Hypertonic induction of aquaporin-5: novel role of hypoxia-inducible factor-1α

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AQP5 by osmotic stress has been reported in a mouse lung epithelial (MLE-15) cell line and in hyperosmolar rats, suggesting possible roles for AQP5 in regulating alveolar surface liquid toxicity and/or maintaining cell volume (17). Effects of hypertonic stress on AQP5 expression in MLE-15 cells were shown to be mediated via an ERK-dependent pathway. Increases in AQP5 were gradual, with levels of AQP5 mRNA and protein peaking at 12 and 24 h, respectively. AQP5 mRNA and protein both increased without a change in mRNA stability, consistent with transcriptional activation of AQP5. However, a 1.5-kb AQP5 proximal promoter/enhancer could not be activated by hypertonicity in transient transfection assays.

Isolated rat AT2 cells in primary culture lose their characteristic phenotypic hallmarks, such as lamellar bodies and production of surfactant-associated proteins, and change morphologically to resemble AT1 cells (10). Concurrently, they gradually express phenotypic markers characteristic of AT1 cells in situ, including expression of AQP5, suggesting that AT2 cells in primary culture transdifferentiate toward an AT1

Aquaporins constitute a family of water channel proteins that increase water permeability to osmotic gradients in a variety of secretory and absorptive cells (2, 20). Aquaporin-5 (AQP5) is a member of this family that is expressed at high levels in lung, salivary gland, and lacrimal tissues (26). In adult alveolar epithelium, AQP5 is selectively expressed on the apical surface of alveolar epithelial type I (AT1) cells (3, 25). AT1 cells isolated from rat lungs demonstrate very high osmotic permeability to water compared with other mammalian cells, including alveolar epithelial type II (AT2) cells (11). While AQP5−/− mice do not exhibit a lethal phenotype or a decrease

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cell-like phenotype in vitro (4, 6). When grown on semipermeable supports, AEC form polarized high-resistance monolayers and exhibit active sodium transport, reflecting properties of the alveolar epithelium in vivo (7). AT2 cells in primary culture therefore constitute a well-characterized model with which to investigate functional properties of the alveolar epithelium.

Hypoxia-inducible factor-1 (HIF-1) is a transcription factor that mediates adaptive responses to changes in tissue oxygenation. It regulates the transcription of numerous genes involved in vascular development, glucose and energy metabolism, iron metabolism, and cell proliferation and viability. HIF-1 is a basic helix-loop-helix transcription factor that is composed of two subunits, HIF-1α and HIF-1β (30). HIF-1β is constitutively expressed, whereas the level of HIF-1α is subjected to regulation by hypoxia and iron chelators. In addition, we and others (5, 39) have shown that HIF-1 is activated in response to stimulation of signaling pathways or treatment with growth factors, leading to induction of several genes under normoxia, including vascular endothelial growth factor (VEGF).

In this report, we utilized AEC in primary culture in conjunction with a previously cloned 4.5-kb fragment of the rat AQP5 promoter/enhancer (3) to investigate molecular mechanisms governing transcriptional regulation in response to hypertonic stress. In searching for transcription factor(s) that mediate hypertonic induction of AQP5 in both primary rat AEC and MLE-15 cells, HIF-1α has been identified as a transcription factor that is induced to activate transcription of AQP5 and VEGF by hypertonicity. These findings suggest that HIF-1α plays a novel role in governing cellular responses to hypertonic stress and that signaling pathways regulating hypoxic response may overlap, at least in part, with those activated during hyperosmotic stress.

METHODS

Cell isolation and preparation of AEC monolayers. AT2 cells were isolated from adult male Sprague-Dawley rats (~125–150 g) by disaggregation with elastase (2.0–2.5 U/ml; Worthington Biochemical, Freehold, NJ), followed by differential adherence on IgG-coated bacteriological plates. All animals were treated in accordance with the guidelines and approval of the University of Southern California Institutional Animal Care and Use Committee. Freshly isolated AT2 cells were plated in minimal defined serum-free medium (MDSF) consisting of Dulbecco’s modified Eagle’s medium and Ham’s F-12 nutrient mixture in a 1:1 ratio, supplemented with 1.25 mg/ml bovine serum albumin, 10 mM HEPES, 0.1 mM nonessential amino acids, 2.0 mM glutamine, 100 U/ml sodium penicillin G, and 100 μg/ml streptomycin. Cells were seeded onto tissue culture-treated polycarbonate filter cups (Nuclepore, Corning-Costar, Corning, NY; 0.4 μm) at a density of 1.0 × 10⁶ cells/cm² and grown to confluence for RNA isolation and morphological analysis. Cell viability (95%) was measured by trypan blue dye exclusion.

Maintenance of cell lines. MLE-15 cells (J. Whitsett, University of Cincinnati) were cultivated on 35 mm culture dishes (Becton Dickinson) in HITES medium (RPMI 1640; Invitrogen, Carlson, CA) supplemented with 10 nM hydrocortisone, 5 μg/ml insulin, 5 μg/ml human transferrin, 10 nM β-estradiol, 5 μg/ml selenium, 2 mM L-glutamine, 10 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2% FBS. 293T human kidney cells were grown in Dulbecco’s modified Eagle’s medium (Sigma, St. Louis, MO) supplemented with 4 mM L-glutamine, 10% FBS, and 200 U/ml penicillin.

Exposure of primary AEC to hyperosmotic stress. Beginning on day 6 in culture, medium was made hypertonic by adding 200 mM sorbitol to MDSF. Sorbitol was selected to induce hyperosmotic stress based on a previous study demonstrating that AQP5 protein expression was only increased by relatively impermeable solutes such as sorbitol and sucrose (17). Cells were harvested for evaluation of AQP5 mRNA and protein levels by Northern and Western blot analysis, respectively, at intervals up to 24 h. To determine the effects of hypertonicity on mRNA stability, AEC treated with sorbitol on day 6 were exposed to actinomycin D (1 μg/ml) and RNA was harvested at intervals up to 18 h for Northern blot analysis.

Northern blot analysis. Total RNA was harvested according to the method of Chomczynski and Sacchi (9). Briefly, AEC monolayers were washed with cold phosphate-buffered saline (PBS; pH 7.2) and lysed in Trizol D solution (G-Medium containing DTT and 7.2 mM sodium citrate, 0.5% sarkosyl, and freshly added 0.72% 2-mercaptoethanol). RNA was extracted with phenol/chloroform and precipitated with isopropanol twice. RNA (10 μg) was fractionated by denaturing agarose gel electrophoresis and transferred onto Hybond-N+ membrane (Amersham, Piscataway, NJ). Membranes were hybridized with a 1.2-kb AQP5 cDNA probe labeled with [32P]dCTP [Random Prime DNA Labeling Kit (Roche, Indianapolis, IN)]. Signal intensity was normalized to 18S rRNA. AQP5 and 18S rRNA levels were quantified by exposure to a phosphor screen, scanned using a Storm 840 PhosphorImager (Amersham), and analyzed using ImageQuant software (Amersham).

Western blot analysis. Protein was extracted from AEC monolayers in 2% SDS lysis buffer (62.5 mM Tris-HCl, 2% SDS, and 10% glycerol) and lysates were cleared by centrifugation (30 min, 9,500 rpm, 4°C). Samples (10–20 μg) were separated by SDS-PAGE (12.5%) and transferred to Immobilon-P membranes (Millipore, Bedford, MA). Membranes were blocked in 5% nonfat milk solution overnight at 4°C and incubated with an anti-AQP5 polyclonal antibody (Chemicon, Temecula, CA) at a dilution of 1:1,000 in 20 mM Tris-HCl, pH 7.5, 500 mM NaCl, and 0.1% Tween 20 for 2 h at room temperature. After being washed, membranes were incubated with peroxidase-labeled anti-rabbit secondary Ab diluted 1:1,000 (Promega, Madison, WI) for 1 h. Complexes were visualized using enhanced chemiluminescence (ECL; Amersham) according to the manufacturer’s protocol. To ensure equal loading, protein was normalized to eukaryotic initiation factor 2α (eIF2α) using a rabbit anti-eIF2α polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Signal intensity was quantified using the Fluorchem Imaging System (Alpha Innotech, San Leandro, CA).

Production of lentivirus in 293FT cells. A 4.5-kb rat AQP5 promoter fragment was cloned into the lentivirus backbone vector (L. Naldini, Turin, Italy) pRRL.hCMV.Sin.GFP after removal of the CMV promoter. The green fluorescent protein reporter gene was replaced by a luciferase reporter gene from pGL3-Basic (Promega) to create pRRL.AQP5.Sin.Luc. Infectious lentivirus was created by cotransfection of pRRL.AQP5.Sin.Luc plasmid with pΔ8.7 and pMD.G into human 293T cells. The infection cocktail (500 μl), consisting of 10 μg of pRRL.AQP5.Sin.Luc plasmid, 10 μg pCMV.ARB.91, 6 μg pMD.G, 63 μl 2 M CaCl₂, and H₂O₃, was mixed with 500 μl warm HBS (8.18% NaCl, 5.9% HEPES, and 0.2% NaHPO₃; pH 7.2), added dropwise to 293T cells plated on 10 cm culture dishes and incubated at 37°C overnight. The virus was harvested after 48 h, filtered through 0.45 μm filters, and concentrated through a centrifugal concentrator (300 kDa cutoff, Pall Life Science, Ann Arbor, MI) and stored at −80°C.

Lentivirus transduction of MLE-15 cells and primary AEC. One day before infection, MLE-15 cells were trypsinized, counted, and...
plated at $5 \times 10^5$ cells per 60 mm culture dish. On the day of infection, 300 µl of thawed AQ5-Luciferase (AQ5-Luc) virus stock, 1.5 ml of HITES medium and polybrene (final concentration 6 µg/ml) were mixed and added to the cells after removal of culture medium. Cells were incubated at 37°C overnight and changed into HITES media the following day. After 48 h, virus-infected MLE-15 cells were split into 24-well plates and seeded at $10^5$ cells/well. After reaching ~80% confluence, cells were exposed to hypertonic sorbitol solution in RPMI medium containing 0.05% FBS for periods of 12, 24, and 36 h. Control cells were incubated in RMPI medium containing 0.05% FBS. Cells were harvested by the addition of passive lysis buffer (Promega) and shaking at room temperature for 15 min, followed by one freeze/thaw cycle. Luciferase activity was measured using a commercially available kit (Promega) and normalized to total protein measured by the Bio-Rad protein assay kit (Bio-Rad, Hercules, CA).

For transduction of AEC, $10^6$ AT2 cells were plated in each well of a 24-well Primaria plate. On day 3, after being washed twice with MDSF, cells were incubated at 37°C overnight with 100 µl AQ5-Luc virus stock, 100 µl MDSF and polybrene (final concentration 6 µg/ml). The following day, virus was removed and MDSF was added to a total volume of 1 ml per well. On day 5, hypertonic induction and analysis of luciferase activity were performed as described above at intervals up to 36 h.

Preparation of nuclear extracts. AEC were washed with PBS (pH 7.2) and scraped with a cell scraper in PBS. After centrifugation, pellets (from 10 wells) were resuspended in 1 ml hypotonic buffer (10 mM HEPES, pH 7.5, 10 mM KCl, 3 mM MgCl$_2$, 0.05% Nonidet P-40, 1 mM EDTA, pH 8.0, 10 mM NaF, and 0.1 mM Na$_3$VO$_4$) supplemented with protein inhibitor cocktail set III (5 µl/ml, Calbiochem, San Diego, CA) and incubated on ice for 20 min, followed by vortexing for 10 s and centrifugation at 500 g for 10 min at 4°C. Nuclear pellets were washed twice with hypotonic buffer and lysed in 200 µl lysis buffer [100 mM HEPES (pH 7.5), 0.5 M KCl, 5 mM MgCl$_2$, 28% glycerol, and protein inhibitor cocktail set III (5 µl/ml)] for 30 min on ice with shaking. After centrifugation at 20,000 g for 30 min to remove debris, the nuclear extracts were stored at ~80°C.

Hybridization of nuclear extracts from AEC with TranSignal protein/DNA assay. AT2 cells were plated on 6-well plates (2 $\times 10^6$ cells per well). From day 6, cells were exposed to hypertonic sorbitol solution and nuclear extracts harvested at 4 and 24 h following initiation of sorbitol exposure. Hybridization to the DNA array was carried out according to the manufacturer’s protocol (Panomics, Redwood City, CA). Briefly, 10 µg of nuclear extract from AEC in MDSF or sorbitol were incubated with the TranSignal probe (version II) for 30 min at 4°C and hybridized to the TranSignal array. Signal was detected with an alkaline phosphatase conjugated antibody. The gel area containing the protein/DNA complex was excised. Probe in the complexes was extracted with gel extract beads and hybridized to the TranSignal array. Signal was detected with an HRP-based chemiluminescence detection system and analyzed using a FluorChem Imager (Alpha Innotech).

Preparation of AQ5PS and HIF-1β plasmids and transient transfection assays. The 1716-bp rat AQ5PS-luciferase reporter plasmid was previously generated in pGL2-Basic (3). HIF-1α and HIF-1β expression plasmids, pCEM4/HIF-1α and pBMS/Neo/D24–1, were prepared. For transient transfections, MLE-15 cells were seeded at a density of 6 $\times 10^5$ cells per well in 24-well plates the day before transfection. The following day, cells were transfected using SuperFect reagent (Qiagen, Valencia, CA). The 1716-AQ5PS-luciferase reporter construct (0.75 µg) was co-transfected with increasing amounts of HIF-1α and HIF-1β expression plasmids in equal ratios or corresponding empty expression vectors and incubated with 6 µl SuperFect reagent for 10 min at room temperature. Cells were harvested 48 h after transfection, and firefly luciferase activity was determined with the Dual-Luciferase reporter assay system (Promega). Firefly luciferase was normalized to Renilla luciferase activity.

Mutation of HIF-1 binding sites by site-directed mutagenesis. Two putative HIF-1α consensus binding sites were identified in the proximal AQ5 promoter between −419/−414 and −330/−326. Before mutants were generated out of these two sites, a 483-bp fragment of AQ5 spanning from −2 to −484 was generated from the −1716-bp construct by PCR using the forward primer 5’-CGGTGATACCTTC-CAGACGCTGTTGGGAG-3’ and reverse primer 5’-GCAAGCTT-GTGGCCTTGGGCGCGGGTGT-3’, which have KpnI and HindIII sites at each 5’ end, respectively. The PCR product was first cloned into TOPO cloning vector (Invitrogen), sequenced, and cloned into pG2L-2Basic. The two HIF-1α binding sites were mutated using the Quiック Change II Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA), changing the core motifs from −419 to −414 (site 1) and from −330 to −326 (site 2). Primers 5’-GGAGTTCCGACCTTTCGCGCCAGGGAGC-3’ and 5’-CGCCCTCCTCTGCGCGGCGGGCGTCGACCTCGC-3’ were used to mutate site 1 and primers 5’-GGCCGATGCGGCCTGAAAAGGGCGCGCGGCGTCGACCTCGC-3’ and 5’-GGCCCGCAGCGCCCTGTTGACCCACCGCCGGCGTCGACCTCGC-3’ were used to mutate site 2 (mutations are underlined). Clones were sequenced to verify mutations. Transcriptional activity was assessed in transient transfection assays in MLE-15 cells following cotransfection of HIF-1α and HIF-1β expression plasmids in equal ratios or corresponding empty expression vectors and compared with activity of wild-type −484-AQ5PS-luciferase.

Response of VEGF HRE to hypertonicity. To determine whether hypertonic stress regulates hypoxia-responsive genes via the HIF-1 pathway, MLE-15 cells were transiently transfected with a wild-type VEGF hypoxia response element (HRE) linked to a luciferase reporter (WT HRE-luc) or mutant HRE-luc (MT HRE-luc) construct. After exposure to hypertonic sorbitol solution for an additional 24 h, cells were harvested for assay of luciferase activity. Control cells were incubated in RPMI medium containing 0.05% FBS.

Effect of hypertonicity on mRNA expression in AEC by quantitative RT-PCR. RNA was harvested from primary AEC or MLE-15 cells exposed to hypertonic sorbitol solution for 24 h. Samples were pretreated with a DNA-free kit (Ambion, Austin, TX) for removal of contaminating DNA. cDNA was synthesized with Superscript II (Invitrogen) using a mixture of random hexamer and oligo-dT primers in a ratio of 1:10. PCR primers used for amplification of VEGF, HIF-1α, AQ5, and 18S are as follows: VEGF forward 5’-GTC-CATAGTGGACCTGGTT-3’ and reverse 5’-CTATGTCGCGCTGTGGTGA-3’; HIF-1α forward 5’-GCCACGACCATCTCCTG-3’ and reverse 5’-GGCCATGGTAAAGGAAGTG-3’; AQ5 forward 5’-GGCTACGAGCACCAAC-3’ and reverse 5’-GACCGAGAAGCAACATGATAAG-3’; 18S forward 5’-CGTCATGCAAGCACCAGATGTT-3’ and reverse 5’-CGTCATGCAAGCACCAGATGTT-3’. The amplification protocol was set as follows: 95°C denaturation for 30 min, followed by 40 cycles of 10-s denaturation at 95°C, 20-s annealing at 60°C, and 30-s extension at 72°C. Real-time quantitation was carried out with the iCycler iQ Real-Time PCR Detection System (Bio-Rad). Melt-curve analysis was performed immediately after each amplification protocol to ensure specificity of the chosen primer pairs and absence of primer-dimers. Relative expression of VEGF, AQ5, and HIF-1α was calculated according to the comparative method using the formula $2^{-\Delta\Delta Ct} = 2^{-(Ct_{sample} - Ct_{calibrator})}$ to calculate the normalized target gene expression level in the sample. In this experiment, $Ct_{calibrator} = Crsorbit - Ct_{18S}$; $Ct_{calibrator} = Crsorbit - Ct_{18S}$.
hypertonic sorbitol solution containing 0.05% FBS. Control cells were maintained in RPMI medium containing 0.05% FBS. RNA and protein were harvested after 24 h of sorbitol treatment for quantitation of HIF-1α mRNA and AQP5 mRNA and protein by quantitative RT-PCR and Western blotting.

**Statistical analysis.** Data are shown as means ± SE; n is the number of observations. We used z-tests to determine whether the ratioometric data (i.e., normalized against control) are different from control. When appropriate, two-tailed Student’s t-tests were used to determine the significance of differences between two group means. For all other data, we performed one-way ANOVA, followed by post hoc comparisons of group means using the Student-Newman-Keuls procedure. P < 0.05 was considered significant.

**RESULTS**

**Effects of hypertonic stress on expression of AQP5 mRNA and protein.** Effects of hypertonic stress on AQP5 expression were evaluated in primary rat AEC in monolayer culture in serum-free medium. Medium was made hypertonic by the addition of 200 mM sorbitol on day 6, and cells were harvested after 24 h. Northern and Western blot analyses (Fig. 1) demonstrate 54 ± 19% and 128 ± 24% increases in steady-state levels of AQP5 mRNA and protein, respectively, after exposure to hypertonic sorbitol solution.

**Fig. 1.** Effects of hypertonicity on aquaporin-5 (AQP5) mRNA and protein levels. A: representative Northern blot demonstrates an increase in AQP5 mRNA following exposure to hypertonic sorbitol solution. Densitometric analysis indicates an increase of −50% after 24 h. Results are normalized to 18S rRNA. B: representative Western blot demonstrates an increase in AQP5 protein following exposure to hypertonic sorbitol solution. Densitometric analysis indicates an increase of −130% after 24 h. Results are normalized to eukaryotic initiation factor (eIF) levels. Data are expressed as means ± SE (n = 3). *P < 0.05, significantly different from minimal defined serum-free medium (MDSF).

**Effects of hypertonic stress on AQP5 stability.** To assess whether hypertonic induction of AQP5 mRNA was exerted at the transcriptional or posttranscriptional level, AQP5 mRNA stability was evaluated by comparing the half-lives of AQP5 mRNA from primary rat AEC grown in MDSF ± sorbitol, following treatment with actinomycin D. The representative Northern blot shown in Fig. 2A demonstrates parallel reductions in AQP5 mRNA in MDSF ± sorbitol following treatment with actinomycin D. Densitometric analysis (Fig. 2B) indicates similar reductions in AQP5 mRNA relative to t = 0 in MDSF ± sorbitol (51 ± 21% vs. 48 ± 11% at 18 h, respectively), suggesting that the increase in AQP5 following exposure to hypertonic sorbitol solution likely involves transcriptional control mechanisms.

**Fig. 2.** Effects of hypertonicity on AQP5 mRNA stability. A: representative Northern blot demonstrates parallel reductions in AQP5 mRNA in MDSF ± sorbitol. B: densitometric analysis demonstrates similar reductions relative to t = 0 (51 ± 21% vs. 48 ± 11%, respectively, in MDSF vs. MDSF + sorbitol) following exposure to actinomycin D. Results are expressed as means ± SE (n = 3). *P < 0.05, significantly different from MDSF at t = 0. **P < 0.05, significantly different from MDSF + sorbitol at t = 0.

**Hypertonicity-induced AQP5 transcriptional activation in MLE-15 cells and primary rat AEC.** Although results from AQP5 mRNA stability studies suggest that osmotic stress-
regulated AQP5 expression is mediated at the transcriptional level (Fig. 2), we were unable to demonstrate transcriptional activation of AQP5 by hypertonicity in transient transfection assays using \( ^{1716} \text{AQP5-luciferase} \) in MLE-15 cells (data not shown). To overcome the low transfection efficiency barrier in MLE-15 and primary AEC, lentivirus vectors encoding an AQP5-luciferase reporter (pRRL.AQP5.Sin.Luc) were engineered and produced (Fig. 3) to investigate the effects of hypertonic stress on AQP5 transcriptional activity. Following transduction with lentivirus, MLE-15 cells were exposed to hypertonic medium for up to 36 h. As shown in Fig. 4A, exposure to hypertonic sorbitol solution induced a 2-fold increase in luciferase activity in MLE-15 cells after 12 h, with further increases up to 36 h. Transduction of primary AEC with pRRL.AQP5.Sin.Luc on day 3, followed by treatment with sorbitol from day 5, showed an 2.5-fold increase in luciferase activity, which was sustained for 24 h but declined to basal levels after 36 h (Fig. 4B).

Identification of activated transcription factors in nuclear extracts of primary AEC following hypertonic stress. To identify putative transcription factors involved in modulating effects of hypertonicity on AQP5 transcription, nuclear extracts from primary AEC in MDSF were exposed to hypertonic sorbitol for 4 or 24 h were interacted with oligonucleotide probes that recognize specific transcription factor consensus binding sequences. After formation of DNA-protein complexes, complexed probes were separated and hybridized to an array containing consensus binding sequences. As shown in Fig. 5, several transcription factors were identified as being activated as early as 4 h following exposure to hypertonic sorbitol solution, suggesting a potential role for these transcription factors in mediating cellular transcriptional responses to osmotic stress. Since two putative HIF-1α consensus binding sites were identified in the proximal AQP5 promoter, we chose to further characterize interactions of HIF-1 with the AQP5 promoter to elucidate the transcriptional mechanisms mediating responses of AQP5 gene transcription to osmotic stress.

**HIF-1 transactivates the AQP5 proximal promoter.** Cotransfection of MLE-15 cells with an AQP5-luciferase construct spanning from \(-1716 \text{ to } -1716 \text{ bp (}^{1716} \text{AQP5-Luc}) \) and increasing amounts of HIF-1α and HIF-1β expression plasmids, pCEM4/HIF and pBM5/Neo/D24−1, demonstrated transcriptional activation of AQP5 by HIF-1 with concentration-dependent increases in luciferase activity (Fig. 6). Transcriptional activation of \(-1716\text{-AQP5-Luc} \) and \(-484\text{-AQP5-Luc} \) by HIF-1α and HIF-1β did not differ significantly (Fig. 7A), suggesting that the elements responsible for activation of AQP5 by HIF-1 are encompassed by the proximal promoter region, which contains two putative HIF-1 binding sites (Fig. 7B). Transcriptional activation was decreased by 53 ± 8 and 51 ± 8% following transient transfection of HIF-1α and HIF-1β expression plasmids with constructs mutated at either putative HIF-1 binding site 1 or 2, respectively, compared with the parental \(-484\text{-AQP5-Luc} \) construct (Fig. 7C). However, even when both HIF-1 binding sites were mutated, there was no further reduction in luciferase activity, suggesting the presence of additional cryptic binding sites within the proximal promoter and/or the possibility that HIF-1 activates AQP5 through its interactions with other transcription factors.
Induction of VEGF HRE by osmotic stress. To further investigate the role of the HIF-1 pathway in mediating osmotic stress-induced gene expression, effects of hypertonic sorbitol solution on transcriptional activation of the VEGF HRE, a known hypoxia response element, were evaluated. Reporter assays were undertaken in MLE-15 cells transiently transfected with a VEGF HRE-Luc construct or a plasmid, in which this HRE was mutated. Following exposure to hypertonic sorbitol solution, luciferase activity of the wild-type HRE-Luc construct was decreased 3-fold and mutation of the HRE almost completely attenuated the hypertonicity-mediated induction (Fig. 8).

Induction of VEGF expression by osmotic stress. Since VEGF is one of the target genes of HIF-1 in hypoxic conditions (14), we further examined whether hypertonicity modulates expression of VEGF via its effects on HIF-1, using quantitative real-time PCR (Q-PCR) to evaluate VEGF expression in AEC. As shown in Fig. 9, VEGF expression increased 3.20 ± 1.05 fold after exposure to hypertonic sorbitol solution, suggesting that VEGF may also be one of the target genes of hypertonicity-induced HIF-1 activation. AQP5 used as positive control demonstrated a 5.56 ± 0.91-fold increase following hypertonic stress.

Effects of HIF-1α inhibition with HIF-1α siRNA on induction of AQP5 by osmotic stress. To confirm a specific role for HIF-1α in mediating hypertonicity-induced increases in AQP5, inhibition of HIF-1α expression was accomplished using HIF-1α siRNA in MLE-15 cells. As shown in Fig. 10, HIF-1α mRNA is increased ~2-fold following exposure to hypertonic sorbitol solution (Fig. 10A), accompanied by ~4-fold increases in AQP5 mRNA (Fig. 10B) and protein (Fig. 10C). Prevention of HIF-1α induction with HIF-1α siRNA significantly abrogates induction of AQP5 mRNA (Fig. 10B) and protein (Fig. 10C) by sorbitol, further supporting a role for HIF-1α in mediating responses of AQP5 to hypertonicity.

DISCUSSION

Exposure of AEC to hypertonic sorbitol solution leads to increased steady-state levels of both AQP5 mRNA and protein in primary AEC. These effects are not accompanied by changes in AQP5 mRNA half-life, suggesting that hypertonicity-mediated induction is transcriptionally regulated. This was confirmed by transduction of both primary AEC and MLE-15 cells with lentivirus vectors harboring the 4.5-kb rat AQP5 promoter/enhancer region upstream of the luciferase reporter in the presence of a hyperosmotic stimulus. We also identified HIF-1α as a transcription factor whose expression is upregulated by hypertonicity. Cotransfection of HIF-1α and HIF-1β with AQP5 in MLE-15 cells revealed dose-dependent transcriptional activation of the AQP5 promoter by HIF-1, which is partially inhibited by mutagenesis of putative HIF-1α binding sites in the proximal promoter. Consistent with these results, quantitative RT-PCR confirmed increased expression of HIF-1α mRNA following sorbitol treatment. Inhibition of sorbitol-induced increases in HIF-1α mRNA with siRNA significantly prevented induction of AQP5 mRNA and protein by hypertonic stress. Transient transfection of VEGF HRE-luciferase into MLE-15 cells and quantitative RT-PCR analyses of endogenous VEGF expression further support the hypothesis that exposure of AEC to hypertonic sorbitol solution leads to induction of VEGF. Together with increased HIF-1 activity in nuclear extracts from osmotically stressed AEC, these studies suggest a novel role for HIF-1 in mediating adaptive responses of AEC to hypertonicity involving both AQP5 and VEGF.

AQP5 was previously shown to be upregulated by hypertonicity in MLE-15 cells and in the lungs of rodents that were made hypertonic by injection of hyperosmolar saline (17). These effects were found to be mediated, at least in part, through an ERK-dependent pathway. Because of their inability to demonstrate transcriptional activation of an AQP5-luciferase reporter, Hoffert et al. (17) suggested that effects of hyperos-
molarity on AQP5 expression may be indirect. In this study, transcriptional activation of AQP5 by hypertonic stress is demonstrated in parallel with increases in AQP5 mRNA and protein in AEC. This difference may be because lentivirus transduction is more efficient than transient transfections for the reporter assays and/or that there is a requirement for chromatin modification following stable integration of the AQP5 reporter. In a recent study, Sidhaye et al. (34) provided additional evidence to support long-term regulation of AQP5 in lung epithelial cells by showing that cAMP has both acute and chronic effects on AQP5 expression in lung epithelial cells, although cAMP is typically believed to mediate more acute effects.

Several AQP family members have been shown to be regulated by hypertonicity. In the case of AQP1, AQP4, and AQP9, regulation has been shown to be at the transcriptional level (1). A GC-rich hypertonicity response element was demonstrated in AQP1, but the trans-acting hypertonicity response element binding protein has not yet been identified (42). No discrete hypertonicity response element has been identified in AQP4 and AQP9, although regions important for osmotic responsiveness have been localized within these promoters (1). Other hypertonicity-responsive genes, such as aldose reductase, sodium/myo-inositol cotransporter, sodium/chloride/betaine cotransporter, urea transporter, and heat shock protein, have been shown to contain the osmotic response element ORE/TonE, which initiates adaptive accumulation of compatible organic osmolytes by binding of toxicity-response enhancer/osmotic response element-binding protein (TonEBP/OREBP) in response to increased toxicity (16).
Protein/DNA array analyses identified HIF-1α as a transcription factor that was induced by hypertonicity in our current studies, which was further confirmed by qRT-PCR. Further examination of the proximal AQP5 promoter/enhancer region identified two putative cis-acting HRE (located at −419/−414 and −330/−326), leading us to investigate the possibility that effects of hypertonicity are mediated by interactions of HIF-1 with the AQP5 promoter. Consistent with the array findings, co-transfections demonstrated transcriptional activation of the proximal AQP5 promoter by HIF-1, which was partially abrogated by mutagenesis of these two sites, suggesting a role for HIF-1 in osmotic regulation of AQP5. Furthermore, inhibition of HIF-1α induction with HIF-1α siRNA significantly reduced induction of AQP5 by hypertonic stress. These results represent the first demonstration that HIF-1 and HRE are involved in transcriptional regulation of AQP5 and VEGF by hypertonic stress in AEC.

HIF-1-regulated genes play key roles in critical developmental and physiological processes, including angiogenesis, erythropoiesis, glucose transport, glycolysis, iron transport, cell survival, and proliferation (29). More importantly, clinical and laboratory studies have demonstrated that HIF-1 target genes are involved in numerous human diseases, such as myocardial and cerebral ischemia, pulmonary hypertension, pre eclampsia, intrauterine growth retardation, and cancer (31). In addition to oxygen, iron is also required as a cofactor for prolyl hydroxylase, which explains the observed hypoxia-mimetic effects of iron chelators such as desferoxamine (DFO) and iron antagonists such as cobalt chloride (44). Under hypoxia or hypoxia-mimetic conditions, HIF-1α escapes prolyl hydroxylation and degradation, which enables it to dimerize with the constitutively expressed HIF-1β and translocate to the nucleus where the heterodimer exerts gene regulatory activity. The HIF-1 heterodimer binds to a specific HRE, which contains the core sequence RCGTG (29). Although HIF-1 activity is largely regulated in a hypoxia-dependent manner, increasingly it is being recognized that activation of HIF-1 can be mediated by a variety of stimuli, including hormones and cytokines (28), nitric oxide (19), carbon monoxide (12), and mechanical (21) and thermal (33) stress. We report here the novel finding that HIF-1 activity is also regulated by hypertonic stress. Concurrent increases in AQP5 and VEGF expression in response to hypertonicity suggest that the HIF-1-dependent pathway plays an additional role in governing adaptive responses following hypertonic exposure of AEC.

It has been reported that possible mechanisms of HIF-1α activation include HIF-1α stabilization, posttranslational modification, nuclear translocation, dimerization, transcriptional activation and/or interaction with other proteins (46). Upregulation of HIF-1α mRNA by hypertonicity suggests that activation of HIF-1α in this setting is at least in part transcriptionally regulated. Interestingly, treatment of MLE-15 cells with DFO mimics hypertonic induction of HIF-1 but not AQP5 by Western blot analysis (data not shown), indicating that HIF-1 is necessary but not sufficient to induce AQP5 expression. The effects of HIF-1 on AQP5 under hypertonic conditions probably involve mechanisms distinct from those occurring under hypoxic conditions. For example, p38 has been shown to modulate HIF-1 activity through competitive binding of p300 (46), and p38 MAP kinase is required for hypoxic (but not DFO) activation of HIF-1 (13). Differential interactions of HIF-1 with these and other transcription factors likely determine the specific response of target genes to HIF-1 under different stress conditions.

Emerging evidence has suggested that HIF-1 plays an important role in governing cellular processes to enable cells to survive or escape noxious conditions, such as an oxygen deficient environment or toxic insults (38, 43). Within distal lung epithelium, AT1 cells are particularly susceptible to injury. Hence, it is not surprising that HIF-1 is involved in regulation of a differentiation-related gene, AQP5, and its responses to environmental stress. The exact nature of cross-talk between hypoxia-mediated and hypoxia-elicited HIF-1 activation is unclear. Hypertonic activation of aquaporins has been shown to involve activation of several MAP kinase pathways with variable responses, depending on the particular cell type. For example, hypertonic induction of AQP5 required activation of the ERK signaling pathway in MLE-15 cells (17), whereas hypertonic induction of AQP1 in mouse inner medullary collecting duct-3 cells involved all three MAP kinase pathways (40). Induction of AQP4 and
AQP9 by hyperosmolar mannitol in astrocytes activated p38-MAPK (1). Upregulation of several other osmotically responsive genes, including TonE-regulated genes, has been shown to be largely mediated by p38 MAPK, although the other MAP kinases, such as ERK, JNK, and non-receptor-mediated tyrosine kinases, have also been implicated (32). Our current findings suggest HIF-1 as an additional potential downstream target of this pathway. Emerling et al. (13) recently demonstrated that activation of p38/HIF-1 is required for HIF-1 activation, suggesting involvement of a mechanism whereby hypertonic activation of MAP kinase pathways, and particularly p38, may lead to activation of HIF-1. In addition, several reports have shown that the p42/p44(ERK2/ERK1) MAPK can phosphorylate the HIF-1α subunit, leading to enhanced HIF-1 activity (27, 45). Notwithstanding the uncertainty concerning the detailed mechanism(s) underlying this process, our results unequivocally demonstrate a crucial role of HIF-1 in augmenting AQP5 expression. Although it is possible that these events are indirectly linked, they may functionally cooperate downstream under different extracellular stimuli such as hypoxia and hyperosmotic stress. One plausible model is that HIF-1-mediated transactivation is part of a central regulatory circuit in response to both hypoxic and hyperosmotic stresses.

Although the precise physiological role of AQP5 to regulation of epithelial surface liquid in the lung is yet to be clearly defined, conditions in which effects of hyperosmolarity on AQP5 would be relevant in vivo include salt-water drowning and diseases such as cystic fibrosis (in which epithelial surface fluid regulation may be impaired). A recent study by Chen et al. (8) demonstrated that downregulation of AQP5 in a human lung adenocarcinoma cell line led to marked increases in MUC5AC, implicating AQP5 in chronic regulation of mucin genes and mucus secretion, also undoubtedly affecting epithelial surface liquid regulation. In this report, we have demonstrated that hypertonic stress induces AQP5 expression at the transcriptional level and, unexpectedly, HIF-1 is a transcription factor that is activated by hypertonicity. Additionally, hypertonicity is able to activate VEGF HRE and increase endogenous VEGF expression, suggesting that the mechanism(s) mediating hypertonic induction of AQP5 via HIF-1 may be extended to regulation of other genes (including other aquaporins), which

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**Fig. 10.** Prevention of HIF-1α induction with siRNA reduces induction of AQP5 expression by osmotic stress. MLE-15 cells were transfected with HIF-1α small interfering (si)RNA, followed by treatment with sorbitol for 24 h after which cells were harvested for analysis of HIF-1α and AQP5 expression. **A:** quantitative RT-PCR demonstrates that hypertonic induction of HIF-1α mRNA is prevented by HIF-1α siRNA. **B:** quantitative RT-PCR demonstrates that hypertonic induction of AQP5 mRNA is significantly reduced by HIF-1α siRNA. Data represent means ± SE of three different experiments. *P < 0.05, significantly different from scrambled siRNA in RPMI medium; **P < 0.05, significantly different from scrambled siRNA after sorbitol treatment.
together participate in regulation of cell survival under environmental stresses such as hypertonicity.

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