

Development of Cytosolic Hypoxia and Hypoxia-inducible Factor Stabilization Are Facilitated by Aquaporin-1 Expression^{*[5]}

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O₂ is essential for aerobic life, and the classic view is that it diffuses freely across the plasma membrane. However, measurements of O₂ permeability of lipid bilayers have indicated that it is much lower than previously thought, and therefore, the existence of membrane O₂ channels has been suggested. We hypothesized that, besides its role as a water channel, aquaporin-1 (AQP-1) could also work as an O₂ transporter, because this transmembrane protein appears to be CO₂-permeable and is highly expressed in cells with rapid O₂ turnover (erythrocytes and microvessel endothelium). Here we show that in mammalian cells overexpressing AQP-1 and exposed to hypoxia, the loss of cytosolic O₂, as well as stabilization of the O₂-dependent hypoxia-inducible transcription factor and expression of its target genes, is accelerated. In normoxic endothelial cells, knocking down AQP-1 produces induction of hypoxia-inducible genes. Moreover, lung AQP-1 is markedly up-regulated in animals exposed to hypoxia. These data suggest that AQP-1 has O₂ permeability and thus could facilitate O₂ diffusion across the cell membrane.

Oxygen (O₂) is necessary for aerobic life because of its central role in mitochondrial ATP synthesis by oxidative phosphorylation. Traditionally, O₂ is considered to diffuse freely across the plasma membrane (1, 2); however, recent studies have shown that O₂ permeability of lipid bilayers is some orders of magnitude lower than previously thought (3). Therefore, it has been suggested that there exist yet unknown plasmalemmal O₂ channels to ensure the fluxes required for O₂ uptake in conditions of high demand or limited O₂ availability. Good candidates are aquaporins, widely distributed intrinsic membrane proteins that form water-permeable complexes (3, 4). Mammalian aquaporin-1 (AQP-1)⁴ is highly expressed in cells with rapid gas

(O₂/CO₂) turnover such as erythrocytes (5) and microvessel endothelium (6, 7), and experiments performed with recombinant AQP-1 expressed in *Xenopus* oocytes have suggested that it confers upon the cells increased membrane CO₂ permeability (8–10). In addition, it has been shown that the AQP-1 tobacco plant homolog Nt-AQP-1 facilitates CO₂ transport, particularly in conditions of small transmembrane CO₂ gradient, and has a significant function in photosynthesis and in stomatal opening (11). Against a possible role of AQP-1 as a gas channel is that AQP-1 null mice do not show any obvious sign of respiratory distress or alteration of lung or erythrocyte CO₂ transport (12, 13). This observation could, however, be explained if other aquaporins can compensate, at least partially, for the lack of AQP-1. In fact, AQP-1 functions as a well established water channel, but AQP-1 null humans (Colton-null blood group) (14) and AQP-1-deficient mice (12) have only subtle changes of erythrocyte water diffusion or renal urine concentration. A recent study shows that after subcutaneous or intracranial malignant cell implantation, AQP-1 null animals present impaired tumor growth and vascularity (15), alterations that are compatible with reduced O₂ uptake in extreme conditions by tumor cells. We performed experiments designed to test the hypothesis that AQP-1 regulates transmembrane O₂ transport. Here, we show that in mammalian cells stably transfected with AQP-1 and exposed to hypoxia, the loss of intracellular O₂ is accelerated, thus leading to faster stabilization of the hypoxia-inducible transcription factor (HIF) and up-regulation of HIF-dependent genes. Redistribution of intracellular O₂ in hypoxia by mitochondrial inhibitors (16) is blunted in cells with high AQP-1 content. Moreover, inhibition of native AQP-1 expression with a small interfering RNA (siRNA) leads to up-regulation of hypoxia-inducible genes. Finally, we report that AQP-1 gene expression in lung *in vivo* is markedly induced by hypoxia. These data indicate that AQP-1 might have a role in O₂ homeostasis by facilitating O₂ diffusion across the cell membrane.

EXPERIMENTAL PROCEDURES

Cell Culture, Transfections, siRNA, and in Vitro Hypoxic Treatments—Rat cDNAs for AQP-1 and AQP-3 were cloned into pcDNA3 (Invitrogen). PC12 cells were cultured in Dul-

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[5] The on-line version of this article (available at <http://www.jbc.org>) contains supplemental Fig. 1.

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⁴ The abbreviations used are: AQP, aquaporin; HIF, hypoxia inducible factor; siRNA, small interfering RNA; TH, tyrosine hydroxylase; PGK1, phospho-

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glycerate kinase 1; VEGF, vascular endothelial growth factor; DETA-NO, (Z)-1-[N-(2-aminoethyl)-N-(2-ammonioethyl)amino]diazene-1-ium-1,2-diolate; HAQP, high AQP-1 expression PC12 clone; LAQP, low AQP-1 expression PC12 clone.

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becco's modified Eagle's medium (Invitrogen) supplemented with 5% fetal bovine serum, 10% horse serum, and 1% penicillin/streptomycin (Invitrogen) in a CO₂ (10%) incubator at 37 °C. To obtain stable subclones, PC12 cells were transfected with 20 μg of pcDNA3-AQP1 or pcDNA3-AQP3. Screening of positive clones was done by Northern blot and *in situ* hybridization analysis. From 40 clones analyzed, 20 were positive for either AQP-1 or AQP-3 with variable levels of expression.

siRNA experiments were done in an endothelial murine cell line derived from a mixed hemangioendothelioma (EOMA cells, ATCC catalog No. CRL-2586). Transfections were performed using Lipofectamine 2000 and an AQP-1-designed oligonucleotide (100 nM; ID 48361, Ambion, Austin, TX), with a scrambled oligonucleotide as a negative control. After 48 h of inhibition, levels of distinct mRNAs were analyzed by real-time reverse transcription and polymerase chain reaction.

Hypoxia experiments performed to analyze mRNA levels of tyrosine hydroxylase (*TH*), phosphoglycerate kinase 1 (*PGK1*), or vascular endothelial growth factor (*VEGF*) were done on a cell incubator with 1–10% O₂ (CO₂ 10%) (17). Experiments designed to measure stabilization of HIF-2α protein were done in a sealed glove box anaerobic workstation (Coy Laboratory Products Inc., Grass Lake, MI) with O₂ maintained at 1–2%. Mitochondrial respiratory inhibitor myxothiazol (Sigma-Aldrich) and the NO donor DETA-NO (Alexis Biochemicals, San Diego, CA) were prepared fresh and added to the culture medium.

In Vivo Hypoxic Treatments—Rats were maintained either in normal conditions or in a hypoxia incubator (Coy Laboratory Products) for 24–48 h at 10% O₂. Animals were anesthetized by injection of 350 mg/kg chloral hydrate and then sacrificed following the animal care protocols approved by our institution. Tissues were removed either to extract RNA or for immunohistochemistry studies.

Pimonidazole Staining of Hypoxic PC12 Clones—PC12 cells were grown on coverslips 24 h prior to the hypoxia treatment. Cells were then transferred to hypoxia (3% O₂) for 0.5, 1, or 2 h, or let in normoxia, and 200 μM pimonidazole was added to the medium for 30 min. After washing with PBS, cells were fixed with paraformaldehyde (3%) for 10 min at room temperature and subsequently washed again overnight with PBS. After blocking with bovine serum albumin, coverslips were incubated for 30 min with the fluorescein isothiocyanate-labeled HypoxyprobeTM-1 monoclonal antibody 1 (mAb1, 1:100 dilution) from the HypoxyprobeTM-1 Plus kit (Chemicon International, Temecula, CA). As a secondary antibody, the anti-fluorescein isothiocyanate monoclonal antibody conjugated with horseradish peroxidase provided with the kit was used. Coverslips were mounted and immunostained cells were examined on an Olympus Provis (Tokyo) microscope. Densitometric measurements of photographs taken under Normaski optics were performed using the NIH Image software.

Immunohistochemistry—After removal from the animal, tissues were kept overnight in 10% phormol and included on paraffin. Five-μm slices were cut with a microtome and mounted on microscope slides (Superfrost/plus, Fisher Sci-

entific). Rabbit polyclonal anti-AQP-1 (1:500 dilution, Abcam, Cambridge, UK) and biotin peroxidase-conjugated secondary antibody (1:200, Pierce) were used. Sections were developed in diaminobenzidine and photographed using a BX61-Olympus microscope. For cytochemistry, cells were fixed for 10 min with 3% paraformaldehyde at room temperature. A rabbit polyclonal anti-AQP-1 antibody (1:100 dilution, Chemicon) and a fluorescent polyclonal goat anti-rabbit antibody (Alexa Fluor 568, Molecular Probes, Invitrogen) were used. Immunofluorescence was then analyzed with a Leica DM IRBE confocal microscope.

RNA Analysis—Extraction of RNA from PC12 cells was done following the method described by Cathala *et al.* (18). To extract RNA from tissues, the TRIzol reagent (Invitrogen) was used as indicated by the manufacturer. For Northern analysis, 10 μg of total RNA were resolved in agarose/formaldehyde gel and transferred to Hybond-N+ nylon membrane. The Ultrahyb solution (Ambion) was used for hybridization with the specific ³²P probes (19, 20). Results were visualized using a PhosphorImager (Typhoon 9400, Amersham Biosciences). The reverse transcription reaction was performed immediately after the mRNA isolation using SuperScript II RNase H⁻ reverse transcriptase (Invitrogen).

Real-time PCR analysis was performed in an ABI Prism 7500 Sequence Detection System (Applied Biosystems, Warrington, UK) using SYBR Green PCR Master mix (Applied Biosystems) and the thermocycler conditions recommended by the manufacturer. Amplification of 18 S ribosomal or cyclophilin RNA was done to normalize for RNA input amounts. Primers were designed using the Primer Express software (Applied Biosystems). For AQP-1, the primers were: forward (F), 5'-CCA TTG ACC ACT GGC ATA GTA CA-3' and reverse (R), 5'-AGT GTC CTG ACC TGT GAA GTG AGT A-3'; for *TH*: F, 5'-TCG GAA GCT GAT TGC AGA GA-3' and R, 5'-TTC CGC TGT GTA TTC CAC ATG-3'; for *VEGF*: F, 5'-CGC AAG AAA TCC CGG TTT AA-3' and R, 5'-CAA ATG CTT TCT CCG CTC TGA-3'; for 18 S ribosomal RNA: F, 5'-AAC GAG ACT CTG GCA TGC TAA CTA-3' and R, 5'-GCC ACT TGT CCC TCT AAG AAG T-3'; for *PGK1*: F, 5'-AGA GCC CAC AGT TCC ATG GT-3' and R, 5'-GCA AAG TAG TTC AGC TCC TTC TTC A-3'; for *β-actin*: F, 5'-GGC CCA GAG CAA GAG AGG TA-3' and R, 5'-CAT GTC GTCCAGTT GGT AAC A-3'; and for *cyclophilin*: F, 5'-GCA CTG GTG GCA AGT CCA T-3' and R, 5'-GCC AGG ACC TGT ATG CTT CAG-3'. Melting curve analysis showed a single sharp peak with the expected *T_m* for all samples.

Western Blotting—Cells were washed with cold PBS, scraped in 1 ml of cold PBS, and centrifuged at 165 × *g* for 5 min at 4 °C. For whole-cell protein extract, the pellet was lysed in 150–300 μl of homogenization buffer: 50 mM Hepes (pH 7.3), 5 mM EDTA, 250 mM NaCl, 5 mM dithiothreitol, 0.2% (v/v) Nonidet P-40 (Sigma-Aldrich), and 1% (v/v) of complete protease inhibitors mixture (Sigma-Aldrich). The resuspended pellet was left on ice for 5 min, vortexed, and then centrifuged at 16,000 × *g* for 15 min at 4 °C. For cell-membrane protein extract, cells were scraped and collected in 1 ml of homogenization buffer containing 320 mM sucrose, 5 mM Hepes, and 1% (v/v) of complete protease inhibitors mixture. Samples were

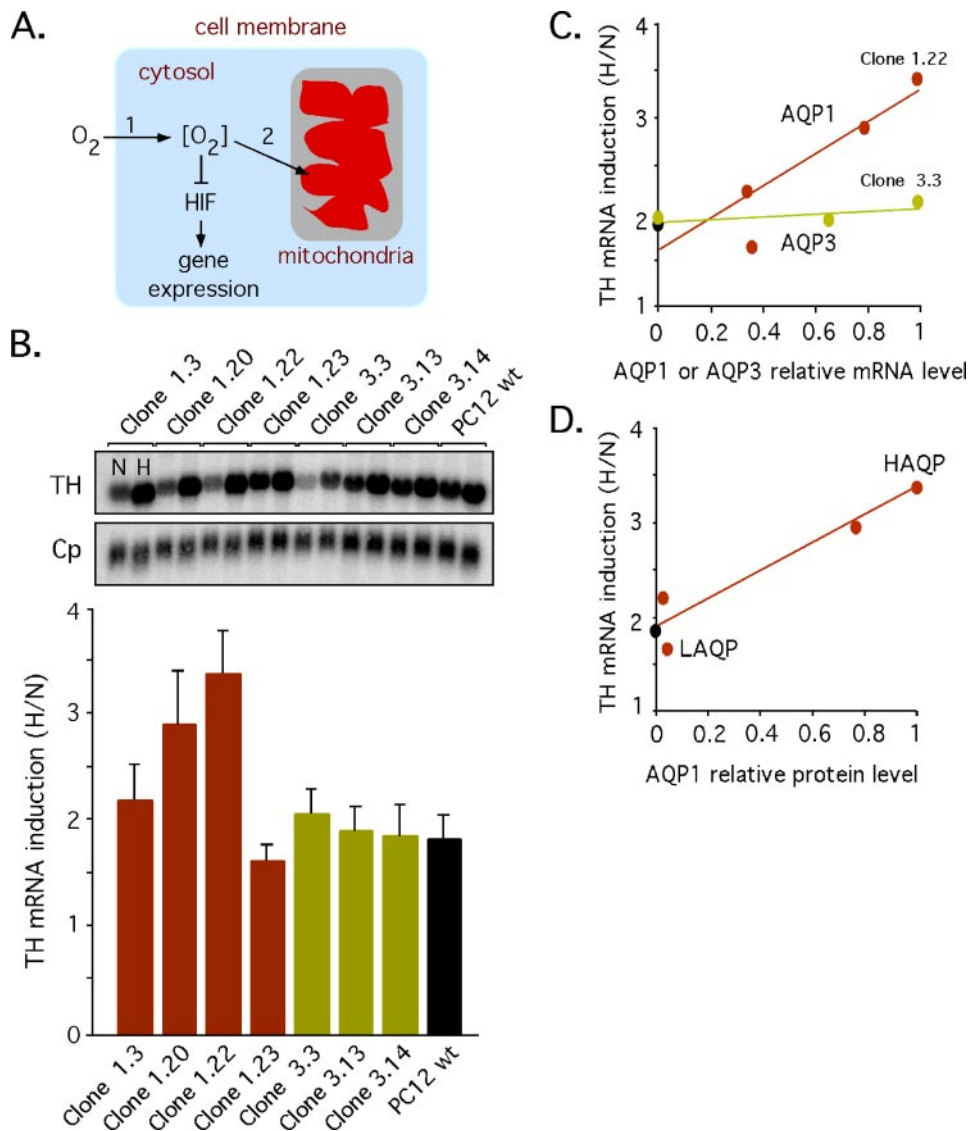


FIGURE 1. AQP-1 expression facilitates the hypoxia-dependent induction of TH gene. *A*, schematic illustration of cytosolic O₂ concentration resulting from the equilibrium between transmembrane influx (1) and uptake by mitochondria (2). HIF destabilization by O₂ and gene expression in hypoxia are represented. *B*, PC12 cell lines stably transfected with AQP-1 and AQP-3 and effect on the hypoxia-inducible TH gene. *Top*, Northern blot analysis of TH mRNA in AQP-1 (clones 1) and AQP-3 (clones 3) expressing cell clones and wild type PC12 cells exposed to normoxia (N, 21% O₂) or hypoxia (H, 6% O₂) for 12 h. Cyclophilin (Cp) mRNA is used as a load control. *Bottom*, bar diagram representing average values (mean \pm S.E.) from 7–9 separate experiments. *C*, data from the bar diagram in *B* (hypoxic mRNA TH induction) expressed as a function of the amount of AQP-1 and AQP-3 mRNA expressed in the various PC12 cell clones permanently transfected with either AQP-1 or AQP-3 gene. The black symbol indicates the value in wild type PC-12 cells, which do not express either AQP-1 or AQP-3. Note that for AQP-1-expressing clones, TH mRNA induction increases linearly ($r^2 = 0.82$) with the level of AQP-1 expression. *D*, hypoxic (6% O₂ for 12 h) TH mRNA expression as a function of the amount of AQP-1 protein detected by Western blot in the different cell clones. TH mRNA induction increases linearly ($r^2 = 0.92$) with the level of AQP-1 protein expression. The black symbol indicates the value in wild type PC-12 cells. The cell clones selected for further studies (HAQP and LAQP) are indicated in the figure.

homogenized with a Polytron and centrifuged at 2000 \times *g* for 5 min at 4 $^{\circ}$ C, and then supernatant was centrifuged at 30,000 \times *g* for 30 min at 4 $^{\circ}$ C. The pellet was resuspended in homogenization buffer plus 1% SDS. Protein concentration was analyzed with the Bradford method (Bio-Rad Protein Assay) for whole-cell extract and with the Lowry method (Bio-Rad DC Protein Assay) for cell membrane protein extraction, and kept at -20 $^{\circ}$ C until Western blot assay (21). Twenty μ g of whole-cell extracts were resolved by SDS-PAGE (6%) for HIF2 α . For

AQP-1, 40 μ g of cell membrane proteins were resolved in 10% SDS-PAGE. After electrophoresis, proteins were transferred into polyvinylidene difluoride membranes (Hybond-P, Amersham Biosciences) using a Novex apparatus (Novel Experimental Technology, San Diego, CA). Membranes were probed with 1:1000 anti-HIF2 α (Abcam), 1:2000 anti-HIF1 β (Abcam), 1:1000 anti-AQP-1 (Chemicon), and 1:10,000 anti- β -tubulin (Sigma). Immuno-reactive bands were developed with the ECL system (Amersham Biosciences) and visualized using a PhosphorImager (Typhoon 9400, Amersham Biosciences).

Water Permeability Measurements—Cells grown on coverslips were loaded for 5 min with 1 μ M calcein acetoxymethyl ester (Molecular Probes) and mounted in a small (\sim 250 μ l) perfusion chamber that allowed rapid exchange of a solution with 300 (isosmotic) to another with 150 (hyposmotic) mmol/kg. The rate of change in calcein fluorescence was monitored as described (22). The isosmotic solution used for these experiments contained in mM: 140 NaCl, 4.5 KCl, 2.5 CaCl₂, 1 MgCl₂, 10 HEPES, and 10 glucose (pH: 7.4). The hyposmotic solution was obtained by water dilution.

Statistical Analysis—Data were presented as mean \pm S.E. and were analyzed with either paired Student's *t* test or the one-way analysis of variance followed by Tukey's test.

RESULTS

Responsiveness to Hypoxia Is Accelerated in Cells with High Levels of AQP-1

To investigate the role of AQP-1 in plasmalemmal O₂ transport, we generated several subclones of PC12 cells stably transfected with either AQP-1 or AQP-3, representative members of the two major classes of aquaporins (23, 24). Because direct measurement of O₂ fluxes at the cellular level is not technically feasible, in our initial experiments the expression of HIF-dependent genes was monitored as a readout of cytosolic O₂ concentration (Fig. 1A). HIF, a master regulator of the responses of tissues to low O₂ tension (25–27), is composed of α - and β -subunits; the stability of the α -subunit is regulated by a family of O₂-dependent prolyl hydroxylases (26, 28). At low O₂ tensions,

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cellular hydroxylase activity is inhibited, and HIF α accumulates to heterodimerize with HIF β . The heterodimeric HIF activates the expression of numerous genes. In *AQP-1* or *AQP-3*-transfected clones we tested the hypoxic induction of *TH*, an O₂-dependent gene up-regulated by even mild hypoxia in PC12 cells (29). The rationale behind these experiments was that if AQP-1 is O₂-permeant, development of cytosolic hypoxia and subsequent cumulative induction of TH mRNA should be faster in cells expressing high levels of AQP-1 than in wild type PC12 cells. Hypoxic TH mRNA induction in the seven clones studied besides wild type PC12 cells is shown in Fig. 1*B* (top). Although the basal TH mRNA levels varied among the different clones and in separate experiments on a given cell clone, we compared for each cell type the relation of TH mRNA in hypoxia versus the values in normoxia. A quantitative summary of TH mRNA induction after 12 h of hypoxic (6% O₂) exposure in several clones of *AQP-1*- and *AQP-3*-transfected cells is shown in Fig. 1*B* (bottom) and C. Although for *AQP-1*-expressing clones there was a correlation between hypoxic TH mRNA induction and the level of AQP-1 expression, the response of *AQP-3* clones (either with high or low expression levels) was indistinguishable from that of wild type PC12 cells. Differences between *AQP-1*-expressing clones were also seen with shorter (6 h) hypoxic (6%) exposure (supplemental Fig. 1*A*). In the case of *AQP-1* clones there was a lineal correlation between TH mRNA expression and the level of AQP-1 in the various cell types as determined by Western blot (Fig. 1*D*). As result of this initial screening, two *AQP-1*-transfected clones, one with high level of AQP-1 and highly responsive to hypoxia (clone HAQP) and another with low level of AQP-1 and low responsiveness to hypoxia (clone LAQP) similar to wild type cells (Fig. 1*D*), were selected for further analysis. The high expression of AQP-1 in HAQP in comparison with LAQP or wild type cells was further confirmed by immunocytochemistry (Fig. 2*A*). In addition, we showed that HAQP cells responded more rapidly to an hypotonic challenge than LAQP cells (Fig. 2*B*), thus indicating that recombinant AQP-1 expressed in the PC12 cell membrane was functional as a water-permeable channel. We also used in some experiments a highly expressing *AQP-3* clone (clone 3.3 in Fig. 1, *B* and *C*), which behaves similar to the wild type PC12 cells (supplemental Fig. 1, *B–D*), to make comparisons with the highly expressing *AQP-1* cells.

The differential behavior of HAQP and LAQP cells that was clear when they were assayed in mild hypoxia (6% O₂) (Fig. 3*A*, left) became even more apparent in conditions of extreme hypoxia. After 12 h in 1% O₂, TH mRNA accumulation in HAQP was ~3–4-fold higher than in LAQP cells (Fig. 3*A*, right), indicating that the loss of cytosolic O₂ was accelerated by AQP-1. Contrarily, TH mRNA induction by cobalt, an agent that stabilizes HIF independent of O₂ tension (25, 27), was the same in the two cell clones (Fig. 3*B*). Similar to TH, the hypoxic mRNA up-regulation of phosphoglycerate kinase 1 (PGK1) and vascular endothelial growth factor (VEGF), two other O₂-sensitive genes (25, 27), was higher in HAQP than in LAQP or wild type cells (Fig. 3, *C* and *D*). Cobalt *TH* induction and hypoxic induction of *PGK1* in the *AQP-3* clone was similar to the values obtained in PC12 cells (supplemental Fig. 1, *C* and *D*). Altogether these data supported the hypothesis that

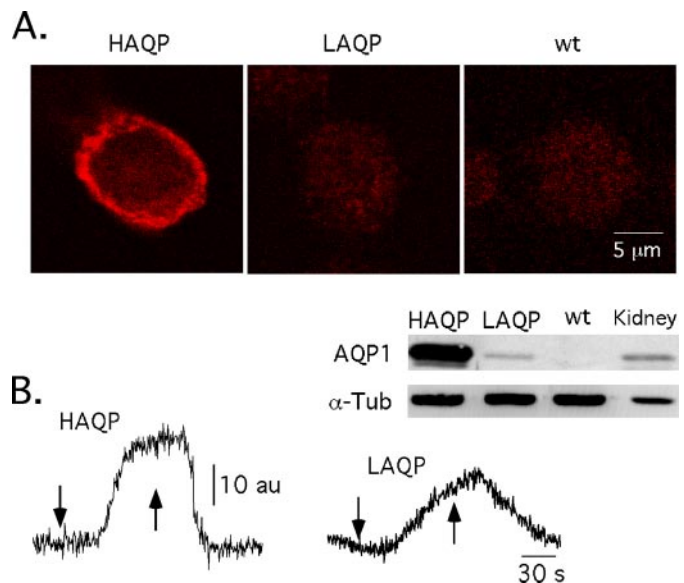


FIGURE 2. *A* (top), confocal images illustrating the immunocytochemical localization of AQP-1 in the indicated cell types. *Bottom*, Western blot analysis of AQP-1 protein expression in the cells studied in comparison with kidney homogenates. Note the high level of AQP-1 expression in HAQP cells. α -Tubulin is used as a load control. *B*, change of volume of individual HAQP and LAQP cells in response to a hypotonic shock measured by calcein fluorescence (a.u., arbitrary units). Switching to solutions of different osmolality is indicated by downward (150 mmol/kg) and upward (300 mmol/kg) arrows. wt, wild type.

AQP-1 expression confers increased O₂ permeability upon PC12 cell membrane.

HIF-2 α Stabilization in Hypoxia Depends on the Level of AQP-1 Expression—Because the expression of the O₂-sensitive genes depends on the stabilization of HIF, we investigated whether the regulation of the two components of this heterodimeric transcription factor (HIF α and HIF β) was influenced by the presence of functional AQP-1. In fair agreement with the results on the O₂-dependent genes studied (*TH*, *PGK1*, and *VEGF*), the accumulation of HIF-2 α (the most abundant O₂-dependent isoform in PC12 cells (21, 30)) during the first few hours after exposure to hypoxia was significantly greater in HAQP than in LAQP or wild type cells. As an internal control, the levels of HIF-1 β (a constitutive protein that dimerizes with all HIF isoforms (25)) were unaltered by hypoxia (Fig. 4, *A* and *B*). To check whether the presence of AQP-1 has a bidirectional impact on transmembrane O₂ transport (*i.e.* it also facilitates O₂ influx), we induced HIF-2 α accumulation in hypoxia, and after rapidly switching to normoxia, HIF-2 α degradation time course was monitored. As proteasomal degradation of accumulated HIF-2 α could be nonspecifically influenced by the amount of proteins existing in the cells, we compared in these experiments clones with high levels of aquaporin expression (clones 1.22 and 3.3, Fig. 1 and supplemental Fig. 1*B*). Degradation of HIF-2 α in normoxia was clearly faster in cells expressing AQP-1 (HAQP1) than in cells with AQP-3 (HAQP3), thus supporting the view that AQP-1 increases transmembrane O₂ transport (Fig. 4, *C* and *D*).

To further analyze the alterations in membrane O₂ transport caused by AQP-1 expression, we studied the differential response of HAQP and LAQP cells to redistribution of intra-

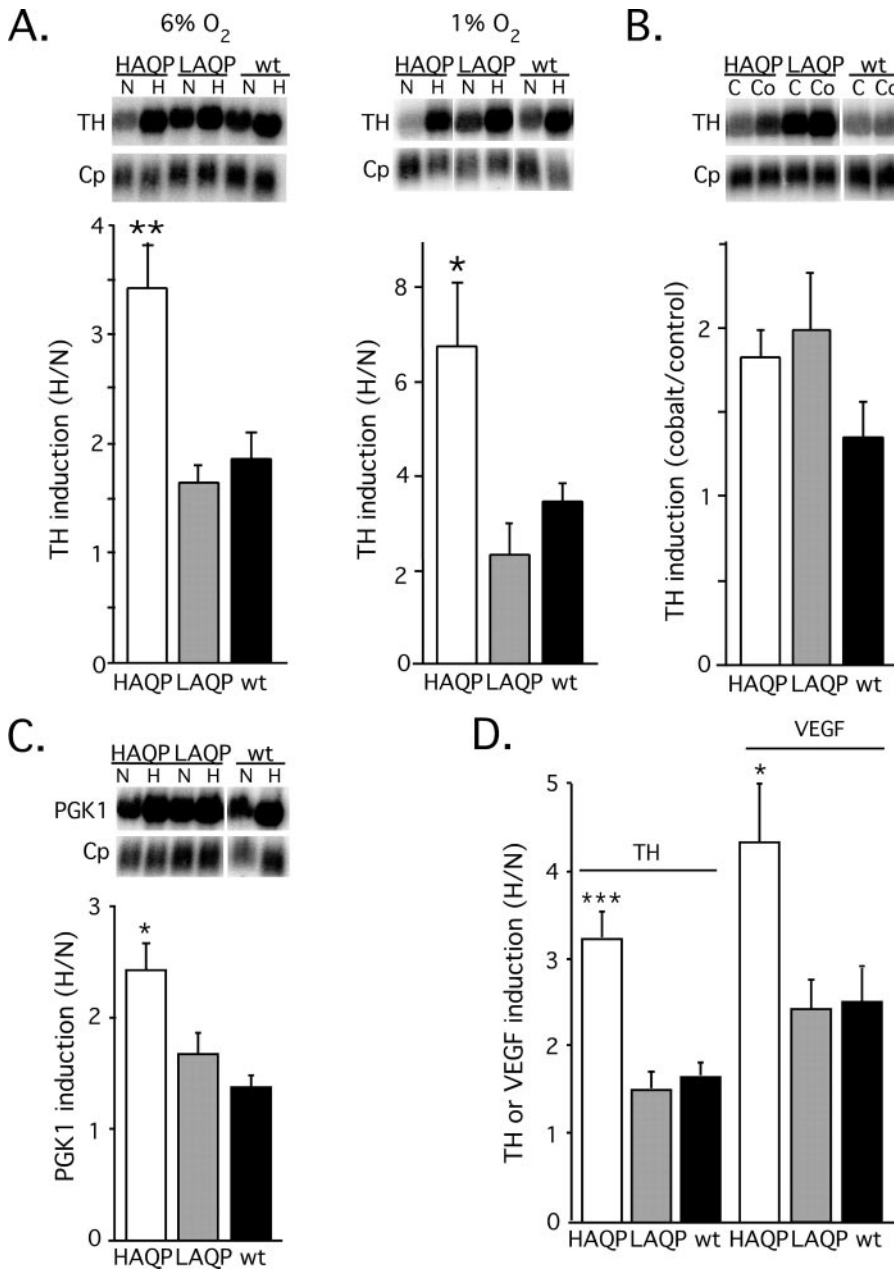


FIGURE 3. Induction of oxygen-sensitive genes in AQP-1-expressing cells. *A* (left), Northern blot analysis of TH mRNA expression in HAQP, LAQP, and wild type (wt) PC12 cells exposed to normoxia (N, 21% O₂) or hypoxia (H, 6% O₂ for 12 h). Cyclophilin (Cp) mRNA is used as a load control. The bar diagram represents values (mean \pm S.E.) from 7–9 separate experiments. *Right*, similar experiment using lower O₂ tension (H, 1% O₂ for 12 h). Average values from three separate experiments. *B*, Northern blot analysis of TH mRNA in normoxic HAQP, LAQP, and wild type cells treated with 0.1 mM cobalt chloride (Co) for 12 h (C, control). Average values from three separate experiments. *C*, Northern blot analysis of phosphoglycerate kinase 1 (PGK1) mRNA expression in AQP-1-expressing cell clones HAQP, LAQP, and wild type PC12 cells exposed to normoxia (N) or hypoxia (H, 6% O₂ for 12 h). Cyclophilin (Cp) mRNA is used as a load control. The bar diagram represents values (mean \pm S.E.) from 5 separate experiments. *D*, TH and VEGF mRNA analysis by real time PCR in the cell clones indicated exposed to normoxia (N) or hypoxia (H, 6% O₂ for 12 h). Note that the level of hypoxic TH mRNA induction is similar to that estimated by Northern blot (panel A, left). Mean \pm S.E. of 5 experiments. *, $p < 0.05$; ***, $p < 0.001$.

cellular O₂ by mitochondrial inhibition. As reported before (16, 31), inhibition of mitochondrial electron transport in wild type cells with myxothiazol (a complex III blocker) or DETA-NO (an NO donor that inhibits cytochrome *c* oxidase) lead to destabilization of HIF-2 α in hypoxic (1% O₂) cells (Fig. 5). It has been proposed that HIF destabilization upon inhibition of mitochondrial respiration in hypoxic cells (16, 31) is caused, among

other mechanisms (32, 33), by intracellular O₂ redistribution toward nonrespiratory O₂-dependent targets such as prolyl hydroxylases, so that they do not register hypoxia (16). Quite interestingly, HIF-2 α destabilization produced by myxothiazol and by DETA-NO was less pronounced in HAQP in comparison with LAQP or wild type cells (Fig. 5). These observations further support the view that AQP-1 increases O₂ permeability in HAQP cells, thereby facilitating the transmembrane efflux of intracellular O₂ spared by inhibition of respiration. Thus, upon mitochondrial inhibition hypoxic HAQP cells can maintain a more severe cytosolic hypoxia and higher HIF-2 α content than LAQP or wild type cells.

Cytosolic Loss of O₂ in Hypoxia Is Facilitated by AQP-1 Expression—To obtain more direct evidence that AQP-1 facilitates the development of cytosolic hypoxia, and to rule out that overexpression of AQP-1 activates HIF through an oxygen-independent signal transduction pathway, we studied the transmembrane fluxes of O₂ in HAQP and LAQP cells using pimonidazole staining, a broadly used hypoxia marker that forms intracellular protein adducts at low O₂ tension (31, 34, 35). With this technique, the loss of cytosolic O₂ upon exposure to hypoxia was clearly faster in HAQP than in LAQP cells (Fig. 6). These differences estimated with the Hypoxyprobe method were similar to those inferred from the HIF-2 α experiments (Fig. 4B), thus suggesting that the distinct time courses of HIF-2 α stabilization observed in HAQP and LAQP cells reflect, indeed, differences in membrane O₂ transport between the two cell types.

Knocking Down Native AQP-1 with siRNA Results in Up-regulation of Hypoxia-inducible Genes—Besides the studies on PC12 cells overexpressing AQP-1, we also performed loss of function experiments using a nonmetastatic murine hemangioendothelioma cell line (EOMA cells), which behave *in vitro* in a manner similar to microvascular endothelial cells (36, 37). First, we investigated whether, as in microvessel endothelium (6, 7), EOMA cells also express AQP-1 as well as hypoxia-dependent

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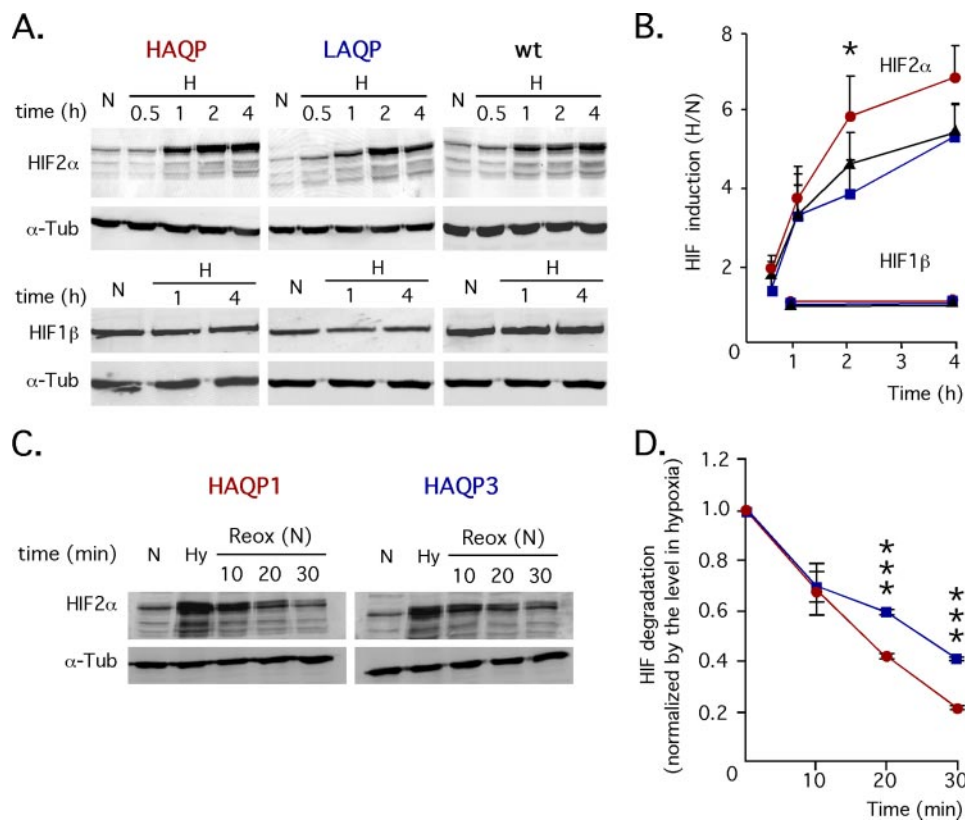


FIGURE 4. Regulation of HIF in AQP-expressing cells. *A*, Western blot analysis of HIF2 α and HIF1 β in the cell clones studied exposed to normoxia (N, 21% O₂) or hypoxia (H, 1% O₂) for the time indicated. *B*, summary of HIF2 α induction by hypoxia in HAQP (red), LAQP (blue), and wild type (black) cell clones. Data points are mean \pm S.E. values of 5–13 different experiments. After 2 h in hypoxia, HIF2 α value in HAQP cells is statistically different ($p < 0.05$) with respect to that in normoxia. For LAQP or wild type cells, 4 h in hypoxia are needed to reach HIF2 α values statistically different from those in normoxia. *C*, representative Western blot analysis of HIF2 α in cell clones overexpressing AQP-1 (HAQP1) or AQP-3 (HAQP3) exposed to hypoxia (H, 1% O₂) for 4 h and then transferred back to normoxia (N, 21% O₂) for the time indicated. *D*, time course of HIF2 α degradation during reoxygenation (21% O₂). Levels of HIF2 α were normalized in each clone to the value obtained at the end of the hypoxic treatment. Data points are mean \pm S.E. values of 3 different experiments. Degradation of HIF2 α in HAQP1 expressing cells was significantly higher (***, $p < 0.001$) than in high AQP-3 expression PC12 clone after 20 and 30 min of reoxygenation.

genes such as *PGK1* or *VEGF* (Fig. 7A). In these cells the level of *PGK1* mRNA expression was quite insensitive to moderate hypoxia; however, *VEGF* mRNA induction was clearly modulated by moderate levels of hypoxia in an O₂ tension-dependent manner (Fig. 7A). In normoxic EOMA cells treated with specific *AQP-1* siRNA, the level of *AQP-1* expression decreased to ~50–60% of the value in control. In parallel with the decrease of *AQP-1* expression we observed an almost 2-fold increase of *VEGF* mRNA (Fig. 7B). Knocking down *AQP-1* with siRNA had little effect on the levels of either β -actin (a gene independent of O₂ tension) or *PGK1*, a gene that, as shown above, is less sensitive to hypoxia than *VEGF* in EOMA cells (Fig. 7). These data further suggest that the decrease of *AQP-1* expression in endothelial cells diminishes the transmembrane O₂ fluxes, which results in reduced cytosolic O₂ tension and induction of HIF-dependent genes.

AQP-1 Gene Expression Is Up-regulated by Hypoxia in Vivo—Because AQP-1 is expressed in microvessels (6, 7), we searched its up-regulation in lungs of animals subjected to hypoxia. *A priori*, we presumed that this could be an advantageous adaptive response if AQP-1 plays any role in membrane O₂ transport.

Indeed, AQP-1 was expressed in lung endothelial cells (inset in Fig. 8), and the AQP-1 gene was also highly O₂-sensitive in comparison with the more classically studied *PGK1* gene. In animals exposed to mild hypoxia (10% O₂) for 24 h, the level of *PGK1* mRNA was barely altered, but we observed a robust ~3-fold AQP-1 mRNA induction in lung tissue (Fig. 8).

DISCUSSION

The results in this study provide evidence that AQP-1 can accelerate the establishment of cytosolic hypoxia possibly through facilitation of O₂ transport across the plasma membrane. We have also shown that AQP-1 gene expression is regulated exquisitely by O₂ tension. Although we did not directly measure transmembrane O₂ fluxes in PC12 cells because it is not technically feasible, our conclusions are supported by several independent observations. (i) In cells overexpressing AQP-1 (HAQP cells) and exposed to a hypoxic environment, the levels of HIF α - and O₂-dependent genes (*TH*, *PGK1*, and *VEGF*) were higher than in LAQP cells, suggesting that the loss of cytosolic O₂ was accelerated by the presence of AQP-1. It must be noted that this experimental protocol estimates the cumulative stabilization of HIF (and mRNA induction of HIF-dependent genes) during the development of hypoxia rather than the level of cytosolic O₂ in steady state conditions. (ii) HIF-2 α degradation elicited by reoxygenation of hypoxic cells was faster in cells expressing AQP-1 than in cells highly expressing AQP-3. (iii) The differential behavior exhibited by HAQP and LAQP cells during exposure to low O₂ tension was confirmed when the development of cytosolic hypoxia was estimated by the cumulative staining of pimonidazole, an HIF-independent hypoxia marker (34, 35). (iv) In hypoxic PC12 cells, inhibition of mitochondrial respiration produced HIF-2 α destabilization (16), which was less pronounced in HAQP than in LAQP or wild type cells. This finding is compatible with an increased O₂ permeability in HAQP cells, thereby facilitating the transmembrane efflux of intracellular O₂ spared by inhibition of respiration. (v) In normoxic endothelial cells, lowering *AQP-1* expression with a siRNA blunted aquaporin mRNA expression by ~50–60% and selectively up-regulated *VEGF*, a gene induced in these cells by even moderate levels of hypoxia. Altogether, these data strongly support the view that AQP-1 is involved in O₂ homeostasis and facilitates transmembrane O₂ transport.

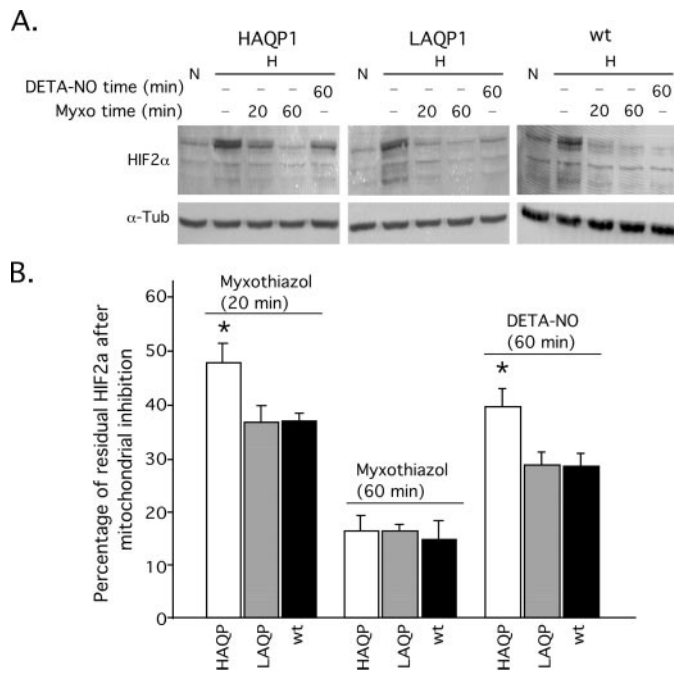


FIGURE 5. Differential response by AQP-1-expressing clones to mitochondrial inhibition. *A*, induction of HIF2 α in hypoxic (1% O₂ for 4 h) cells and its degradation after treatment with myxothiazol (*myxo*, 1 μ M) or DETA-NO (100 μ M) for the indicated time periods. A representative example from 7–8 separate experiments with α -tubulin used as a load control. *B*, summary of residual HIF2 α levels after treatment with mitochondrial inhibitors. Values (mean \pm S.E. from 7–8 different experiments) in the *bar diagrams* are expressed as the percentage of the maximum level (100%) of HIF2 α reached after 4 h of hypoxia. Destabilization of HIF2 α after treatment with myxothiazol (20 min) and DETA-NO (60 min) was significantly less (*, $p < 0.05$) in HAQP cells than in LAQP or wild type (*wt*) cells.

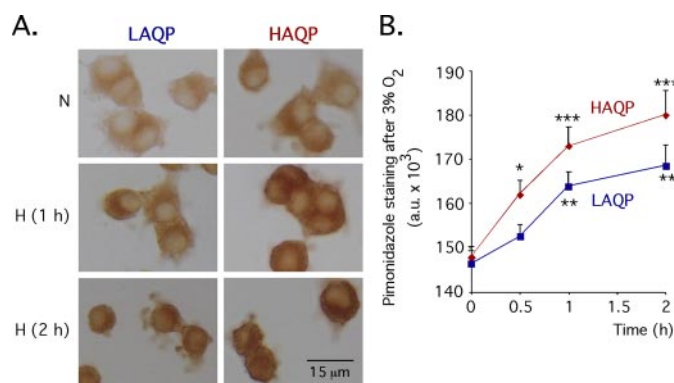


FIGURE 6. Pimonidazole staining of hypoxic PC12 cell clones. *A*, microphotographs of pimonidazole staining in the cell clones studied exposed to normoxia (*N*, 21% O₂) or hypoxia (*H*, 3% O₂) for the time indicated. Pimonidazole (200 μ M) was added to the cells for the last 30 min of incubation, and afterward the immunostaining was performed. *B*, summary of the densitometric measurements of the pimonidazole staining in HAQP (*red*) and LAQP (*blue*) cells. Data points are mean \pm S.E. values of seven different experiments. After 0.5 h in hypoxia, the increase in pimonidazole staining in HAQP cells is statistically different ($p < 0.05$) with respect to that in normoxia. In contrast, for LAQP cells 1 h in hypoxia is needed to reach a value statistically different from the one in normoxia (*a.u.*, arbitrary units). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Our proposal that AQP-1 could function as an O₂ channel is in fair agreement with previous reports suggesting that AQP-1 increases membrane CO₂ permeability (8–11). Recently, it has been proposed that AQP-1 also transports NO across endothelial cells and contributes to the regulation of vascular tone (38, 39). Although our experiments suggest that AQP-3 is not

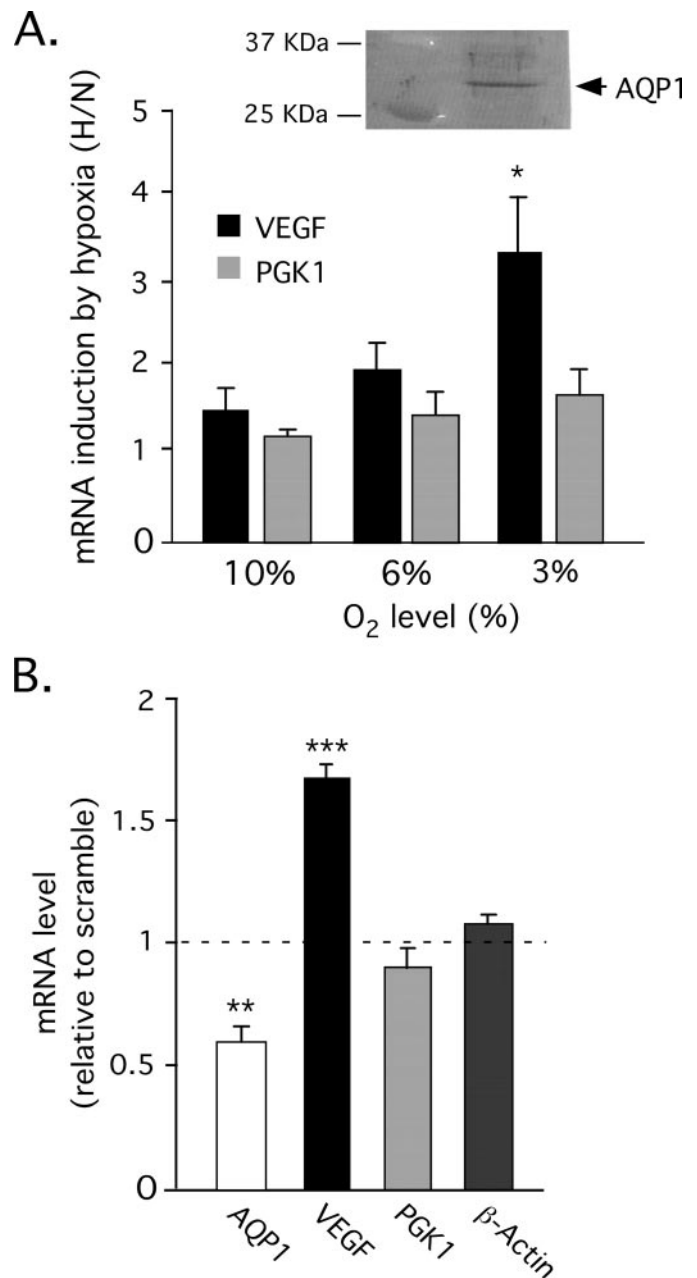


FIGURE 7. Knocking down the level of native AQP-1 in EOMA cells modulates the expression of endogenous O₂-sensitive genes. *A*, levels of mRNA expression of VEGF and PGK1 analyzed by quantitative real time PCR after 24 h of incubation of the cells in different hypoxic conditions (*H*, 10, 6, and 3% O₂) normalized to values in normoxia (*N*, 21% O₂). *Bars* represent mean \pm S.E. values of 6–9 different experiments. Note that whereas the expression of both genes was induced by hypoxia, the induction of VEGF was clearly higher to that of PGK1. The *inset* shows a Western blot analysis confirming the expression of AQP-1 protein in endothelial EOMA cells. *B*, effect of blunting AQP-1 expression by siRNA on the mRNA expression of O₂-sensitive genes (VEGF, PGK1, and β -actin). Note that inhibition of AQP-1 expression by about 50% produced an almost 2-fold induction of the highly O₂-sensitive gene VEGF, whereas the others genes remained unaffected. *Bars* represent mean \pm S.E. values of seven different experiments. For endogenous normalization of the PCR amplifications, cyclophilin levels were used as load control. mRNA levels are normalized to values obtained with scramble siRNA. Statistical analysis is referred to levels of β -actin expression. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

O₂-permeable, there are several other aquaporins that overlap AQP-1 distribution and could partially compensate for the AQP-1 deficiency (24). For example, in alveolar epithelial type I

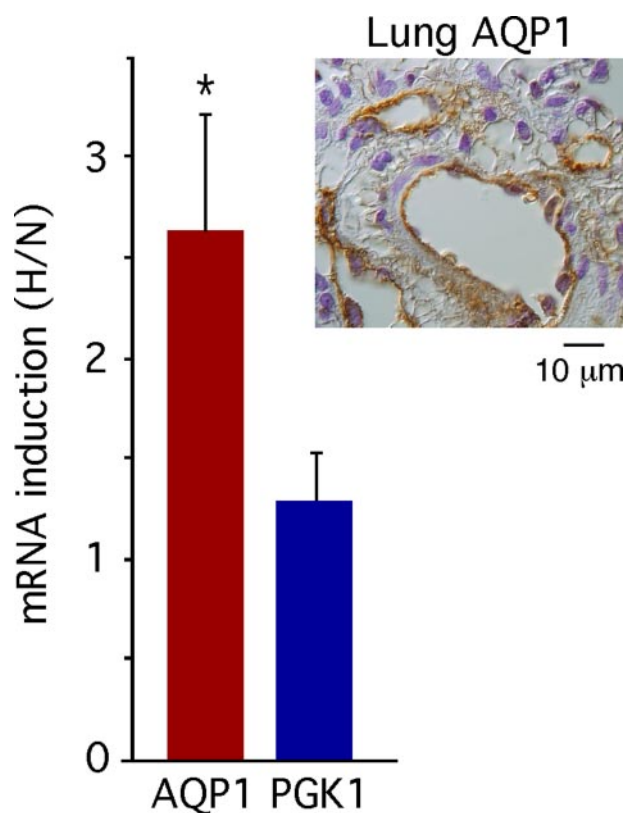


FIGURE 8. Hypoxic up-regulation of the AQP-1 gene *in vivo*. Real-time PCR analysis showing induction of AQP-1 and PGK1 mRNA in lung homogenates of rats exposed to hypoxia (10% O₂) for 24 h. Mean \pm S.E. of 7 experiments. *, $p < 0.05$. The inset is a microphotograph illustrating the AQP-1 protein expression in rat lungs after 48 h in hypoxia (10% O₂). Immunostaining of AQP-1 appears as brown deposits preferentially located at the microvessel endothelium.

cells the AQP-5 gene is up-regulated by HIF through interaction with hypoxia-responsive elements in the promoter (40), and AQP-4 mRNA has also been shown to be up-regulated by hypoxia in astrocytes (41). This could explain why humans (14) and mice (12, 13) lacking AQP-1 do not exhibit major gross physiological alterations related with either water or O₂ metabolism. In addition, besides aquaporins, other membrane proteins could also contribute to membrane O₂ transport (3, 10).

The novel role of AQP-1 in O₂ homeostasis proposed here is compatible with its expression in erythrocytes (5) or vascular endothelium (6, 7). AQP-1 is also overexpressed in cells of proliferating microvessels (8), as well as in aberrant cells of human and rat tumors (42–44), situations in which a limited O₂ availability could have induced AQP-1 expression to facilitate O₂ uptake by the cells. In fact, AQP-1 null mice present impaired tumor growth after subcutaneous or intracranial malignant cell implantation, with reduced tumor vascularity and extensive necrosis (15). Migration of AQP-1-deficient aortic endothelial cells *in vitro* is greatly decreased, and it has been suggested that the lack of AQP-1 alters water fluxes required for rapid turnover of cell membrane protrusions at the leading edge of migrating cells (15). The presence of AQP-1 could also be critical for aerobic ATP synthesis, particularly in membranes of low gas permeability (4) or when transmembrane O₂ gradients are small, as it occurs in cells exposed to environments with extremely low O₂ concentrations or in multilayered diffusion

barriers such as that existing between the lung alveoli and blood. In conclusion, our observations support a new physiologic role for aquaporins as O₂ transporters (4) besides their canonical function as water-permeable channels. AQP-1 pharmacology could be of potential use in conditions of altered O₂ exchange or to control O₂-dependent angiogenesis and tumor cell growth.

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