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ORIGINAL ARTICLE

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Aquaporin-1 expression and angiogenesis in rabbit chronic myocardial ischemia is decreased by acetazolamide

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Abstract Aquaporin-1 (AQP1) is a water channel protein expressed in endothelial and epithelial cells of many tissues, including the vasculature, where it serves to increase water permeability of the cell membrane. Prior studies have also reported that AQP1 plays a central role in tumor angiogenesis by promoting endothelial cell migration. To investigate whether AQP1 might also influence vascular angiogenesis in ischemic myocardium, the expression level of AQP1 for 21 days post myocardial infarction in rabbit hearts was observed. Aquaporin-1 mRNA and protein levels increased on day 3, and peaked on day 7 post surgery. This correlated well with the pattern of neovascularization and increased water content of infarct border tissue, and suggested that AQP1 may be involved in myocardial angiogenesis in response to ischemia injury. These AQP1-induced changes were tempered by acetazolamide, a carbonic anhydrase inhibitor, which acted by downregulating AQP1 expression. Acetazolamide treatment did not significantly affect the expression of vascular endothelial growth factor in the tissues studied. Our findings indicate a novel role for AQP1 in postnatal angiogenesis, which has implications in diverse pathophysiological conditions including wound healing, tumor metastasis, and organ regeneration.

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Key words Aquaporin-1 · Angiogenesis · Acetazolamide · Myocardial infarction · Vascular endothelial growth factor

Introduction

The aquaporins (AQPs) are a family of transmembrane channel-forming glycoproteins, which serve as molecular pathways for the rapid transport of water across biological membranes.[1] Aquaporin-1 is expressed in many mammalian tissues and organs, and localizes to the endothelial cells of the continuous nonfenestrated capillaries and venules in blood vessels where it plays a crucial role in osmotically driven transendothelial water movement.[2,3] Prior studies have shown that AQP1 is expressed in the endothelium[4,5] and myocytes[6,7] of the heart. It was also found in the endocardium of very early stage fetal sheep heart[8] and was expressed in other vascular sites later in gestation.[9] Other recent studies have suggested that AQP1 promotes microvascular generation by accelerating cell migration in tumor models.[10]

Acetazolamide is a kind of sulfanilamide serving as carbonic anhydrase (CA) inhibitor, which has been found to inhibit osmosis-initiated water permeability of AQP1-cRNA injected oocyte.[11] Moreover, some other studies directly revealed that acetazolamide inhibits the expression of AQP1 protein, and also has the capability of repressing tumor angiogenesis and metastasis,[12] but the effects of acetazolamide on AQP1 protein expression and angiogenesis in a rabbit model of chronic myocardial ischemia has not been reported.

Since the primary function of AQP1 in the heart is unknown, we decided to study whether AQP1 promotes angiogenesis or other pathophysiological processes under conditions of ischemia, as observed in tumor. In the present study, we examine the influence of AQP1 on angiogenesis in a rabbit model of chronic myocardial ischemia.

Materials and methods

In situ coronary ligation model

We used a well-characterized rabbit model of regional cardiac ischemia injury.[13] All research protocols were approved by the Animal Care Committee of the University of Sichuan, China. Animals received humane care according to the Guide for the Care and Use of Laboratory Animals prepared by the Institute of Laboratory Animal Resources (National Research Council, USA, 1996). To generate the model, adult New Zealand White male rabbits weighing 2.5–3.0 kg were anesthetized with an intravenous bolus of sodium methohexital (10 mg/kg) to allow intubation with an orotracheal tube: a pediatric laryngoscope. Animals were endotracheally intubated (3 mm internal diameter, Aire-Cuff Veterinary endotracheal tube, Chenggong, Chengdu, China) and mechanically ventilated with 100% oxygen at a rate of 18–20 breaths per minute with a tidal volume of 45 ml using a small animal respirator (Rongke, Chengdu, China). Rabbits were ventilated with a positive-pressure respirator using room air supplemented with 100% oxygen. Anesthesia was maintained with sodium pentobarbital (35 mg/kg intravenous). Additional doses of pentobarbital were given during surgery as needed.

Under sterile conditions, a thoracotomy was performed through the fourth left intercostal space to expose the heart, and a 6-0 polypropylene suture was passed with tapered needle under the left anterior descending (LAD) coronary artery just below the tip of the left auricle. Myocardial infarction (MI) was produced by permanent LAD occlusion. The rabbits in the sham group underwent the same procedure except for the LAD ligation. Chest wall was closed in layers after surgical protocols were completed.

After application of buprenorphine (0.1 mg/kg subcutaneous), and weaning from the respirator, the rabbits were placed on a heating pad while recovering from anesthesia. A bipolar lead was sutured to the chest wall to record the ECG. Antibiotics were administered intramuscularly before surgery and for 2 days thereafter (gentamicin, 0.7 mg/kg once daily). All rabbits were allowed to recover on day 1 following surgery according to the design of the study.

Animal grouping, drug preparation, and treatment

The rabbits were divided into four groups of 30 animals each: (1) sham operation; (2) control MI (C-MI): rabbit underwent MI without acetazolamide treatment; (3) low-dose acetazolamide with MI (AT-MIa): rabbits underwent MI with a 150 mg/kg per day dose of acetazolamide; and (4) high-dose acetazolamide with MI (AT-MIb): rabbits underwent MI with a 300 mg/kg per day dose of acetazolamide. The chosen dosage of acetazolamide (Sigma, NO# A9842; Sigma, St. Louis, MO, USA) was determined by our preliminary experiment, and was administered intramuscularly beginning 12 h post surgery and maintained for a period of up to 21 days. The C-MI group received the same volume of 0.9% sterile sodium chloride solution in the same administration pattern.

Determination of infarct size

At 3, 7, 14, or 21 days post surgery, hearts were removed from the euthanized animals and placed on a Langendorff apparatus. Hearts were then arrested in diastole with the administration of 10% potassium chloride through the right carotid artery. The coronary arteries were perfused with a 0.9% normal saline solution containing 2.5 mM $CaCl_2$. Two milliliters of 10% Evans blue dye was injected into the aorta, then perfused with the saline solution to wash out excess dye. Hearts were then removed from the Langendorff apparatus and frozen. Four to six 1-mm thick sections were taken of the left ventricle from apex to base. The slices were incubated in 1% triphenyltetrazolium chloride (TTC) solution in isotonic phosphate buffer, pH 7.4, at 37°C for 20 m. The TTC reacts with reduced nicotinamide adenine dinucleotide (NADH) in the presence of dehydrogenase enzymes so that viable cells stain red. The myocardium at risk was determined by negative staining with Evans blue, and the infarcted myocardium was gray-white. Computer morphometry was used to calculate the left ventricular area, risk area, and infarct area. Infarct area was then expressed as a percentage of the risk area. Left ventricular cavity and papillary muscle area were excluded.

For preparation of tissue and immunohistochemical localization of AQP1 and CD31 On days 1, 3, 7, 14, or 21 after myocardial infarction, the animals were euthanized with an overdose of phenobarbital. Multiple tissue samples were taken from areas of infarction, areas at risk of infarction, and ischemia-free areas for histological examination to seek evidence of neovascularization. Based on prior studies,[14,15] we assumed that neovascularization of ischemic regions would proceed from the circumflex and nonoccluded LAD distributions and the tissue stimulus for neovascularization would be intense in tissue adjacent to the infarct zone. Samples were taken from the area at risk midway between the edges of the macroscopically infarcted myocardium and the junction of the LAD and circumflex territories (the "border-zone"). After fixation in 10% formalin, samples were embedded in paraffin. For each heart specimen, 6–7 slices of 4 μm thickness were taken at 2-mm intervals perpendicularly to the long axis of the heart.

These sections were immunohistochemically stained using an avidin-biotin-peroxidase complex (Dako, Carpinteria, CA, USA) to localize AQP1. After deparaffinization, the sections were immersed in 0.3% hydrogen peroxide in methanol for 30 min, then incubated overnight at 4°C with the primary antibody RSP53, rabbit polyclonal anti-AQP1 (BA0648, Boster, Wuhan, China). The slides were washed in phosphate-buffered saline (PBS) and incubated with the secondary antibody, biotinylated swine anti-rabbit immunoglobulin (Boster), for 1 h at room temperature. Finally, the slides were washed in PBS and incubated for 30 min with avidin-biotin-peroxidase complex, and the peroxidase color reaction was developed. For microscopic examina-

tion, the slides were counterstained with Harris hematoxylin. A similar dilution of rabbit IgG was used as the primary antibody for the negative control. Immunohistochemical reactions were considered positive if more than 15% nuclei were stained.

Capillary and arteriolar numerical densities

The determination of capillary and arteriolar numerical densities was carried out as described previously.[14] Endothelial cells were detected with anti-CD31 antibody (Boster). Vessels were identified as capillaries if vessel diameter was not exceeding 10 μm and presented no outer layers, and vessel lumen presented no more than one endothelial nucleus. Vessels were identified as arterioles if the vessel had one or two layers of smooth muscle and diameter was 19–50 μm.[14] Ten nonoverlapping fields within the border zone of the MI and in nonischemic myocardium of each animal from both AT-MI groups and the control MI group, as well as left ventricle myocardial tissue from a similar anatomic position of animals in the sham group, were analyzed at 100× magnification. The capillary density was determined as the mean number of capillaries per square millimeter.[16,17] Arteriolar density was similarly determined as the mean number of arterioles per square millimeter in ten nonoverlapping fields for each rabbit using 40× magnification.

Percentage of water content in left ventricle

The left ventricle from animals in all four groups (sham, C-MI, AT-MIa, and AT-MIb) was removed at 1, 3, 7, 14, or 21 days post surgery and analyzed for water content as previously described.[18] Briefly, the atria and great vessels were removed, and the left ventricle (including septum) and right ventricle were separated. Left ventricular water content was calculated as percentage difference between initial wet weight and dry weight after 24 h desiccation at 80°C.

Western blot analysis

Samples collected from remote myocardium (ischemia-free area) and infarct border of the MI animals as well as left ventricle of sham group animals were subjected to Western blot analysis to detect AQP1. Total protein was extracted using RIPA solution (50 mM Tris–HCl pH 7.4, 1% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM ethylenediamine tetraacetic acid, 1 mM phenylmethanesulfonyl fluoride, 1 μg/ml each of aprotinin, leupeptin, and pepstatin, 1 mM Na_3VO_4, 1 mM NaF). Thirty micrograms of each sample was subjected to 12% sodium dodecyl sulfate gel electrophoresis and transferred to polyvinylidene fluoride membrane (Roche, China). After blocking with PBS containing 0.1% Tween 20 (PBS-T) and 3% bovine serum albumin for 1 h at room temperature, the membrane was incubated with primary antibody for 1.5 h. After washing with PBS-T, the membrane was incubated with horseradish peroxidase-conjugated secondary antibody followed by further washing with PBS-T. Signals were detected using the ECL Western blot analysis system (Amersham Pharmacia, Piscataway, NJ, USA) and X-ray films.

Quantitative real-time reverse transcription–polymerase chain reaction (RT-PCR)

Myocardium samples were taken from the infarct border of animals in the control MI group and both AT-MI groups. Left ventricle myocardium of animals from the sham group was also collected as mock-control. Total RNA was extracted from each sample using Trizol according to the manufacturer's protocol (Labsystem, Vantaa, Finland). Two micrograms of DNaseI-treated total RNA was reverse-transcribed by Moloney murine leukemia virus reverse transcriptase enzyme in a 20 μl reaction mix containing 100 pmol oligo(dT) for 1 h at 42°C. Hepatitis B virus DNA and reverse-transcribed cDNA were quantified by real-time PCR using AQP1 primers and probe specific for the rabbit AQP1 gene: sense 5′-ACC GAC TAC CTC ATG AAG ATC CT-3′, antisense 5′-TCC TTG ATG TCC CGC ACG AT-3′; Taqman probe: 5′-(FAM) CGG CTA CAG CTT CAC CAC CAC GGC (Eclipse)-3′. Amplification and detection were performed with an ABI 7700 (Applied Biosystems, Foster City, CA, USA) sequence detection system. Briefly, real-time PCR was done in 50-μl volumes containing 2 μl DNA or reverse-transcribed cDNA template.

Values of cDNA were normalized to that of GAPDH, which was amplified with rabbit β-actin-specific primers and probe: sense 5′-GAT CGA CTA CAC TGG CTG TGG-3′; antisense 5′-GGT TGT TGA AGT TGT GGG TGA G-3′. Taqman probe: 5′-(FAM) ACC GCC GAG CCG AAG GAC CGA (Eclipse)-3′. The PCR conditions were as follows: 50°C for 2 min; 95°C for 10 min; 40 cycles of 95°C for 15 s, 58°C for 1 min; and 72°C for 5 min. Three independent experiments were performed on each sample and the amount of AQP1 in the myocardium sample was expressed as a percentage of the mock-treated controls. Results were analyzed with SDS 2.0 software (Applied Biosystems).

Statistical analysis

Results are expressed as mean ± standard error of the mean (SEM). Differences between groups were tested for statistical significance by two-way analysis of variance (ANOVA) and Student's *t*-test ($P < 0.05$).

Results

Immunohistochemical detection of AQP1 in myocardium

Anti-AQP1 antibody staining of a sample of nonischemic myocardium showed that AQP1 was expressed in endothelial cells of artery, vein, and capillary. In myocytes, AQP1 was mainly expressed on the cell membrane and showed

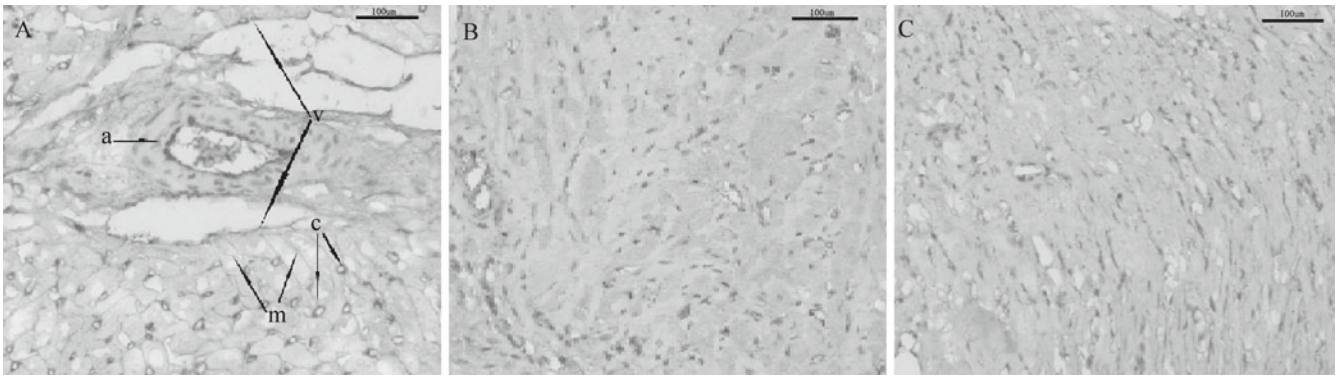


Fig. 1A–C. Aquaporin-1 (AQP-1) immunohistochemical staining characteristics in representative samples of myocardium (×100). AQP1 protein (brown) stained using peroxidase method. **A** Localization of AQP1 in nonischemic myocardium shows AQP1 in capillary (*c*), small artery (*a*), vein (*v*), and myocytes (*m*). **B** Localization of AQP1 in infarct border tissue 3 days post surgery shows AQP1 in endothelial cells of blood vessels including capillary. **C** Necrotic myocardium at 21 days post myocardial infarction shows AQP1 scattered irregularly throughout the tissue sample

lighter staining compared to the heavy staining in endothelium of vessels (Fig. 1A). In comparison, ischemic tissue showed AQP1 primarily in endothelial cells of blood vessels including capillary (Fig 1B). At 21 days post MI, extensive collagen deposition had occurred in the central infarction region and very little AQP1 expression was observed in this type of tissue. As the result of myocyte necrosis, AQP1 was scattered irregularly throughout the tissue sample (Fig. 1C).

Ischemia-induced elevation of AQP1 expression is tempered by acetazolamide treatment

Infarct border myocardium samples collected from animals that underwent control MI (C-MI), low-dose acetazolamide treatment with MI (AT-MIa), and high-dose acetazolamide treatment with MI (AT-MIb) all show positive immunohistochemical staining against AQP1, with weaker staining observed in acetazolamide-treated groups (Fig. 2A). Staining differences became obvious on day 3 post MI. A similar immunohistochemical staining pattern was observed even in nonischemic tissue in the acetazolamide-treated groups (Fig. 2B).

Expression of AQP1 relative to β-actin was characterized using densitometry of Western blots. Infarct border and nonischemic tissue from C-MI, AT-MIa, and AT-MIb groups, and nonischemic tissue from sham-operated groups were analyzed. Aquaporin-1 protein levels in all infarct border tissue samples were similar to that found in nonischemic tissue from the sham group on day 1 post surgery. Beginning on day 3 post surgery, an increase ($P < 0.05$) in AQP1 protein levels compared to sham was observed in tissues from all MI groups. The response in C-MI was greatest ($P < 0.05$), and increased AQP1 levels were maintained through day 21 post surgery. In AT-MIa and AT-MIb groups the response was not as great, and increased AQP1 protein levels were maintained through days 14 and 7 post surgery, respectively (Fig. 3A).

The effect of acetazolamide treatment on AQP1 levels was also seen in nonischemic tissue samples. In this case, AQP1 protein levels were similar across all four groups at day 1 post surgery (Fig 3B) and remained relatively constant through day 21 post surgery in sham and C-MI groups. Nonischemic tissue samples from the acetazolamide-treated groups, however, showed a dose-dependent decrease ($P < 0.05$) in AQP1 protein levels starting at day 3 post surgery, and continued to drop through day 21 (Fig. 3B).

This phenomenon was also observed in mRNA levels as measured using quantitative real-time PCR. Total RNA from infarct border tissue from C-MI, AT-MIa, and AT-MIb groups, and from nonischemic tissue from sham-operated groups were analyzed. Aquaporin-1 mRNA levels were significantly ($P < 0.05$) increased in infarct border tissue from the C-MI group compared to nonischemic tissue from the sham-operated group between days 3 and 21 post surgery, peaking around day 7, then dropping through day 21. A similar, albeit lower, response was seen in the acetazolamide-treated groups in a dose-dependent manner (Fig. 4).

Downregulation of AQP1 expression was related to reduced myocardial water content and capillary density and increased ratio of infarct size : area at risk

Myocardial water content was computed by comparing wet and dry weights of left ventricular nonischemic myocardium collected from animals in all four groups (sham, C-MI, AT-MIa, and AT-MIb) on days 1, 3, 7, 14, and 21 post surgery. Among the hearts that underwent coronary ligation, water content in myocardium of C-MI hearts was greater than that in acetazolamide-treated hearts ($P < 0.05$) in a dose-dependent manner (Fig. 5).

Capillary counting was performed in tissue from the infarct border region from all coronary ligation groups, and in left ventricle myocardial tissue in a similar anatomic position from sham operation group. Deparaffinized tissue

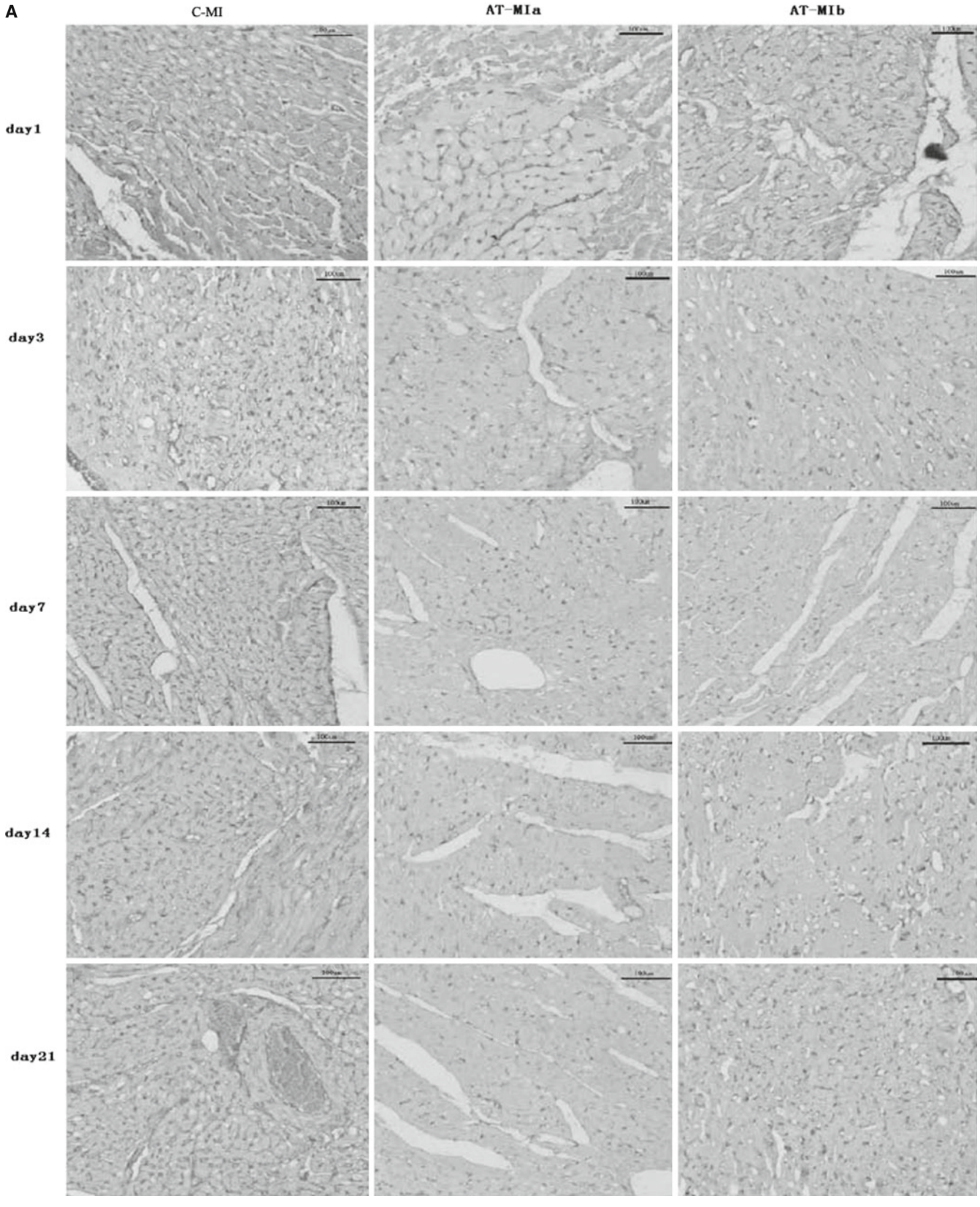
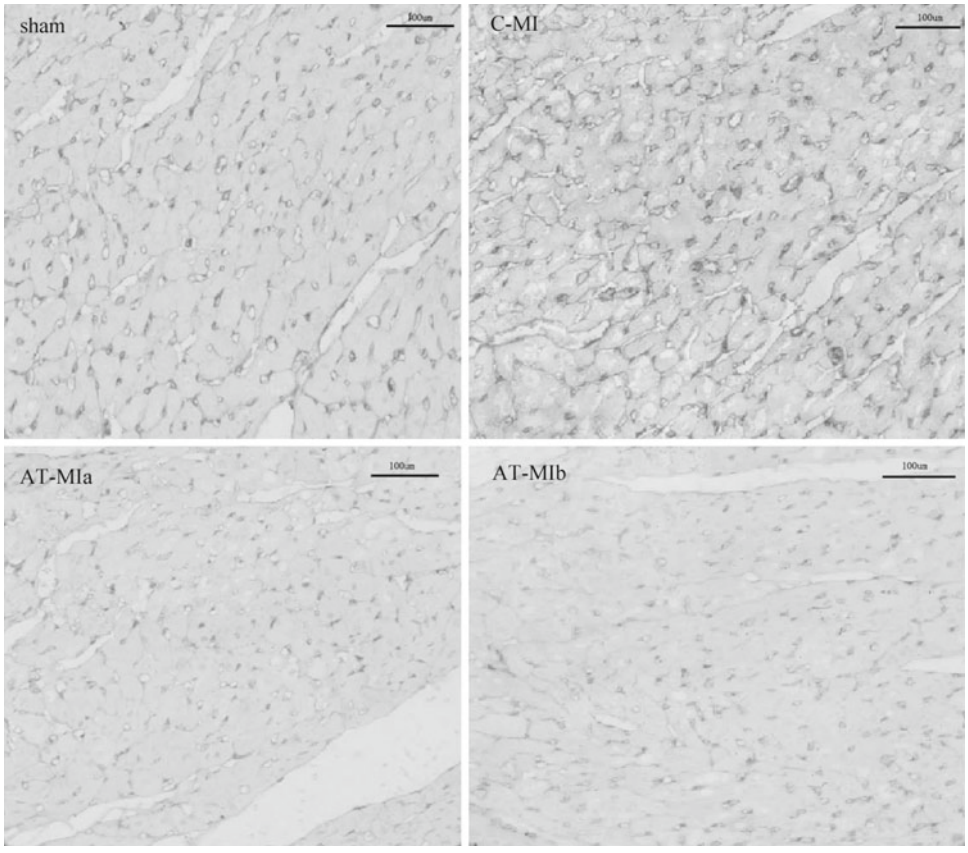


Fig. 2A,B. AQP1 expression in myocardium tissue in response to MI and acetazolamide treatment shown by immunohistochemical staining (×100). **A** Infarct border myocardium from control MI (*C-MI*), low-dose acetazolamide with MI (*AT-MIa*), and high-dose acetazolamide with MI (*AT-MIb*) groups collected on days 1, 3, 7, 14, and 21 post surgery shows weaker AQP1 staining in acetazolamide-treated tissue starting on day 3 post surgery. **B** Nonischemic myocardium collected on day 21 post surgery from sham, C-MI, AT-MIa, and AT-MIb groups showing weaker staining in the acetazolamide-treated tissue samples

Fig. 2. *Continued*



slices were stained with anti-CD31 antibody and examined under optical microscope at 100× magnification. The AT-MI groups showed a statistically significant ($P < 0.05$) decrease in capillary density in a dose-dependent manner compared to the C-MI group, starting on day 7 and continuing through day 21 post surgery (Fig. 6).

The effect of acetazolamide treatment on myocardial angiogenesis was further evaluated by comparing the ratios of infarction area to risk area from hearts in the C-MI group to hearts in the AT-MI groups. Harvested hearts from animals that underwent coronary ligation were perfused with Evans blue solution to determine the total area at risk of infarction. Staining of myocardium slices by TTC was used to demarcate viable cells. Computer morphometry was used to calculate the ratio of infarct size to total risk area in hearts collected on days 3, 7, 14, and 21 post surgery. The acetazolamide-treated hearts had a statistically significantly higher infarct:risk area ratio ($P < 0.05$) than C-MI hearts collected on day 7 through day 21 post surgery (Fig. 7).

Reduced capillary density in acetazolamide treated myocardial tissue was accompanied by intact temporal pattern of vascular endothelial growth factor expression

Vascular endothelial growth factor (VEGF) protein level relative to β-actin was determined by Western blot in infarct border tissue samples collected from all coronary ligation groups (Fig. 8). At each time point, there was no significant difference in VEGF protein levels between low-dose acetazolamide, high-dose acetazolamide, or untreated MI myocardium ($P > 0.05$).

Discussion

Several recent studies have provided evidence that AQP1 is an intrinsic osmolarity-driven water channel that may have a role in tumor angiogenesis,[10,19] but no studies have investigated the expression pattern of AQP1 under conditions of chronic ischemia. In the present study, we observed expression of AQP1 in ischemic heart tissue of rabbits for 21 days post infarction. Immunohistochemistry studies showed that AQP1 was highly expressed in myocytes and endothelial cells of blood vessels in myocardium, and that expression levels of AQP1 mRNA and protein were significantly increased in infarct border tissue starting on day 3 post surgery, reached a peak on day 7, then fell through day 21 to basal levels. Interestingly, this temporal pattern of AQP1 expression correlates with the pattern of myocardial angiogenesis post ischemia observed in prior studies.[20–22] Furthermore, we demonstrated that acetazolamide treatment resulted in downregulation of AQP1 mRNA and protein expression, which was accompanied by impaired neovascularization. These results suggest that AQP1 is upregulated in postischemic myocardium and positively influences angiogenesis.

The role of AQP1 in angiogenesis has been investigated in several recent studies. One such study suggested that

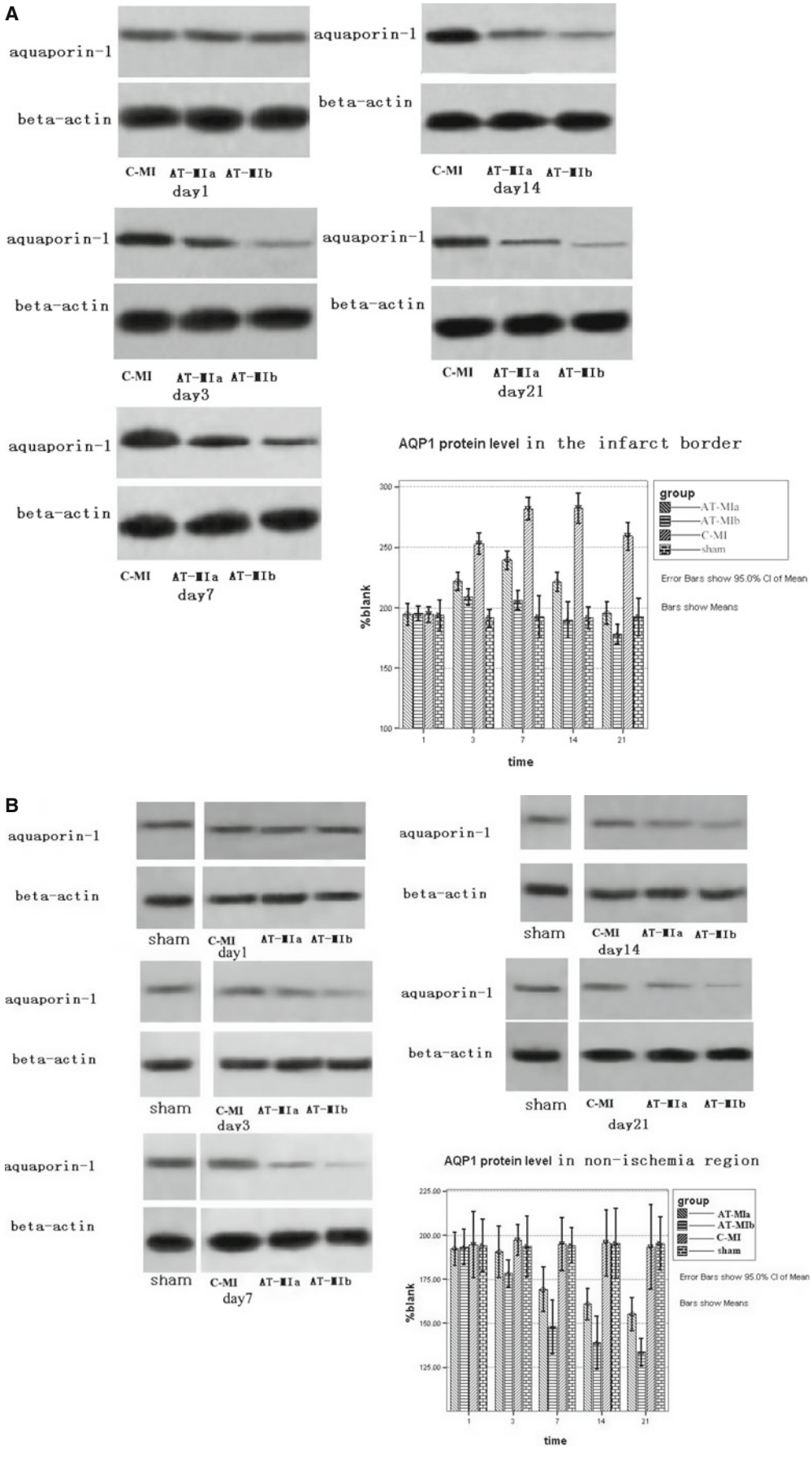


Fig. 3A,B. Western blot of infarct border and nonischemic tissue from control MI (*C-MI*), low-dose acetazolamide with MI (*AT-MIa*), and high-dose acetazolamide with MI (*AT-MIb*) groups, and nonischemic tissue from sham-operated groups showing AQP1 protein levels relative to β-actin. **A** Representative blot from infarct border tissue collected from all coronary ligation groups on days 1, 3, 7, 14, and 21 post surgery. *Bar graph* shows AQP1 protein level relative to β-actin as determined by densitometry. AQP1 protein levels increased on day 3 post surgery for all MI groups compared to sham and was greatest in C-MI. A lower, dose-dependent response was seen in acetazolamide-treated groups. **B** Representative blot from nonischemic tissue collected from all groups on days 1, 3, 7, 14, and 21 post surgery. *Bar graph* shows AQP1 protein level relative to β-actin as determined by densitometry. AQP1 protein levels remained relatively constant for sham and C-MI groups through day 21 post surgery. AQP1 protein levels dropped in a dose-dependent manner in the acetazolamide-treated groups

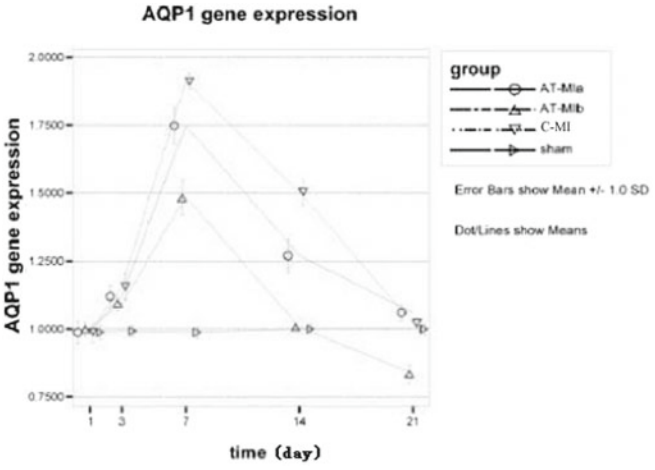
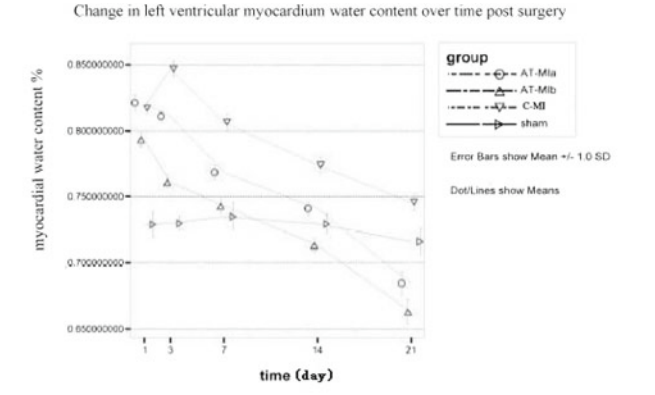


Fig. 4. AQP1 mRNA levels in infarct border tissue from control MI (*C-MI*), low-dose acetazolamide with MI (*AT-MIa*), and high-dose acetazolamide with MI (*AT-MIb*) groups, and nonischemic tissue from sham-operated groups was determined using quantitative real-time polymerase chain reaction in samples collected on days 1, 3, 7, 14, and 21 post surgery. AQP1 gene expression increased in all coronary ligation groups compared to sham, starting on day 3 post surgery. Acetazolamide treatment inhibited the expression of AQP1 in a dose-dependent manner



Water content in left ventricular myocardium measured at various days post surgery(Mean +/- SD)

	Sham	C-MI	AT-MIa	AT-MIb
1day	0.7297±0.0028	0.8170±0.0021	0.8212±0.0028	0.7933±0.0026
3day	0.7348±0.0049	0.8469±0.0027	0.8111±0.0019	0.7607±0.0019
7day	0.7294±0.0034	0.8065±0.0029	0.7682±0.0027	0.7430±0.0029
14day	0.7160±0.0047	0.7736±0.0029	0.7410±0.0025	0.7131±0.0026
21day	0.7290±0.0046	0.7456±0.0031	0.6845±0.0043	0.6628±0.0044

Fig. 5. Mean unit (mg) water content in left ventricular myocardium over time post surgery. Water content in left ventricular myocardium was measured on days 1, 3, 7, 14, and 21 post surgery. Water content was significantly reduced in a dose-dependent manner in acetazolamide-treated hearts compared to C-MI hearts ($P < 0.05$)

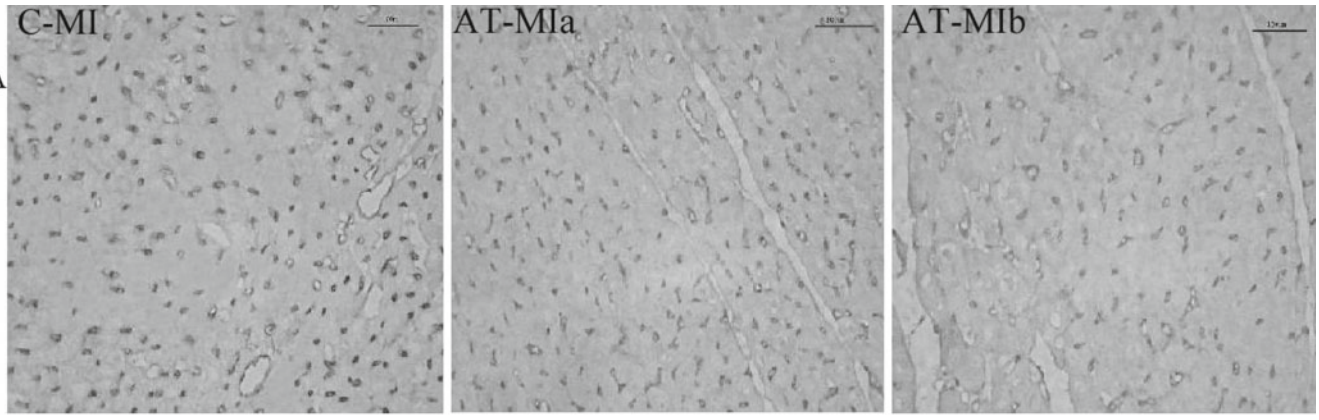
AQP1 promotes microvascular generation by accelerating cell migration in tumor models.[10] Similarly, silence of AQP1 has been shown to inhibit angiogenesis in vivo and in vitro.[23,24] In this study, we observed increased angiogenesis in response to increased AQP1 expression under conditions of chronic ischemia. This response was tempered by acetazolamide in a dose-dependent manner. Taken together, these results suggest that acetazolamide may not be directly associated with decreased angiogenesis, but may influence the expression of AQP1 through an unknown mechanism under ischemic conditions. Further exploration of the effect of acetazolamide on AQP1 expression under ischemic conditions is warranted. Our results indicated that acetazolamide impaired angiogenesis and decreased myocardial water content by downregulation of APQ1 protein expression, which might be similar with those by use of an AQP1 knockout animal or in culture with siRNA against AQP1. However, as acetazolamide had no effect on the mRNA expression of AQP1, the loss of AQP1 activity might be related to its degradation and/or inhibition induced by acetazolamide.

The role of AQP1 in water transport has been well characterized. Under physiological conditions, AQPs represent a major route of water transport in guinea pig and rat heart cells.[25] Under conditions of ischemia, however, we have little information about whether APQ1 plays a predominant role in handling water movement. Furthermore, lens epithelial water permeability was decreased in aquaporin-1-deficient mice.[26] Acetazolamide also has an affect on AQP1-mediated water transport, as studies have shown that acetazolamide treatment results in inhibition of osmosis-initiated water permeability in oocytes overexpressing AQP1.[11,27] Our study of infarcted rabbit myocardium demonstrated a sharp increase in water content to the peri-infarction region. It is likely that APQ1 plays a predominant role in handling water movement when there is an inwardly directed osmotic gradient as a result of cellular events such as ischemia, in which accumulation of lactate osmotically draws water into the cell. Our observation that acetazolamide influenced downregulation of AQP1 expression concomitant with decreased water content and angiogenesis in infarct border myocardium correlates with recent data that AQP1 deprivation resulted in impaired endothelial migration and subsequent decreased angiogenesis.[10,28] In addition, the observed decrease in water content in acetazolamide-treated myocardium might be related to reduction of the microvascular angiogenesis caused by downregulation of AQP1 expression.

Vascular endothelial growth factor expression increases in response to ischemia, resulting in angiogenesis.[29–31] Under certain conditions, however, neovascularization occurs without VEGF.[32,33] Aquaporin-1-induced angiogenesis, in fact, may be independent of the VEGF signaling pathway.[24] Our experiment demonstrated that while downregulation of AQP1 in the presence of acetazolamide led to impaired angiogenesis in ischemic tissue, there was no change in VEGF expression levels. Furthermore, VEGF expression levels over 21 days post surgery in infarct border tissue was similar in the C-MI and the acetazolamide-treated MI groups. These observations support the hypothesis that AQP1 acts through a mechanism that is independent of the VEGF signaling pathway.

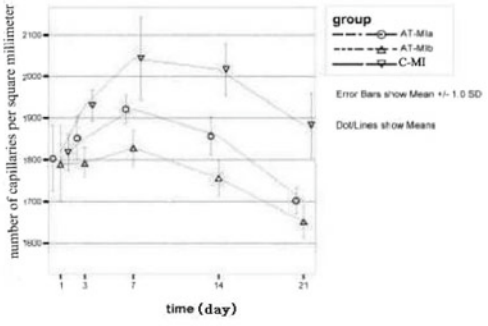
Our results offer new insights into the temporal fluctuation and angiogenic effect of AQP1 expression in myocardium under conditions of chronic ischemia. We also show that downregulation of AQP1 expression by acetazolamide

A



B

Capillary density in acetazolamide treated and untreated infarct border tissue from coronary ligated hearts at various days post surgery



Mean capillary density in infarct border tissue from sham operated and coronary ligated hearts at various times post surgery

	Sham	C-MI	AT-MIa	AT-MIb
1day	1788.7±25.9	1818.4±13.2	1802.9±23.6	1790.4±26.6
3day	1757±24.7	1930.0±11.1	1852.8±15.4	1792.2±11.2
7day	1765±33.2	2043.0±30.3	1920.6±10.1	1829.4±12.6
14day	1755.3±23.8	2016.2±18.8	1857.3±13.5	1756.5±12.5
21day	1752.5±21.8	1882.4±23.1	1701.1±9.5	1651.1±11.6

C

Mean number of capillariy density in nonischemic heart tissue

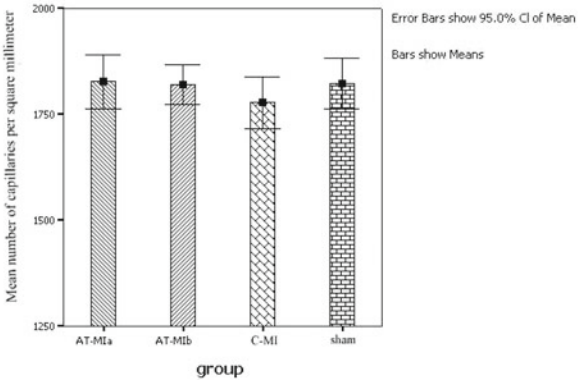


Fig. 6A–C. Capillary density calculations show reduced angiogenesis in infarct border myocardial tissue of acetazolamide-treated hearts compared to C-MI hearts. **A** Anti-CD31 staining of infarct border tissue was used to count number of capillaries/mm^2 (capillary density) in samples from sham-operated and acetazolamide-treated or untreated coronary ligation hearts on days 1, 3, 7, 14, and 21 post surgery (×100). **B** Tabulation and graphical representation of the data. Capillary density in infarct border tissue samples was highest for C-MI heart tissue and decreased in a dose-dependent manner in the acetazolamide treated heart tissue. **C** Capillary density in nonischemic tissue was similar across all groups

treatment results in impaired angiogenesis, further confirming the role of AQP1 in angiogenesis. Aquaporin-1, therefore, may be a potential target site of therapeutic angiogenesis under conditions of myocardial ischemia.

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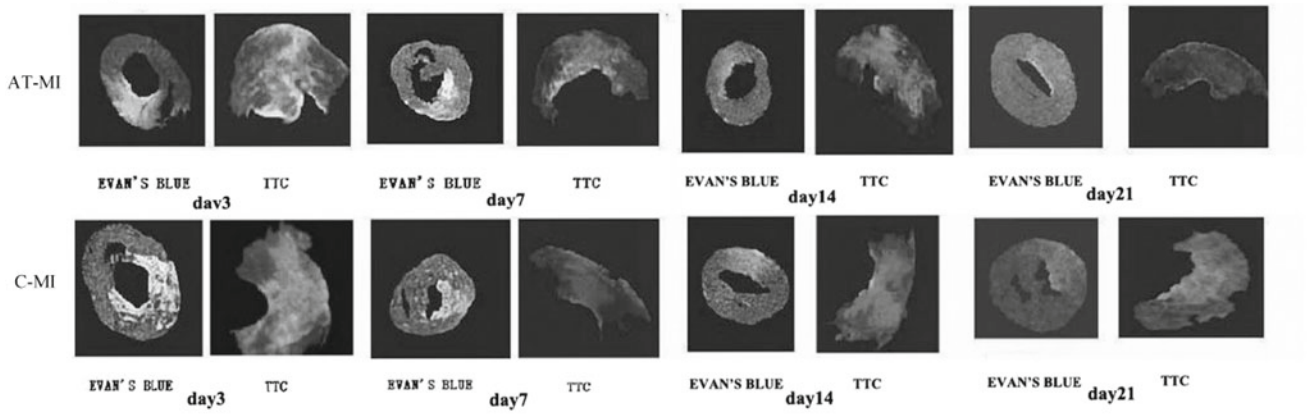
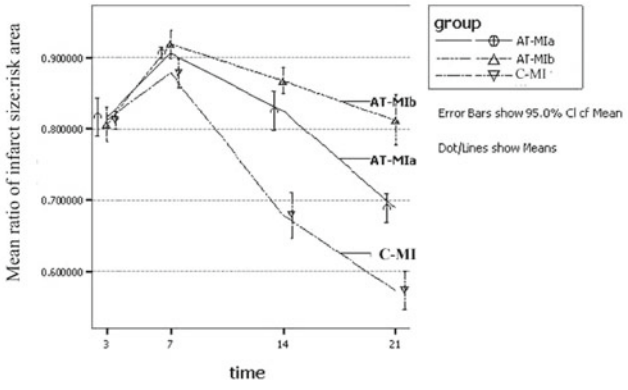
A. The slices of left ventricular tissue with(AT-MI) or without acetazolamide treatment on(C-MI) 3, 7, 14, and 21 days post surgery were stained with TTC and perfused with Evan's blue solution



B. Ratio of infarct size:risk area in acetazolamide treated or untreated coronary ligated hearts at various days post surgery (mean +/- SD)



	C-MI	AT-MIa	AT-MIb
3day	0.8122±0.0039	0.8163±0.0084	0.8059±0.0076
7days	0.8789±0.0061	0.9061±0.0024	0.9187±0.0060
14days	0.6784±0.0095	0.8254±0.0083	0.8676±0.0054
21days	0.5735±0.0079	0.6882±0.0060	0.8120±0.0108

Fig. 7A,B. Measurement of infarct size in acetazolamide-treated and untreated coronary ligated hearts. **A** Hearts harvested from animals that underwent coronary ligation with (*AT-MI*) or without acetazolamide treatment (*C-MI*) on days 3, 7, 14, and 21 post surgery were perfused with Evans blue solution, and slices of left ventricular tissue were stained with triphenyltetrazolium chloride (*TTC*), then subjected to computer morphometry analysis to calculate ratio of infarct size to total risk area. **B** Tabulation and graphical representation of the data. Ratios were higher in acetazolamide-treated hearts compared to C-MI hearts in a dose-dependent manner

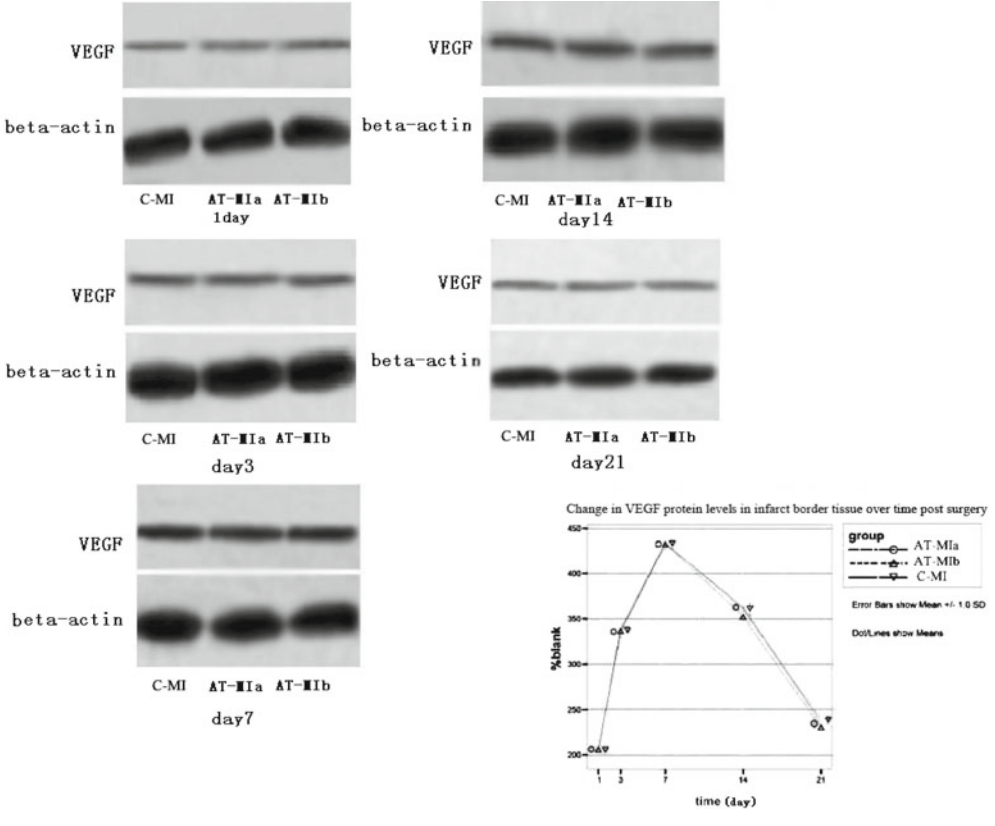


Fig. 8. Representative western blots of infarct border tissue from control MI (*C-MI*), low-dose acetazolamide with MI (*AT-MIa*), and high-dose acetazolamide with MI (*AT-MIb*) groups collected on days 1, 3, 7, 14, and 21 post surgery, showing vascular endothelial growth factor (*VEGF*) protein levels relative to β-actin. Expression levels were determined by densitometry and plotted. There was no significant difference in VEGF protein levels between all coronary ligation groups

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