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Original article

Aquaporin-1 in cardiac endothelial cells is downregulated in ischemia, hypoxia and cardioplegia

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ABSTRACT

Aquaporin-1 (AQP1) is expressed in human and mouse hearts, but little is known about its cellular and subcellular localization and regulation. The aim of this study was to investigate the localization of AQP1 in the mouse heart and to determine the effects of ischemia and hypoxia on its expression. Mouse myocardial cells were freshly isolated and split into cardiomyocyte and non-cardiomyocyte fractions. Isolated, Langendorff-perfused C57Bl6 mouse hearts (n = 46) were harvested with no intervention, subjected to 35 min of ischemia or ischemia followed by 60 min of reperfusion. Eleven mouse hearts were perfusion-fixed for electron microscopy. Forty C57Bl6 mice were exposed to normobaric hypoxia for one or two weeks (n = 12). Needle biopsies of human left ventricular myocardium were sampled (n = 30) during coronary artery bypass surgery before cardioplegia and after 30 min of reperfusion. Human umbilical vein endothelial cells (HUVECs) were subjected to 4 h of hypoxia with reoxygenation for either 4 or 24 h. AQP1 expression was studied by electron microscopy with immunogold labeling, Western blot, and qPCR. Expression of miR-214 and miR-320 in HUVECs with hypoxia was studied with qPCR. HUVECs were then transfected with precursors and inhibitors of miR-214. AQP1 expression was confined to cardiac endothelial cells, with no signal in cardiomyocytes or cardiac fibroblasts. Immunogold electron microscopy showed AQP1 expression in endothelial caveolae with equal distribution along the basal and apical membranes. Ischemia and reperfusion tended to decrease AQP1 mRNA expression in mouse hearts by 37 ± 9% ($p = 0.06$), while glycosylated AQP1 protein was reduced by 16 ± 9% ($p = 0.03$). No difference in expression was found between ischemia alone and ischemia-reperfusion. In human left ventricles AQP1 mRNA expression was reduced following cardioplegia and reperfusion ($p = 0.008$). Hypoxia in mice reduced AQP1 mRNA expression by 20 ± 7% ($p < 0.0001$), as well as both glycosylated (−47 ± 10%, $p = 0.03$) and glycan-free protein (−34 ± 16%, $p = 0.05$). Hypoxia and reoxygenation in HUVECs downregulated glycan-free AQP1 protein (−34 ± 24%, $p = 0.04$) and upregulated miR-214 (+287 ± 52%, $p < 0.05$). HUVECs transfected with anti-miR-214 had increased glycosylated (1.5 fold) and glycan-free (2 fold) AQP1. AQP1 in mouse hearts is localized to endothelial cell membranes and caveolae. Cardioplegia, ischemia and hypoxia decrease AQP1 mRNA as well as total protein expression and glycosylation, possibly regulated by miR-214.

1. Introduction

Of the 13 aquaporin (AQP) subtypes that have been identified in mammals, AQP1 was the first to be described [1]. AQP1 is expressed in many tissues, the primary function being transmembrane water flux. AQP1 knockout mice have impaired urine concentration [2] and reduced water movement across the peritoneum [3] and lung microvessels [4]. In the brain AQP1 participates in generation of cerebrospinal fluid [5].

Both mouse and human hearts express AQP1, but its cellular and subcellular localization is controversial [6–8]. AQP1 mRNA is observed in the fetal mouse heart in endocardial cushions, primordial valves and

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septae, and its level decreases after birth [9]. Expression of AQP1 in adult mammalian hearts has not been clearly established. Endothelial cells, cardiomyocytes, smooth muscle cells and fibroblasts have all been reported to express AQP1. Some differences in expression pattern and functional importance of AQP1 were found between mice, guinea pigs, rabbits and humans [10–12].

The site of AQP1 expression may be an important determinant of its role in myocardial water balance in health and disease. Myocardial ischemia leads to a pronounced tissue edema [13,14] predominantly of cytotoxic nature, secondary to lactate hyper-production and calcium influx [15] combined with increased membrane fragility, which leads to increased cell osmolarity and water influx. Myocardial edema impairs systolic and diastolic function [16] and causes additional ischemia of cardiomyocytes due to an increased diffusion distance for oxygen [17]. If AQP1 is expressed in cardiomyocytes, it may facilitate water accumulation and clearance from these cells. If it is found in endothelial cells, it may mediate water flux between the vasculature and the interstitium. One purpose of the current study was to investigate the precise cellular and subcellular localization of AQP1 in the mouse heart.

In a number of tissues AQP1 is transcriptionally regulated during ischemia. Expression of AQP1 is induced by ischemia in the brain [18] and in retinal cells [19], but it is decreased in the ischemic kidney [20,21]. The regulation of AQP1 expression in the heart is not well characterized. In sheep, cardiopulmonary bypass with cardioplegia [14] or anemia [22] increased mRNA of AQP1. Ischemia had no effect on AQP1 mRNA expression in rat hearts [10]. Another main purpose of the present investigation was to study the effects of ischemia alone, ischemia and reperfusion, cardioplegia, and hypoxia on AQP1 expression in the heart. Such regulation may be of major pathophysiological relevance, as it modifies myocardial water balance in acute and chronic settings and thus may influence function and metabolism as well as the outcome of ischemic and hypoxic injury of the heart.

MicroRNAs are small non-canonical endogenous inhibitors of gene expression, operating with high specificity to block protein translation. Inhibition begins with sequence-specific interaction of the microRNA molecule with target messenger RNA [23]. MicroRNAs have been recognized as major regulators of protein expression in hypoxia and ischemia, and their effect on aquaporin expression has been suggested as well [24]. We therefore aimed at investigating whether such regulation may be relevant for AQP1.

2. Materials and methods

2.1. Ethical standards

All animal experiments conform to the guidelines for the use and care of laboratory animals ("Principles of laboratory animal care", NIH publication No. 86-23. Revised 1996) and the study was approved by the Norwegian Animal Health Authority. C57Bl6 male mice weighing 25 ± 3 g (Scanbur AS, Oslo) were used. All animals had a conventional microbiological status. They had free access to food (RM3 from Scanbur BK AS, Norway) and water and were kept at a 12 hour light/12 hour dark cycle in rooms where the temperature was set to 23 °C and humidity to 55–60%. All animals were acclimatized for at least five days before the experiments.

Needle biopsies of the left ventricle were obtained from patients undergoing coronary artery bypass surgery with cardiopulmonary bypass and cardioplegia. The study was designed according to the ethical standards laid down in the 1964 Declaration of Helsinki, and was accepted by the ethics committee of Tampere University Hospital, Finland. Informed consent was obtained from all patients.

Human umbilical vein endothelial cells (HUVEC) were isolated from umbilical cords supplied by the department of Obstetrics, Oslo University Hospital-Rikshospitalet, Oslo. Ethical approval was obtained from the Regional Human Research Ethical Committee.

2.2. Isolation of cardiomyocytes

Cardiomyocytes were isolated from five C57Bl6 mouse hearts and cultured following the modified procedure described by O'Connell et al. [25]. Briefly, the hearts were excised and mounted on a Langendorff apparatus and perfused briefly with Krebs–Henseleit buffer (KHB) (mmol/l: NaCl 118.5; $NaHCO_3$ 25; KCl 4.7; KH_2PO_4 1.2; $MgSO_4 * 7H_2O$ 1.2; glucose $* 1H_2O$ 11.1; $CaCl_2$ 2.4) to wash away the blood. Digestion of extracellular matrix was performed with the use of Collagenase II (Worthington Biochemical, Lakewood, NJ). Digested myocardial tissue was cut from the perfusion apparatus and mechanically disrupted. Cardiomyocytes were separated from the rest of the cells by differential centrifugation followed by stepwise reintroduction of calcium, and grown in laminin-coated culture plates. The remaining cells were grown in non-coated plates. All the cells were incubated for 4 h at 37 °C and harvested using a cell scraper. To assess the cellular content of the two populations, RNA was isolated from the cells as described below, and qPCR was conducted with primers specific for the different myocardial cell types: cardiomyocytes — troponin I; fibroblasts — vimentin, endothelial cells — cadherin, smooth muscle cells — smooth muscle actin. Primer sequences are presented in Table 1. The cardiomyocyte fraction contained predominantly cardiomyocytes with some smooth muscle cells, while the remaining fraction was positive only for the markers of fibroblasts and endothelial cells.

2.3. Human cardiac fibroblasts

Primary human adult ventricular fibroblasts were purchased from 3H Biomedical, Uppsala, Sweden, Cat.# SC6310. The cells were thawed from the cryopreserved stock (passage 5) in liquid nitrogen and plated in cell flasks coated with Poly-L-Lysine (Cat.# SC04113, 3H Biomedical) using the fibroblast medium with supplements (Cat.# SC2311, 3H Biomedical). The cells were grown at 37 °C in 5% CO_2. Upon reaching approximately 80% confluence the cells were harvested for Western blot analysis for AQP1 by washing twice with PBS and adding the lysis buffer directly into the cell flask.

2.4. Human umbilical vein endothelial cells (HUVECs)

Harvesting of HUVECs was performed as described by Jaffe et al. [26]. HUVECs were grown in MDCB-131 medium (Cat.# M8537, Sigma-Aldrich) with 7% fetal calf serum, epidermal growth factor (10 ng/ml), fibroblast growth factor (1 ng/ml), hydrocortisone (1 μg/ml), gentamicin (50 μg/ml), and fungizone (0.25 μg/ml). The cells were routinely grown to 80% confluence at 37 °C in 5% CO_2, and cells from passage 5 were used in all experiments. For Western blot analysis of AQP1 the cells were washed twice with PBS and treated with the cell lysis buffer.

Table 1
Primers used for real-time qPCR. The primers were designed to span the exon-exon junctions to be mRNA-specific. SYBR green was used where probe is not listed.

Specificity	Direction	Sequence
AQP1, human. Taqman	Forward	TTGGCTCCGCGGTGAT
	Reverse	GGCTCCCCCGATGAATG
	Probe	ACTTCAGCAACCACTGGATTTTCTGGGTG
AQP1, mouse. Taqman	Forward	CACCTCCTCCCTAGTCGACAATT
	Reverse	TACCAGCTGCAGAGTGCCAAT
	Probe	CTTGGCCGCAATGACCTGGCTCAC
Acta2	Forward	TCCTGACGCTGAAGTATCCGATA
(Mus musculus actin, alpha2)	Reverse	GGTGCCAGATCTTTTCCATGTC
Cdh5	Forward	TGGCCAAAGACCCTGACAAG
(Mus musculus cadherin)	Reverse	TCGGAAGAATTGGCCTCTGT
Tnni3	Forward	CAGATGGAACGAGAGGCAGAA
(Mus musculus troponin I)	Reverse	CGGCATAAGTCCTGAAGCTCTT
Vim	Forward	CCCTGAACCTGAGAGAAACTAACC
(Mus musculus vimentin)	Reverse	GTCTCATTGATCACCTGTCCATCT