Original Research

Arginine Promotes the Expression of Aquaporin-3 and Water Transport in Porcine Trophectoderm Cells Through NO- and cAMP-Dependent Mechanisms

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Abstract

Background: Dietary supplementation with L-arginine (Arg) has been shown to increase the volume of fetal fluids in gestating swine. Aquaporins (AQPs), known as water channel proteins, are essential for embryonic growth and development. It was not known if Arg mediates water transport through AQPs in porcine conceptus trophectoderm (pTr2) cells. **Methods**: pTr2 cells derived from pregnant gilts on day 12 of gestation were cultured in customized Arg-free Dulbecco's modified Eagle's Ham medium (DMEM) supplemented with either 0.00, 0.25, or 0.50 mM Arg. **Results**: Arg treatment increased water transport and the expression of AQP3, which was abundantly expressed in pTr2 cells at both the mRNA and protein levels. Arg also increased the expression of iNOS and the synthesis of nitric oxide (NO) in pTr2 cells. The presence of N^{ω}-nitro-L-arginine methyl ester hydrochloride (L-NAME; an inhibitor of NO synthase) significantly attenuated the Arg-induced expression of AQP3. Furthermore, 0.50 mM Arg increased the concentrations of cAMP and the abundances of phosphorylated cAMP-dependent protein kinase A (PKA), phosphorylated PKA $\alpha/\beta/\gamma$, and phosphorylated CREB. These effects of Arg were mimicked by Forskolin (a cell-permeable activator of adenylyl cyclase), but inhibited by H-89 (an inhibitor of cAMP-dependent protein kinase). **Conclusions**: The results of this study demonstrate that Arg regulates AQP3 expression and promotes water transport in pTr2 cells through NO- and cAMP-dependent signaling pathways.

Keywords: arginine; aquaporin-3; pig; placenta; pregnancy; cAMP pathway

1. Introduction

Embryonic and fetal losses remain a significant problem in mammals. L-Arginine (Arg), as a conditionally essential amino acid for these animals, plays an important role in embryonic and fetal development [1,2]. Supplementation with 0.4% or 0.8% Arg between days 14 and 25 of gestation ameliorated porcine embryonic loss, possibly involving an increase in total volume of fetal amniotic and allantoic fluids [3]. Moreover, dietary Arg supplementation enhances placental growth and uterine function to increase litter size in pigs [4,5]. The beneficial effects of Arg for the improvement of reproductive performance in gestating pigs may be mediated through the synthesis of nitric oxide (NO), polyamines, and creatine [6,7]. Moreover, Arg serves as an important substrate for NO synthesis in porcine placentae to stimulate placental vascular development and angiogenesis [8].

The placenta is responsible for the transport of nutrients and waste products between conceptus (fetus and placenta) and mother, therefore providing a protective and stable environment for the conceptus to develop and grow [9]. Importantly, water is quantitatively the most abundant nutrient required for fetal growth. The vast and rapid transport of water across the placenta is facilitated by water channel proteins termed aquaporins (AQPs) [10]. To date, 13 AQPs (AQP0-AQP12) have been found in mammals [11,12]. AQP3, in particular, may be involved in the regulation of embryonic growth and uterine fluid homeostasis during the periods of conceptus implantation and parturition [10,13,14]. Understanding the effects of Arg on AQP-mediated water transport in the placenta would provide important insights into the nutritional regulation of early embryonic growth and development in mammals. Results of our recent study indicated that dietary Arg supplementation stimulates the expression of selective AQPs in porcine placentae for enhancing water transport from mother to fetus during pregnancy [15].

AQP expression may be regulated by the cyclic adenosine monophosphate (cAMP) signaling pathway in a human amnion epithelia-derived cell line [16,17], in kidney epithelial-cells [18] and in Chinese hamster ovary

cells [19]. Furthermore, cAMP up-regulated expression of AQP1, AQP8, and AQP9 mRNAs in human amnion via the protein kinase A (PKA)-independent pathway [20]. Moreover, NO may function as a modulator of water and electrolyte transport by tissues such as the intestine [21] and salivary glands [22]. Increasing evidence has also shown that NO can regulate the expression of AQPs 1, 2, 4 and 5 in the heart, kidney, and lung of rodents [23–26]. However, whether the cAMP and NO signaling pathways could mediate the ability of Arg to regulate the expression of AQP3 in porcine trophectoderm (pTr2) cells is unknown. Interestingly, AQP3 has been shown to play an important role in embryonic growth and development in rodents [13]. Thus, this study was conducted to test the hypothesis that Arg regulates the expression of AQP3 and promotes water transport in pTr2 cells.

2. Materials and Methods

2.1 Materials

The following chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA): D-(+)–glucose (G-5767), sodium bicarbonate (S5761), L-arginine (Arg, A-6969), L-glutamine (G-5792), L-leucine (L-8000), L-proline (P-0380), human insulin (I3536), N^{ω}-nitro-L-arginine methyl ester hydrochloride (L-NAME, N5751), forskolin (F3917), Sp-Adenosine 3′, 5′-cyclic monophosphorothioate triethylammonium salt hydrate [sp-cAMP (an analogue of cAMP), A166] and H-89 dihydrochloride hydrate [H-89 (an inhibitor of PKA), B1427]. The DMEM/F12 (Gibco, Merelbeke, Belgium), custom-made Arg-free Dulbecco's modified Eagle's Ham medium (DMEM) (Formula, #08-5009EF), penicillin/streptomycin solution (P/S), fetal bovine serum (FBS), 0.25% trypsin-ethylene diamine tetraacetic acid (EDTA), and phosphate buffered saline (PBS) were obtained from Gibco. The Arg-free DMEM contained concentrations of other amino acids that are normally present in the plasma of gestating swine [3]. Isotope water stock (^3H_2O, stock concentration at 1 mCi/mL) was purchased from American Radiolabeled Chemicals.

2.2 Cell Culture and Treatments

Porcine trophectoderm (pTr2) cells were derived from elongated porcine blastocysts obtained from gilts on day 12 of pregnancy [27]. Cells were cultured in 75-cm^2 flasks (Corning, NY, USA) in DMEM/F12 medium supplemented with 5% fetal bovine serum (FBS), 1% penicillin/streptomycin (PS) and 0.10 U/mL insulin at 37 °C under an atmosphere of 5% CO_2 in air. The medium was changed every 2 days.

At confluence, the cells were collected using 0.25% trypsin-EDTA, and seeded at a density of 1×10^5 cells/mL in 6-well, or 2×10^4 cells/mL in four-well culture plates (Lab-Tek® Chamber Slide™ System, Nunc International). Cells were cultured overnight (16 h) in complete DMEM/F-12 containing 5% FBS, 1% PS and 0.10 U/mL insulin, and

then incubated with the Arg-free DMEM containing 5% FBS, 1% PS and 0.10 U/mL insulin for 6 h, as previously described [27]. To determine an appropriate concentration of Arg supplementation, cells were treated with 0.00 mM (control), 0.25 mM, or 0.50 mM Arg for 24 or 48 h, respectively. The Arg concentrations used in the present study were selected based on the previous study that Arg concentrations in the plasma of non-supplemented gilts are 240 μM (approximately 0.25 mM) at 1 h after feeding and reach a peak value of 500 μM (0.50 mM) at 1 h after feeding in arginine-supplemented gilts [28].

To investigate the potential role of NO, L-NAME, an inhibitor of NO synthase (NOS), was added to inhibit the Arg-NO pathway. Briefly, pTr2 cells were cultured in Arg-free DMEM containing 5% FBS, 1% PS and 0.10 U/mL insulin for 6 h, and then in fresh medium supplemented with either 0.50 mM Arg or 0.50 mM Arg plus 3.00 mM L-NAME (0.50 mM Arg + L-NAME) for 24 h.

Additionally, Forskolin (10 μM; a cell-permeable activator of adenylyl cyclase), a cAMP analogue (sp-cAMP, 50 μM), or H-89 [10 μM; which inhibits cAMP-dependent protein kinase (PKA)] was used to determine the role of the cAMP signaling pathway in mediating the effect of Arg on AQP expression, as previously described [29]. Briefly, the pTr2 cells were cultured in Arg-free DMEM containing 5% FBS, 1% PS and 0.10 U/mL insulin for 6 h, and then in fresh medium supplemented with 10 μM Forskolin, 50 μM sp-cAMP, or 10 μM H-89 (H-89) for 2 h. Thereafter, the cells were cultured in fresh medium containing 0.50 mM Arg for another 48 h. Thus, the four treatments were Control (0.50 mM Arg), Forskolin (10 μM Forskolin + 0.50 mM Arg), sp-cAMP (50 μM sp-cAMP + 0.50 mM Arg), and H-89 (10 μM H-89 + 0.50 mM Arg). The treated cells were harvested for the determination of mRNA expression, protein expression, NO production, and cAMP concentrations (see below). All experiments were performed in triplicate using cells from passages 10–20.

2.3 Quantitative Real-Time PCR

Total RNA was extracted from pTr2 cells using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The concentrations and purity of the RNA samples were determined using a NanoDrop 2000 UV-Vis spectrophotometer (Thermo, Wilmington, MA, USA). The integrity of the RNA was assessed with 1.5% agarose gel in Tris acetate-EDTA buffer. The ratio of absorbance at 260 nm and 280 nm was approximately 2.0 for the total RNA isolated from pTr2 cells. Total RNA was reverse-transcribed into cDNA using the PrimerScript® RT reagent Kit with gDNA Eraser (Takara Biotechnology Co., Dalian, China). Real-time PCR was performed in the CFX Connect TM Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) using the SYBR® Premix Ex Taq TM II (Takara Bio, Otsu, Japan). The specific sequences of

forward and reverse primers and targeted amplicons for AQP-1 (221 bp), AQP-3 (212 bp), AQP-9 (62 bp), and AQP-11 (229 bp), as well as the housekeeping gene β-actin (208 bp) are provided as previously described [30]: AQP1:5′-TGACCTTCGCTATGTGCTTCC′ and 5′-GTCCAAGTGTCCAGAGGGGTAG-3′, AQP3: 5′-TGACCTTCGCTATGTGCTTCC-3′ and 5′- GTCCAAGTGTCCAGAGGGGTAG-3′, AQP9: 5′-TGTCATTGGCCTCCTGATTG-3′ and 5′-TGGCACAGCCACTGTTCATC-3′, AQP11: 5′-CGTCTTGGAGTTTCTGGCTACC-3′ and 5′-CCTGTCCCTGACGTGATACTTG-3′, and β-actin: 5′-TCCACCGCAAATGCTTCTAG-3′ and 5′-TGCTGTCACCTTCACCGTTC-3′, respectively. The primers were synthesized by Sangon Biotech Co. (Shanghai, China). The relative expression of mRNAs for targeted genes after treatments was calculated using the $2^{-\Delta\Delta Ct}$ method, while the specific expression of AQPs in pTr2 cells cultured without Arg was calculated using the $2^{-\Delta Ct}$ method. The experiments were run in triplicates and the relative expression of targeted genes were normalized to the expression of the housekeeping gene (β-actin).

2.4 Ussing Chambers for Measuring Water Transport

For the measurements of water transport, the pTr2 cells were cultured on a snap-well insert (polycarbonate with a pore size of 4 microns; Cat. no. P3901, Physiologic Instruments) to 100% confluency. The snap-well insert with a confluent monolayer of cells was mounted to a special slider (Cat. no. P2305, Physiologic Instruments), and then transferred to four sets of Ussing chambers. Both sides of the chambers in each set contained the same volume of Krebs biocarbonate buffer (pH 7.4, 37 °C) and were constantly gassed with 95% O_2 and 5% CO_2. Krebs biocarbonate buffer contained 119 mM NaCl, 4.8 mM KCl, 2.5 mM $CaCl_2.2H_2O$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, 20 mM Hepes, and 5 mM glucose. Before assays, the quality of electrodes used in the chambers was assessed with a blank snap-well insert mounted into the slider to ensure the electric current of 60–68 μA. Thereafter, the blank insert was replaced with a snap-well insert containing confluent cells. Water transport was measured by adding 20 μL of a ^3H_2O solution [prepared by mixing 500 μL Krebs biocarbonate buffer with 50 μL ^3H_2O stock (American Radiolabeled Chemicals Inc)] to 5 mL of oxygenated (95% O_2/5% CO_2) Krebs bicarbonate medium (containing 5 mM D-glucose and 0.00, 0.25, or 0.50 mM Arg) in the "mucosal side" of the Ussing chamber. A sample (50 μL) was collected from the "serosal side" of the Ussing chamber every 5 min over a 20-min period, and measured for radioactivity by a liquid scintillation spectrometer (Perkinelmer, USA). The specific radioactivity of ^3H_2O in the solution of the "mucosal side" was used to calculate the rate of water transport per well (the confluent monolayer of cells).

2.5 Immunofluorescence Assay

For immunofluorescence assays, pTr2 cells were seeded at 2×10^4 cells/mL into four-well dishes (Lab-Tek, Nunc). After treatment, cells were fixed with 2% paraformaldehyde for 15 min, and then permeabilized with 1% Triton X-100 for 10 min, washed twice with PBS, and blocked with 5% BSA for 30 min. The samples were incubated with a primary antibody overnight at 4 °C. The primary antibodies were polyclonal rabbit anti-AQP-1 (1:200, 101AP, FabGennix, USA), anti-AQP-3 (1:200, bs-1253R, Bioss, China), anti-AQP-9 (1:50, OABF00907, Aviva Systems Biology, USA), anti-AQP-11 (1:50, 1101AP, FabGennix, USA), anti-iNOS (1:100, ab15323, Abcam, USA), anti-cAMP protein kinase catalytic subunit (1:1000, ab26322, Abcam, USA), anti-PKA $\alpha/\beta/\gamma$ (1:200, sc-98951, Santa Cruz Technology, USA) and anti-p-PKA $\alpha/\beta/\gamma$ (1:200, sc-32968, Santa Cruz Technology, USA). Subsequently, the stained cells were washed three times with PBS and incubated with the corresponding secondary antibodies at 1:200 (Alexa Fluor 594 goat anti-rabbit-IgG, Life Technology, USA; Alex Fluor 488 goat anti- rabbit-IgG, Life Technology, USA) at 25 °C for 2 h in the dark. The samples were subsequently counterstained with DAPI/anti-fade mounting medium (ProLong® Gold anti-fade reagent with DAPI, Life Technologies, Carlsbad, CA, USA). Fluorescence signals were observed using a fluorescence microscope (Axio Observer A1, Carl Zeiss, Jena, Germany) and a Confocal Laser Scanning Microscope (LSM 710, Carl Zeiss, Heidenheim, Germany).

2.6 Western Blotting Analysis

After treatment, cells were washed twice with cold PBS and lysed in a lysis buffer (RIPA, BioTeke, Beijing, China) containing 1 mM phenyl-methylsulfonyl fluoride (PMSF) (Sigma, St. Louis, MO, USA), followed by centrifugation at $14,000 \times g$ at 4 °C for 15 min. The concentrations of protein in the supernatant fluid were determined using a BCA Protein Assay Reagent Kit (Thermo Fisher Scientific, Rockford, USA). After boiling for 10 min, the samples (15 μg each) were separated by 12% SDS-polyacrylamide gel electrophoresis (PAGE) gels and then transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After being blocked with 5% BSA in Tris-Tween-buffered saline (TBST) at 25 °C for 3 h, the membranes were incubated with a primary antibody at 4 °C overnight with gentle rocking. The membranes were then incubated with the secondary antibody for 2 h at room temperature. The information on all antibodies used in this study is provided in Table 1. The membranes were treated with the Clarity™ Western ECL Substrate (Bio-Rad, Irvine, CA, USA), and protein bands were visualized and quantitated using the ChemiDoc XRS imaging system and the Image Lab software (version 3.0, National Institutes of Health, Bethesda, MD, USA), as previously described [30].

Table 1. Antibodies and dilutions used for western blotting analysis.

Antibody	Supplier[1]	Cat. no.	Dilution
Rabbit polyclonal to AQP3	Bioss	bs-1253R	1:500
Rabbit polyclonal to AQP9	AVIVA System Biology	OABF00907	1:1000
Rabbit polyclonal to AQP11	FabGennix	1101AP	1:500
Rabbit polyclonal to PKA $\alpha/\beta/\gamma$	SCT	sc-98951	1:1000
Rabbit polyclonal to p-PKA $\alpha/\beta/\gamma$(Thr198)	SCT	sc-32968	1:1000
Rabbit polyclonal to PKA C-α	CST	4782	1:1000
Rabbit polyclonal to p-PKA C-α(Thr197)	CST	4781	1:1000
Rabbit polyclonal to PKA $\alpha/\beta/\gamma$	CST	sc-98951	1:1000
Rabbit polyclonal to p-PKA $\alpha/\beta/\gamma$(Thr198)	CST	sc-32968	1:1000
Rabbit monoclonal to CREB(48H2)	CST	9197	1:1000
Rabbit monoclonal to p-CREB(Ser133)	CST	9198	1:1000
Rabbit polyclonal to cAMP-dependent protein kinase catalytic subunit	Abcam	ab26322	1:1000
Mouse monoclonal to β-actin	Abcam	ab8224	1:4000
HRP-conjugated goat-anti-rabbit IgG	Abcam	ab6721	1:4000
HRP-conjugated goat-anti-mouse IgG	Abcam	ab6789	1:4000

[1]SCT, Santa Cruz Technology; CST, Cell Signaling Technology.

2.7 Determination of Intracellular Concentrations of Cyclic Adenosine Monophosphate (cAMP)

The pTr2 cells were incubated in ice-cold lysis buffer (0.1 M HCl) for 5 min, and then centrifuged at 1000 $\times$ g for 3 min at 4 °C. The concentrations of protein in the cell lysates were determined using the BCA Protein Assay Reagent Kit. The concentrations of cAMP in cell lysates were determined using a commercial enzyme-linked immunosorbent assay (ELISA) kit (Cat. no. CA200, Sigma-Aldrich, Munich, Germany), according to the manufacturer's guidelines. The intracellular concentrations of cAMP are expressed as pmoL/μg protein.

2.8 Measurement of Nitrite and Nitrate as Indicators of NO Production

Concentrations of nitrite and nitrate (stable oxidation products of NO) in the culture medium of pTr2 cells were determined using a commercial NO assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Briefly, the pTr2 cells were seeded in a 6-well plate at a density of 1 $\times$ 10^5 cells/mL and the culture medium was used to determine the concentrations of nitrite and nitrate according to the instructions of the manufacturer. The cell lysates were also analyzed for the concentrations of protein as noted previously. The concentrations of nitrite and nitrate in culture medium are expressed as pmoL/μg protein.

2.9 Statistical Analysis

Data from at least three independent experiments were analyzed by the SPSS software (version 17.0, IBM, Chicago IL, USA). The results are expressed as means $\pm$ SEM. The Student's paired t-test was used to determine differences between two treatment groups. Differences among three or more treatment groups were determined using one-way ANOVA, followed by the Duncan's multiple-range test. In the case of obtaining results over different time points, the data were analyzed by using ANOVA for repeated measurements. Differences in means among treatments were considered statistically significant at $p < 0.05$.

3. Results

3.1 Expression of AQPs in pTr2 Cells

The expression of mRNAs and proteins for AQP1, AQP3, AQP9, and AQP11 in pTr2 cells were determined by quantitative RT-PCR and immunofluorescence analyses, respectively (Fig. 1). AQP1 was localized mainly to the nucleus, whereas AQP3, AQP9 and AQP11 were localized mainly in the plasma membrane of pTr2 cells (Fig. 1B). Importantly, the expression of AQP3 mRNA in pTr2 cells was greater than that for AQP1, AQP9 and AQP11 ($p < 0.05$), indicating a potential role of the abundantly expressed AQP3 in mediating water transport by pTr2 cells (Fig. 1). Therefore, AQP3 was the focus of our subsequent experiments.

3.2 Arg Enhanced Water Transport by Up-regulatiing Expression of AQP3 in pTr2 Cells

The transport of water across the apical and basolateral membranes of pTr2 cells was linear within a 20 min period (Fig. 2). Compared with the control group (0.00 mM Arg), 0.25 and 0.50 mM Arg increased ($p < 0.05$) the transport of water by pTr2 cells during the 20 min period. The rate of water transport was about 30% greater ($p < 0.05$) in the presence of 0.50 mM Arg, as compared to 0.25 mM Arg. For consistency, we mainly used the 0.50 mM Arg concentration for other assays.
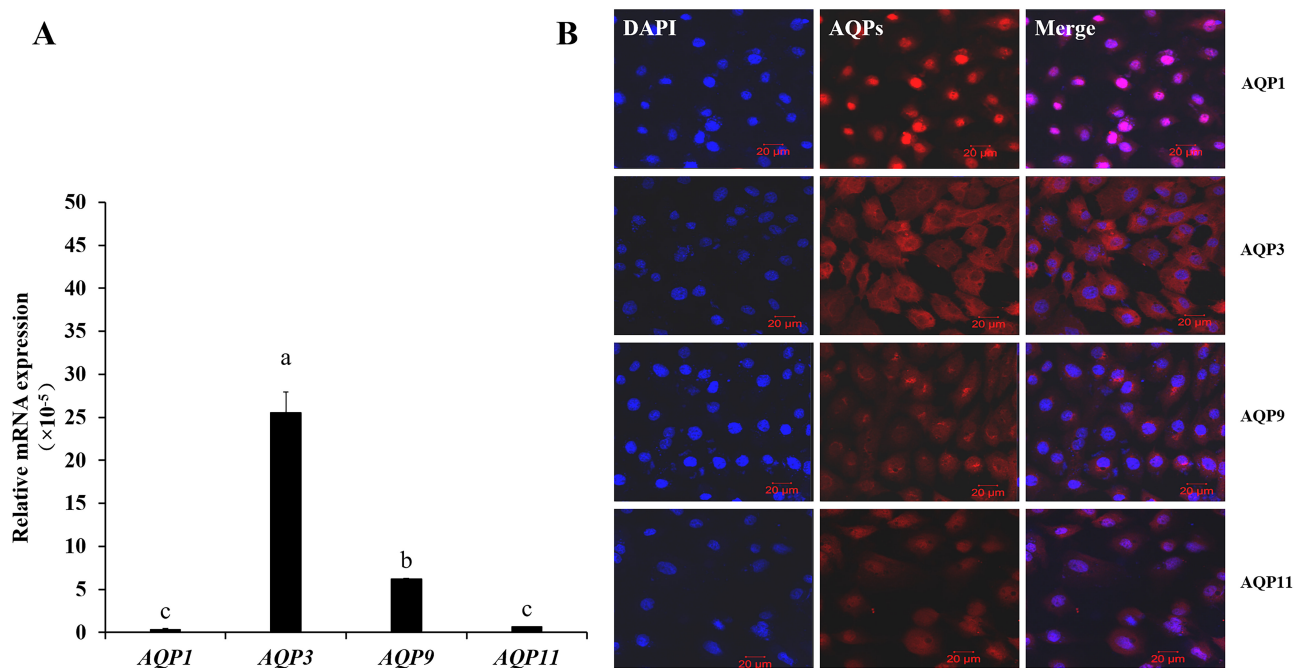
Fig. 1. The expression of AQPs in pTr2 cells. (A) pTr2 cells were cultured in DMEM/F12 containing 5% FBS, 1% penicillin/streptomycin solution (PS) and 0.10 U/mL insulin for 48 h. (A) The expression of AQP1, AQP3, AQP9, and AQP11 mRNAs in pTr2 cells (n = 6). The relative mRNA expression of AQPs was calculated using the $2^{-\Delta Ct}$ method. Data were analyzed by one-way ANOVA followed by the Duncan's multiple comparison using the SPSS software (version 17.0). The bars represent means $\pm$ SEM and different letters (a–c) indicate significant differences ($p < 0.05$). (B) Immunofluorescent localization of AQP proteins in pTr2 cells was determined using a confocal laser scanning microscope. Representative immunofluorescence images are shown for three independent experiments in which nuclei were labeled with DAPI (blue) and AQPs detected by specific antibodies (red). Scale bars: 20 μm. The original magnification was at 400$\times$.

More importantly, 0.50 mM Arg increased the mRNA expression of AQP3 in pTr2 cells, as compared with 0.00 mM and 0.25 mM Arg (Fig. 3A; $p < 0.05$). Based on western blotting and immunofluorescence analyses, AQP3 protein was also increased in response to the treatment of pTr2 cells with Arg (Fig. 3B and C; $p < 0.05$). These results indicated that Arg up-regulated AQP3 expression to enhance water transport by pTr2 cells. Moreover, because AQP3 and other AQPs may work together to regulate water transport by pTr2 cells, the expression of other AQPs has also been shown in **Supplementary Fig. 1**. We found that the AQP11 mRNA expression was increased ($p < 0.05$) in pTr2 cells treated with 0.25 and 0.50 mM Arg (**Supplementary Fig. 1**). However, mRNA levels for both AQP1 and AQP9 mRNAs were not significantly affected by 0.25 or 0.50 mM Arg ($p > 0.05$).

3.3 Arg Regulated Expression of AQP3 Partly via an NO-dependent Mechanism

It was not known if Arg increased expression of AQP3 via an NO-dependent pathway. Results of the present study revealed that 0.50 mM Arg significantly increased NO production by pTr2 cells after 48 h of treatment (Fig. 4A; $p < 0.05$). NO synthesis by pTr2 cells was significantly de-creased by the addition of L-NAME plus Arg (0.50 mM Arg + L-NAME group) when compared with the 0.50 mM Arg group (Fig. 4B; $p < 0.05$). Compared with the control group, 0.50 mM Arg enhanced the fluorescence signaling of iNOS in pTr2 cells (**Supplementary Fig. 2**). L-NAME treatment markedly decreased ($p < 0.05$) the fluorescence intensity of the labeled iNOS protein in the cells (**Supplementary Fig. 3**).

When compared with the 0.50 mM Arg group, pTr2 cells cultured in the presence of 0.50 mM Arg + L-NAME had lower levels of AQP3 mRNA and protein based on results from the qRT-PCR, western blotting, and immunofluorescence analyses (Fig. 5; $p < 0.05$). Collectively, these results indicate that Arg increased AQP3 expression in pTr2 cells partially via an NO-dependent mechanism.

3.4 The Role of the cAMP-PKA Pathway in Mediating Arg-induced Increases in Expression of AQP3 in pTr2 Cells

To further investigate the role of the cAMP-PKA signaling pathway in the Arg-induced increase in AQP3 expression in pTr2 cells, the cAMP signaling pathway was examined. Arg (0.50 mM) increased the abundance of the PKA protein in pTr2 cells as shown by immunofluores-
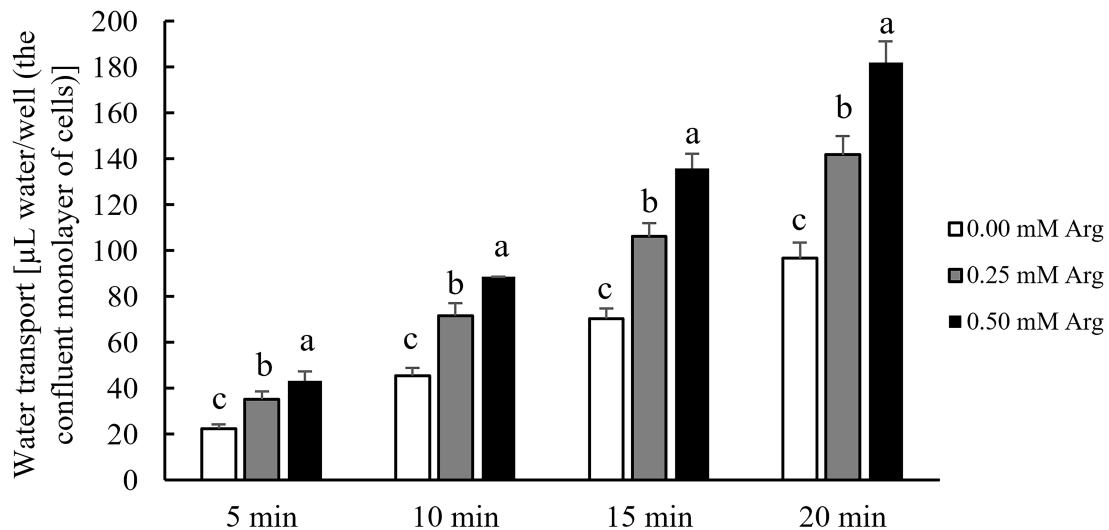
Fig. 2. Effects of arginine on water transport by pTr2 cells. Cells were cultured on a snap-well insert (Physiologic Instruments) until reaching 100% confluency, and then transferred to four sets of Ussing chambers. Water transport was measured over a 20 min period by adding ^3H_2O to 5 mL of oxygenated (95% O_2/5% CO_2) Krebs bicarbonate medium [containing 5 mM D-glucose and 0.00, 0.25, or 0.50 mM L-arginine (Arg)] in the "mucosal side" of the Ussing Chamber. Data were analyzed by one-way ANOVA for repeated measurements, followed by the Duncan's multiple comparison using the SPSS software (version 17.0). Values, expressed as μL water/well (monolayer of cells), are means $\pm$ SEM, n = 6. Different letters (a–c) indicate significant differences ($p < 0.05$). At each time point, increasing the concentration of Arg from 0.00 to 0.25 and to 0.50 mM in the solution of the "mucosal side" increased water transport by pTr2 cells in a dose-dependent manner ($p < 0.05$).

cence analyses (Fig. 6A). Of note, treatment with 0.50 mM Arg induced nuclear translocation, confirming the induction of active intracellular signaling. Further, concentrations of intracellular cAMP were increased in pTr2 cells by 0.50 mM Arg when compared to control values (Fig. 6B; $p < 0.05$). Furthermore, when compared with 0.00 mM Arg, the abundances of the PKA protein's catalytic subunit (Fig. 6C), phosphorylated PKA, phosphorylated PKA $\alpha/\beta/\gamma$, and phosphorylated CREB (Fig. 6D) were all increased in response to 0.50 mM Arg ($p < 0.05$).

The effects of Forskolin, sp-cAMP, and H-89 confirmed that changes in the expression of AQP3 protein were coordinate with changes in the PKA catalytic subunit as determined using immunofluorescence microscopy and western blot analyses (Fig. 7). Additional evidence showed that cAMP concentrations in pTr2 cells after the treatment with 0.50 mM Arg were increased ($p < 0.05$) by Forskolin, but was decreased ($p < 0.05$) by H89 (**Supplementary Fig. 4**). These results indicate that the cAMP-PKA signaling pathway positively enhanced the expression of AQP3 mRNA and protein in pTr2 in response to Arg treatment that stimulated the transport of water by these cells.

4. Discussion

Maternal nutrition plays an important role in offspring metabolism and growth [31,32]. Notably, increasing dietary Arg supplementation improves reproductive performance and fetal development in pigs [33,35–38], rats [39]

and sheep [40]. The expression of AQPs in placentae are associated with changes in the volumes of amniotic fluid and allantoic fluids of pigs during gestation [41–44]. Moreover, dietary supplementation with Arg between days 14 and 25 of gestation enhances porcine embryonic development and survival in association with an increase in the volume of amniotic fluid by 36 to 61% [3]. In Arg-supplemented gilts, the increase in the placental transport of water from mother to fetus during the period of placentation results from increases in the expression of selective AQPs [15]. However, the mechanism(s) responsible for mediating effects of Arg on fetal fluids remained to be defined.

Ussing chambers provide a useful system for measurement of the transport of electrolytes, organic nutrients, water, and drugs across the small intestine, placenta, and other epithelial tissues [45]. The quantity of water exchange across the placenta can be demonstrated using the ^3H_2O tracer [46]. Membrane permeability studies suggested that the structure of the placental trophoblast layer may serve to regulate water flow across the placenta [47]. Placentation is epitheliochorial in pigs in which porcine placental trophectoderm/chorion cells directly attach to the uterine luminal epithelium, and these two epithelia serve as the conduit for maternal support (including water provision) for conceptus growth and development [48]. In the present study, we used ^3H_2O in the Ussing chamber system to determine water transport by pTr2 cells and found that the addition of physiological concentrations of Arg (0.25 and 0.50 mM) to
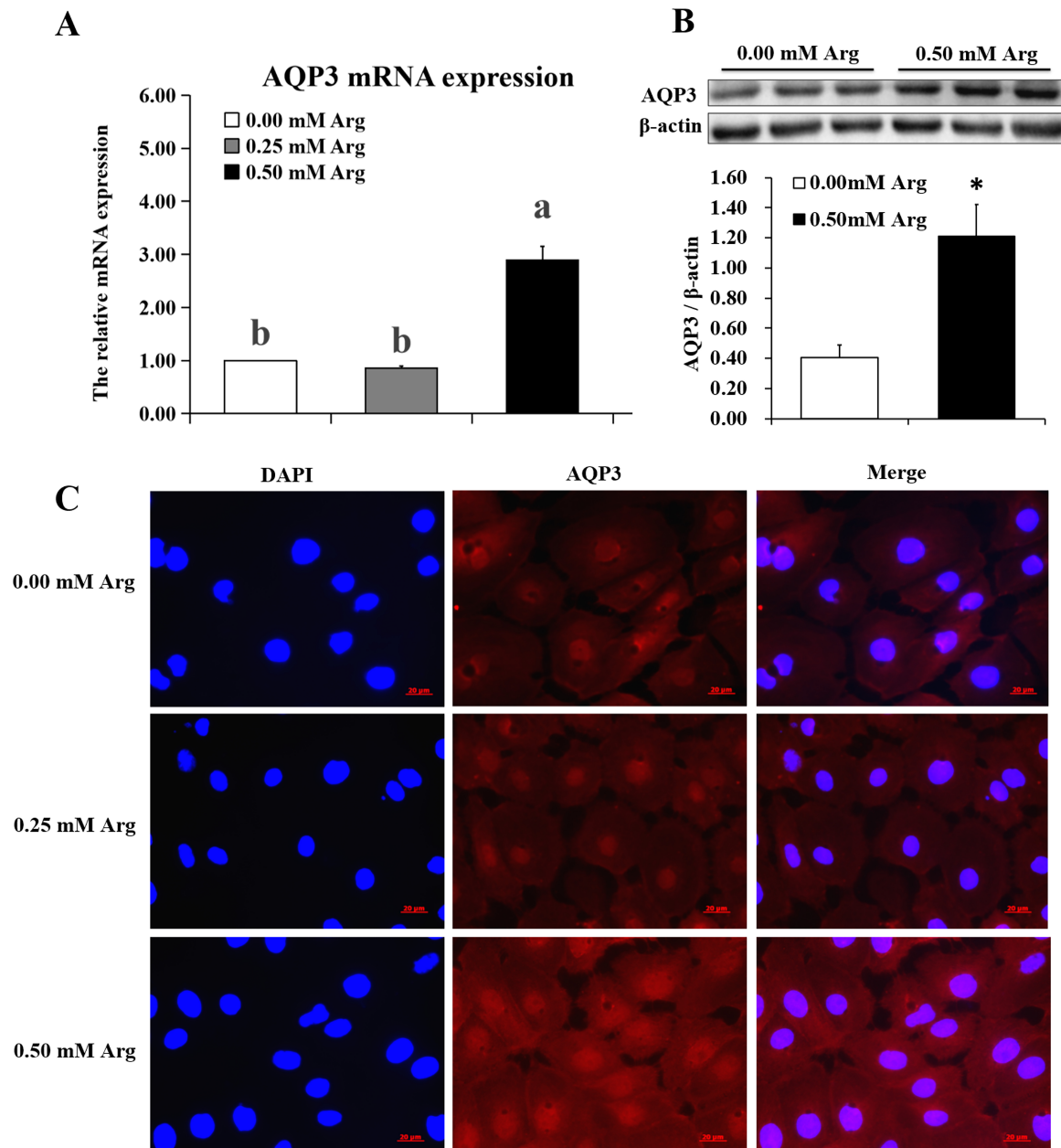
Fig. 3. Effects of arginine on the expression of AQP3 in pTr2 cells. Cells were cultured for 48 h in arginine (Arg)-free DMEM medium containing 5% FBS, 1% penicillin/streptomycin solution (P/S) and 0.10 U/mL insulin supplemented with 0.00 mM (control), 0.25 mM, and 0.50 mM arginine (Arg). (A) Expression of the AQP3 mRNA in pTr2 cells at 48 h after treatment (n = 6). Data were analyzed by one-way ANOVA. The bars represent means $\pm$ SEM. Different letters (a-b) indicate significant differences ($p < 0.05$). (B) After treatment for 48 h, the abundance of the AQP3 protein was analyzed by western blotting. Data were analyzed by the Student's paired t-test. Representative results of three independent experiments are shown as means $\pm$ SEM; * $p < 0.05$. (C) Representative immunofluorescence images of cells at 48 h after treatment, labeled with DAPI (blue, nuclei) and AQP3 antibody (red). Scale bars: 20 μm. Original magnification, 400$\times$.

the "mucosal side" dose-dependently increased water transport by the cells across their apical and basolateral membranes. This indicates an increase in the net flux of water from the maternal to the fetal side of the placenta. These results were consistent with those of our recent study indicating that water transport from mother to fetus was greatly facilitated by AQPs in the placentae of gilts receiving dietary Arg supplementation [15], and help to elucidate the mechanism whereby supplementing Arg to the diet of gestating swine enhances the volumes of amniotic and allantoic fluids, embryonic survival and fetal development [36,49,50], as well as the growth performance of piglets [51].
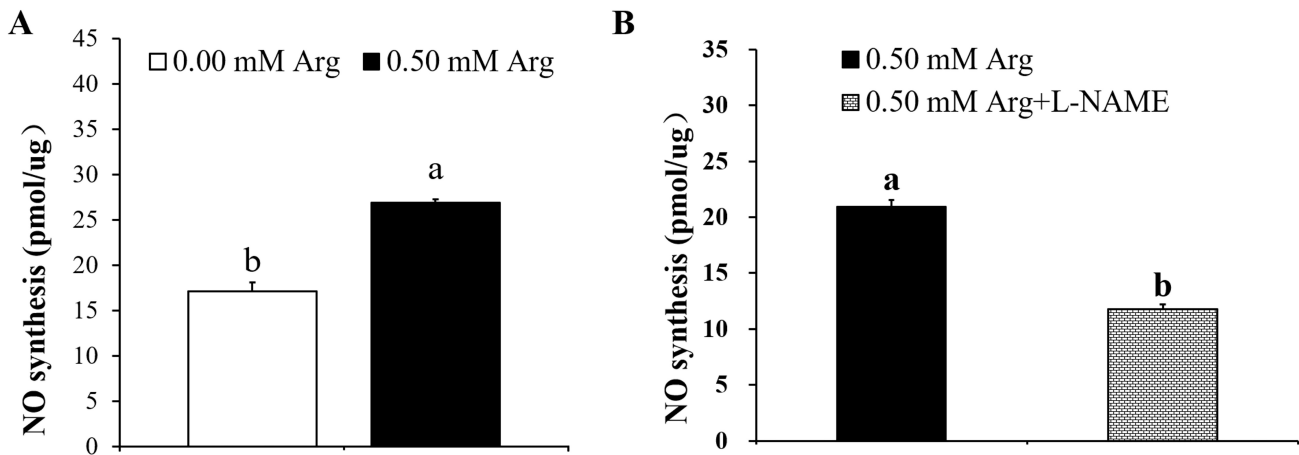
Fig. 4. Measurement of NO production by cultured pTr2 cells. (A) Arginine (Arg) supplementation increased NO synthesis by pTr2 cells. (B) The effect of L-NAME supplementation to medium was to decrease NO synthesis by pTr2 cells. Data were analyzed by the Student's paired t-test. Representative results of three independent experiments are shown as means $\pm$ SEM. Different letters indicate significant differences ($p < 0.05$). L-NAME, N^ω-nitro-L-arginine methyl ester hydrochloride (an inhibitor of NO synthase).

A recent study indicated that dietary supplementation of cystine upregulates AQP3 expression in intestinal cells of postnatal piglets, implicating AQP3 as a promising target for regulating water transport in epithelial cells [33]. Moreover, results of the present work clearly showed that AQP3 was expressed abundantly in pTr2 cells, as reported for placentae of other mammals [52,53]. Conversely, a deficiency of AQP3 in embryos from the 2-cell stage onward impaired embryonic development [54]. In addition, increased expression of AQP3 in the placenta contributes to an enhanced flux of water from other to fetus [53]. Likewise, AQP3 promotes the proliferation of human gastric carcinoma cells [55,56] and keratinocyte cells [57]. Particularly, AQP3 facilitated water transport across the membranes of mammalian cells, thereby reshaping cellular protrusions essential for cell migration [58]. Thus, AQP3 was the focus of our current work as the addition of Arg to culture medium increased AQP3 mRNA and protein abundances in pTr2 cells.

We also investigated mechanisms involved in the regulation of AQP3 expression by Arg in pTr2 cells. Arg stimulates proliferation, migration, and protein synthesis in an established ovine trophectoderm cell line via effects on the synthesis of NO [59–61], and Arg increases cell migration via an NO-dependent mechanism in rat crypt cells [62]. NO acts by stimulating the production of cGMP from GTP by guanylate cyclase [44]. In addition, Arg enhances the production of NO and polyamines and activates the mTOR-signaling pathway to stimulate the proliferation of porcine [27] and ovine [61] trophectoderm cells. However, results of other studies with porcine jejunal epithelial cells [63] and pTr2 cells [27] indicated that Arg activates protein synthesis and the mTOR pathway via both NO-dependent and NO-independent mechanisms. Previous studies have shown that NO regulates the mRNA levels for AQP1, AQP2, AQP4,

and AQP5 [23–26], but a role of NO in modulating AQP3 expression has not been reported for animal cells. Therefore, to define the role of NO in regulating the expression of AQP3, we determined the expression of iNOS and NO synthesis in pTr2 cells treated with or without Arg. Consistently, Arg increased iNOS expression and stimulated NO synthesis in pTr2 cells, and this effect of Arg was attenuated by L-NAME (an inhibitor of NOS). Accordingly, up-regulation of AQP3 expression by Arg may enhance water transport by pTr2 cells via an NO-dependent mechanism. Collectively, our results indicate that an Arg-induced increase in AQP3 expression was attenuated by L-NAME, supporting a role for NO in contributing to placental water transport to facilitate the embryonic development at least partially through the modulation of AQP3 expression. These results were consistent with those from earlier studies demonstrating that NO modulated the transport of water and electrolytes by cells of the intestine [21,64].

The ubiquitous second messenger, cAMP, is a key regulator of cell migration, proliferation, metabolism, and differentiation [65]. Interestingly, dietary Arg supplementation increases the concentrations of cAMP in the skeletal muscle, white adipose, and brown adipose tissue of rats [66]. The cAMP-PKA-dependent cell signaling is crucial for regulating the expression of AQPs in placentae [14,43,44]. For example, AQP1 gene expression was up-regulated by Arg vasopressin and cAMP agonists in trophoblast cells [67]. Moreover, AQP3 expression was stimulated by cAMP agonists in human amnion cells in culture, suggesting a role for cAMP in amniotic fluid homeostasis [17]. To identify a role of the cAMP sigaling in mediating AQP3 expression in Arg-supplemented pTr2 cells, we examined the expression of PKA and its downstream cell signaling pathway upon cAMP activation. As shown in Fig. 6, the concentrations of cAMP, as well as the abundances of
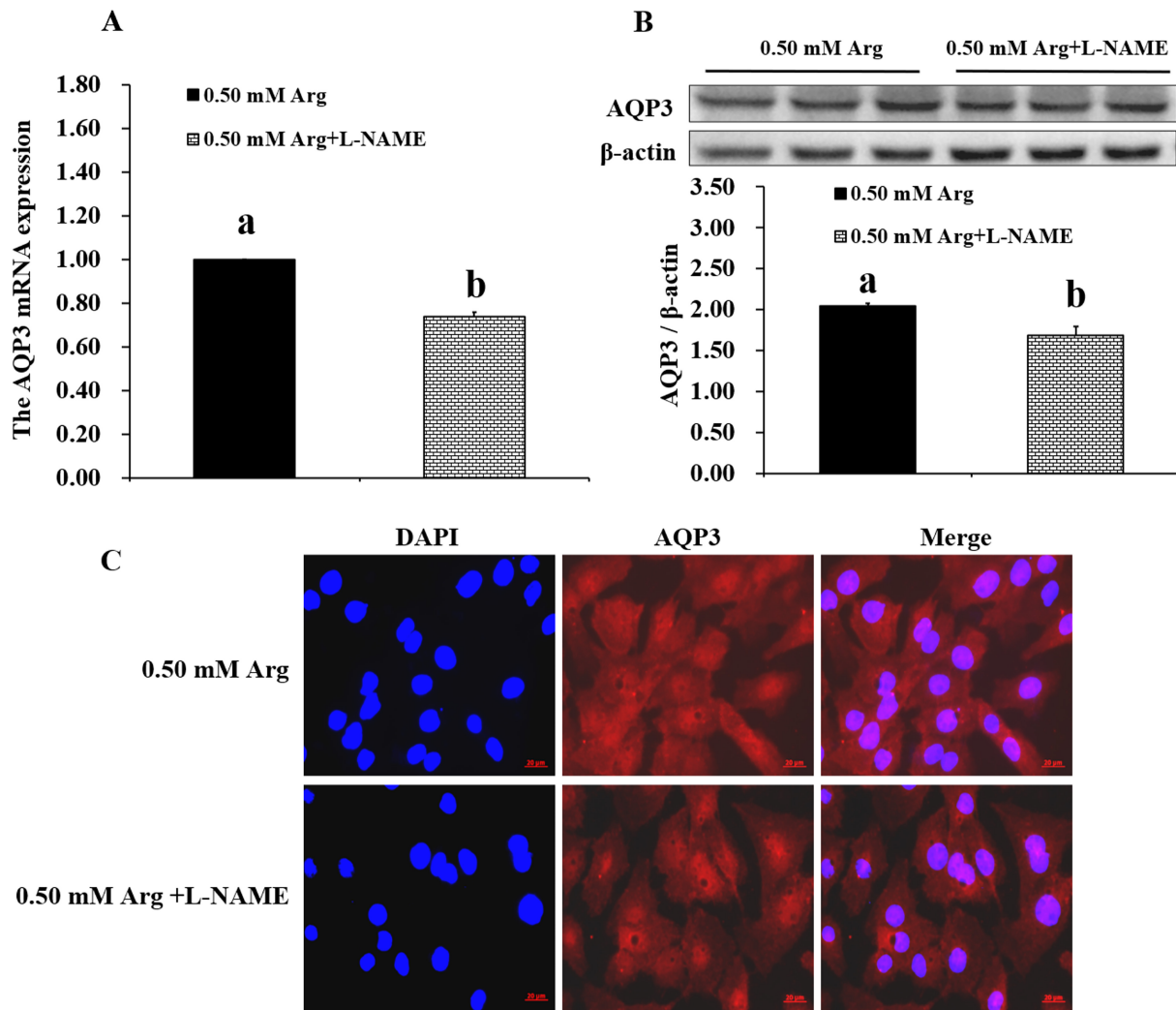
Fig. 5. The NO-dependent effect of arginine to increase the expression of AQP3 in pTr2 cells. (A) The expression of AQP3 mRNA in pTr2 cells at 24 h after treatment (n = 6) was decreased ($p < 0.05$) in the presence of L-NAME that inhibited the production of NO from arginine (Arg). (B) After a 24 h treatment period, the abundance of AQP3 protein was decreased ($p < 0.05$) in cells treated with 0.50 mM Arg + L-NAME versus 0.50 mM Arg alone. Data were analyzed by the Student's paired t-test. (C) Representative immunofluorescence images of cells at 24 h after treatment labeled with DAPI (blue, nuclei) and antibody to AQP3 (red) also suggest a decrease in AQP3 protein in cells cultured in the presence of 0.50 mM Arg + L-NAME as compared to treatment with 0.50 mM Arg alone. Scale bars: 20 μm. Original magnification, 400×. L-NAME, N$^{\omega}$-nitro-L-arginine methyl ester hydrochloride (an inhibitor of NO synthase).

phosphorylated PKA, CREB, and AQP3 increased in response to Arg supplementation. These results supported the notion that the cAMP-PKA-CREB pathway plays an important role in mediating the stimulatory effect of Arg on AQP3 expression and water transport by pTr2 cells. Consistent with this notion, AQP3 mRNA expression was increased after the addition of Forskolin to increase cellular cAMP concentration in amnion epithelial cell cultures for 3 to 6 h [17]. Moreover, Forskolin greatly increased AQP3 expression at 2 h and the elevation remained for 10 h, but returned to the baseline level at 20 h [47]. In accordance with these results, we found that Forskolin increased cAMP levels and the abundance in the proteins of its downstream target PKA and AQP3 in pTr2 cells, while H-89 (an inhibitor of PKA) markedly reduced the abundance of the AQP3 protein in pTr2 cells. Collectively, our results indicated that Arg up-regulated AQP3 expression in pTr2 cells partly via the activation of the NO and cAMP-PKA signaling pathway, therefore promoting placental water transport and embryonic/fetal growth and development. In support of this view, dietary supplementation with Arg (a truly functional amino acid in nutrition [68]) to gilts between days 14 and 25 of gestation increased NO synthesis by placentae [69].
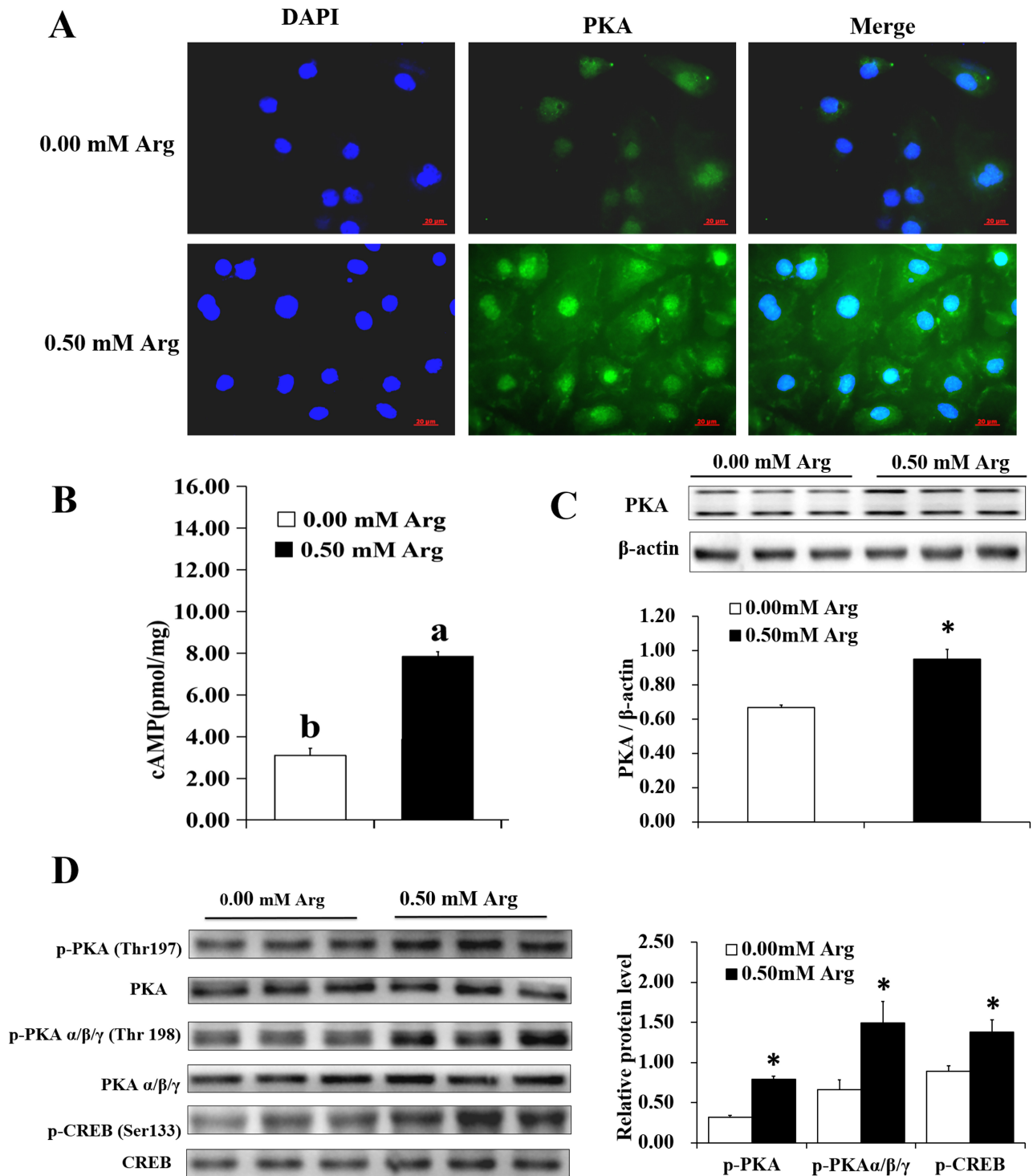
Fig. 6. Effects of arginine on the cAMP signaling pathway in pTr2 cells. Cells were cultured in arginine (Arg)-free DMEM [containing 5% FBS, 1% penicillin/streptomycin solution (P/S) and 0.10 U/mL insulin] supplemented with 0.00 mM Arg (control) or 0.50 mM Arg. (A) Representative immunofluorescence images of increases in PKA in pTr2 cells at 48 h after treatment, labeled with DAPI (blue, nuclei) and the PKA catalytic subunit antibody (green). Scale bars: 20 μm. Original magnification, 400×. (B) cAMP concentrations in pTr2 cells increased in a dose-dependent manner ($p < 0.05$) at 48 h after treatment, measured by enzyme-linked immuno sorbent assay (ELISA). Different letters (a-b) indicate significant differences ($p < 0.05$). (C,D) The expression of cAMP-dependent protein kinase (PKA) catalytic subunit (panel C) and components of the PKA/CREB pathway related proteins (panel D) increased ($p < 0.05$) in pTr2 cells after 48 h treatment as determined by western blotting analysis. Data were analyzed by the Student's paired t-test. Representative results of three independent experiments are shown as means $\pm$ SEM; * $p < 0.05$.
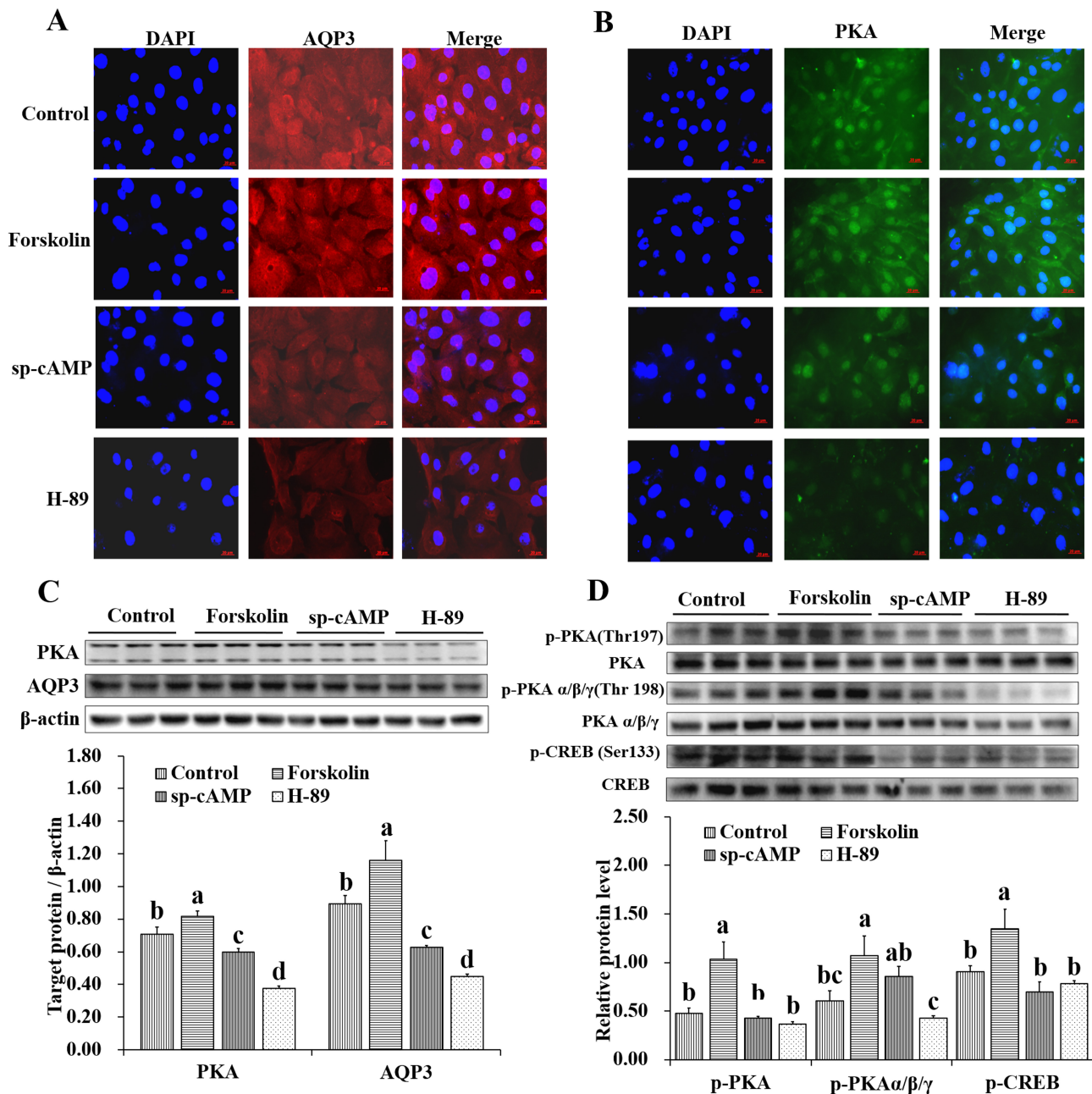
Fig. 7. Effects of the cAMP signaling pathway on AQP3 expression in pTr2 cells. Cells were cultured in arginine (Arg)-free DMEM containing 5% FBS, 1% penicillin/streptomycin solution (P/S) and 0.10 U/mL insulin for 6 h, followed by culture for 2 h in the presence of fresh medium supplemented with 10 μM Forskolin (a cell-permeable activator of adenylyl cyclase), 50 μM sp-cAMP (an analogue of cAMP), or 10 μM H-89 (an inhibitor of PKA). Thereafter, cells were cultured in the presence of 0.50 mM Arg for 48 h. Thus, the four treatments were Control (0.50 mM Arg), Forskolin (10 μM Forskolin + 0.50 mM Arg), sp-cAMP (50 μM sp-cAMP + 0.50 mM Arg), and H-89 (10 μM H-89 + 0.50 mM Arg). (A,B) Representative immunofluorescence images of cells after the treatment, labeled with DAPI (blue) and AQP3 antibody (red) (A) and PKA catalytic subunit antibody (green) (B), are shown. Scale bars: 20 μm. Original magnification, 400$\times$. (C) The abundances of PKA catalytic subunit protein and AQP3 were determined by western blotting analysis and revealed significant effects of treatments on the expression of AQP3. The bars represent means $\pm$ SEM. Different letters (a–d) indicate significant differences ($p < 0.05$). (D) Western blotting analysis of proteins related to the PKA/CREB signaling pathway in pTr2 cells revealed significant effects of treatment on the expression of AQP3. Data were analyzed by one-way ANOVA followed by the Duncan's multiple comparison test using the SPSS software (version 17.0). Representative results of three independent experiments are shown as means $\pm$ SEM. Means not sharing the same letter differ ($p < 0.05$).

5. Conclusions

Results from tracer flux, as well as RT-PCR, immunofluorescence microscopy, and western blotting analyses indicated that Arg up-regulated AQP3 expression and enhanced water transport in pTr2 cells via NO- and cAMP-dependent mechanisms. Further investigations are needed to determine the roles of NO and cAMP in the expression of the AQP3 gene at both the transcriptional and translational levels. Furthermore, it is imperative to define the functional roles of modifications in the placental expression of AQP3 and other AQPs in gilts and sows fed Arg-supplemented diets during gestation.

Author Contributions

CZ, ZJ, JY and GW designed the research. CZ, JY and CT performed the research. GW, FB, GAJ, ZJ, SH and YB contributed to data interpretation. CZ, JY and CT analyzed the data. CZ and JY wrote the manuscript. GW, FWB, GAJ and JY revised the manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript.

Ethics Approval and Consent to Participate

Not applicable.

Conflict of Interest

The authors declare no conflict of interest. GW is serving as one of the Section Editor-in-Chief of this journal. We declare that GW had no involvement in the peer review of this article and has no access to information regarding its peer review. Full responsibility for the editorial process for this article was delegated to GP.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://www.imrpress.com/journal/FBL/27/3/10.31083/j.fbl2703083.

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