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Hypoxia-induced Up-regulation of Aquaporin-1 Protein in Prostate Cancer Cells in a p38-dependent Manner

Lu Tie[1]*, Ning Lu[1]*, Xue-Yang Pan[1], Yan Pan[1], Yu An[1], Jun-Wei Gao[1], Yan-Hua Lin[1], He-Ming Yu[2] and Xue-Jun Li[1]

[1]State Key Laboratory of Natural & Biomimetic Drugs, Department of Pharmacology, School of Basic Medical Sciences and Institute of System Biomedicine, Peking University, Beijing, [2]National Research Institute for Family Planning, Beijing, *contributed equally to this study

Key Words

Aquaporin-1 (AQP1) • Hypoxia • p38 • MAPK • PKC • Calcium • Cancer cells

Abstract

Background/Aims: Aquaporin-1 (AQP1) is a glycoprotein that mediates osmotic water transport, its expression has been found to correlate with tumour stage in some tumours. However, the mechanism by which AQP1 protein expression is regulated in tumor cells remains to be fully elucidated. We hypothesized that hypoxia might play an important role in AQP1 induction during tumorigenesis and at the late stages of tumor development. Methods: Isotonic and serum-free hypoxic models were used to investigate AQP1 expression in PC-3M human prostate cancer cells. Results: AQP1 expression was up-regulated by density-induced pericellular hypoxia and cobalt(II) chloride ($CoCl_2$)-induced hypoxia at the transcriptional level. Moreover, phosphorylation of p38 mitogen-activated protein kinase (MAPK) was induced by density-induced pericellular hypoxia and $CoCl_2$-induced hypoxia, specific inhibitors of p38 MAPK could concentration-dependently block those effects of hypoxia on AQP1 expression. Intracellular calcium ion (Ca^{2+}) and protein kinase C (PKC) were shown to be responsible for the activation of p38 MAPK pathway. In addition, AQP1 induction in dense cultures was dependent on lowered oxygen (O_2) tension. In high cell density culture, certain secretory proteins might induce AQP1 expression indirectly. Conclusion: These findings suggest that AQP1 could be induced by hypoxia at transcription level, and the regulation of AQP1 in PC-3M cells is dependent on calcium, PKC and p38 MAPK, as well as low oxygen tension.

Introduction

The aquaporins (AQPs) are a family of channel-forming membrane glycoprotein, originally confirmed to mediate the cellular water-transportation driven by a transmembrane osmotic gradient [1, 2]. The first member aquaporin-1 (AQP1) is present throughout many cell types such as epithelial, endothelial, microvascular endothelial beds outside of the brain, as well as a variety of tumor cells [3]. Recently, intensive studies have been focused on the modulation of AQP1 expression in tumor cells and its effects on tumorigenesis, tumor growth and progression

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Xue-Jun Li
Department of Pharmacology
School of Basic Medical Sciences, Peking University
38 Xueyuan Road, Beijing, 100191 (China)
Tel. +8610 82802863, Fax +8610 82802863, E-Mail xjli@bjmu.edu.cn

[4, 5]. According to clinical analysis, AQP1 expression might be regarded as a new molecular prognostic marker in patients with renal cell carcinoma [6]. Moreover, impaired tumour growth, reduced tumour vascularity and extensive necrosis are found in AQP1-null mice [7]. Our previous study demonstrated that a carbonic anhydrase inhibitor acetazolamide could inhibit AQP1 protein expression and angiogenesis in tumor tissues [8, 9]. However, the mechanism of AQP1 over-expression in tumor regions has not been previously determined.

In comparison with normal tissues, tumors are poorly oxygenated and contain hypoxic regions [10]. Aggressive tumors often have insufficient blood supply, partly because tumor cells grow faster than endothelial cells and partly because a newly formed vascular supply is disorganized [11], and therefore, hypoxia condition always exists among fast-growing aggressive tumors [12]. When the tumor cells are exposed to hypoxia, the transcription factor hypoxia-inducible factor-1 (HIF-1) is activated and promotes the transcription of several genes by binding to specific sites in their promoter regions entitled hypoxia response element (HRE). Although it is important, HIF-1 does not participate in all the signal pathways which are responsible for tumor adaptation to hypoxia and the regulation of downstream proteins involved [13, 14]. Other signal pathways are also activated by hypoxia, and they could potentially regulate AQP1 expression. Mitogen-activated protein kinases (MAPKs) are responsible for the induction of several types of AQPs [15-17], and MAPKs could be activated by hypoxia [18]. The purpose of this study was to test the hypothesis that hypoxia might be able to regulate AQP1 expression through the activation of MAPKs in tumor cells.

Therefore, in the current study we investigated oxygen (O_2)-related mechanisms of inducible AQP1 expression by introducing human prostate cancer PC-3M cells. Experimental models of pericellular hypoxia induced by dense cultures [19, 20], as well as severe hypoxia mimicked by cobalt(II) chloride ($CoCl_2$) [19-21] were used. In addition, we used dialysis membrane to counterbalance changes in the culture medium, such as osmotic pressures, which would influence AQP1 expression.

Materials and Methods

Materials

Lipofectamine 2000 was purchased from Invitrogen (Carlsbad, CA, USA). pBluescript expression plasmid containing rat AQP1 cDNA was a generous gift from Dr. J. Fischbarg (Columbia University, New York, USA). Anti-AQP1 antibody was a gift from Dr. B.X. Yang (University of California, San Francisco, USA). Anti-p38 MAPK antibody and anti-phospho-p38 MAPK (p-p38) antibody were purchased from Santa Cruz (California, CA, USA). Fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit immunoglobulin G (IgG) and tetramethylrhodamine isothiocyanate (TRITC)-conjugated goat anti-rabbit IgG were purchased from Becton Dickinson (Franklin Lakes, NJ, USA). pGL3-Basic vector and the pRL-TK plasmid were purchased from Promega (Madison, WI, USA). Fast mutagenesis system kit was purchased from TransGen Biotech, Beijing, China). Dual-luciferase reporter assay system was purchased from Promega. RPMI 1640 medium, $CoCl_2$ and propidium iodide (PI) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Other chemicals were purchased from the Chinese Chemical Co.

Plasmid constructions

AQP1 plasmid constructions were prepared as previously described [22]. Briefly, the pBluescript expression plasmid containing rat AQP1 cDNA was digested with *Eco*RI/*Apa*I to extract AQP1 cDNA that was then subcloned into pEGFP-C3 vector at *Eco*RI and *Apa*I restriction sites, named pEGFP/AQP1. The correctness of insertion was confirmed by digestion with *Eco*RI/*Apa*I and DNA sequencing on an ABI-373A automated DNA sequencer.

Cell culture, density assay and transfection

PC-3M human prostate cancer cells line was cultured according to our previous procedure [23]. Briefly, cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum at 1×10^4 cells/cm^2 and maintained in a 5% CO_2 incubator with room air (i.e. 21% Oxygen). Then cell density assay was performed as described [19]. Cells were plated at a concentration of 1×10^6 cells in 100 mm plates (to simulate a 'sparse' condition since at this cell number there was essentially no cell-cell contact) and in 34.8 mm plates (to simulate a 'dense' condition since at this cell number cells were confluent and there was complete cell-cell contact) with serum-free medium. The culture plates were dialyzed against serum-free RPMI 1640 medium to ensure cells were cultured under isotonic conditions. In addition, cells cultured under both conditions were treated with 240 uM $CoCl_2$ for 24 h to mimic hypoxia. For transient transfection of PC-3M cells with the AQP1 cDNA, Lipofectamine 2000 was used according to the manufacturer's instruction.

Immunofluorescence and confocal imaging

Immunofluorescence and confocal imaging of AQP1, p38 MAPK and p-p38 MAPK was performed as previously described [24]. Briefly, cells plated on glass coverslips were washed with 0.1 M phosphate-buffered saline (PBS) pH 7.4, fixed with 4% paraformaldehyde for 15 min and pretreated with 0.5% Triton X-100 for 10 min at room temperature. Nonspecific binding sites in the cells were blocked with 5% normal goat serum for 30 min and incubated overnight at 4°C with the anti-AQP1 antibody (1/500), anti-p38 MAPK antibody (1/500) or anti-p-p38 MAPK antibody (1/500). The following day cells

were washed with PBS and incubated for 1 h with the TRITC-conjugated goat anti-rabbit IgG (1/100) or FITC-conjugated goat anti-rabbit IgG (1/100) for 30 min in the dark at 37°C. After secondary antibody was added, both groups were incubated with 10 μg/ml PI for 10 min at room temperature. Fluorescent signals emitted by FITC-conjugated secondary antibody (λ_{ex}= 488 nm, λ_{em}= 515 nm), TRITC-conjugated secondary antibody (λ_{ex}= 568nm, λ_{em}= 585nm) and PI (λ_{ex}= 488 nm, λ_{em}=605 nm) were observed by using a Leica TCS SP2 confocal microscope.

Intracellular quantification of AQP1, p38 MAPK and p-p38 MAPK by flow cytometry

Quantification of the expression of AQP1, p38 MAPK and p-p38 MAPK was performed by flow cytometer (FCM) (Becton-Dickinson, Franklin Lakes, NJ, USA) as previously described [23]. After being treated as required, cells washed with cold PBS (3×10 min) and centrifuged at 800 rpm for 5 min at 4°C. After that, cells were fixed with 4% paraformaldehyde in PBS for 15 min at room temperature, and then permeabilized in 0.1% Triton X-100 for 5 min at room temperature. Cells were treated with 10% normal goat serum for 5 min to block nonspecific binding sites, then incubated with anti-AQP1 antibody, anti-p38 MAPK antibody or anti-p-p38 MAPK antibody which was diluted at 1:1000 in PBS/1.5% BSA for 1 h at room temperature. After being washed and centrifuged as above, cells were incubated with TRITC or FITC-conjugated goat anti-rabbit IgG which was diluted at 1:100 in PBS/1.5% bovine serum albumin (BSA) for 1.5 h at room temperature. The cells then were analyzed with FCM after being washed with cold 0.1 M PBS (3×10 min) and centrifuged at 800 rpm for 5 min at 4°C. Negative controls were set without the presence of the primary antibody. The data were expressed as protein expression which was analyzed and compared to the internal standard by the software automatically in FCM.

Dual-luciferase reporter Assay

For analysis of promoter activities of AQP1, segment (-1519/+78) from the 2 kb sequences 5' upstream of the first exon of human AQP1 (GeneBank, NCBI reference sequence: NC_000007.13) was cloned and inserted into pGL3-Basic vector upstream to the luciferase cassette. The construct (designated as pGL3-1519) was employed to test the promoter segment potentially involved in the AQP1 regulation by $CoCl_2$ induced hypoxia. Site-directed mutagenesis of the putative HRE binding site, located between nucleotides -1293 to -1286 from the transcription initiation site of the AQP1 gene ($^{-1293}$AGC **ACG** TT^{-1286} → $^{-1293}$AGC **ATT** TT^{-1286}) [25, 26], was obtained by using the fast mutagenesis system kit. All constructs were verified by sequencing. A dual-luciferase reporter assay system was used to assess the luciferase activity. Briefly, PC-3M cells were transfected with pGL3-promoter construct or the mutant construct and co-transfected with pRL-TK control plasmid. After transfection cells were treated with 240 μM $CoCl_2$ for 24 h to mimic hypoxia. Later, cells were lysed and processed following the manufacturer instruction. The luciferase activity was measured by a FlexStation 3 microplate reader (Molecular Devices, Sunnyvale, CA,USA). For each sample, the pGL3 firefly luciferase activity was normalized by the *Renilla* luciferase activity from the pRL-TK control.

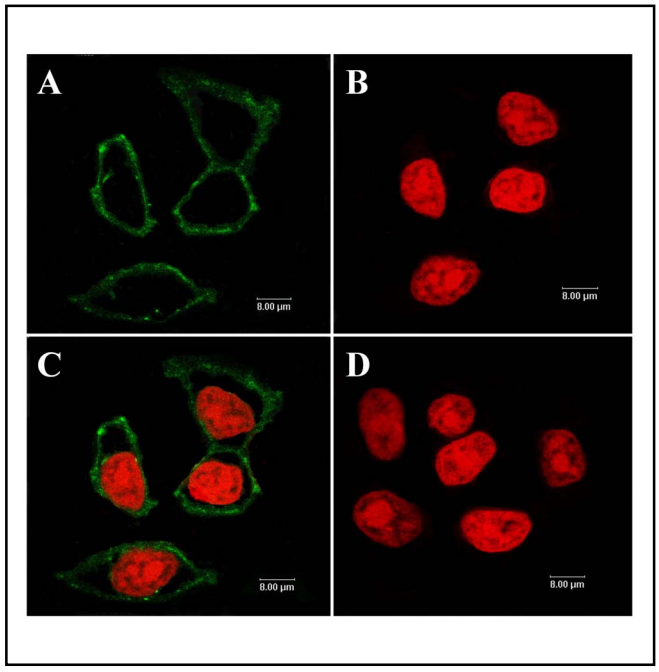


Fig. 1. Confocal images of AQP1 expression in PC-3M cells with anti-AQP1 antibody and PI. Picture (A) represented AQP1 expression (green). Picture (B) represented nucleus (red). Picture (C) was an overlaid image of A and B. Picture (D) the negative control was set without the presence of the primary antibody.

Statistical analysis

Data were expressed as means ± SD. The significance of differences was evaluated with one-way ANOVA followed by Student-Newman-Keuls test. A probability level of $P<0.05$ was considered statistically significant.

Results

The expression of AQP1 in PC-3M cells

Previous studies indicated that AQP1 was expressed exclusively in several tumor cells, such as renal carcinomas, erythroleukemia cell lines, glioma cell lines. To characterize the expression and localization of the AQP1 in PC-3M cells, laser confocal fluorescence microscopic analysis was carried out by using AQP1 antibody. As shown in Fig. 1, AQP1 was expressed on membrane of PC-3M cells.

AQP1 protein expression is regulated by cell density

It is well established that change of osmotic pressure may affect the expression of AQP1 [15, 27], and the

osmosis in culture medium might fluctuate because of cell metabolism and the release of certain factors under hypoxic conditions. To eliminate the possible involvement of osmosis in current cell models, the dialysis membrane was used to counterbalance the change of osmotic pressure during experiment. Serum, as a stimulator, could induce AQP1 expression [28], so serum-free culture medium was used in the following study. As shown in Fig. 2A, AQP1 level was up-regulated in culture medium with serum. In accordance, serum resulted in an increase in cell proliferation and total cellular protein content (data not shown). Glucose is another factor which affects the level of AQP1 [15], but since it could traverse the dialysis membrane freely, the effect of glucose in the current model could be neglected (Fig. 2B).

AQP1 expression under pericelluar hypoxia and $CoCl_2$ induced hypoxia

Cells cultured in the presence of serum reached a higher density than those growing in serum-free medium, and AQP1 induction may partly reflect differences of cell density. To determine whether cell density could evoke the increase of AQP1 level, the density assay was used. As shown in Fig. 3, AQP1 level was markedly elevated in cells cultured in 34.8 mm plates compared with those grown in 100 mm plates after being seeded 24 h in isotonic and serum-free medium.

Pericellular hypoxia could activate certain signal transduction pathways, through which a variety of proteins are regulated, however, not all of these pathways are overlapped with those stimulated by hypoxia mimic reagents [20, 21]. Hypoxia mimic agent $CoCl_2$, by competing with Fe^{2+} for binding the O_2 sensor, produces a state equivalent to that of chronic hypoxia even in the presence of normoxic levels of O_2 [19]. After being treated with $CoCl_2$ for 24 h, a significant increase of AQP1 expression was observed in cells cultured in both 34.8 mm plates and 100 mm plates (Fig. 3). Furthermore, $CoCl_2$-induced AQP1 expression and cell density-induced AQP1 expression were possibly additive, indicating that $CoCl_2$ mimic hypoxia may involve particular signal pathways other than those induced by cell density.

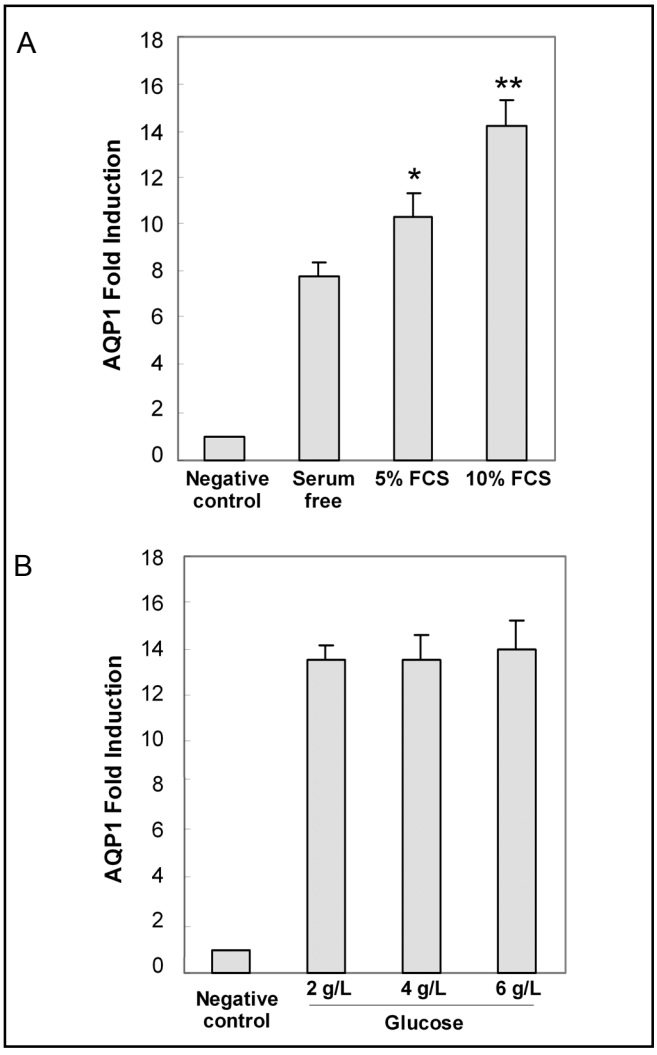
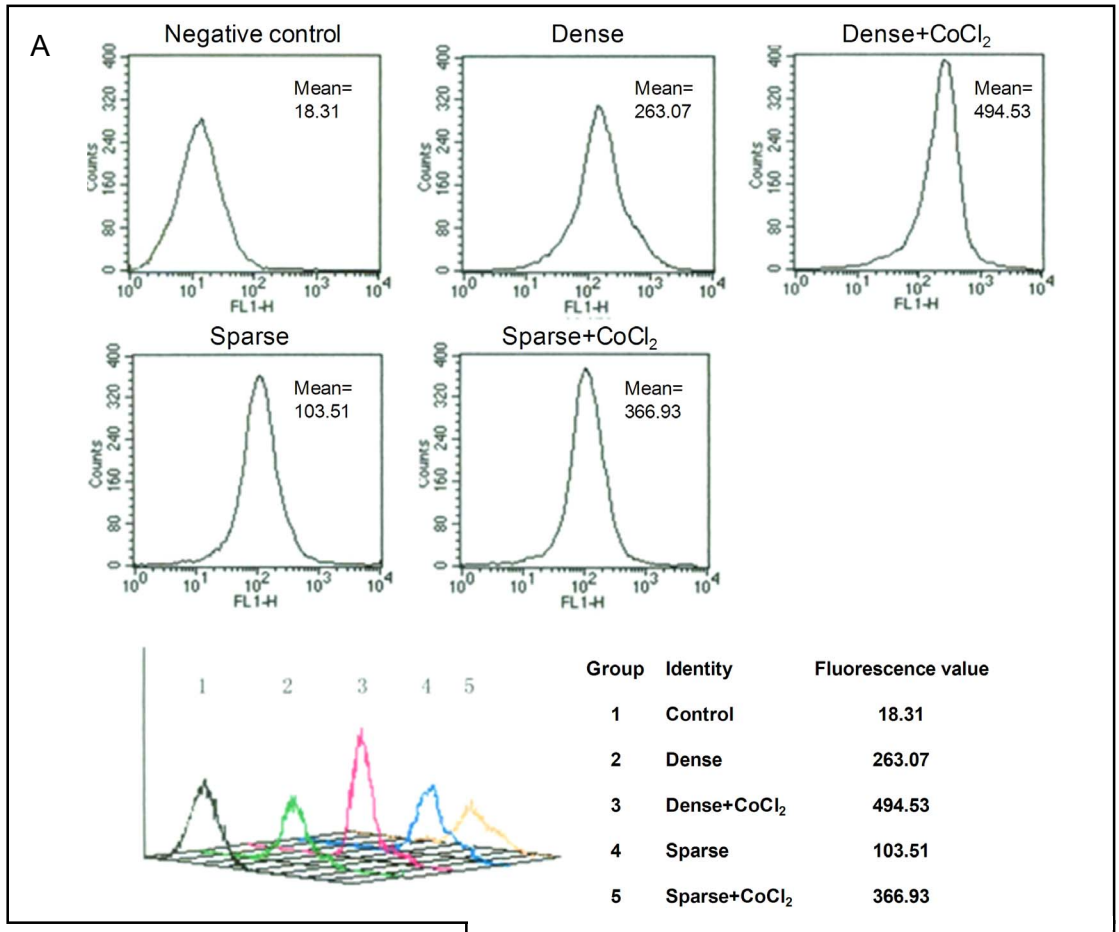


Fig. 2. Effects of serum (A) and glucose (B) on AQP1 expression in isotonic culture models. Cells were cultured under experimental conditions for 24 h, then fixed, permeated, incubated with primary antibody and FITC-conjugated secondary antibody, and analyzed by FCM (λ_{ex}= 488 nm, λ_{em}= 515 nm). Data shown were mean ± SD derived from three independent experiments and of the fold change with respect to negative controls. (A, $^{*}P<0.05$ compared with cells incubated with serum free medium; $^{**}P<0.01$ compared with cells incubated with serum free medium; B, $P>0.05$ compared with cells cultured with 2 g/L glucose).

Transcriptional and post-transcriptional regulations of hypoxia-induced AQP1 expression

Hypoxia-induced AQP1 expression may be caused by mRNA induction, stabilization, translation efficiency enhancement, or protein stability increase. To elucidate the mechanisms underlying, PC-3M cells were pretreated with the RNA synthesis inhibitor actinomycin D or protein synthesis inhibitor cycloheximide, and then incubated under normal, density-induced hypoxic and $CoCl_2$-induced hypoxic conditions. As shown in Fig. 4A, actinomycin D or cycloheximide abolished the stimulatory effects of hypoxia on AQP1 expression, suggesting the induction of AQP1 expression by hypoxia might result from mRNA induction or translation efficiency enhancement.



To explore the mediating mechanism, we constructed the expression plasmid of pEGFp-AQP1-C3. The expression of AQP1 at the protein level could be evaluated by detecting fluorescent value in PC-3M cells transfected with the plasmid. Because of lacking the native AQP1 promoter, the plasmid could also serve to examine whether the native AQP1 promoter was involved in hypoxia-induced AQP1 expression. As shown in Fig. 4B, the expression of EGFP-AQP1 was affected neither by density-induced pericellular hypoxia nor by $CoCl_2$-induced hypoxia. Thus, some elements in the promoter region might be responsible for hypoxia induced AQP1 expression at the transcription level.

p38 MAPK signal pathway, PKC and intracellular Ca^{2+} in AQP1 induction

MAPKs comprise a family of protein-serine/threonine kinases, consisting of three subgroups: extracellular signal-regulated kinases (ERKs), p38 and c-Jun NH_2-terminal kinases (JNKs). They have been shown to participate in signal transduction pathways that control intracellular events including acute responses to hormones and major developmental changes in organisms [29].

Fig. 3. Effects of pericelluar hypoxia and $CoCl_2$ induced hypoxia on AQP1 expression. 1×10^6 cells were cultured in 100 mm (sparse) and 34.8 mm (dense) plates separately with or without $CoCl_2$ for 24 h to mimic hypoxic condition. Immunofluorescence assay of AQP1 was analyzed by FCM. (A) Histogram showed fluorescence intensity detected by FCM (one representative experiment). (B) Graphs shown were mean of fluorescence intensity ± SD derived from three independent experiments and of the fold change with respect to negative controls. ($^{**}P<0.01$ compared with sparse cells; $^{***}P<0.001$ compared with sparse cells).

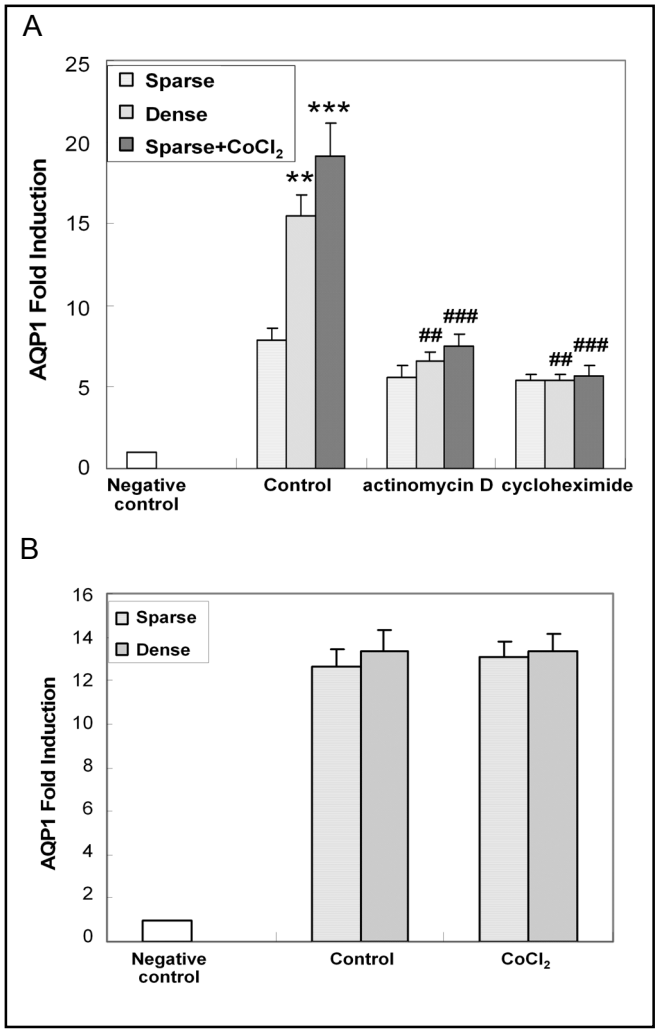


Fig. 4. Transcription and post-translation regulation of AQP1 expression. (A) Cells were incubated with or without 240 μM $CoCl_2$ in sparse and dense culture for 24 h in the presence or absence of actinomycin D (5 μg/ml) or cycloheximide (2 μg/ml). Immunofluorescence assay of AQP1 was analyzed by FCM. Data shown were mean ± SD derived from three independent experiments and of the fold change with respect to negative controls. ($^{**}P$<0.01 compared with sparse cells; $^{***}P$<0.001 compared with sparse cells; $^{\#\#}P$<0.01 compared with dense cells; $^{\#\#\#}P$<0.001 compared with sparse cells treated with $CoCl_2$). (B) Cells were transfected with native AQP1 promotor deficient plasmid pEGFP- AQP1-C3 and then seeded in sparse or dense cultures with or without $CoCl_2$ for an additional 24 h. Immunofluorescence assay of AQP1 was analyzed by FCM. Data shown were mean ± SD derived from three independent experiments and of the fold change with respect to negative controls.

Recent evidence suggests that activation of p38 MAPK by cellular stress could be involved in diverse responses [18]. We hypothesized that p38 MAPK might be a potent factor in hypoxia-induced AQP1 over-expression. Specific antibodies against p38 MAPK and p-p38 MAPK were used. As shown in Fig. 5A and 5B, p38 MAPK was markedly activated by dense or $CoCl_2$. To examine the role of p38 MAPK in hypoxia-induced AQP1 expression, cells were pretreated with different concentrations of p38 MAPK inhibitor (SB203580) for 30 min and then incubated under the condition of dense or with $CoCl_2$ for 24 h Pretreatment of cells with the p38 MAPK inhibitor SB203580 prevented the AQP1 expression induced by dense or $CoCl_2$ in a concentration-dependent manner, whereas there was no significant difference in AQP1 expression under normal conditions (Fig. 5C).

It has been reported that PKC and intracellular Ca^{2+} could lead to the activation of p38 MAPK under hypoxia [14, 30]. To examine whether PKC and intracellular Ca^{2+} accounted for the regulation of AQP1, PKC inhibitor (calphostin C) and a calcium channel blocker (verapamil) were used. At first, we pretreated cells with different concentrations of calphostin C or verapamil and testified p38 MAPK activation after incubating cells with or without $CoCl_2$ in sparse culture and without $CoCl_2$ in dense culture. Data obtained from Fig. 6A and 6B showed that PKC inhibitor calphostin C and the calcium channel blocker verapamil attenuated phosphorylation of p38 MAPK induced by density or $CoCl_2$ in a concentration-dependent manner. In addition, cells were pretreated for 30 min with 1 μM calphostin C, 1 μM verapamil, or 1 μM SB203580 and then incubated as required for 24 h. AQP1 expression induced by dense or $CoCl_2$ was significantly suppressed by PKC inhibitor calphostin C and the calcium channel blocker verapamil (Fig. 6C).

Hypoxia-mediated AQP1 expression is regulated by HRE binding site

It has been well documented that HRE could bind HIF-1 under hypoxia and has been assigned a critical part in the regulation of a bunch of genes [31, 32]. Through sequence analysis, a typical HRE was identified on the promoter of human AQP1, 5'-AGCACGTT-3', corresponding to nucleotides -1293 to -1286 of human AQP1 transcription initiation site. To examine whether this element was involved in regulating AQP1 expression under hypoxia, a mutant was designed at this site (nucleotides -1290 to -1288) as the pGL3-1519-Mut-Luc construct. $CoCl_2$-induced hypoxic treatment resulted in a 1.8-fold enhancement of the activity of the pGL3-1519-Luc construct, as compared with the untreated control (Fig. 7). Mutation in the HRE binding site abolished the stimulatory effects induced by $CoCl_2$.

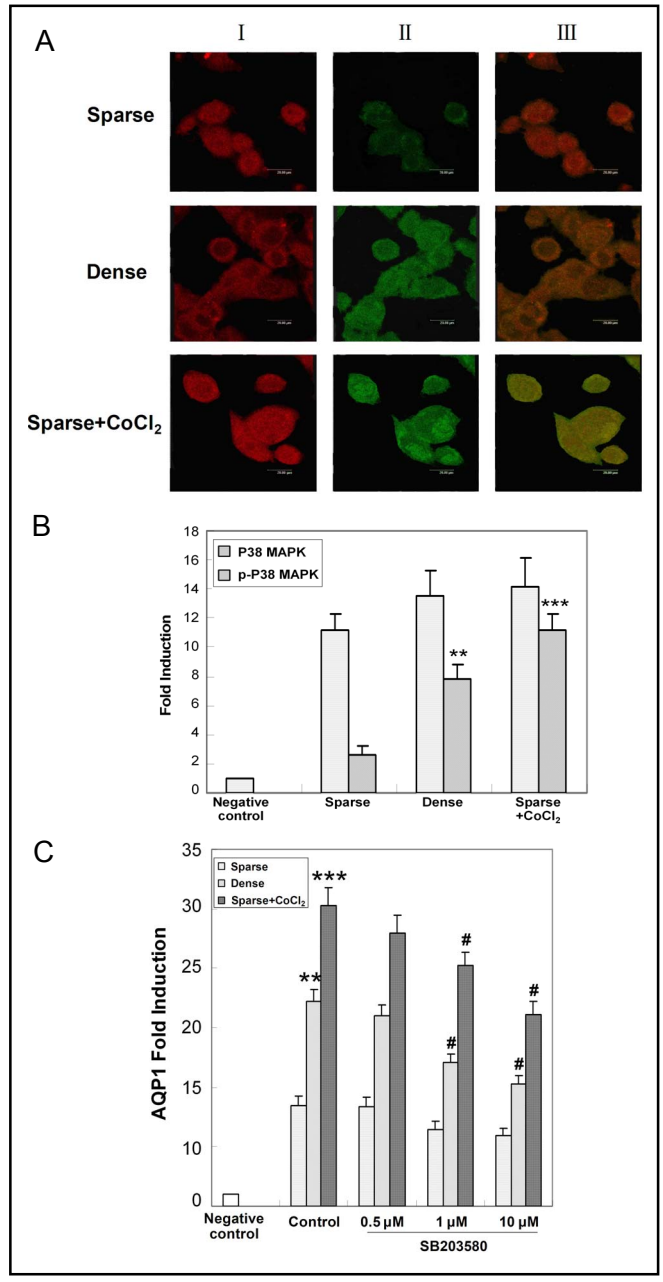


Fig. 5. Effects of pericelluar hypoxia and $CoCl_2$ induced hypoxia on p38 activation. (A) Cells were incubated with or without 240 μM $CoCl_2$ in sparse and dense culture for 24 h. In the following immunofluorescence and confocal imaging, p38 MAPK was detected by TRITC-conjugated anti-IgG antibody as revealed by group I (red), and p-p38 MAPK was detected by FITC-conjugated anti-IgG antibody as represented by group II (green). Group III were overlaid images of I and II. (B) After being cultured for 24 h as required, cells were harvested and incubated with anti-p38 MAPK antibody or anti-p-p38 MAPK antibody. Immunofluorescence assay was analyzed by FCM. Data shown were mean ± SD derived from three independent experiments and of the fold change with respect to negative controls. ($^{**}P<0.01$ compared with sparse cells; $^{***}P<0.001$ compared with sparse cells). (C) Cells were pretreated with different concentrations of SB203580 for 30 min, and then incubated with or without 240 μM $CoCl_2$ in sparse and dense culture for 24 h. Immunofluorescence assay of AQP1 was analyzed by FCM. Data shown were mean ± SD derived from three independent experiments and of the fold change with respect to negative controls. ($^{**}P<0.01$ compared with sparse cells; $^{***}P<0.001$ compared with sparse cells; $^{\#}P<0.05$ compared with dense cells or sparse cells treated with $CoCl_2$).

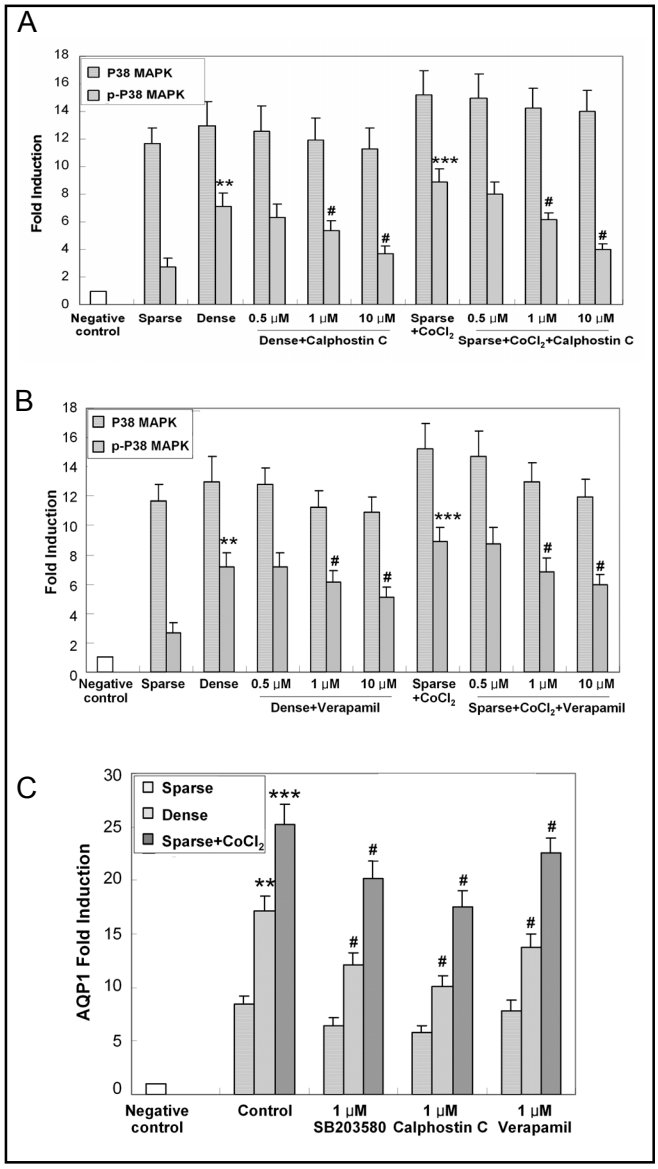
AQP1 expression in dense cultures is dependent on O_2 deprivation

To determine whether the density-induced AQP1 expression was dependent on O_2 deprivation, the possible involvement of O_2 was investigated in the current model. Sheta and colleagues [19] demonstrated that pO_2 close to the surface of plated cells 48 h after plating was 13% (96 mmHg) for sparse and 9% (70 mmHg) for dense LNCaP human prostate carcinoma cells whereas, pO_2 in equilibrated medium is 16% (116 mmHg). A pO_2 gradient was generated by incubating 1×10^6 cells in 34.8 mm plates with varying volumes of medium [33, 34]. AQP1 expression was enhanced as cells were incubated with more medium, indicating that O_2 deprivation played a role in cell density-mediated induction of AQP1 (Fig. 8). In addition, effects of reoxygenation on AQP1 expression were also examined by gentle shaking [19, 34]. 1×10^6 cells were seeded in 34.8 mm plates and incubated with 2 ml medium, allowed to attach for 4 h, and left under shaking for an additional 20 h. Reoxygenation by shaking significantly decreased AQP1 induction (Fig. 8), suggesting the role of a pO_2 gradient in cell density-dependent AQP1 expression.

Effect of pericellular hypoxic conditioned medium on AQP1 expression

Reduction of oxygen level in hypoxia could be captured by putative oxygen sensors and then various signal molecules were activated, which would lead to numerous alterations in gene transcription, translation and protein stability. Those changes might in return affect hypoxia and expression of other proteins. To examine whether the cell release induced by pericellular hypoxia was involved in the regulation of AQP1 expression, 1×10^6 cells were seeded in 100 mm plates and then treated with conditioned medium for 24 h. The conditional medium was obtained from 34.8 mm plates, in which 1×10^6 cells

Fig. 6. Effects of PKC and intracellular calcium inhibitors on p38 MAPK activation and AQP1 induction under hypoxic conditions. (A) Cells were pretreated with different concentrations of PKC inhibitors (calphostin C) for 30 min and then inbubated under different conditions as required for 24 h. Cells were harvested and incubated with anti-p38 MAPK antibody or anti-p-p38 MAPK antibody. Immunofluorescence assay was analyzed by FCM. Data shown were mean ± SD derived from three independent experiments and of the fold change with respect to negative controls. ($^{**}P$<0.01 compared with sparse cells; $^{***}P$<0.001 compared with sparse cells; $^{\#}P$<0.05 compared with dense cells or sparse cells treated with $CoCl_2$). (B) Cells were pretreated with different concentrations of a calcium channel blocker verapamil for 30 min and then inbubated under different conditions as required for 24 h. Immunofluorescence assay was analyzed by FCM. Data shown were mean ± SD derived from three independent experiments and of the fold change with respect to negative controls. ($^{**}P$<0.01 compared with sparse cells; $^{***}P$<0.001 compared with sparse cells; $^{\#}P$<0.05 compared with dense cells or sparse cells treated with $CoCl_2$). (C) Cells were pretreated with 1 μM SB203580, 1 μM calphostin C or 1 μM verapamil for 30 min and then inbubated under different conditions as required for 24 h. Immunofluorescence assay was analyzed by FCM. Data shown were mean ± SD derived from three independent experiments and of the fold change with respect to negative controls. ($^{**}P$<0.01 compared with sparse cells; $^{***}P$<0.001 compared with sparse cells; $^{\#}P$<0.05 compared with dense cells or sparse cells treated with $CoCl_2$).



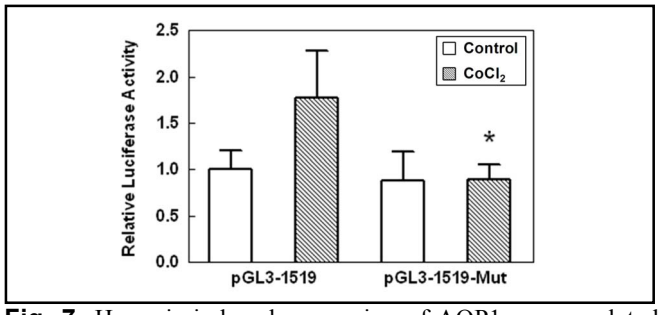


Fig. 7. Hypoxia-induced expression of AQP1 was regulated by HRE binding site. PC-3M cells were transfected with pGL3-promoter construct or the mutant construct and co-transfected with pRL-TK control plasmid, and luciferase activities were measured after 24 h of $CoCl_2$ treatment. Data shown were mean ± SD derived from three independent experiments and of the fold change with respect to cells transfected with pGL3-promoter construct without $CoCl_2$ treatment. ($^{*}P$<0.05 compared with cells transfected with pGL3-promoter construct with $CoCl_2$ treatment).

were cultured for 24 h. Compared with the control group, AQP1 expression was enhanced, but did not reach the level in cells cultured in 34.8 mm plates (Fig. 9), indicating that the up-regulation of AQP1 expression was due in part to some soluble factors secreted by cells under pericellular hypoxia.

Discussion

The major new findings in the present study are: (1) Cell density mediated pericellular hypoxia and $CoCl_2$ induced hypoxia evoked the up-regulation of AQP1 expression in PC-3M cells at transcriptional level; (2) *in vitro* inhibition of p38, PKC and intracellular Ca^{2+} prevented the increase of AQP1 expression induced by hypoxia; (3) pO_2 gradient and cell releases under pericellular hypoxia could partially modulate AQP1 expression.

Protein expression could be regulated on the levels of transcription, translation, and protein stability. In this

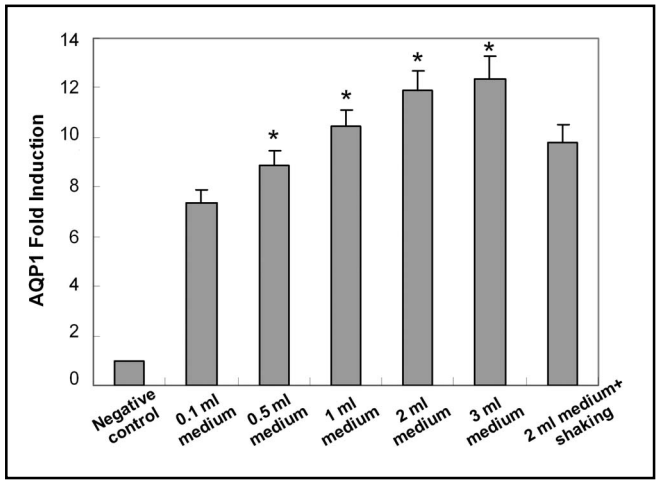


Fig. 8. AQP1 expression in dense culture was dependent on pO_2. 1×10^6 cells were cultured in 34.8 mm plates with 0.1 ml, 0.5 ml, 1 ml, 2 ml or 3 ml medium respectively without shaking for 24 h, in the meantime 1×10^6 cells were cultured in 34.8 mm plate with 2 ml medium, allowed them to attach for 4 h before shaking for an additional 20 h. Cells were harvested and immunofluorescence assay was analyzed by FCM. Data shown were mean ± SD derived from three independent experiments and of the fold change with respect to negative controls. ($^*P<0.05$ compared with cells cultured with 0.1 ml medium).

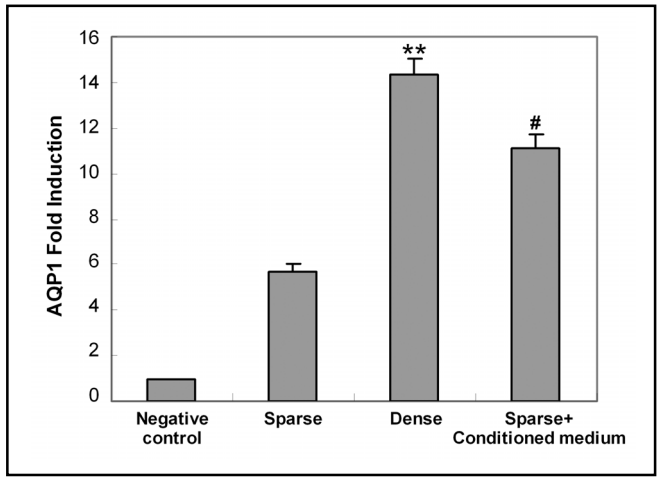


Fig. 9. Effects of pericellular hypoxic conditioned medium on AQP1 expression. 1×10^6 cells were cultured in 100 mm plates and treated with conditioned medium obtained from 34.8 mm plates for an additional 24 h, in which 1×10^6 cells were seeded 24 h ago. AQP1 expression was determined by FCM. Data shown were mean ± SD derived from three independent experiments and of the fold change with respect to negative controls. ($^{**}P<0.01$ compared with sparse cells; $^{\#}P<0.05$ compared with dense cells.

study, it seemed transcription level was solely responsible for hypoxia induction. However, in other situations, AQP1 was found to be regulated at other levels. Leitch [35] reported the decrease of AQP1 ubiquitination and instability in hypertonic stress. Umenishi [36] demonstrated that MAPK pathways were activated under hypertonic stress and could induce AQP1. But no concrete information, concerning whether MAPKs could stabilize AQP1, is currently available. Here, we provided evidence that the stability of AQP1 was not influenced by hypoxia, despite the activation of p38 MAPK. In addition, regulation of AQP1 in pericellular hypoxia and $CoCl_2$ mimic hypoxia displayed additive effects. It has been well documented that HRE could bind HIF-1 under hypoxia and has been assigned a critical part in the regulation of a bunch of genes [31, 32]. Our current study found that the enhancer activity induced by hypoxia mimicked by $CoCl_2$ was lost by a mutation in the HRE binding site, so this element might, at least in part, be involved in regulating AQP1 expression under hypoxia. However, Kaluz [34] reported that increased cell density did not affect HIF-1α levels in HeLa Cells, and that similar observation was made by Sheta [28] in PC-3 cells, whereas positive effects of $CoCl_2$ on HIF-1 expression have been well elucidated [37]. Accordingly, AQP1 might be at least partially up-regulated in $CoCl_2$ mimic hypoxia by HIF-1.

Hypoxia, according to some researches, activated all three MAPKs, and might also regulated AQP1 through mechanisms other than them [38]. Umenishi [36] demonstrated the importance of MAPKs and the HRE, and revealed that transcription factors induced by MAPKs acted by interacting with the HRE. Other signals activated by hypertonic stress had not been isolated, and the joint efforts of MAPKs might be ultimately responsible to induce transcription factors related to the hypertonic element. It was possible that hypoxia activated MAPKs which induced transcription factors alone or joined with other signals, and then worked by binding to HRE. It was equally possible that MAPKs, together with other signals possessed solely by hypoxia, induced certain transcription factors interacting with an element different from the hypertonic response element. Sequence analysis between AQP1 and other proteins induced under hypoxia and in which MAPKs were at least partially responsible in their induction might give us some hints for searching of such putative elements.

Involvements of PKC in the activation of p38 MAPK under hypoxia were documented by some research groups, but the underlying mechanisms remain

unclear. In contrast the positive effects of PKC on the activation of ERK MAPK have been well elucidated. Some isoforms of PKC are activated by DAG and the intracellular Ca^{2+} released as a result of inositol 1, 4, 5-trisphosphate (IP3) production after the activation of Gq receptor. PKC may then regulate protein kinase Raf through direct phosphorylation and eventually the activities ERK MAPK [39]. Moreover, Yang [40] and Umenishi [36] reported that p38 and ERK MAPKs shared common signals leading to their activation, though identities of those signals remained to be classified. Umenishi [36] demonstrated, under conditions of hypertonic stress, all three MAPKs signal transducers were indispensable in the regulation of AQP1, and therefore, it seemed that more than one substrate was involved towards the final regulation of AQP1. In the current study we could not tell whether PKC and intracellular Ca^{2+} up-regulated AQP1 expression solely through p38 MAPK, or through ERK MAPK, and further efforts should be made concerning signal transductions between p38, ERK, JNK MAPKs and the final over-expression of AQP1.

Signals produced by hypoxia might be captured by putative oxygen sensors and then amplified and passed on to their specific effectors. In this study, we showed that hypoxia regulated AQP1 expression via MAPKs, whereas such signals would also be utilized to generate other responses that might, in turn, featured particular effects on AQP1 expression as well. Moreover, AQP1 expression could be induced indirectly by hypoxia, for instance, cell releases under hypoxia were shown to affect AQP1 induction. So several cascades were relied on to regulate AQP1 under hypoxic conditions, and some of them might also function via MAPKs. Although we could not tell whether cell-cell contacts and transductions activated thereby appeared to be important for AQP1 induction, we proved the importance of some of the micro-environmental factors prevailing in dense cultures. Micro-environmental factors included acidosis, glucose deprivation, lowered oxygen concentrations, cell releases, osmotic pressure changes, and some of them were reported to have effects on expressions of a variety of proteins [41]. With the help of dialysis membrane, the possible effects exerted by some of them were excluded. Gently shaking proved the importance of low oxygen level toward AQP1 induction. The mere fact that conditioned medium of densely-cultured cells could induce AQP1 expression indicated a positive role played by cell release, which should be secretory proteins since the existence of dialysis membrane eliminated influences of small molecules.

Previous studies have shown that AQP1 is one of the protein families involved in cell cycle control known as delayed early-response genes [42]. It was believed that this gene might have a potential role in uncontrolled cell replication, as in cancer [28, 43]. There is a chance that by accumulating mutations inside the critical motifs in the channel structures, AQP1 as well as some other AQPs can gain novel functions other than of a water channel, which might facilitate tumor growth [28]. Preliminary investigations points out that AQP1 has oncogenic properties [28], and AQP1 deficient mice did undergo a slower tumor growth rate [7]. According to our data, in dense culture, where tumor cells grew and proliferated more rapidly, cells expressed higher level of AQP1, and so did cells growing under severe hypoxia. Also, tumor hypoxia appears to be strongly associated with tumor propagation, malignant progression, and resistance to therapy [33]. Thus, there could be a positive loop between tumor growth and progression and AQP1 expression, and AQP1 might be a therapeutic target for cancer therapy [8, 28]. Nevertheless, our current models only dealt with conditions, under which osmotic pressures remained the same. However, as hypoxia and cell metabolisms went on *in vivo*, osmotic pressures were sure to fluctuate, which would complicate the situations.

Conclusions

The findings of this study demonstrate that hypoxia could induce AQP1 expression at transcriptional level in PC-3M tumor cells, via intracellular Ca^{2+}, protein kinase C and p38 MAPK signaling pathways. These findings may provide a mechanistic basis for targeting AQP1 as a potential therapeutic strategy in cancer.

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