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Expression of Na-K-Cl cotransporter and edema formation are age dependent after ischemic stroke

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Abstract

Age is the most important independent risk factor for stroke; however aging animals are rarely used in stroke studies. Previous work demonstrated that young male mice had more edema formation after an induced stroke than aging animals. An important contributor to cerebral edema formation is the Na-K-Cl cotransporter (NKCC). We examined the expression of NKCC in young (10-12 week) and aging (15-16 months) C57BL6 male mice after middle cerebral artery occlusion (MCAO) and investigated the effect of pharmacological inhibition of NKCC with Bumetanide on cerebral edema formation. Both immunofluorescent staining and Western blotting analysis showed that NKCC expression was significantly higher in the ischemic penumbra of young compared to aging mice after stroke. Edema formation was significantly more robust in young mice and was reduced with Bumetanide. Bumetanide had no effect on cerebral edema in aging mice after MCAO. This suggests that NKCC expression and edema formation are age dependent after ischemic stroke.

Keywords

Aging; Bumetanide; cerebral edema; ischemic stroke; middle cerebral artery; mice; Na-K-Cl cotransporter

Introduction

Age is the most important independent risk factor for stroke. With increasing life expectancy, elderly patients will soon constitute the majority of stroke victims (Rojas et al., 2007). Numerous neurochemical and physiological changes occur with aging (Anyanwu, 2007) making it critical to model age-related diseases appropriately at the bench. However, aging animals are rarely used in stroke studies due to significant costs of animal care, the increased complexity of surgical procedures, and lower survival rates compared to young animals. As the physiological response to stroke may differ in the aging brain, therapies that

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are efficacious in young animals may not be effective in aging. This could contribute to the failure of translation of promising neuroprotective therapies to patients at the highest risk for stroke, the elderly.

Cerebral edema is one of the most common complications following stroke and leads to an increase in death and disability (Johnston et al., 1998). Edema formation is induced by the net uptake of brain cations and water into the brain. This process is initiated well before blood brain barrier (BBB) breakdown occurs (Betz, 1996). During an ischemic insult, the BBB secretes Na⁺, Cl⁻ and water into the brain, leading to astrocytic swelling in an attempt to take up these ions from the interstitial fluid (Iadecola, 1999, Kimelberg, 1999). NKCC, the electroneutral Na-K-Cl cotransporter, is a membrane protein that transports Na⁺, K⁺, and Cl⁻ ions into and out of a wide variety of epithelial and non-epithelial cells (Haas and Forbush, 1998), and has been shown previously to participate in ischemia-induced cerebral edema formation (O'Donnell et al., 2004). Two isoforms of the NKCC channel have been identified to date, NKCC1 is expressed throughout the brain and NKCC2 is found only in the kidney (Yan et al., 2001b, Yan et al., 2003).

The formation of cerebral edema appears to differ in young and aging brains after stroke. It is increasingly recognized that the structure and function of components of the BBB change with age (Shah and Mooradian, 1997). Aging of the normal brain is accompanied by changes in its structure, function and metabolism. With increasing age, a number of synaptosomal and membrane linked enzymes decrease, including synaptosomal bound hexokinase and phosphofructokinase, ATP citrate-lyase, acetyl Co-A carboxylase, fatty acid synthase, and Na⁺K⁺ ATPase (Baquer et al., 2009). We have previously found that young male mice have enhanced edema formation compared to aging animals 24 hours after experimental stroke (Liu et al., 2009). This suggests that molecular mechanisms contributing to edema formation may differ in young and aging animals after ischemic insults. The NKCC inhibitor bumetanide was reported to reduce edema formation after MCAO in rats (O'Donnell et al., 2004) but the response in aging animals is unknown. In this study, we examined NKCC expression in both young and aging mice after stroke, and administered bumetanide to investigate whether NKCC contributes to differential edema formation in young and aging animals after stroke.

Methods & Materials

Animals

The present study was conducted in accordance to the National Institutes of Health guidelines for the care and use of animals in research, and under the protocols approved by the University of Connecticut Animal Care and Use Committee. C57BL6 male mice were purchased from Charles River Laboratories (Wilmington, MA). Both young male mice (10-12 weeks; 22 to 25g) and aging male mice (16 months; 30-40g) were used in this study.

Ischemic Model

Focal transient cerebral ischemia was induced by MCAO (0.21mm silicone coated suture for young and 0.23 mm for aging) for 90 minutes followed by reperfusion under Isoflurane anesthesia as described previously (McCullough et al., 2005b, Liu et al., 2009). Rectal muscle temperatures were maintained at an automated temperature control feedback system. Sham animals were subjected to surgery with sutures of the same size but the suture was not advanced into the middle cerebral artery. Cerebral blood flow (CBF) was measured by laser Doppler flowmetry (LDF, Moor Instruments Ltd, England) in all animals as previously described (McCullough et al., 2005b). Only mice in which CBF in the MCA distribution showed a sharp drop of over 85% of control immediately after MCAO were included. For

bumetanide treatment, sham or MCAO mice were administered bumetanide (30.4 mg/kg in two doses of 15.2 mg/kg iv) immediately after MCAO and at reperfusion. Bumetanide (MP Biomedicals, Solon, OH) was prepared as a fresh stock solution for each experiment by dissolving 18mg in 0.5ml of 0.5N NaOH solution, and then diluted in a 0.5% saline solution containing albumin to a desired concentration and adjusted to a pH of 7 using 1N HCl solution (O'Donnell et al., 2004).

Neurological deficits were confirmed and scored as follows: 0, no deficit; 1, forelimb weakness and torso turning to the ipsilateral side when held by tail; 2, circling to affected side; 3, unable to bear weight on affected side; and 4, no spontaneous locomotor activity or barrel rolling. Monitoring of physiological variables was performed in companion cohorts for all groups prior to MCAO and 60 minutes after reperfusion as described previously (McCullough et al., 2005b).

Terminal Histopathology

24 hours after stroke, mice were euthanized and the brains were removed and cut into five 2-mm slices. The slices were stained with 1.5% 2, 3, 5-Triphenyltetrazolium Chloride (TTC) solution at 37°C for 10 minutes and then fixed with 4% formalin, images were digitalized, and the infarct volumes (corrected for edema) were analyzed using computer software (Sigmascan Pro5) as previously described (Liu et al., 2009).

Measurement of Edema

At 24h of stroke, brain edema was measured as previously described (Liu et al., 2009). Briefly, the brain was quickly removed after the animal was sacrificed. Then the brain was blotted to remove residual absorbent moisture, and dissected through the interhemispheric fissure into right and left hemisphere. The wet weight is determined with a resolution of 0.1mg. The dry weight was measured after the hemispheres are dried for 3 days at 100°C in a drying oven. The tissue water content was then calculated as % H₂O = (1-dry wt/wet wt) × 100% (Liu et al., 2008).

Immunohistochemistry

Floating brain sections were prepared as described previously (McCullough et al., 2005a). Briefly, free-floating sections were washed in 0.1M Borate Buffer and then blocked in Normal Goat Serum Block Solution for 30 minutes at room temperature followed by incubation with anti-Na-K-Cl cotransporter antibody (T4, 1:500, DSHB, IA), which was obtained from the Developmental Studies Hybridoma Bank, developed under the auspices of the NICHD, and maintained by The University of Iowa, Department of Biology, Iowa City, IA 52242, and Von Willebrand Factor (VWF) (1:100, Santa Cruz, CA) in phosphate-buffered saline containing 1% BSA overnight at 4°C. The secondary antibodies (1:1000, Invitrogen, Carlsbad, CA) were either goat anti-mouse or goat anti-rabbit depending on the primary antibody and were incubated for 2 hours at room temperature. 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI, 1:1000, Invitrogen, Carlsbad, CA) was added to the sample together with the secondary antibodies. The signal was visualized with immunofluorescence confocal microscopy using Zeiss image acquisition software (Zeiss LSM 510). All slices were the same distance from bregma and four 20× fields/animal (n= 5 animals/gp) were analyzed in the penumbral area of the infarct. NKCC positive cells were counted using MacBiophotonics ImageJ software (NIH) with a DAPI counterstain. For each animal, the total number of cells was averaged across fields of view. These averages (avg.# cells/field of view) were used for statistical analysis.

Western Blots

24 hours after stroke, the brain samples were dissected into core and penumbral regions, and the protein concentration was determined by BCATM Protein Assay Kit (Thermo Fisher Scientific Inc., Rockford, IL) and subjected to Western Blotting as previously described (McCullough et al., 2005a, Liu et al., 2009). Sample proteins were resolved on 4% to 20% SDS electrophoresis gels and transferred to a polyvinylidene difluoride membrane. NKCC was detected using the anti-NaK-Cl cotransporter antibody (T4) (1:500; DSHB, IA). β -actin (1:5000; Sigma) was used as a loading control. All blots were incubated overnight in primary antibodies at 4°C in TBS containing 4% BSA and 0.1% Tween 20. Secondary antibodies (goat anti-mouse IgG 1:5000, donkey anti-goat IgG 1:1000; Santa Cruz) were diluted and ECL detection kit (Amersham Biosciences) was used for signal detection. The densitometry of Western Blotting images was performed with computer software (ImageJ NIH).

Statistics

All values are expressed as mean $\pm$ SEM and analyzed with a t-test for two groups, and one-way analysis of variance (ANOVA, with Bonferroni post hoc correction, when appropriate) for the comparison of the means between the experimental groups except the neurological deficit scores, which were done by Mann-Whitney U test. All assessments were performed by a blinded investigator except mouse age. Due to the higher body weights and severe behavioral deficits in aging mice, experimenters were aware of the different age groups (aging vs. young). The criterion for statistical significance was $P < 0.05$.

Results

NKCC expression was higher after stroke in young mice

In order to determine the cell type expressing NKCC, we co-labeled brain slices with NKCC and VWF antibodies plus DAPI staining. VWF is synthesized by endothelial cells and is an endothelium-specific glycoprotein (Yokota, 2007). NKCC extensively colabeled with VWF suggesting that NKCC is found primarily in endothelial cells in both young and aging mice (Fig. 1A). To examine whether stroke and age have any effect on NKCC expression, we examined the amount of NKCC labeling by immunofluorescent staining in the penumbra. Both young and aging mice had an increase in NKCC expression by MCAO compared to shams (Fig. 1B). Young mice had higher levels of stroke-induced penumbral NKCC expression compared with aging mice, (Fig. 1A & B). In order to confirm that NKCC was higher in young mouse brain after stroke, we performed Western Blotting on brain homogenates. Young mice had significantly higher levels of NKCC protein in the penumbra compared to aging mice after stroke, consistent with immunofluorescence findings (Fig. 2A & B).

Physiological parameters were not significantly different between vehicle and bumetanide treated mice

To evaluate whether bumetanide can cause changes in physiological parameters in mice, we monitored physiological variables of animals before stroke and 60 minutes after reperfusion. No differences of physiological parameters were found between vehicle and drug treated mice of both age prior to and 60 minutes after reperfusion. The LDF showed a sharp drop to 15% of baseline after occlusion, and returned to above 90% of baseline 60 minutes after reperfusion in all groups.

Bumetanide treatment decreased edema formation in young mice after stroke

Consistent with previous work (Liu et al., 2009), young vehicle-treated male mice had significantly more edema in the ipsilateral hemisphere compared to aging males 24h after stroke (Fig, 3A & B). Administration of bumetanide decreased edema formation significantly compared to vehicle treated mice in young cohort. In aging males, there was no significant difference in the absolute water content in drug-treated males compared to vehicle treated mice.

Stroke outcomes were not significantly improved by bumetanide administration

To evaluate the effect of inhibition of NKCC, we examined behavior with the neurological deficit score (NDS) and infarct volume by TTC staining. The NDS was scored at 1.5h and 24h after stroke. Neurological deficits were significantly improved at 24h in young mice in both vehicle and drug treated groups compared to the deficits seen at 1.5h of stroke, and the mortality at 24h of stroke was 15%. Aging mice did not show the same pattern of behavioral improvement at 24h (Fig, 4E), with no change in NDS over time and a higher mortality of 25% compared to that of young mice. No differences in NDS were seen between vehicle and bumetanide treated groups in either age group. Histological analysis with TTC showed that vehicle treated aging mice had smaller total infarct volumes than their young counterparts, which is consistent with several previous reports (Shapira et al., 2002, Liu et al., 2009) (Fig, 4A-D). Surprisingly, although treatment with bumetanide significantly decreased cerebral edema in young mice after stroke, it had no effect on infarct volumes at this time point. Bumetanide also had no effect on infarct size in aging mice.

Discussion

The present study examined NKCC expression, edema formation and infarct volumes after stroke in young and aging mice, and shows several important new findings. Firstly, NKCC is expressed in vascular endothelial cells in the brain after stroke. In contrast to a previous report from non-ischemic rat brain that demonstrated NKCC expression in astrocytes (Yan et al., 2001a), we found robust colabeling with the endothelial marker VWF, and not with the astrocytic marker GFAP. NKCC was primarily expressed in vascular endothelial cells after the brain was exposed to an ischemic insult. This is in agreement with work by O'Connell and colleagues which demonstrated with electron microscopy that NKCC is located on the luminal membrane of BBB endothelial cells in both sham and ischemic brains (Yerby et al., 1997, Brillault et al., 2008) and consistent with its role in the secretion of Na⁺ and water across the BBB from the blood into the brain during early cerebral ischemia (Brillault et al., 2008). Secondly, NKCC expression after stroke differs in aging mice compared to young cohorts. Although both young and aging mice had increased expression of brain NKCC after stroke compared to shams, these effects were significantly more robust in the young male brain. Interestingly, these age-related differences in NKCC were only seen under ischemic conditions, as there were no significant differences in NKCC expression with aging in sham brains. Thirdly, as seen in our previous work (Liu et al., 2009), young mice had more robust edema formation after an induced stroke than aging mice. Administration of the selective NKCC inhibitor bumetanide led to a significant reduction in stroke-induced edema in young mice compared with vehicle-treated young mice, but no effect was seen in aging mice. In other words, less cerebral edema formed after stroke in the aging brain, and what little was formed was not decreased by inhibition of NKCC. Finally, in contrast with previous reports, inhibition of NKCC with bumetanide had no beneficial effect on stroke outcome in either young or aging mice. This was somewhat surprising as other investigators have shown that inhibition of NKCC (Yan et al., 2003), or genetic deletion of NKCC1 is neuroprotective (Chen et al., 2005). As has been seen previously, young vehicle-treated mice had significantly larger total infarct volumes

compared to aging male mice, despite the more severe short-term functional deficits seen in the aging mice (Shapira et al., 2002, Liu et al., 2009).

Our result that NKCC expression is upregulated after stroke is consistent with previous work that showed an increase in NKCC expression as early as 2h after ischemia which remained elevated throughout 24h of reperfusion (Yan et al., 2003). This increase in NKCC is stimulated by protein phosphorylation in response to both cAMP and cell shrinkage induced by oxidative stress (Lytle and Forbush, 1992, Tan et al., 1998); therefore, it was not surprising that an upregulated expression of NKCC was seen exclusively in stroke animals. Stroke-induced NKCC expression is age dependent, the mechanism for this muted response in the aging brain remains elusive. However, NKCC activation occurs in conjunction with the activity of abluminal Na⁺K⁺ ATPase, which by generating an inwardly directed electrochemical gradient for Na⁺, provides the driving force for NKCC to facilitate ions transportation (Schettino and Lionetto, 2003). It has recently been shown that activity of the Na⁺K⁺ ATPase is decreased with aging (Baquer et al., 2009) which could contribute to the differential edema formation across the lifespan.

It is widely accepted that NKCC plays an important role in cerebral edema formation after ischemia (Chen and Sun, 2005, Kumar et al., 2006). NKCC can be activated by decreasing pHi and contributes to acidosis-induced cytotoxic brain edema (Ringel et al., 2000). In previous studies, no age-related differences were found in Aquaporin 1 or 4 expression (Liu et al., 2009), leading us to consider NKCC as a potential contributor to age related differences in edema. Accumulating studies (O'Donnell et al., 2004, Chen and Sun, 2005, Jayakumar et al., 2008) have reported that the NKCC inhibitor bumetanide decreased edema formation after ischemic stroke, consistent with our findings in young male mice. However, inhibition of NKCC did not have any effect on edema formation in the aging brain, possibly due to the lower ischemia-induced expression of NKCC. The finding that aging animals had less edema formation after stroke has evidential support from clinical post-mortem studies (Jaramillo et al., 2006) which have confirmed more robust edema formation in the young brain after stroke and is in part the rationale for proposing an upper age limit of 60 for hemispherectomy (Hofmeijer et al., 2008). The differential edema formation seen in aging could have important clinical consequences, as the vast majority of stroke patients are over 65.

Edema formation after ischemia can cause an increase in brain mass and is believed to contribute to secondary damage of brain tissue (Ringel et al., 2000). Surprisingly, in the present study no differences of infarct volumes or neurological deficits were seen between vehicle and bumetanide treated mice in either young or aging cohorts, despite that administration of bumetanide significantly decreased edema formation in young mice. Former studies in rats (Yan et al., 2003, O'Donnell et al., 2004) reported that bumetanide reduces both edema formation and histological neuronal damage induced by ischemic injuries. However ischemic lesion volume and edema formation differ among animal species and strains. For example, in Wistar rats, reperfusion did not cause any changes in ischemic lesion volume, but edema formation was significantly reduced; in Sprague-Dawley rats, however, reperfusion caused a significant reduction of ischemic lesion volume while did not modify edema formation (Walberer et al., 2006). In this study, inhibition of NKCC with bumetanide did significantly reduce edema formation but this did not translate into a reduction in infarct volumes in young mice, indicating that edema formation may not correlate well with infarction after ischemic injury, but may instead be age dependent.

This study has several limitations, and the results should be interpreted with these in mind. We did not examine the expression of tight or gap junction proteins in BBB after stroke, although our former study (Liu et al., 2009) showed that changes in BBB integrity after

stroke correlated with infarction volume, but not with edema formation. The wet-dry method utilized in this study measures absolute water content measurement and cannot differentiate cytotoxic and vasogenic edema, which could predominate at different times after ischemic injury, and may show different chronology in the aging brain. It is unlikely that cerebral edema peaks at later time points in the aging brain, as infarction is complete by 24 hours in both young and aging animals in this model; future studies will examine later time points after injury to determine if reducing cerebral edema is a viable goal in the aging brain.

Collectively, aging mice have a different edema response to stroke compared to young mice. This may be due in part to the decrease in NKCC expression with a subsequent reduction in edema formation in the aging brain after ischemic stroke. Importantly, these findings also draw attention to the potential limitations of pre-clinical studies that are performed only in young animals.

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Figure 1. Immunofluorescent staining of NKCC, VWF, and DAPI in the brains of young and aging mice after MCAO. **A.** 100x fields demonstrate the co-localization of NKCC and VWF (top panel); coronal section of mouse brain stained with NKCC (green), VWF (red), and DAPI (blue). Lower two panels show 20x view of the penumbral areas of the infarct. **B.** Semi-quantification of NKCC cells. n=5 animals/group; *P<0.05 versus young mice. Scale bar = 5 μ m (100x), 20 μ m (20x).

Figure 2. Western blots of NKCC in brains of young and aging mice. **A.** NKCC was probed from brain homogenates of sham (Sh) animals, core (C) and penumbral (P) regions of infarct in the ipsilateral hemisphere. β -actin was used as a loading control. **B.** The optical density of samples were expressed as the ratio of the NKCC bands to control bands (β -actin); statistical analysis was performed from three separate experiments. n=6 animals/group; *P<0.05 versus penumbra in young mice.

Figure 3. Edema formation in brain after stroke. **A.** The absolute water content in ipsilateral (ip) and contralateral (ct) hemispheres of young and aging mice. * $P < 0.05$ versus the ipsilateral hemisphere in young vehicle treated group. **B.** The edema index (EI) was expressed as the ratio of the absolute water content in the ipsilateral hemisphere over that in the contralateral hemisphere. * $P < 0.05$ versus the vehicle treated young mice; $n = 6$ animals/gp.

Figure 4.

Stroke outcomes in young and aging mice. **A.** A representative TTC stained coronal slice (slice three of five of 2-mm slices made from the olfactory bulb to the Cerebellum) in each group. **B & C & D.** Quantification of infarct volumes based on TTC staining in cortex (B), striatum (C), and total hemisphere (D). * $P < 0.05$ versus young mice; $n = 6 \sim 9$ animals/gp. **E.** Neurological deficit scores (NDS) at 1.5h and 24h of stroke. * $P < 0.01$ versus 1.5h in vehicle treated young mice. ** $P < 0.05$ versus 1.5h in drug treated young mice; $n = 6 \sim 9$ animals/gp. Y, young; A, aging.