Published in final edited form as:

Neurosci Lett. 2017 November 20; 661: 126–131. doi:10.1016/j.neulet.2017.09.062.

Transgenic expression of HuR increases vasogenic edema and impedes functional recovery in rodent ischemic stroke

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DISCLOSURES

None.

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Abstract

Background and Purpose—Ischemic stroke produces significant morbidity and mortality, and acute interventions are limited by short therapeutic windows. Novel approaches to neuroprotection and neurorepair are necessary. HuR is an RNA binding protein (RBP) which modulates RNA stability and translational efficiency of genes linked to ischemic stroke injury.

Methods—Using a transgenic (Tg) mouse model, we examined the impact of ectopic HuR expression in astrocytes on acute injury evolution after transient middle cerebral artery occlusion (tMCAO).

Results—HuR transgene expression was detected in astrocytes in perilesional regions and contralaterally. HuR Tg mice did not improve neurologically 72 hours after injury, whereas littermate controls did. In Tg mice, increased cerebral vascular permeability and edema were observed. Infarct volume was not affected by the presence of the transgene.

Conclusions—Ectopic expression of HuR in astrocytes worsens outcome after transient ischemic stroke in mice in part by increasing vasogenic cerebral edema. These findings suggest that HuR could be a therapeutic target in cerebral ischemia/reperfusion.

KEY TERMS

Ischemic stroke; Transgenic mice; Brain edema; Middle cerebral artery occlusion; Vascular permeability

INTRODUCTION

Ischemic stroke is a significant cause of morbidity and mortality world-wide, and only a fraction of acute stroke patients is eligible for thrombolysis or thrombectomy [15]. Despite promising preclinical results, there are no proven neuroprotective agents for acute ischemic stroke [34]. This clinical problem provides the rationale for identifying acutely activated molecular pathways which modulate secondary tissue injury or neural repair.

We investigated post-transcriptional gene regulation as a novel pathway in cerebral ischemia/reperfusion. Genes linked to secondary tissue injury including pro-inflammatory cytokines (e.g., IL-1 β and TNF- α) and matrix metalloproteinases (e.g., MMP-9 and 12) increase in the hyperacute phase and are modulated post-transcriptionally [3, 10, 11, 13, 16, 21, 26, 28, 32]. The key elements in post-transcriptional gene regulation are adenine- and uridine-rich elements (AREs) in the 3' and/or 5' untranslated regions of mRNA [1, 3, 7]. AREs, through interaction with RNA-binding proteins (RBPs), modulate mRNA stability and translational efficiency. RBPs such as KH-type splicing regulatory protein (KSRP) and tristetraprolin (TTP) are negative regulators, whereas HuR is generally a positive regulator. RBPs can dramatically affect target mRNA and protein levels. In astrocytes, knockdown of HuR led to a 30–40% attenuation of factors relevant to ischemia such as TNF- α , IL-1 β , and

MMP-12, whereas knockout of KSRP led to significant increases [13, 20, 21, 24, 26]. These observations provided the basis for our hypothesis that HuR affects secondary tissue injury in experimental ischemic stroke. We used a transgenic (Tg) mouse model in which human HuR is selectively expressed in astrocytes using the glial fibrillary acidic protein (GFAP) promoter [37]. After exposure to transient cerebral ischemia, we observed increases in perilesional HuR transgene expression, vascular permeability, and cerebral edema. Tg mice exhibited worse short-term neurologic outcomes. These findings highlight the importance of post-transcriptional regulation in ischemic stroke and identify HuR as a potential therapeutic target.

MATERIALS AND METHODS

Animals. Procedures were approved by the University of Chicago Institutional Animal Care and Use Committee and were in accordance with NIH guidelines for the use of animals in research. The Tg mouse line was previously described [37]: the GFAP promoter was utilized to direct expression of the human HuR gene coupled with the FLAG octapeptide tag in C57Bl/6J mice. Animals were housed in cages with littermates on a 6AM – 6PM light cycle. Mice were fed Teklad Diet 2918 (Harlan Laboratories, Madison, WI) *ad libitum*.

Blinding, allocation, group size, and endpoints. Technicians and investigators were blinded to the genetic background of the mice. Wild-type (Wt) and Tg mice are externally indistinguishable. Mice were randomly tagged with sequentially numbered ear tags at weaning and underwent experimental ischemic stroke when 8 – 10 weeks old. Male mice are reported in this manuscript; female mice were also studied but are not reported here. Animals housed in each cage (between three to six mice) underwent transient middle cerebral artery occlusion (tMCAO) on the same day. All cages housed Wt with Tg mice. There were four cohorts of animals: Wt, 24-hour survival (final n = 20); Tg, 24-hour survival (final n = 20); Wt, 72-hour survival (final n = 26); and Tg, 72-hour survival (final n = 23). Target cohort size of 20 was calculated based on a 15% difference in infarct volume 24 hours after tMCAO in Wt mice. Target cohort size was inflated by 20% to account for expected increased mortality by 72 hours.

tMCAO, sham tMCAO, and neurologic score. Mice were anesthetized with isoflurane (induction: 5%; maintenance: 1.5 – 2%) in oxygen (0.05L/min) and medical air (1L/min). After sterile preparation and draping, tMCAO was performed as previously described [5] with minor modifications. Briefly, the right common carotid artery (CCA) was exposed, and a 7-0 nylon suture tipped with dental adhesive was introduced into the CCA until laser Doppler signal (Periflux System 5000, Perimed, Inc., Ardmore, PA) from a probe positioned over the MCA territory dropped by 80%. During MCAO, rectal temperature was maintained between 36.5 and 37.5°C with a homeothermic system (Harvard Apparatus, Holliston, MA). After 30 min, the occluding thread was removed, and the neck was sutured. One hour after reperfusion, the neurologic score was determined: 0 = no deficit; 1 = twists when suspended by the tail; 1.5 = veers while walking; 2 = circles while walking; 3 = rolls; 4 = no movement. Euthanasia was performed 24 or 72 hours later. For sham tMCAO, the occluding thread was not advanced into the MCA.

Immunolabeling. 8µm-thick frozen sections at bregma 0.86 were fixed in 4% paraformaldehyde and labeled with anti-FLAG (F7425, Sigma-Aldrich Corp., St. Louis, MO) and anti-GFAP (ab5541, EMD Millipore, Billerica, MA) antibodies, per manufacturer's protocol. Cy3-conjugated (AP194C, Millipore) and Alexa Fluor 488-conjugated (A11008, Life Technologies, Grand Island, NY) secondary antibodies were used. Regions of interest (ROI), were digitally imaged (10× objective; BX41 microscope and DP72 digital camera, Olympus America Inc., Lombard, IL; Data in Brief) and FLAG-positive cells were counted per ROI and averaged for three tissue sections/animal.

Additional 8µm-thick sections were fixed with 10% buffered formalin and labeled with an anti-GFAP antibody (Z0334, Dako, Carpinteria, CA), 1:5, 000 followed by Alexa Fluor 488-conjugated secondary antibodies (A11001, Life Technologies). Sections were then counterstained with Hoechst 33258 (Sigma) and double-labeled for MAP2 (ab5392, Abcam, Cambridge, MA) followed by a Cy3-conjugated secondary (AP194C, Millipore).

Additional 8µm-thick sections were fixed with 4% paraformaldehyde and immunolabeled with anti-FLAG and anti-MAP2 (ab5392, Abcam) antibodies followed by the secondary antibodies described above.

Infarct volume and swelling. Additional 8µm-thick frozen sections from mouse brains at bregma levels 1.10, 0.86, 0.50, -0.70, -1.22, -1.94, -2.54, and -3.64 were stained with hematoxylin and eosin (HE) and digitally imaged (1.25× objective plus in-line 0.5× video adapter, Olympus). Metamorph Offline, v7.7 (Molecular Devices Corp., Sunnyvale, CA) was utilized to outline remnant (normal neuropil) ipsilateral areas and contralateral and ipsilateral hemispheres. Lesional volumes were calculated in mm³ and expressed as a percentage: (contralateral hemisphere volume – ipsilateral remnant volume) / contralateral hemisphere volume * 100%. Hemispheric swelling was calculated as: (ipsilateral hemisphere volume - contralateral hemisphere volume) / contralateral hemisphere volume * 100%.

Evans Blue content. Mice were anesthetized, and 4 µl/g of filter-sterilized 2% Evans Blue in normal saline was injected intravenously. Mice were euthanized two hours later; 25 ml of cold (4°C) phosphate-buffered saline (PBS) was systemically perfused via cardiac puncture. Brains were dissected into left and right hemispheres, and the olfactory bulbs and cerebella were removed. Hemispheres were homogenized in cold PBS (Omni Tissue Homogenizer, Omni International, Kennesaw, GA) and extracted with trichloroacetic acid (Sigma). Supernatants were assayed at 620 nm excitation/690 nm emission (Tecan Safire 2, Tecan Group Ltd., Männedorf, Switzerland).

qRT-PCR. Ten 8µm-thick frozen sections per mouse brain were collected at bregma levels 1.10 to -1.94. Infarcts were visible to the naked eye and infarcted tissue was scraped off the slides with a sterile microblade (#37-6100, Roboz Surgical Instrument Co., Inc., Gaithersburg, MD) and homogenized in 600 µl RLT lysis buffer (#74104, Qiagen, Boston, MA). Symmetrical contralateral (non-infarcted) tissue was similarly processed. Total RNA was isolated from homogenates using RNeasy Mini Kit (#74104, Qiagen,) per manufacturer's protocol. 100 ng of total RNA per sample was reverse-transcribed into

cDNA using Quantitect Reverse Transcription Kit (#205311, Qiagen). The cDNA was used for qRT-PCR using fluorogenic 5'-nuclease assay technology with probes and primers for the housekeeping gene peptidylprolyl isomerase A (PPIA; Integrated DNA Technologies, Coralville, IA) and TaqMan® Gene Expression Assay Reagents (#4331182, Life Technologies, Carlsbad, CA) for mouse VEGFa. Individual gene amplification was performed in duplicate with the CFX96 Touch Real-time PCR Detection System (185-5096, Biorad, Des Plaines, IL, USA) using Universal PCR Master Mix AmpliTaq Gold DNA Polymerase with Buffer II and MgCl₂ (N8080247_3620536626, Life Technologies). The threshold cycle, C_T, was determined using CFX Manager software v3.1 (Biorad). Statistical analysis. Data are presented as mean ± SD except where otherwise indicated. Student's *t*-test, Mann-Whitney rank sum test, Wilcoxon signed rank sum test, or Fisher's exact test were used.

RESULTS

The transient ischemic stroke model performs as expected in Tg mice. Cerebral vascular anatomy, operative physiology and mortality, infarct distribution and histology were not different between Wt and Tg mice (Data in Brief).

Ischemia-reperfusion augments HuR transgene expression in astrocytes. Ischemia-reperfusion affects endogenous RBPs and Hu antigens in Wt mice and rats (Data in Brief). For Hu antigens, we observed an overall attenuation in ischemic tissue, and a transition from nuclear predominance in the non-ischemic control tissue to cytoplasmic predominance in the injury core (Data in Brief). This cytoplasmic relocation may relate to a stress response or ischemia-related nuclear membrane permeability in dying cells. In contrast, KSRP, a predominantly nuclear RBP that regulates mRNA degradation [8], was only weakly detected in the borderzone and undetectable in the injury core (Data in Brief). Taken together, these data suggest that endogenous RBPs have different kinetics and/or translational patterns in response to ischemic injury.

At 72 hours after injury, an increase in GFAP immunoreactivity was observed at the infarct periphery (Figure 1 A – C) which is consistent with typical injury-mediated astrocyte activation [9]. In Tg mice, an enrichment of FLAG-positive astrocytes was observed 72 hours after ischemia (Figure 1 D, E; Figure 2). In ROIs selected in the contralateral hemisphere to match the location of perilesional ROIs (refer to Data in Brief for the schematic), the number of FLAG-positive cells after tMCAO was approximately doubled compared to intact animals (Figure 2 A, D). In ipsilateral ROIs, however, the number of FLAG-positive cells was approximately four times that of intact animals 24 hours, and five times that of intact animals 72 hours, after tMCAO (Figure 2 B, D). Tg HuR-expressing cells were similarly increased in the contralateral and ipsilateral subventricular zones (data not shown). No FLAG-positive cells were present in the infarct core (Figure 2 C). In summary, FLAG-HuR was delivered to regions where astrocytes (and the GFAP promoter) were activated after tMCAO.

Tg mice have worse neurologic outcomes. To determine whether the outcome from experimental ischemic stroke differed between Tg and Wt mice, neurologic function of each

mouse was evaluated longitudinally (i.e., post-operatively and at euthanasia). More Tg mice worsened or remained dysfunctional 72 hours after tMCAO than Wt mice ($p = 0.034$; Figure 3).

Swelling and vascular permeability are increased in Tg mice. To evaluate the pathophysiologic substrate of the difference in functional outcome between Tg and Wt mice, infarct volume and swelling were determined. There was a ~ 2-fold increase in cerebral swelling in Tg versus Wt mice at 72 hours (Figure 4 A, B), but there was no difference in infarct size (determined as a subtraction of uninjured tissue volume from intact hemisphere volume): 24h, $42 \pm 14\%$ (Wt) vs. $44 \pm 9\%$ (Tg), t -test, non-significant; 72h, $38 \pm 17\%$ (Wt) vs. $46 \pm 10\%$ (Tg), t -test, non-significant. The disproportionate increase in swelling suggested that the cause may have been an increase in vasogenic, rather than cytotoxic, edema, which was confirmed by a ~ 4-fold increase in vascular permeability in Tg mice 72 hours after injury (Figure 4 C). The rostro-caudal gradient of edema evolution differed between Wt and Tg animals. In Tg mice, rostral (bregma 1.10 to -0.70) as well as caudal regions (bregma -1.22 to -3.64) showed increasing edema, whereas in Wt mice, the increase in swelling was limited to rostral regions (Figure 4 D). To evaluate a potential molecular cause of increased vascular permeability, cerebral VEGFa mRNA levels were quantitated, but no transgene-related differences were detected at either survival time point (data not shown).

DISCUSSION

We showed that transgenic expression of HuR in perilesional astrocytes enhanced vasogenic cerebral edema in an ischemia/reperfusion mouse model of stroke. The significantly worse short-term functional outcome of HuR Tg mice is consistent with current thinking that vasogenic edema is a major contributor to post-ischemic morbidity [19]. The mechanism may be increased mass effect on adjacent uninjured tissue or otherwise disrupted perilesional neural function and/or repair.

Astrocytes play key roles in normal brain function [2, 31]. Upon central nervous system (CNS) injury, astrocytes act as first responders and become activated in injured tissue where they play different, and at times conflicting, roles [2, 29, 31]. On the one hand, astrocytes provide energy substrates to neurons, remove excitotoxins in the microenvironment, and promote recovery; on the other, once activated, they initiate an inflammatory response [9, 13, 31] which becomes amplified through the production of chemokines and recruitment of additional glial cells and immune cells [13, 35]. Following ischemia, activated astrocytes are present at the infarct borderzone within the first week, during the peak inflammatory response [9]. Since in our study the FLAG-HuR transgene is under the control of the GFAP promoter, the increase in FLAG-HuR immunoreactivity in perilesional regions likely resulted from astrocyte activation during typical injury evolution.

The rapid induction of inflammatory mediators in activated astrocytes reflects their remarkable plasticity in response to injury. The molecular response is facilitated by post-transcriptional gene regulation in conjunction with transcription [4]. A large number of inflammatory mediators increased in ischemic stroke is regulated post-transcriptionally

through AREs in the 3' UTR [4, 7, 11, 28, 32]. We have previously shown that many of these targets are positively regulated by HuR in astrocytes and microglia, including TNF- α , IL-6, IL-1 β and MMP-12 [22, 25]. In the acute phase, astrocyte activation can disrupt the blood-brain barrier and/or facilitate the development of edema through the secretion of these factors [2, 6, 13, 17, 31]. In the current study, we observed increased vascular permeability and hemispheric edema in HuR Tg mice, despite the lack of a transgene-mediated increase in infarct size. This correlative finding suggests that transgenic HuR in perilesional astrocytes disrupts vascular permeability through its regulation of downstream mediators such as the ones mentioned above. Interestingly, in a model of traumatic spinal cord injury in the same Tg mouse line, we also observed increased vascular permeability in HuR Tg mice compared to Wt littermates [22]. When the two studies are considered together, ectopic HuR appears to modulate common downstream pathways in two remarkably different CNS injury models [36].

More definitive proof of the role of HuR in vasogenic edema in ischemic stroke will require identification of specific HuR-regulated factors producing this effect, and demonstrating an attenuation of these factors and vasogenic edema after genetic deletion of astrocytic HuR. We did evaluate VEGFa, a key mediator of vascular permeability [6, 30] regulated by HuR and secreted by astrocytes [23, 27], but found no increase in VEGFa mRNA in Tg mouse brains. This does not exclude VEGF as a key mediator since the lack of the expected effect could be due to limitations in detecting mRNA changes in a small number of cells expressing the transgene or to HuR effects at the protein level through modulation of translational efficiency rather than RNA stabilization [7, 33]. This has been observed with HuR targets including cytochrome C, XIAP, and TDP-43 [14, 18, 33].

CONCLUSION

This study suggests that HuR expression in astrocytes can exacerbate vasogenic cerebral edema and worsen functional outcome in acute ischemic stroke. HuR very likely plays a broad role in cerebral responses to ischemia because of its ubiquitous presence. It is possible, for example, that an altered secretome produced by expression of HuR in astrocytes affects the function of Hu proteins in other components of the neurovascular unit such as endothelial cells or neurons. As recently reviewed, the landscape of post-transcriptional regulation becomes complex after cerebral ischemia, and the exact localization and activity of RBPs in different cell types may influence overall cell survival and post-stroke recovery [12] (Data in Brief). This complexity will come into play when determining whether HuR could be a therapeutic target in acute ischemic stroke.

Acknowledgments

This manuscript is dedicated to the memory of Piotr Kulesza, MD, PhD.

FUNDING

This work was supported by: 1) the American Heart Association [Grant-in-Aid 11GRNT7640017 (AAA)]; 2) the National Institutes of Health [NS064133, R21NS081743, R21NS085497 (PHK)]; 3) the Department of Veterans Affairs [Merit Review Award BX0011480, (PHK)]; 4) the University of Alabama at Birmingham NIH Neuroscience Blueprint Core Grant NS57098 (PHK); and 5) the University of Chicago Clinical and Translational Science Award UL1RR024999 (AAA).

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Highlights

- Transgenic expression of HuR in astrocytes worsens short-term stroke outcome
- Brains of transgenic mice with stroke exhibit more edema compared to littermates
- Cerebral vascular permeability is increased in transgenic mice with stroke

Figure 1.

Immunolabeling of glial acidic fibrillary protein (GFAP) and Map2 (a marker of healthy neuropil) in a Wt mouse (A – C), and GFAP/FLAG in a Tg mouse (D – E), 72 hours after tMCAO; Hoechst 33258 nuclear stain (blue); constant camera settings in each set of images. (A) Sparse GFAP (green) in tissue contralateral to the infarct. (B) Augmented GFAP and decreased Map2 (red) immunoreactivity in the perilesional region to the left of the dashed line. The infarct core is to the right of the dashed line. (C) Lack of GFAP and Map2 immunoreactivity in the infarct core. Scale bar = 40 μ m. (D) Scant GFAP expression with interspersed FLAG-positive cells in contralateral tissue. (E) Augmented GFAP expression and FLAG-positive cells in the perilesional region (arrows). Asterisk: infarct core. Insert: magnification of the FLAG-positive cells indicated by the right-most arrow. Scale bar = 100 μ m.

Figure 2.

Enrichment of HuR transgene-expressing cells in perilesional regions in Tg mice 72 hours after tMCAO. Images in panels A–C were obtained using the same camera settings. (A) Contralateral nuclear FLAG immunoreactivity (green; arrows); (B) Lesion borderzone with FLAG immunoreactivity (arrows); (C) Lesion core devoid of FLAG and Map2 immunoreactivity. Scale bar = 40 μ m. (D) FLAG counts in intact Tg mice (pre-tMCAO); Tg mice subjected to sham tMCAO; and Tg mice subjected to tMCAO with 24 or 72-hour survival, mean $\pm$ SEM, #p = 0.018, *p = 0.008, **p < 0.001 (Student's t-test).

Figure 3.

Worse neurologic outcomes in Tg mice. More Wt than Tg mice improved functionally (50 vs. 32%, respectively) in 72 hours, Wilcoxon signed rank sum test, $p = 0.034$.

Figure 4.

Augmented cerebral vasogenic edema in HuR Tg mice. (A) Increased hemispheric swelling in a Tg mouse compared to Wt mouse 72 hours after tMCAO, hematoxylin/eosin. Scale bar = 2 mm. (B) Swelling (percent difference between the ipsilateral and contralateral hemisphere volumes in histologic sections), mean $\pm$ SEM, * p = 0.001 (Student's t-test). (C) Vascular permeability (Evans Blue extravasation) 72 hours after tMCAO, mean $\pm$ SEM, $\wedge p$ < 0.05 (Student's t-test). (D) Rostro-caudal gradient in swelling (24-hour Wt value set to 1),

* $p < 0.05$ Tg 72h vs. Tg 24h, # $p < 0.05$ Wt 72h vs. Wt 24h, ^ $p < 0.05$ Tg vs. Wt, (Student's t-test).

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