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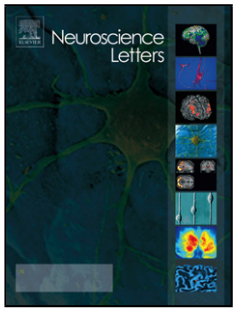

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Acetazolamide Lowers Intracranial Pressure and Modulates the Cerebrospinal Fluid Secretion Pathway in Healthy Rats

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Highlights

- Acetazolamide lowers intracranial pressure in healthy rats
- Acetazolamide inhibits the activity of the Na/K ATPase
- Acetazolamide increase aquaporin 1 in the membrane of choroid plexus
- Acetazolamide has a dual action in choroid plexus affecting both ion transport and protein expression

Abstract

Acetazolamide is one of the most widely used drugs for lowering intracranial pressure (ICP) and is believed to reduce cerebrospinal fluid (CSF) secretion via its action on the choroid plexus (CP). In the CP the main driving force for CSF secretion is primarily active transport of Na^+ ions facilitated by the Na/K ATPase. Transmembrane water channels, known as aquaporins (AQP), are also present in the CP and play an important role in the movement of water. In the present study, we investigated the effect of a single dose acetazolamide on the activity of the Na/K ATPase and ICP. Furthermore, we investigated the expression of Na/K ATPase, AQP1 and AQP4 in the CP tissue following acetazolamide treatment.

12 female Sprague Dawley rats were randomized into two groups; one group received 200 mg acetazolamide and the other vehicle treatment. All animals were subjected to ICP recordings and the CP tissue was collected for qPCR and western blot analysis. The effect of acetazolamide on the Na/K ATPase activity was evaluated in an in vitro assay of primary CP epithelial cells isolated from rats.

Acetazolamide significantly lowered ICP within 10 minutes of injection compared to the vehicle group ($P<0.05$), reaching a maximum reduction at 55 minutes 66 ± 4% ($P<0.00001$). Acetazolamide also significantly decreased the activity of the Na/K ATPase in CP epithelial cells compared to vehicle ($P=0.0022$).

Acetazolamide did not change the AQP1, AQP4 or Na/K ATPase mRNA content in the CP tissue. However, we did record an increase in the amount of AQP1 ($p=0.0152$) and Na/K ATPase ($p=0.0411$) protein in the membrane fraction of the CP, but not AQP4 ($p=0.0649$).

A single dose of acetazolamide lowers ICP and modulates the CSF secretion pathway in healthy rats - Firstly, by inhibiting the Na/K ATPase to slow the CSF production, secondly, by increasing AQP1 and Na/K ATPase protein in the membrane of the CP epithelial cells.

Introduction

Acetazolamide is one of the most widely used drugs for lowering intracranial pressure (ICP) in disabling conditions like idiopathic intracranial hypertension (IIH)[24]. The high ICP associated with IIH often leads to chronic severe headaches, visual disturbances and papilloedema[9, 14, 15], which causes permanent blindness in 20% of cases[3, 23]. Currently, management of IIH aims primarily to avoid visual damage by reducing ICP. Acetazolamide is a carbonic anhydrase inhibitor that is believed to reduce cerebrospinal fluid (CSF) secretion[2, 7, 25] and ICP[11, 12] via its action on the choroid plexus (CP). The largest clinical study evaluating the efficacy of acetazolamide on ICP in IIH patients demonstrated a beneficial effect on ICP compared to placebo[12], whilst another randomized controlled trial of acetazolamide highlighted a very high drug withdrawal rate due to side effects[4]. The most commonly reported side effects of acetazolamide by IIH patient are paresthesia, nausea, and fatigue[24]. Currently, a detailed molecular mechanism of action of acetazolamide in CP has yet to be elucidated.

In the CP the main driving force for CSF secretion is primarily active transport of Na^+ ions into the brain ventricles facilitated by the Na/K ATPase[5, 6]. Inhibitors of this pump, such as ouabain, have been shown to efficiently reduce CSF secretion and the movement of Na^+ into the CSF[7, 18, 26]. The Na/K ATPase is predominantly expressed in the apical membrane of the CP epithelial cells[8]. This luminal localization is crucial in driving Na^+ transport and CSF secretion.

Aquaporin 1 (AQP1), a transmembrane water channel, also has an important role in movement of water across the CP epithelial cells. AQP1 is highly expressed in the apical membrane of the CP epithelium and hence have been implicated to have a key role in CSF secretion[16, 21]. For many years, no direct evidence for this was established. However, in 2004 it was shown that AQP1 null

mice had significantly impaired CSF production and lower ICP compared to wild-type mice[17]. Furthermore, AQP1 expression and water movement are decreased after acetazolamide treatment in primary CP epithelial cells isolated from rats[1].

Another water channel is also present in CP; Aquaporin 4 (AQP4) expression has been shown in CP and in a cell culture of CP to be located in the cytoplasm of the cells[21]. This localization suggests that AQP4 is not normally involved in water movement across the CP epithelium. However, since the only known function of the AQP4 is water transport it might potentially be translocated to the CP epithelia cell membrane and play a role in CSF secretion in response to drugs or pathological conditions.

Studies of ICP in rodent models can be challenging, mainly due to the lack of commercial transducer systems specifically designed for small rodents. Many established methods involve penetration of the dura mater and implementation of cannulas in the lateral ventricles or in the cisterna magna[22]. These procedures introduce a risk of disturbing the CSF system and/or the CP. However, we recently developed and validated a novel method for reliable and minimally invasive repeated ICP measurements in the epidural space of rats[22]. In the present study, we used this methodology to investigate the effect of a single dose acetazolamide on ICP in healthy rats. Furthermore, we investigated the expression of Na/K ATPase, AQP1 and AQP4 in the CP tissue in the same animals. Finally, we employed an in vitro assay to establish the effect of acetazolamide on the activity of the Na/K ATPase as an indirect measure for CSF secretion.

Material and methods

Animals

For the in *vivo work*, 230-260g female Sprague-Dawley rats (Taconic, DK) were used. The rats were group housed in the animal facility at the Research Institute, Rigshospitalet Glostrup, and kept under a 12 h light/dark cycle with free access to food and water ad libitum. All experimental procedures were approved by the Danish Animal Experiments Inspectorate (license number 2014-15-0201-00256).

For the *in vitro* work, 150-200g female Sprague-Dawley rats (Charles River, UK) were used. The rats were housed at the University of Birmingham in accordance with the Animals and Scientific Procedures Act 1986, licensed by the UK Home Office and approved by the University of Birmingham Ethics Committee.

Study design

Initially 3 rats were used to identify the time point of maximum acetazolamide (Diamox®, Mercury pharma, Germany) effect on ICP as well as its duration. The animals were anesthetized and underwent ICP guide cannula implantation (described in detail below) after which the baseline ICP was recorded over 30 minutes. Then 200 mg acetazolamide dissolved in sterile saline was administered by i.p. injection. The recording was continued until the ICP returned to baseline (Figure 1A, top panel). For the main experiment, 12 rats were randomized into two groups with 6 rats in each; the treatment group received 200 mg acetazolamide dissolved in sterile saline and the vehicle group received sterile saline, both groups by i.p. injection. All animals were subjected to the same procedure as above except ICP was only recorded for 60 minutes post dosing. The rats were then euthanized with an overdose of pentobarbital, transcardially perfused with ice cold PBS and the CP tissue was collected 1½ hours post acetazolamide or vehicle treatment.

ICP

The surgical procedure and the actual ICP recording technique were recently published as a methodological paper describing all technical details[22]. In brief, all rats were anaesthetized with a mixture of Hypnorm® (Vetapharma, UK) and midazolam (B. Braun, Germany): 1.25 mg/mL midazolam, 2.5 mg/mL fluanisone and 0.079 mg/mL fentanylcitrate; 2.7 mL/kg by subcutaneously injections. Eyes were covered with Viscotears® eye gel (Novartis Healthcare, Copenhagen, Denmark) and the rat was placed in a stereotactic frame (David Kopf Instruments, US). A heating pad with connected rectal thermometer was used to keep the body temperature at 37˚C. Then a two-centimeter midline incision was made on top of the skull and the bone was exposed by retraction of skin and soft tissue.

A dental drill was used to make four burr holes in the skull; one to enable implementation of the epidural guide cannula (PlasticsOne, C313G, US) and three to fit small anchoring screws to the skull. At the site of the epidural guide cannula placement the dura mater was exposed and bone residues removed in an atraumatic and very careful procedure to avoid penetration or damage to the dura mater and associated blood vessels. Then the epidural guide cannula and the anchoring screws were placed and aligned with the interior surface of the skull and secured using dental resin-cement (Clearfil SA Cement, RH Dental, Denmark). Next the ICP guide cannula and the transducer (DTX-Plus™, Argon Medical Devices, US) were connected by a polyethylene tube filled with sterile water. Air bubbles in the tubing or transducer were eliminated if observed. All ICP recordings were initiated with confirmation of proper signal by observing an ICP increase induced by jugular vein compression (Queckenstedt test) (Figure 1). The pressure signal was visualized and recorded using Perisoft for Windows v.2.5.5 (Perimed, Sweden).

Na/K ATPase activity in primary CP cell culture

Choroid plexus tissue from lateral and 4th ventricles were isolated and incubated with 0.25% trypsin solution for 2.5 hours at 4°C followed by 30 minutes at 37°C. Trypsin digestion was stopped by the addition of newborn calf serum and the cell suspension was centrifuged at 20 g for 10 minutes. Cells were re-suspended in DMEM/F12 (Gibco, Thermo Fisher Scientific) supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, 4mM L-glutamine, 200ng/ml hydrocortisone, 5ng/ml sodium selenite and 10ng/ml EGF. 20µM cytosine arabinoside was used for the first 5 days in culture to limit the growth of fibroblasts[10]. Initially the cells were seeded onto laminin coated flasks (20µg/ml) and allowed to grow for 2 days before being transferred to 12 well inserts (Greiner Bio-One Ltd). On day 4 the media was replaced with DMEM/F12 supplemented with 10% FBS and 1% penicillin/streptomycin and refreshed every 2-3 days. After reaching confluency, CP epithelial cells were serum deprived for 3 days prior to the beginning of the studies (Day 10-14 after initial CP tissue isolation).The effect of acetazolamide on Na/K ATPase activity in the CP epithelial cells was evaluated by the colorimetric measurement of phosphate released from ATP with the use of a phosphate assay kit (ab65622, Abcam). The total Na/K ATPase activity was defined as the portion of phosphate production that was inhibited by 1mM ouabain. The cells were incubated with aCSF for 1 hour at 37^{o}C before incubation in aCSF containing; 100µM acetazolamide or saline-, in the presence and absence of 1mM ouabain for 30 minutes at 37^{o}C. The cells were then lysed with NP-40 lysis buffer on ice and spun at 13,000g to remove cell debris. Phosphate was measured per manufacturer's instructions. Briefly a reaction mix was added to the sample and incubated at room temperature for 60 minutes before the plate was analyzed at 650nm. Na/K ATPase activity was calculated as the difference between the amount of phosphate produced in the presence and absence of ouabain for each treatment.

qPCR and Western blot of CP membrane fractions

Choroid plexus from lateral and 4th ventricles were collected immediately following transcardial perfusion with ice cold PBS. The tissue was then stored in -80 degrees until analysis. RNA was purified using the GenElute™ Mammalian Total RNA Miniprep Kit (Sigma-Aldrich). Following elution the RNA yield and purity was assessed spectrophotometrically (Nanodrop 200C, Thermo Fisher Scientific Inc.). cDNA was synthesized from 200 ng RNA using the iScript cDNA Synthesis Kit (Bio-Rad, CA, USA). Then qPCR analysis was performed using the QuantiTect SYBR Green PCR system (Qiagen, Hilden Germany) per manufacturer's instructions; QuantiFast SYBR Green I dye (Qiagen), primers and cDNA from each sample were mixed in wells of a qPCR plate in duplicates. The plate was then analyzed using the Quant studio 12K Flex Real-Time PCR System (applied biosystems by LifeTechnologies) and the resulting Ct value for each sample was obtained. The relative abundance of mRNA transcripts in each experimental sample was established by calculating the detection ratios between each target gene (AQP1, AQP4 and Na/K ATPase) and the reference gene (GAPDH). The primers used were all pre-validated gene-specific primers from Qiagen's QuantiTect Primer Assay: AQP1 (QT00173789). AQP4(QT01084580). Na/K ATPase alpha1 subunit (QT02384046). GAPDH (QT00199633).

For western blotting the protein membrane fraction was extracted by lysing the CP tissue with the Mem-PER™Plus Membrane Protein Extraction Kit (Catalog number 89842, Thermo Scientific) according to manufactures instructions. The final protein concentration of each sample was evaluated using Bradford protein assay (BioRad DC™ protein Assay, BioRad). 10 µg of total protein were separated on a 4-12% SDS-PAGE gel (PAGEgel Inc.). The proteins were then transferred via dry blotting using the iBlot2 system (LifeTechnologies) onto a PVDF membrane (Invitrogen) and blocked with 5% BSA in TBST. The membranes were then incubated overnight (5°C) with appropriate primary antibodies and subsequently washed and incubated for 1 hour with

secondary antibodies at room temperature. The protein bands were visualized using an ECL-reagent (ECL select^{TM}, GEHealthcare) and a digital image of the blot were obtained with the LAS-4000 imaging system (Fujifilm Life Science). Primary antibodies: Na/K ATPase alpha1 subunit (#3010, Cell Signaling). AQP1 (ab168387, Abcam). AQP4 (ABN411, Merk Millipore). Pan-cadherin (ab51034, abcam). Secondary antibody: Goat-anti-rabbit (P0448, DAKO).

Data Analysis and Statistics

Data is presented as mean ± SEM. All data were processed using GraphPad Prism 5.02. P-values below 0.05 were considered statistically significant. The repeated measures of ICP were analyzed using 2-way ANOVA followed by Bonferroni multiple comparison post-test. The remaining data were analyzed using Mann-Whitney tests.

Results

ICP

To determine the effect of acetazolamide on ICP in healthy rats, the rats were anesthetized and ICP measured before and after an i.p. injection of either 200mg acetazolamide or vehicle. Representative examples of the ICP traces following acetazolamide and saline treatment are presented in figure 1A. A single i.p. dose of 200 mg acetazolamide significantly lowered ICP within 10 minutes of the injection 18 ± 5%, compared to the saline group 5 ± 2% ($P<0.05$), reaching a maximum reduction at 55 minutes 66 ± 4% ($P<0.00001$), (Figure 1B). The maximum response to acetazolamide ranged from 56-85% (Figure 1B) and was sustained for approximately 30 minutes after which the ICP began to normalize and returned to baseline levels 5 hours post dosing (Figure 1A). Saline had no significant effect on ICP (Figure 1A, lower panel and 1B).

Na/K ATPase activity

Total Na/K ATPase activity was determined as the concentration of inorganic phosphate generated by the hydrolysis of ATP that was sensitive to ouabain treatment. This show how much of the measured phosphate that originates from Na/K ATPase. Thirty minutes of 100 µM acetazolamide treatment significantly reduced the level of inorganic phosphate production by the Na/K ATPase to 16 ± 3% compared to the control 47 ± 6% (P=0.0022). Our data confirms that acetazolamide acts on the CP epithelial cells to reduced Na/K ATPase activity by approximately 65%. (Figure 2).

qPCR

To determine if the ICP decrease could be connected to alterations in transcription of protein encoding mRNA for AQP1, AQP4 and Na/K ATPase, this was analyzed by qPCR. The CP was isolated 1½ hours post administration of either acetazolamide or vehicle. At this time point we observed the maximal ICP decrease that had been sustained for 30 minutes (figure 1A, top panel). No significant difference was seen between the two groups for any of the target genes; AQP1 (p=0.699), AQP4 (p=0.421) and Na/K ATPase (p=0.937). (Figure 3).

Western blot of membrane fraction

Since acetazolamide did not affect the transcription of the protein encoding mRNA for any of the three target genes mentioned above we did not expect to find any changes on the protein levels at this early time point either. Instead we speculated if the CSF pathway could be affected not by increased transcription and subsequent translation of mRNA into protein in the entire CP tissue, but maybe just in by altered protein levels in the membrane of the CP tissue where the water transport takes place.

We found that both AQP1 (p=0.0152) and Na/K ATPase (p=0.0411) protein was significantly increased in the CP epithelial membrane 1½ hours after a single dose of acetazolamide. The amount of AQP4 protein in the extracted membrane fraction was not significantly different between the vehicle and acetazolamide treated animals (p=0.0649). (Figure 4).

Discussion

In this study we have demonstrated that a single dose of acetazolamide lowers ICP in healthy rats, with the maximum effect occurring roughly one hour after dosing. The low ICP is maintained for 30 minutes before slowly returning towards the baseline value. The main mechanism of action of acetazolamide is to inhibit the carbonic anhydrase enzyme, which normally catalyzes the conversion of H_2O and CO_2 to HCO_3^- and H^+. Carbonic anhydrase is not directly involved in water or ion transport in the CP; though its inhibition by acetazolamide has been shown to reduce CSF secretion[2, 25] and ICP[11, 12]. CSF secretion by CP is governed by a number of ion transporters specifically located on either the basolateral or the apical surface of the CP epithelial cells. The net flow of ions across the CP facilitates the movement of water and thus CSF secretion[6]. Na/K ATPase in the apical surface is the main driving force for transporting Na^+ into the CSF against its concentration gradient[6], therefore its activity is directly linked to CSF production by the CP. Hence, we find it credible that the drop in ICP is facilitated by the inhibition of the Na/K ATPase activity by acetazolamide. This means that Na+ transport across the CP epithelia is suppressed and the rate of CSF production will slow down and lead to a fall in ICP. Our finding that acetazolamide inhibits the activity of this pump in primary cultures of CP epithelial cells fits well with other studies showing that the Na/K ATPase activity is inhibited and secretion of CSF reduced in the absence of CO_2/HCO_3^-[19], which is substrate and product of the enzymatic reaction enabled by

carbonic anhydrase. The connection between carbonic anhydrase and Na/K ATPase in the CP is not well established, but in other tissues acetazolamide is believed to facilitate its effect by reducing the amount of H^+ ions available for the Na/H exchanger[13, 27]. This in turn limits the amount of Na^+ to be transported by the Na/K ATPase and thus lowers its activity. We believe that a similar mechanism exists in the CP where the Na/H exchanger is also present. This is supported by studies done in rats showing that acetazolamide reduces CSF production and that the mechanism of action is not simply a direct inhibition of Na/K ATPase[20]. However, to fully verify this hypothesis, additional studies of ion transport and interplay between various transporters would be needed.

Furthermore, we have for the first time shown an increased expression of AQP1 and Na/K ATPase proteins in the membrane fraction of CP 1½ hours after in vivo administration of acetazolamide. Both AQP1 and Na/K ATPase are key players in the CSF secretion pathway across the CP cells. The expectation would therefore be that a decrease in the amount of these two proteins would lead to a decrease in CSF production with subsequent decrease of ICP and vice versa. Interestingly, our study shows that both proteins are increased in the cell membrane after acetazolamide treatment. Similar results have previously been published by other groups[1, 28] and might provide an explanation for this contradiction; in studies performed in kidney tissue and cells it was demonstrated that acetazolamide treatment leads to a degradation of AQP1[28]. The study showed that AQP1 is translocated to the plasma membrane of the cells as an acute response to acetazolamide, and then subsequently tagged for degradation by ubiquitination and degraded by proteasomes[28]. This indicated that acetazolamide does in fact decrease AQP1 protein content, but at later time points than investigated in our study. The decrease in AQP1 is not caused by reducing mRNA and protein synthesis, but by promoting translocation to the membrane and tagging the protein for subsequent degradation. A similar mechanism could potentially be present

in the CP, however due to our relatively early time point post treatment we do not see AQP1 degradation, but only the increase of AQP1 in the membrane fraction. This hypothesis is supported by the fact that even though we see increased protein levels in the membrane in CP, we do not record any change in the synthesis of mRNA, which indicates that the increased amount of protein in the membrane does not originate from increased mRNA translation. A similar mechanism might exist for Na/K ATPase. But the increase in membrane AQP1 and Na/K ATPase could also be a compensatory mechanism to the inhibition of Na^+ transport activity and CSF secretion caused by acetazolamide. Since both AQP1 and Na/K ATPase are essential for CSF secretion, the increased membrane content of the two proteins could be a way to try to maintain the necessary level of Na^+ and water transport into the brain ventricles.

In 2004 Oshio et al[17] showed that acetazolamide reduces CSF secretion in mice (ICP was not recorded) but interestingly, the reduction was similar in wild type and AQP1 null mice. This indicates that the acute ICP decrease seen in the present response to acetazolamide treatment may circumvent the AQP1 route. This would explain why ICP is decreased rapidly by acetazolamide and seems to have no direct correlation with the amount of AQP1 present in the membrane of CP. This means that the acute ICP decrease seen after acetazolamide treatment is most likely linked to the inhibition of Na/K ATPase and that the increase in membrane AQP1 and Na/K ATPase is less important for the acute effect of acetazolamide.

Other studies suggest that acetazolamide could also have a direct inhibitory effect on AQP1 besides the effect seen on carbonic anhydrases[1, 28]. However, the experiment performed in AQP1 null and control mice showing that AQP1 null mice also have significantly decreased ICP contradicts this theory[17].

AQP4 mRNA and membrane protein were unchanged following a single dose of acetazolamide in rats. According to the literature and our own observations (data not shown), AQP4 should not be present in the membrane of the CP. Hence, we speculated if this water channel could potentially be translocated to the cell membrane following acetazolamide treatment and facilitate an increased CSF secretion as a compensatory mechanism to the reduction in ICP. However, we did not see any indications for this. The minor signal seen in the western blot analysis is most likely from cytoplasmic contamination of the membrane fraction. The manufacturer of the *Mem-PER^{TM}Plus Membrane Protein Extraction Kit* informs that there will be a contamination of 10% or less from the cytosolic to the membrane fraction. However, since no more AQP4 is present after acetazolamide treatment compared to control, we believe that AQP4 is most likely unlinked to the observed ICP change. Still the presence of AQP4 and especially its location in the CP is puzzling and it is likely that AQP4 could be involved in the CSF secretion pathway during pathological conditions.

In conclusion, we have employed both in vivo and in vitro techniques to demonstrate that a single dose of acetazolamide lowers ICP and modulates the CSF secretion pathway in healthy rats. Firstly, inhibiting the Na/K ATPase reduces the Na^+ flux into the brain ventricles, which slows the production of CSF. Secondly, AQP1 and Na/K ATPase protein is increased in the membrane of the CP epithelial cells, which may represent an early compensatory mechanism for the reduced ICP. Further studies are warranted to elaborate on the detailed molecular mechanism behind these observations. This could include administration of acetazolamide in an IIH model to establish the molecular response during a pathological condition. Furthermore, it would be interesting to study the long term effect after a single dose as well as repeated dosing of acetazolamide of the CP.

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Figure legends

1. Rats were anesthetized and ICP measured before and after an i.p. injection of either 200mg acetazolamide (n=6) or vehicle (n=6).

A: Examples of ICP traces following acetazolamide and saline treatment are presented. The maximum response to acetazolamide ranged from 56-85% and was sustained for approximately 30 minutes after which the ICP began to normalize and returned to baseline levels 5 hours post dosing. Saline had no significant effect on ICP.

B: A single dose of 200 mg acetazolamide significantly lowered ICP within 10 minutes of the injection 18 ± 5%, compared to the saline group 5 ± 2% ($P<0.05$), reaching a maximum reduction at 55 minutes 66 ± 4% ($P<0.00001$).

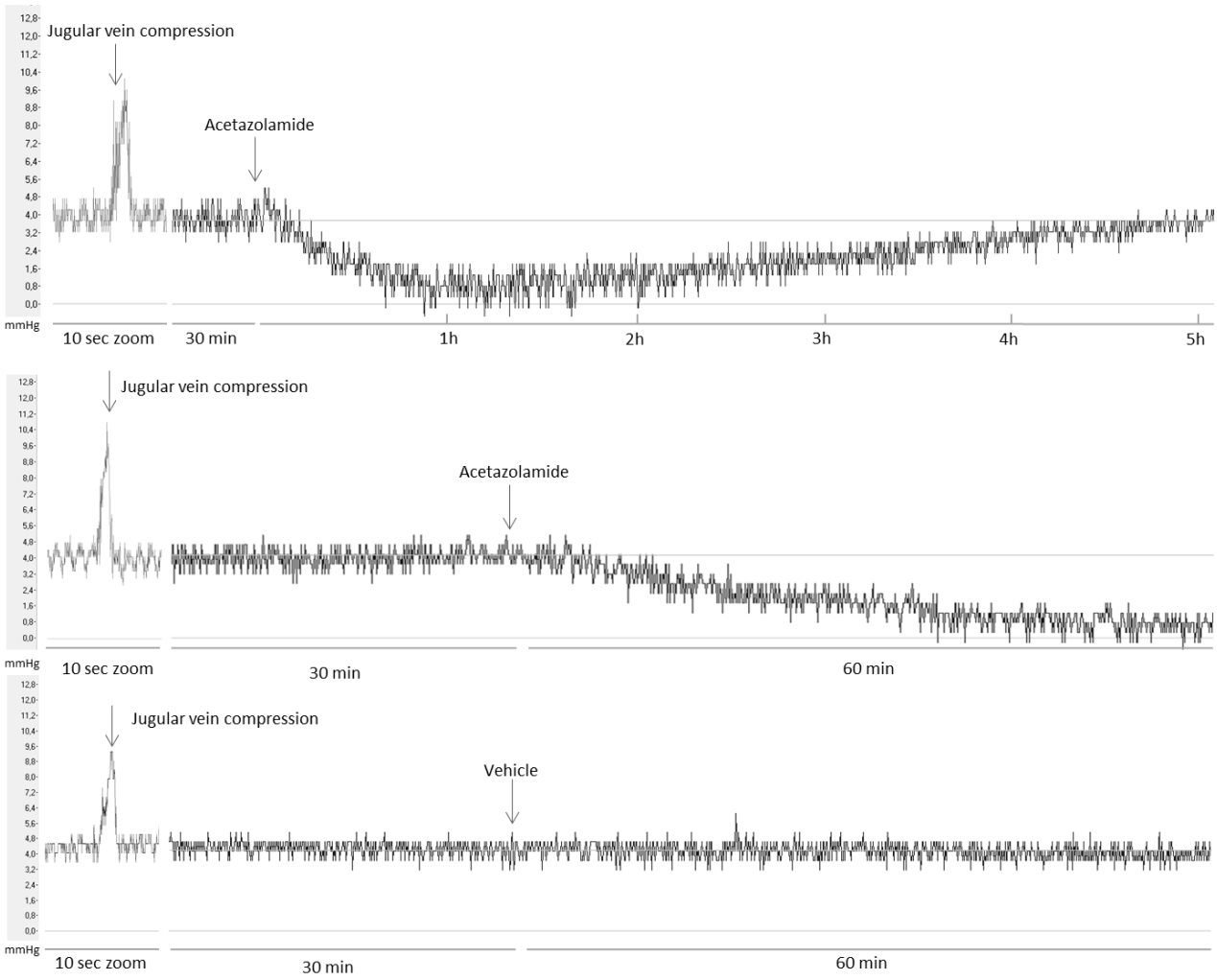
2. Total Na/K ATPase activity was measured by determining the concentration of inorganic phosphate generated by the hydrolysis of ATP that was sensitive to ouabain (a Na/K ATPase inhibitor). In these studies primary rat CP epithelial cells were treated for 30 minutes with aCSF (n=6) or 100µM acetazolamide (n=6), in the presence or absence of 1mM Ouabain. The graph presents the ouabain-sensitive Na/K ATPase activity ± SEM (% change from ouabain control). Acetazolamide significantly ($P<0.01$) reduced Na/K ATPase activity in the CP compared to vehicle.

3. To determine if the ICP decrease could be connected to alterations in transcription of protein encoding mRNA for AQP1, AQP4 and Na/K ATPase, this was analyzed by qPCR. No significant difference was seen between the two groups for any of the target genes; (**A**) AQP1 ($p=0.699$), (**B**) AQP4 ($p=0.421$) and (**C**) Na/K ATPase ($p=0.937$).

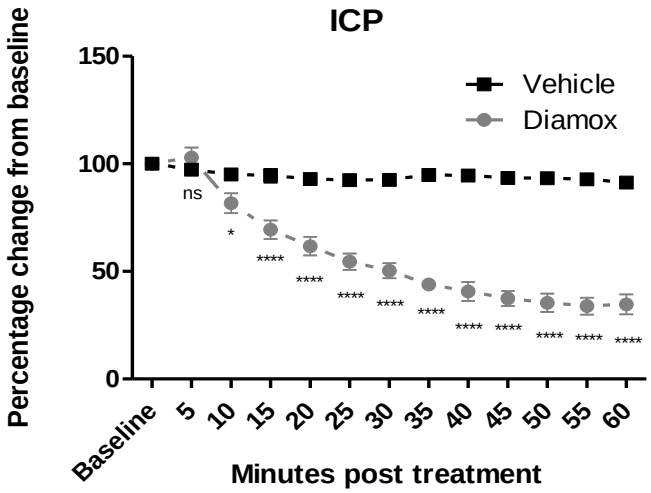
4. We speculated if the CSF pathway could be affected by altered protein levels in the membrane of the CP tissue where the water transport takes place. We found that both (**A**) AQP1 (p=0.0152) and (**B**) Na/K ATPase (p=0.0411) protein was significantly increased in the CP epithelial membrane 1½ hours after a single dose of acetazolamide. (**C**) The amount of AQP4 protein in the extracted membrane fraction was not significantly different between the vehicle and acetazolamide treated animals (p=0.0649).

Figures

1A



1B



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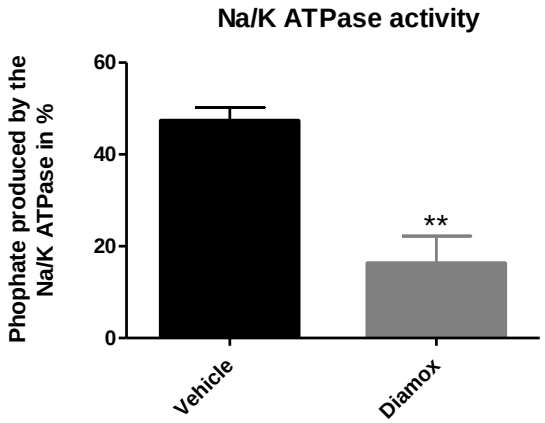

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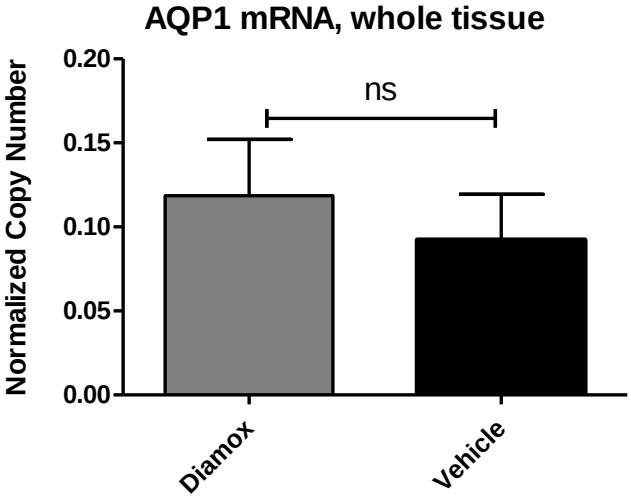

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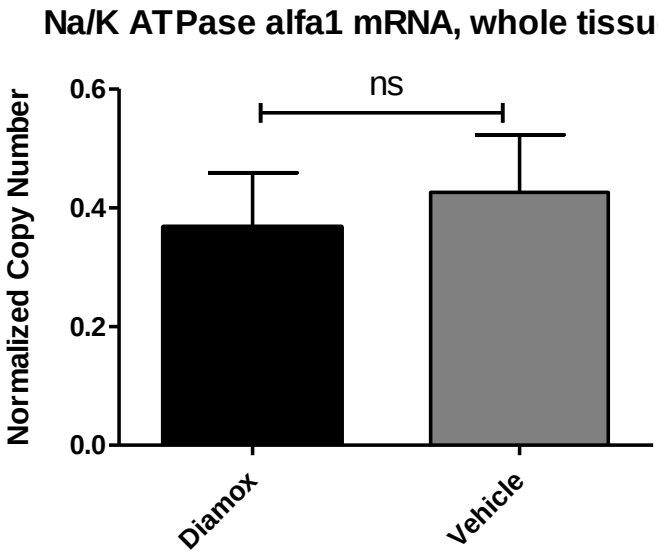

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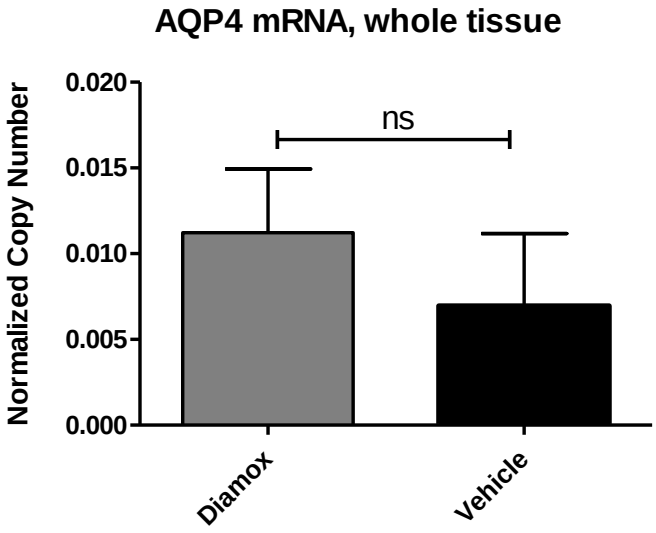

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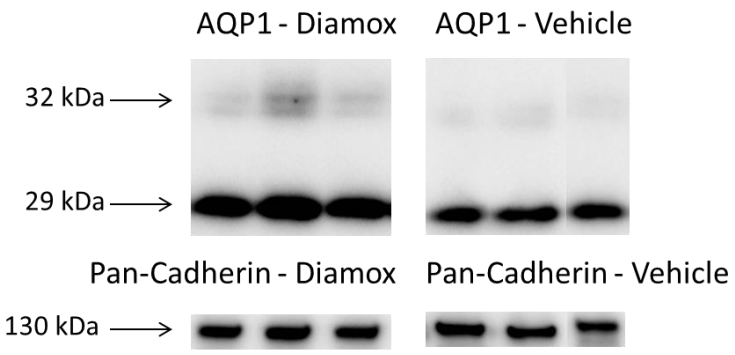
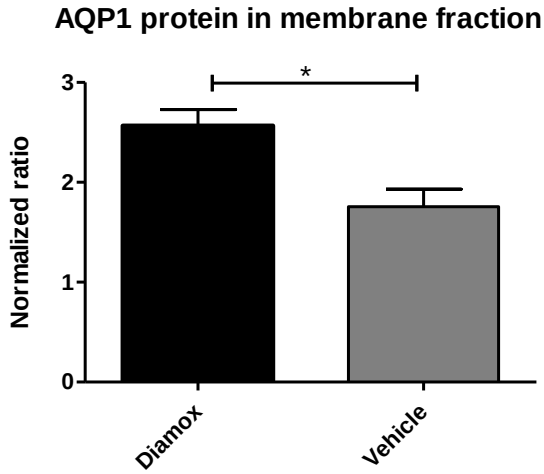
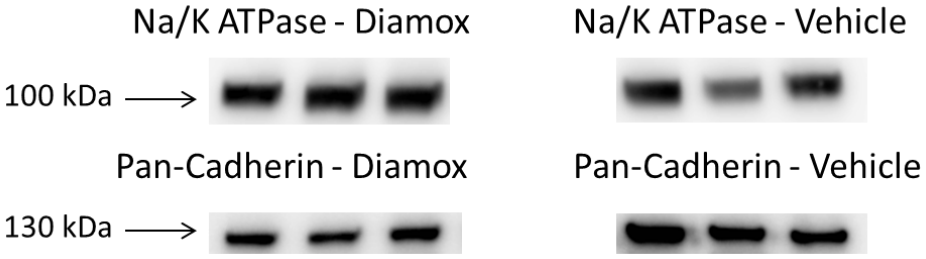
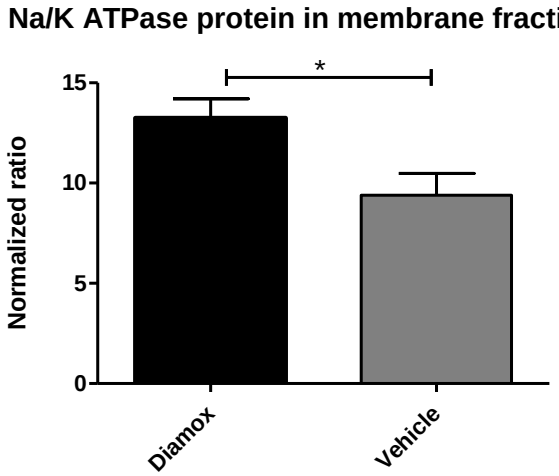




4B





4C