ORIGINAL RESEARCH

Oxygen-dependent regulation of aquaporin-3 expression

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Abstract: The purpose of this study was to investigate whether aquaporin-3 (AQP3) expression is altered in hypoxia and whether hypoxia-inducible transcription factor (HIF)-1 regulates the hypoxic expression. AQP3 mRNA expression was studied in L929 fibrosarcoma cells and in several tissues derived from mice that were subjected to hypoxia. Computational analysis of the AQP3 promoter revealed conserved HIF binding sites within close proximity to the translational start site, and chromatin immunoprecipitation assays confirmed binding of HIF-1α to the endogenous hypoxia response elements. Furthermore, hypoxia resulted in increased expression of AQP3 mRNA in L929 fibrosarcoma cells. Consistently, shRNA-mediated knockdown of HIF-1α greatly reduced the hypoxic induction of AQP3. In addition, mRNA analysis of organs from mice exposed to inspiratory hypoxia demonstrated pronounced hypoxia-inducible expression of AQP3 in the kidney. Overall, our findings suggest that AQP3 expression can be regulated at the transcriptional level and that AQP3 represents a novel HIF-1 target gene.

Keywords: transcriptional regulation, oxygen, hypoxia-inducible factor, hypoxia response element

Introduction

Aquaporins (AQP) are integral membrane proteins that form pores on the membranes of cells.[1] There are 13 known types of AQP in mammals. They transport water molecules in and out of the cells along an osmotic gradient, preventing the passage of ions and other solutes. AQPs are classified into three major subfamilies, ie, classical AQPs (AQPs 0, 1, 2, 4, and 5), aquaglyceroporins (AQPs 3, 7, 9, and 10), and unorthodox AQPs (AQPs 6, 8, 11, and 12).[2] In contrast to the classical "water channels", aquaglyceroporins are permeated by small uncharged molecules like glycerol and urea in addition to water; the function of the unorthodox AQP is currently being elucidated. Genetic defects involving *AQP* genes have been associated with several human diseases like congenital cataracts,[3] nephrogenic diabetes insipidus,[4] or neuromyelitis optica.[5]

Although some of the AQPs are expressed constitutively, there is growing knowledge of stimulus or context-dependent expression of specific AQP isoforms. The expression of AQP1 and 4 has been linked to changes in oxygen availability.[6–8] If oxygen supply does not meet oxygen demand, the affected cells become hypoxic. Cells respond to hypoxia by activating a gene expression program, which is mediated by the hypoxia-inducible transcription factor (HIF). HIF contains two subunits, ie, the constitutively expressed β-subunit (HIFβ) and the oxygen-dependently regulated α-subunit (HIFα).[9] Among the three described HIFα subunits (HIF-1α, HIF-2α, and HIF-3α), the HIF-1α

subunit is most widely expressed. In normoxia, HIFα undergoes oxygen-dependent prolyl hydroxylation, which triggers the subsequent von Hippel Lindau-mediated ubiquitination and proteasomal degradation.[10] The hydroxylation is facilitated by the three described prolyl-4-hydroxylase domain (PHD) enzymes, which are members of the oxoglutarate-dependent dioxygenase family.[11,12] Compared to other members of this family, PHDs have a low affinity toward oxygen, which results in a substrate/oxygen-dependent enzymatic activity within the range of physiological pO_2.[13] In hypoxia, the HIFα subunits escape PHD-dependent hydroxylation and subsequent degradation. As a result, HIFα is stabilized, heterodimerizes with HIFβ, and induces HIF-dependent gene expression. For AQP1, a direct transcriptional regulation of the AQP1 promoter by HIF-1 has been demonstrated.[14] The oxygen-dependent expression of the water-permeable AQP1 complex goes along with the rationale that it also contributes to the transmembrane gas permeation for CO_2, NO, H_2O, NH_3 and most likely, also O_2 in several cell types.[15–17]

In humans, AQP3 was found to be expressed in a variety of tissues. However, expression levels varied considerably, with weak expression in the heart, skeletal muscle, and central nervous tissue; moderate expression in the respiratory system; and high expression levels in the kidney.[18] In the kidney, AQP3 constitutively localizes to the basolateral plasma membrane of collecting duct principal cells, connecting tubule cells, and inner medullary collecting duct cells. Treatment of human retinal pigment cells with $CoCl_2$ results in increased AQP3 mRNA levels.[19] $CoCl_2$ is sometimes described as an inducer of a "chemical hypoxia", because it can induce a HIF-response by inhibiting PHDs. The inhibitory action of cobaltous ions is thought to be based on replacement of catalytic Fe (II) and/or the effects on the availability of ascorbate.[20] However, this is a PHD nonspecific effect and would also affect other Fe (II) and ascorbate-dependent processes. Therefore, we analyzed the response of AQP3 mRNA levels upon exposure to hypoxia in vitro and in vivo and tested if the expression of AQP3 is mediated by HIF-1.

Material and methods
Cell lines and cell culture
L929 cells (ATCC # CCL-1, ATCC, Manassas, VA, USA), derived from mouse, were cultivated in high glucose modified Eagle's medium (Pan Biotech GmbH, Aidenbach, Germany) containing 10% fetal calf serum (Biochrom Ltd, Berlin, Germany), 50 units/mL penicillin G, and 50 µg/mL streptomycin (Pan Biotech). Cells were cultivated in a humidified 5% CO_2, 95% air atmosphere at 37°C. For hypoxic conditions,

O_2 levels were decreased to 1% with N_2 in an in vivo 400 workstation or SCITIVE workstation (Ruskinn Technology Ltd, Pencoed, UK). The establishment and characterization of the HIF-1α knockdown cell line was described recently.[21]

Hypoxic exposure of mice
Animal housing, hypoxic exposure, and organ excision for RNA isolation in male C57BL/6 mice have been described previously.[22] Briefly, groups of three male C57BL/6 mice were exposed to 8% O_2 inspiratory normobaric hypoxia for 0, 12, and 30 hours. All animal experiments were performed according to FELASA (Federation of Laboratory Animal Science Associations) category B and GV-SOLAS (Society for Laboratory Animal Science/Society of Laboratory Animals) standard guidelines and approved by the German animal welfare committee (Regierungspräsidium Karlsruhe, Karlsruhe, Germany) as described previously.[22]

RNA extraction and RT-qPCR
For RNA isolation, $2×10^5$ L929 cells were seeded in 2 mL of culture medium and incubated for 1–48 hours at 37°C and 5% CO_2 in normoxia (21% O_2) or hypoxia (1% O_2). RNA was isolated, and transcript levels were analyzed as described previously.[23] For mouse tissue samples, 2 µg of total RNA was reverse transcribed (RT) using AffinityScript reverse transcriptase (Agilent Technologies, Santa Clara, CA, USA), and the cDNA levels were estimated by quantitative polymerase chain reaction (qPCR) using a SybrGreen qPCR reagent kit (Sigma-Aldrich, St Louis, MO, USA) in a MX3000P light cycler (Agilent Technologies). The ribosomal protein S12 (mS12) was used as a housekeeping gene. The fold change in gene expression was determined by using the delta-delta-CT data analysis method.

Primers were as follows: AQP3 (qPCR on L929 cell extracts) forward 5′-TGCCTTGCGCTAGCTACTTT-3′, AQP3 reverse 5′-AGGCCCAGATTGCATCGTAG-3′; AQP3 (qPCR on tissue extracts) forward 5′-GCTGTGACCTTCGCAATGTG-3, AQP3 reverse 5′-GTACACGAAGACACCAGCGA-3′; Glut1 forward 5′-TGGCCTTGCTGGAACGGCTG-3′, Glut1 reverse 5′-TCCTTGGGCTGCAGGGAGCA-3′; mS12 forward, 5′-GAAGCTGCCAAGGCCTTAGA-3′, mS12 reverse 5′-AACTGCAACCAACCACCTTC-3′; PHD3 forward 5′-GGCCGCTGTATCACCTGTA T-3′, PHD3 reverse 5′-TTCTGCCCTTTCTTCAGCAT-3′.

Chromatin immunoprecipitation assay
A total of $3×10^6$ cells were incubated under normoxic (20% O_2) or hypoxic (1% O_2, 14 hours) conditions. Cells

were treated with 1% formaldehyde for 30 minutes at room temperature, followed by the addition of glycine to a final concentration of 0.125 M. Cells were washed in 4°C phosphate-buffered saline and pelleted. Cell pellets were resuspended in 600 µL of cold cell lysis buffer (50 mM Tris-Cl, pH 8.1, 10 mM ethylenediaminetetraacetic acid [EDTA], 1% sodium dodecyl sulfate [SDS], and protease inhibitors). Cell lysates were sonicated (Bandelin Sonopuls, BANDELIN electronic GmbH and Co. KG, Berlin, Germany) for five 20-second intervals on ice to shear DNA to fragments. Cell debris was removed by centrifugation. The supernatants were diluted 10-fold in IP buffer (16.7 mM Tris [pH 8.1], 1.1% Triton X-100, 1.2 mM EDTA, 0.01% sodium dodecyl sulfate [SDS], and protease inhibitors). Chromatin solutions were precleared with protein A/G Plus Agarose slurry (Santa Cruz Biotechnology, Dallas, TX, USA) while rotating at 4°C for 2 hours. An input sample control (500 µL) was taken from the precleared supernatant, and the remaining supernatant was divided into three parts and incubated with anti-HIF-1α (NB100-479, Novus Biologicals LLC, Littleton, CO, USA), anti-Histone (ab12179, Abcam, Cambridge, UK), or anti-Tubulin (ab6046, Abcam)-antibodies overnight at 4°C. Chromatin/protein complexes were isolated with 60 µL protein A/G Agarose. Pellets were washed with low salt, high salt buffer series (low salt buffer: 20 mM Tris [pH 8.1], 150 mM NaCl, 1% Triton X-100, 2 mM EDTA, 0.1% SDS; high salt buffer: 20 mM Tris [pH 8.1], 500 mM NaCl, 1% Triton X-100, 2 mM EDTA, 0.1% SDS), LiCl buffer (10 mM Tris [pH 8.1], 0.25 mM LiCl, 1 mM EDTA, 1% NP-40, 1% Na-desoxycholate), and TE. DNA–protein complexes were eluted with 1% SDS, 0.1 M NaHCO$_3$. DNA–protein crosslinks were reversed with 5 M NaCl at 65°C for 4 hours. Immunoprecipitated DNA segments were purified with phenol–chloroform–isoamylalcohol. PCR analysis was performed, as described in Zheng et al's[24] study, of the following gene region spanning the hypoxia response element (HRE) consensus: mGlut1 (from −2,906 to −2,689), mAQP3 (from −573 to −1,201). The sequences of the primers used in the PCRs were as follows: mGlut1prom (forward), 5′-CCGGGCTGTCTTACTCACTCTTACTCC-3′, mGlut1prom (reverse), 5′-GGGCTGTGTTCAAGCT-GCGCC-3′; mAQP3prom (forward), 5′-GGAGGCA-GAGACAGGTAGATCT-3′, mAQP3prom (reverse) 5′-GGAACATGTCATCACATGTTGCATG-3′.

Western blot

Western blot experiments were performed as described by Vogler et al.[21] Briefly, cells were lysed in 400 mM NaCl, 1 mM EDTA, 10 mM Tris/HCl, pH 8.0, and 0.1% Triton X-100 including the protease inhibitor cocktail complete mini (Roche Applied Science, Mannheim, Germany). Protein concentrations were quantified with the Bradford method using bovine serum albumin as a standard. Protein extracts were electrophoresed through SDS–polyacrylamide gels and electrotransferred onto nitrocellulose membranes (Amersham, Freiburg, Germany) by semi-dry blotting (PeqLab, Erlangen, Germany). For detection of specific proteins, the following primary and secondary antibodies were used: anti-HIF-1α (NB100-479, Novus Biologicals LLC), anti-β-tubulin (ab6046, Abcam), and HRP-labeled anti-rabbit (Sc-2004, Santa Cruz).

Statistical analysis

Results are expressed as mean values ± standard error of the mean of at least three independent experiments. For statistical comparisons, results were analyzed by one-way analysis of variance followed by Bonferroni's correction. Values of $P<0.05$ (*), $P<0.01$ (**), $P<0.001$ (***) were considered statistically significant.

Results

L929 fibrosarcoma cells were subjected to hypoxia for 1–48 hours (Figure 1). In this time-course experiment, stabilization of HIF-1α is clearly identifiable after 1 hour of hypoxia (Figure 1A), and a significant induction in AQP3 mRNA expression was observed after 4 hours ($P<0.001$; Figure 1B). The AQP3 mRNA expression peaked at about 24 hours of hypoxic incubation ($P<0.001$) and succeeded the induction of the well-established HIF-1 target gene glucose transporter-1 (Glut1). The expression of Glut1 peaked at 4 hours ($P<0.001$) following hypoxic cell incubation, and then declined.

To analyze whether the hypoxic induction of AQP3 mRNA expression levels is HIF-1-dependent, we employed two independent HIF-1α knockdown clones (shC1 and shC2). Generation and characterization of these stable shRNA-mediated knockdown clones have been described by Vogler et al.[21] Similar to the HIF-1 target genes Glut1 and PHD3, knockdown of HIF-1α in L929 cells prevented the hypoxia-stimulated induction of AQP3 in both HIF-1α-deficient clones. However, as seen before, in the wild-type cells, AQP3, Glut1, and PHD3 mRNA expression levels are significantly induced after hypoxic incubation ($P<0.001$) (Figure 2).

In silico analysis of the mouse *AQP3* gene identified a region close to the translational start site containing two core
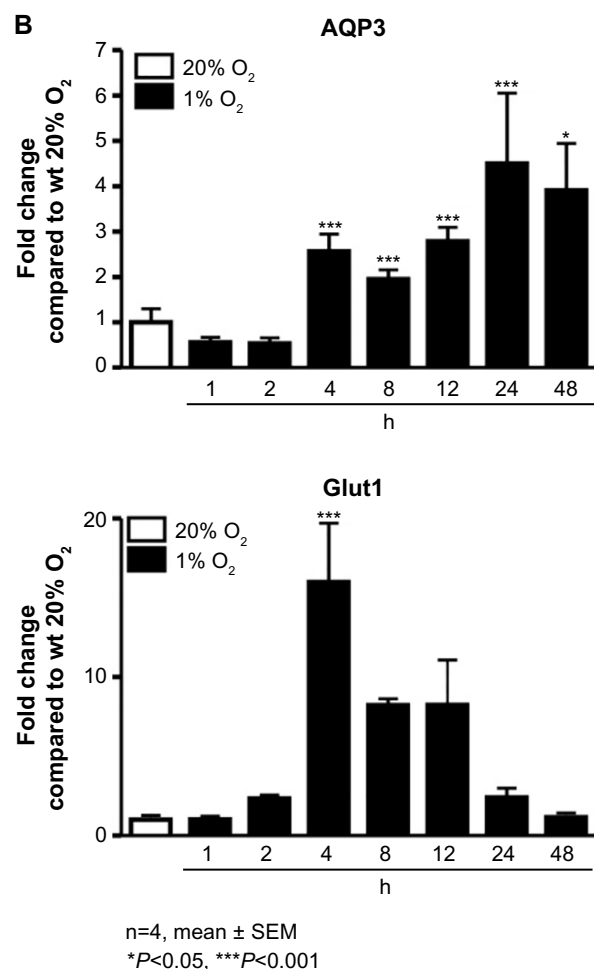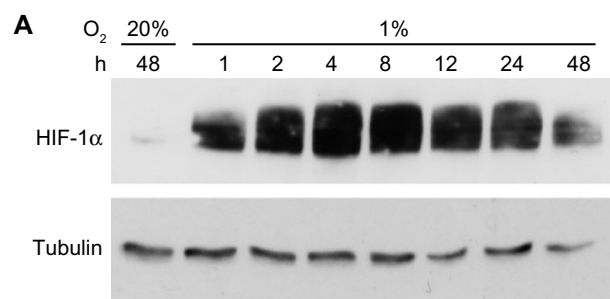
Figure 1 Hypoxia increases the mRNA expression levels of AQP3 in L929 cells.
Notes: Cells were exposed to hypoxia (1% O$_2$) for indicated time points. Cell lysates were probed for HIF-1α protein stabilization by Western blot. Tubulin served as a loading control (**A**). mRNA levels of AQP3 and the established HIF-1 target gene Glut1 were analyzed using RT-qPCR (**B**).
Abbreviations: AQP3, aquaporin 3; Glut1, glucose transporter 1; HIF, hypoxia-inducible factor; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; SEM, standard error of the mean.

sequences of a consensus HIF-1 response element (HRE) 5′-RCGTG-3[25] located at –647 and –916 (Figure 3). To test whether HIF-1α can bind to these HREs in vivo, chromatin immunoprecipitation assays were performed. L929 cells cultured at either 1% O$_2$ or 20% O$_2$ were fixed with formaldehyde, and sonicated cell lysates were immunoprecipitated with anti-HIF-1α antibodies. Precipitated DNA was used to
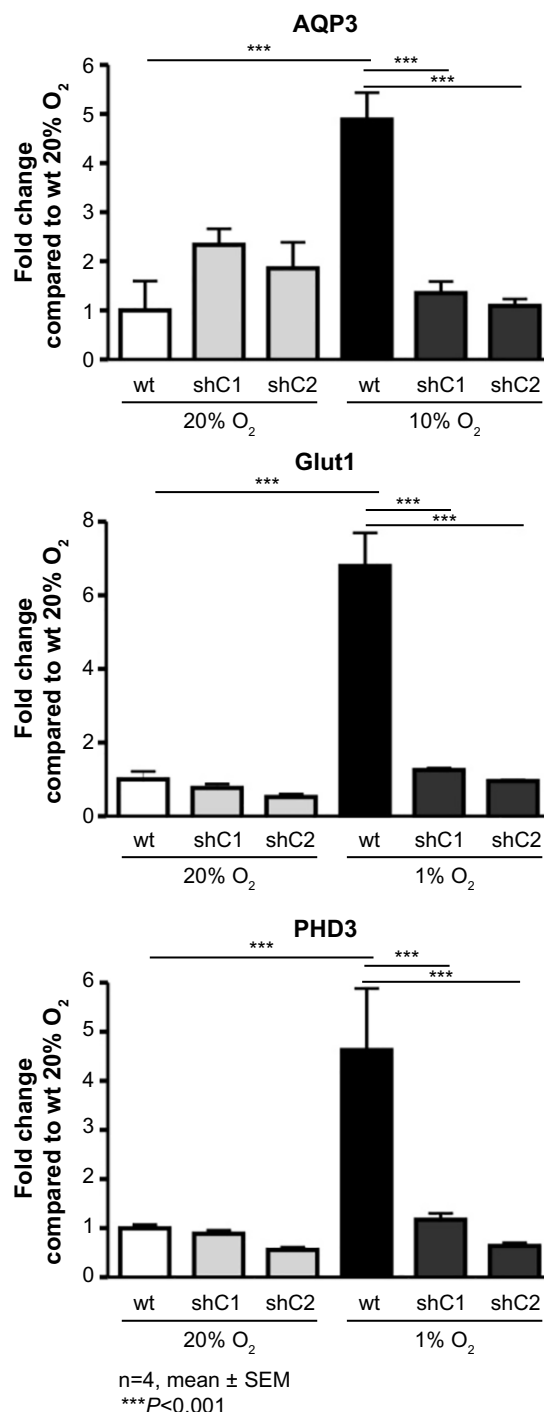

Figure 2 The hypoxic induction of AQP3 mRNA expression is HIF-1 dependent.
Notes: HIF-1α knockdown clones (shC1 and shC2) and wt L929 cells were cultivated for 24 hours at 20% O$_2$ and 1% O$_2$. AQP3, PHD3, and Glut1 mRNA expression levels were analyzed using RT-qPCR.
Abbreviations: AQP3, aquaporin 3; Glut1, glucose transporter 1; HIF, hypoxia-inducible factor; PHD3, prolyl-4-hydroxylase domain-containing enzyme 3; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; SEM, standard error of the mean; wt, wild type.

amplify a fragment containing both putative HREs at –647 and –916 of mouse AQP3. The product was only amplified from hypoxia-treated cell samples immunoprecipitated with the HIF-1α antibody. Primers spanning the mouse Glut1 HRE
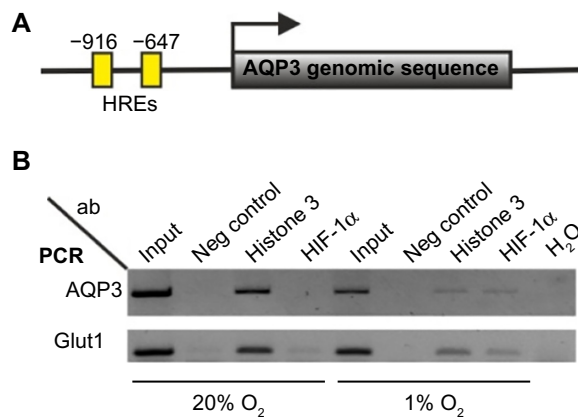
A

B

Figure 3 HREs are located in close proximity to the AQP3 translational start site.
Notes: Target regions of the ChIP assay are indicated on the schematic representation of the AQP3 5′ region, in which the positions of putative HIF-1 binding sites are shown in yellow boxes relative to ATG translational start site (**A**). L929 cells were incubated in normoxic and hypoxic conditions. Cells were treated with formaldehyde for cross-linking, and DNA was extracted and immunoprecipitated with anti-HIF-1α, anti-tubulin (negative control), or anti-Histone 3 antibody (positive control). Definite PCR bands were observed only when HIF-1α or Histone-3 antibodies were used to probe products obtained using primer sets targeting HREs of Glut1 and the putative HIF-1 sites of AQP3 (**B**).
Abbreviations: AQP3, aquaporin 3; ChIP, chromatin immunoprecipitation; Glut1, glucose transporter 1; HIF, hypoxia-inducible factor; HRE, hypoxia response element; PCR, polymerase chain reaction; Neg, negative.

region amplified fragments in a similar pattern and served as a positive control.

To test whether a hypoxic induction of AQP3 can be observed in vivo, mice were exposed to 8% O_2 for 0, 12, and 30 hours. mRNA was isolated from various organs,
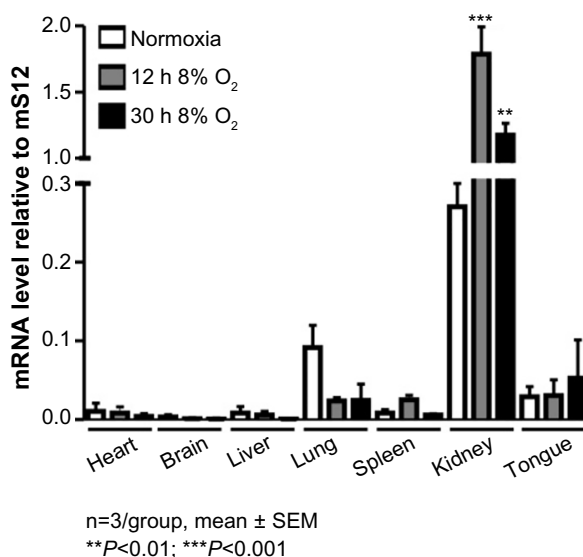

n=3/group, mean ± SEM
**$P<0.01$; ***$P<0.001$

Figure 4 AQP3 is induced in the hypoxic kidneys in vivo.
Notes: Groups of three mice were exposed to an inspiratory O_2 concentration of 8% for 0, 12, and 30 hours as indicated. Organ AQP3 levels were determined by RT-qPCR and displayed as relative mRNA levels, normalized to mS12 transcript levels.
Abbreviations: AQP3, aquaporin 3; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; SEM standard error of the mean.

and AQP3 expression levels were analyzed. Compared to tissues like heart, brain, and liver, we found higher basal levels of AQP3 relative to the housekeeping gene *mS12* in the kidney (Figure 4). Furthermore, in the hypoxia-treated group, a significant induction ($P<0.001$ after 12 hours 8% O_2, $P<0.01$ after 30 hours 8% O_2) of AQP3 was observed in the kidney samples.

Discussion

AQP3 belongs to the family of aquaglyceroporin and thus facilitates the transport of water and small noncharged solutes.[26,27] AQP3 is expressed in the renal collecting duct,[28,29] but can also be found in several extrarenal tissues, including epithelial cells of the skin and retina, and skin fibroblasts.[19,30–32] Furthermore, AQP3 is elevated in various cancers such as lung, prostate, breast, and skin.[33–39] AQP3 adds to tissue hydration, but it has also been discussed to have a role in cell shape, attachment, and migration.[19,39] In cancer, the upregulation of AQP3 expression may promote tumorigenesis and tumor development. Hence, investigating the mechanisms underlying AQP3 expression stood at the focal point of several studies. In skin fibroblasts, AQP3 protein levels are induced by epidermal growth factor,[32] and at the transcriptional level, AQP3 has been described to be regulated under hyperosmotic conditions and by stimulation with platelet-derived growth factor, prostaglandin E_2, arachidonic acid, blood serum, and $CoCl_2$ in retinal pigment epithelial cells.[19] In the promoter of the *AQP3* gene, a functional estrogen response element was identified, which might mediate the AQP3 expression in breast cancer cells.[38]

In the present study, we add to the understanding of the regulation of AQP3 expression, and we show that the AQP3 transcription is induced in hypoxia in vitro and in vivo. This induction is HIF-1α dependent, because HIF-1α deficient cells display a significant reduction in AQP3 mRNA expression levels in hypoxia compared to wild-type cells. Furthermore, our data suggest that the hypoxic AQP3 induction is directly regulated by HIF-1, and we find a cluster of two functional HREs within close proximity to the AQP3 translational start site. Analysis of a panel of murine tissues for AQP3 expression revealed high expression levels of AQP3 in the kidney and substantially lower levels in heart, brain, liver, lung, and tongue, consistent with our own in silico analyses (not shown) and previous studies.[11,18] To the best of our knowledge, hypoxia-dependent regulation of renal AQP3 in mice has not been previously reported. The pronounced hypoxic induction of renal AQP3 in mice observed in the current study substantiates the physiological relevance of

oxygen-dependent regulation of AQP3 expression levels in vivo.

A common characteristic of solid tumors is tissue hypoxia and the activation of the HIF-1 pathway. HIF-1 can play a major role in tumor progression, including proliferation and metastasis. Moreover, there is compelling evidence that HIF-1 is involved in resistance to cancer therapy. However, while many components of the HIF-pathway are protumorigenic, others are antitumorigenic, and the overall consequences for tumor progression and aggressiveness may be cell type- and context-specific.[40] AQP3 has been reported to promote cancer cell motility and invasion.[41] Accordingly, the inhibition of AQP3 reduces proliferation, migration, and invasion of non-small-cell lung cancer cells.[42] Our data reveal that AQP3 is hypoxia-inducible in some cells and tissues, consistent with the hypothesis that AQP3 may represent a protumorigenic component of the HIF-pathway.

Conclusion

Collectively, we show that hypoxia enhances AQP3 expression in L929 fibrosarcoma cells in a HIF-1-dependent manner. These findings should encourage further research evaluating the hypoxic induction of AQP3 in cancer cells.

Acknowledgments

Disclosure

The authors report no conflicts of interest in this work.

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