ml, respectively.

Protein Stability and Half-life Determination—Cellular stability of MyoD was determined by Western blot analysis after treatment of cells with the protein synthesis inhibitor cycloheximide. Briefly, DM-cultured C2C12 cells were treated with 40 μg/ml cycloheximide and incubated under normoxic or hypoxic conditions at 33 °C. At the indicated time total cellular extracts were prepared and analyzed by Western blot with anti-MyoD mAb as described before.

Immunofluorescence—C2C12 cells were transfected with 4 μg of pcDNA3-MyoD together with 1 μg of pcDNA3-GFP as described before and grown in normoxic or hypoxic conditions for 48 h. Cells were then fixed in PBS with 4% paraformaldehyde and permeabilized in PBS with 0.1% Triton X-100. Coverslips were rinsed and blocked for 10 min in PBS with 0.2% bovine serum albumin before incubation with antibodies. Fixed cells were incubated with primary antibodies anti-myogenin mAb (0.3 μg/ml, Dakocytomation, Carpinteria, CA) and anti-MyHC (MF20 mAb; 1:40) for 1 h followed by incubation with rabbit anti-mouse antibody coupled to Texas Red (1:40). The coverslips were mounted and analyzed with a Zeiss microscope equipped for epifluorescence.

Luciferase Assays—C3H10T1/2 cells were transfected with 0.5 μg of the reporter plasmid containing the luciferase gene under the transcriptional control of the myosin creatin kinase promoter (pMCK-Luc) together with 4 μg of pcDNA3-MyoD and 0.5 μg of pcCMV-β-galactosidase (pcCMV-β-gal). Similar amounts of empty vector were used as a control. After transfection cells were seeded in 6-well plates and grown for 24 and 48 h under normoxic or hypoxic conditions. Then cells were harvested and lysed in 100 μl of 1× luciferase lysis buffer (Promega, Madison, WI). Luciferase activity was determined in triplicate and expressed as arbitrary units. Transfection efficiency was normalized by correcting luciferase activity for the levels of β-galactosidase protein.

Statistical Analysis—Data were expressed as the mean ± S.D. or ±S.E. Student’s two-tailed t test was performed, and a p ≤ 0.05 was considered statistically significant.

RESULTS

Hypoxia Reversibly Inhibits Skeletal Muscle Differentiation—To determine whether hypoxia affects myogenic differentiation, proliferating C2C12 myoblasts were cultured in DM for different time periods, either under normoxic or hypoxic conditions, and analyzed for their ability to undergo myogenic differentiation. As expected, at normal oxygen levels C2C12 myoblasts fused into myotubes (Fig. 1A), and MyHC expression increased over time (Fig. 1B). In contrast, C2C12 cells cultured in hypoxia for different time periods showed neither myotube formation nor MyHC protein accumulation (Fig. 1, A and B). A similar effect of hypoxia on myogenic differentiation was also assessed in L6E9 myoblasts rat cell line. Although culture in normoxia conditions for 2 days in DM was permissive for activation of the myogenic program, hypoxic culture conditions inhibited both MyHC accumulation (Fig. 1B) and myotube formation (data not shown).

To analyze whether the myogenic differentiation program was temporarily or permanently inhibited by hypoxia, C2C12 myoblasts were placed in DM in hypoxic conditions for 48 h and harvested, and extracted with radiouclide precipitation in 1 M TCA (pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 1% deoxycholic acid, 0.1% SDS, and 10% glycerol) containing protease and phosphatase inhibitors (2 mM phenethylmethylsulfonyl fluoride, 100 units/ml aprotinin, 10 μg/ml leupeptin and pepstatin 10 mM sodium fluoride, 20 mM sodium vanadate) by rotating for 15 min at 4 °C followed by centrifugation at 14,000 rpm for 10 min. Equal amounts of total cellular proteins (100 μg/ml) were resolved by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane (Amersham Biosciences). Membranes were probed with MyoD rabbit polyclonal antibody (0.4 μg/ml BD Biosciences), myogenin monoclonal antibody (mAb; 0.4 μg/ml, Santa Cruz Biotechnology, Santa Cruz, CA), Myf-5 mAb (0.4 μg/ml, Santa Cruz), myosin heavy chain mAb (MyHC) (1:40, Sigma), g-actin mAb (0.3 μg/ml, Oncogene Science Inc., Cambridge, MA) followed by horseradish peroxidase-coupled secondary antibodies and developed with a chemiluminescence-based detection system (ECL, Amersham Biosciences).

Luciferase Assays—C3H10T1/2 cells were transfected with 0.5 μg of the reporter plasmid containing the luciferase gene under the transcriptional control of the myosin creatin kinase promoter (pMCK-Luc) together with 4 μg of pcDNA3-MyoD and 0.5 μg of pcCMV-β-galactosidase (pcCMV-β-gal). Similar amounts of empty vector were used as a control. After transfection cells were seeded in 6-well plates and grown for 24 and 48 h under normoxic or hypoxic conditions. Then cells were harvested and lysed in 100 μl of 1× luciferase lysis buffer (Promega, Madison, WI). Luciferase activity was determined in triplicate and expressed as arbitrary units. Transfection efficiency was normalized by correcting luciferase activity for the levels of β-galactosidase protein.

Statistical Analysis—Data were expressed as the mean ± S.D. or ±S.E. Student’s two-tailed t test was performed, and a p ≤ 0.05 was considered statistically significant.
hypoxia for 48 h. MyHC was detected by using a specific MF-20 mAb (upper panel). The same filter was probed with anti-α-tubulin mAb to show equal proteins loading (lower panel).

then shifted to normoxic conditions either in GM or in DM. When hypoxic DM was removed and substituted with normoxic GM, C2C12 entered the cell cycle, proliferated (Fig. 2A), and did not express MyHC (Fig. 2B). The addition of normoxic DM to hypoxic C2C12 cells resulted in the induction of myogenic differentiation process, as evidenced by myotube formation (data not shown) and MyHC accumulation (Fig. 2B). These results indicate that myoblast differentiation is not irreversibly inhibited by hypoxia, as myoblasts retain their capacity to proliferate or differentiate when normoxic oxygen levels are restored.

Hypoxic Myoblasts Are Reversibly Arrested in G1—Terminal cell cycle arrest is coupled to muscle differentiation and is required for activation of muscle-specific gene expression (2, 3). Because hypoxia affects, both positively and negatively, cell growth and cell cycle distribution in other cell types (12–15), we sought to investigate the effect of hypoxia on the cell cycle profile of C2C12 myoblasts cultured either in GM and DM at low confluence (<50%) to avoid G1 arrest after cell-cell contact. Hypoxia significantly impaired C2C12 proliferation in GM (Fig. 3A). Cell count per plate remained constant 24 and 48 h after exposure to hypoxia, whereas cells on normoxic control plates proliferated, and their number increased 8-fold within 48 h. Both hypoxic and normoxic cells remained 100% viable as assessed by trypan blue exclusion. Propidium iodide and anti-BrdUrd mAb, as described under “Experimental Procedures.” Then the DNA content was analyzed by flow cytometry. FL1-H, BrdUrd; FL2-A, PI. Representative experiments are shown.

The decision to progress through the cell cycle or to differentiate is taken before the G1 to S phase transition and is controlled by the action of CDKs, their associated cyclins, and the cyclin-CDKI (27). During myogenic differentiation the up-regulation of the CDKIs p21 and p27 ensured terminal growth arrest (6, 7, 28). One of the best characterized molecular events necessary for growth arrest and myogenic program activation is the induction of pRb hypophosphorylation by the CDKIs p21 (4, 7, 8). Given that hypoxia induces accumulation of myoblasts in G1 phase and inhibits differentiation, we monitored the

Fig. 1. Hypoxia inhibits muscle differentiation. A, morphology of normoxic and hypoxic C2C12 cells. GM-cultured C2C12 cells were shifted to DM at 80% confluency and exposed either to normoxia or hypoxia for 72 h. Cells were fixed, and photomicrographs were obtained by a Zeiss light microscope at a magnification of 20×. B, MyHC in normoxic and hypoxic C2C12 and L6E9 cells. Western blot analysis of total extract (40 μg) from C2C12 cells were cultured either in normoxia (N) and hypoxia (H) for the indicated time periods. L6E9 cells were cultured in normoxia and hypoxia for 48 h. MyHC was detected by using a specific MF-20 mAb (upper panel). The same filter was probed with anti-α-tubulin mAb to show equal proteins loading (lower panel).

Fig. 2. Hypoxia-mediated inhibition of myogenic differentiation is reversible. A, C2C12 cells cultured in DM in hypoxia proliferate when transferred to GM in normoxia. DM-cultured C2C12 myoblasts were exposed to hypoxia for 48 h (H48). Hypoxic medium was replaced with GM, and cells were kept to normoxia for 24 h (N24) and 48 h (N48). Then cells were harvested and counted. Results represent the mean ± S.D. of three independent experiments. *, p < 0.01 versus H48; †, p < 0.01 versus N24. B, hypoxic DM-cultured C2C12 express MyHC when transferred to GM in normoxia. DM-cultured C2C12 were left in hypoxia for 48 h and then shifted to normoxia for 4 h either in GM or in DM. Total cellular extracts (100 μg) were resolved by SDS- PAGE, transferred to nitrocellulose filter, and probed with MF-20 mAb to detect MyHC (upper panel). To demonstrate equal loading, the filter was also probed with anti-α-tubulin mAb (lower panel).

Fig. 3. Hypoxia induces growth arrest in C2C12 myoblast cell line. A, hypoxia inhibits C2C12 proliferation. Cells were plated at density of 2 × 10^5/60-mm diameter dish and 16 h later (time 0) exposed to hypoxia. At 0, 24, and 48 h, cells were harvested, and the number of viable cells, as determined by trypan blue exclusion, was counted with a hemacytometer. Results are expressed as the mean ± S.D. of three independent experiments. *, p < 0.01 versus 0, 24, and 48 h. B, hypoxia induces G1 accumulation of C2C12 myoblasts. GM- and DM-cultured C2C12 cells were incubated with 30 μM BrdUrd for the last 30 min of a 48-h incubation in either normoxia or hypoxia. Cells were stained with propidium iodide and an anti-BrdUrd mAb, as described under “Experimental Procedures.” Then the DNA content was analyzed by flow cytometry. FL1-H, BrdUrd; FL2-A, PI. Representative experiments are shown.
expression of cell cycle proteins after placing cells in hypoxic culture conditions. Both GM- and DM-cultured C2C12 myoblasts were grown either in normoxia or hypoxia for 48 h. Then cells were collected, and the expression of proteins important for G1/S phase transition was examined. As previously reported for immortalized cell lines and primary fibroblasts, hypoxia induced pRb hypophosphorylation in GM-cultured C2C12 cells (Fig. 4A). A number of studies suggest that elevated levels of the CDKIs p27 and p21 prevent cyclin/CDK-mediated phosphorylation of pRb (29–31). Western blot analysis revealed increased expression of the CDKI p27 in hypoxic-GM-cultured C2C12 cells as compared with the normoxic counterpart (Fig. 4A). In contrast, p21 levels did not show significant differences in normoxic versus hypoxic GM-cultured cells (Fig. 4A). Although p53 may be induced by hypoxia in a HIF1-dependent manner (32), we did not observe an increase in p53 protein levels in hypoxic conditions despite HIF-1 accumulation (data not shown). In C2C12 cells induced to differentiate, pRb underwent simultaneous dephosphorylation and up-regulation. Interestingly, hypoxia dissociated these two events, with reduced levels of hypophosphorylated pRb as compared with normoxic conditions (Fig. 4B). Moreover, although both p21 and p27 protein levels increased in normoxic DM-cultured C2C12 cells, only p27 accumulated in hypoxic culture conditions (Fig. 4B). These results indicate that a growth arrest program involving Rb dephosphorylation and p27 accumulation is activated in both normoxic and hypoxic DM cultures. The lack of pRb and p21 accumulation, whose transcription is stimulated by MRFs, suggests that hypoxia-cultured C2C12 cells fail to activate the myogenic program after the G1 arrest.  

**Hypoxia Inhibits MRFs Expression**—Successful progression through the skeletal muscle cell differentiation program is marked by MRFs activation, including MyoD, Myf5, and myogenin, which control specific muscle genes expression. The results reported in the previous sections prompted us to investigate whether hypoxia could affect the expression of muscle regulatory genes. Consistent with a previous report (2) MyoD and Myf5 proteins were present both in normoxic GM- and DM-cultured C2C12 cells (Fig. 5A), whereas myogenin was detectable only after the induction of differentiation (Fig. 5A). In hypoxic-cultured C2C12 cells, a marked inhibition of MyoD, Myf5, and myogenin at proteins and mRNA levels were apparent (Fig. 5, A and B). The hypoxia-mediated inhibition of MRFs expression was reversible since both MyoD and Myf5 accumulated when hypoxic C2C12 myoblasts were cultured in normoxia either in GM or in DM (Fig. 5C). Myogenin was detected only in C2C12 cells re-placed in DM after hypoxia (Fig. 5C). Taken together, these results indicate that hypoxia reversibly inhibits MRF expression in myoblasts cultured under differentiation conditions.  

**Hypoxia Inhibits MyoD Protein Accumulation**—As reported above, hypoxia inhibited MyoD mRNA expression and protein accumulation. Because MyoD regulates its own synthesis (33), we sought to investigate whether hypoxia-mediated MyoD down-regulation occurred at transcriptional or post-transcriptional levels. To this end, MyoD expression as well as myogenic differentiation was assessed in MyoD stably transfected C3H10T1/2 cells cultured under hypoxic conditions. It is known that MyoD overexpression in C3H10T1/2 cells induces myogenic differentiation, as evidenced by myotube formation (Fig. 6A) and MyHC accumulation (Fig. 6B) (34). When shifted to hypoxia for 48 h, C3H10T1/2 cells did not show a morphological differentiation as defined by the fusion of single cells into multinucleated myofibers (Fig. 6A). Western blot analysis performed on hypoxic C3H10T1/2 demonstrated the absence of both MyHC and MyoD protein accumulation (Fig. 6A). The marked decrease in MyoD protein was not paralleled by a decrease in its RNA levels (Fig. 6C), suggesting that post-transcriptional modifications are involved in the hypoxia-mediated inhibition of MyoD.  

**Hypoxia Accelerates MyoD Protein Degradation**—Previous reports establish the MyoD half-life at about 40 min (33, 35, 36) and establish that phosphorylation is required for its cell cycle-regulated degradation (36). To analyze whether hypoxia affected MyoD protein stability by increasing its turnover, MyoD accumulation was determined in C2C12 cells after cyclohexi-
confirmed by probing the filter with anti-blot for MyHC and MyoD expression. Equal loading of the lanes was determined in hypoxic culture conditions (Fig. 7, A). MyoD protein levels were determined by Western blot analysis (upper panel). Filters were reprobed with anti-α-tubulin mAb to confirm equal loading of the lanes. MyoD does not modify MyoD mRNA levels in C3H10T1/2 cells. C3H10T1/2 cells stably transfected with MyoD. Morphology of normoxic and hypoxic DM-cultured C3H10T1/2 cells (Fig. 7, B). Hypoxia accelerates MyoD degradation. A—B, hypoxia decreases MyoD protein accumulation in C2C12 myoblasts. C2C12 cells were grown in hypoxic conditions for 48 h and then transferred to hypoxia for 48 h (H). Treatment with proteasome inhibitors Mg132 (50 μM) or lactacystin (25 μM) was performed for the last 6 h of hypoxia. MyoD protein levels were determined by Western blot analysis (upper panel). Filters were reprobed with anti-α-tubulin mAb to confirm equal loading of the lanes (lower panels). B—D, MyoD protein levels normalized for α-tubulin expression were calculated by densitometric analysis. Values are the average of three independent experiments ± S.E. B: †, p < 0.01 versus normoxic conditions for 1 h; †, p < 0.01 versus normoxic conditions for 3 h. D: *, p < 0.001; †, p < 0.05 versus H (without proteasome inhibitors).
expression of MyoD is sufficient to activate a myogenic differentiation program in hypoxic conditions.

**DISCUSSION**

Differention of skeletal muscle involves two major steps, the irreversible withdrawal of myoblasts from cell cycle and the subsequent expression and activation of muscle-specific genes (MRFs).

The myogenic process has been extensively studied. The MRFs Myf5 and MyoD are expressed in early and mid-myogenesis. As muscle cells progress toward a differentiated phenotype, myogenin and additional MRFs are induced and cooperatively establish the irreversible commitment to terminal differentiation by the accumulation of p21 and hypophosphorylated pRb (40), two markers of post-mitotic myoblasts. Many extracellular growth factors and intracellular signaling pathways contribute to the control of muscle cell growth and differentiation. However, the effect of the oxygen tension on myoblast differentiation has not been previously elucidated. In the present work we showed that hypoxia strongly inhibited skeletal muscle differentiation by producing a marked decrease in MyoD, Myf5, and myogenin levels. Previous studies demonstrated that low oxygen levels (6%) increased myogenic cell proliferation (21, 41). However, these data are only apparently in conflict with our results, since the percentage of oxygen in our culture conditions was much lower (less than 1%). It is noteworthy that 6% oxygen is very similar to the oxygen tension present in muscles in physiological conditions (42). Thus, 6% oxygen concentration may not be considered as true hypoxia.

Hypoxia inhibited permanent withdrawal of myoblasts from the cell cycle. C2C12 cells cultured under hypoxic conditions in GM underwent cell cycle arrest with an accumulation predominantly in the G1 phase of the cell cycle. We observed that the CDKI p27 as well as the hypophosphorylated active form of pRb accumulated in GM-cultured C2C12 in the absence of p21 induction. This is in agreement with a previous report demonstrating that hypoxia-induced growth arrest required a functional pRb but not p21 (19). Gardner et al. (19) recently demonstrated that hypoxia-mediated growth arrest involves p27. In their experiments a decrease of p27 by antisense cDNA strategy overcame the hypoxia-mediated inhibition of proliferation of primary fibroblasts. Moreover, p27 was reported to bind cyclin E-CDK complexes and promote pRb dephosphorylation. Similarly, in DM-cultured C2C12 cells, hypoxia induced p27 accumulation. In contrast, neither p21 nor pRb protein accumulation was detected. It is noteworthy that the transcription of pRb and p21 is promoted by MyoD protein (6, 7, 43, 44). The induction of pRb gene transcription by MyoD is a key event in the process of skeletal muscle differentiation because elevated levels of pRb protein are essential for myoblast cell cycle arrest as well as for the terminal differentiation and survival of post-mitotic myocytes (43, 44). Additionally, we showed that ectopic expression of MyoD resulted in the activation of the myogenic differentiation program, identifying MyoD as a relevant target of hypoxia. Thus, hypoxia, by inhibiting MyoD expression, blocked accumulation of early myogenic differentiation markers such as myogenin and p21 as well as pRb and prevented both cell cycle withdrawal and terminal differentiation. In agreement with these observations we found that hypoxia-mediated inhibition of differentiation was reversible. DM-cultured cells removed from hypoxia and grown either in GM or DM preserved their ability to proliferate or differentiate by correctly expressing MRFs as well as cell cycle regulatory factors (data not shown). Interestingly, hypoxia, by inducing G1 accumulation, appeared to favor proliferation when normal oxygen levels were restored. Indeed, when hypoxic cells were shifted in GM and cultured in normoxia, after 48 h the total cell number was three times higher than that observed in normoxic GM-cultured cells for the same time period (Figs. 2A and 3A).

Hypoxia modulated MyoD function at post-transcriptional level by promoting its degradation by the ubiquitin-proteasome pathway. It has been reported that MyoD degradation by the ubiquitin-proteasome pathway represents a tightly controlled process that occurs in late G1 phase of the cell cycle by the cyclin-Cdk complexes (39, 45) and ensures the progression through the S phase (39). Interestingly, hypoxia prevented G1/S phase progression despite reduced levels of MyoD. Moreover MyoD degradation in hypoxic, DM-cultured C3H10T1/2 and C2C12 cells did not represent a secondary effect consequent to the accumulation of the cells in G1/S phase, since low levels of MyoD were detectable after 6 h of incubation in hypoxic culture conditions and this time is not sufficient to induce a significant G1 accumulation (not shown). Thus, hypoxia accelerated MyoD degradation through a cell cycle-independent mechanism.

In conclusion the present study demonstrates that hypoxia elicited MyoD proteasome-degradation may account for a mechanism by which low oxygen levels control myogenic differentiation. Further studies are needed to investigate whether
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other MRFs are regulated in a similar manner. The evidence that myogenic cells L6E9 that express Myf5 but not MyoD (46, 47) failed to differentiate under hypoxic conditions suggests that the inhibition of MRFs could be a general mechanism triggered by hypoxia to control myogenic differentiation in an unfavorable extracellular environment. Recently it has been demonstrated that other cellular stress such as genotoxic stress (48) as well as an alteration of the redox state of the cells (49) modulated myogenic differentiation through different mechanisms, which in turn involve MyoD inhibition. By regulating MRFs expression, extracellular stimuli may prevent differentiation and ensure cell survival. In agreement with this hypothesis we previously reported that hypoxia protected skeletal muscle cells from apoptosis in a vascular endothelial growth factor-dependent manner (50).

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