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Selective Vasopressin-1a receptor antagonist prevents brain edema, reduces astrocytic cell swelling and GFAP, V1aR and AQP4 expression after focal traumatic brain injury

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Abstract

A secondary and often lethal consequence of traumatic brain injury is cellular edema that we posit is due to astrocytic swelling caused by transmembrane water fluxes augmented by vasopressin-regulated aquaporin-4 (AQP4). We therefore tested whether vasopressin 1a receptor (V1aR) inhibition would suppress astrocyte AQP4, reduce astrocytic edema, and thereby diminish TBI-induced edematous changes. V1aR inhibition by SR49059 significantly reduced brain edema after cortical contusion injury (CCI) in rat 5 h post-injury. Injured-hemisphere brain water content (n=6 animals/group) and astrocytic area (n=3/group) were significantly higher in CCI-vehicle (80.5±0.3%; 18.0±1.4 μm²) versus sham groups (78.3±0.1%; 9.5±0.9 μm²), and SR49059 blunted CCI-induced increases in brain edema (79.0±0.2%; 9.4±0.8 μm²). CCI significantly up-regulated GFAP, V1aR and AQP4 protein levels and SR49059 suppressed injury induced up regulation

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COMPETING FINANCIAL INTEREST

The authors declare no competing financial interests.

AUTHOR CONTRIBUTIONS

CRM was involved in the overall designs, direction of the projects and individual experiments, the data analysis and the writing of the full/final manuscript. XL conducted immunohistochemistry and confocal microscopy experiments and analysis. SCH assisted in confocal microscopy experiments, analysis and manuscript preparation. SP performed protein experiments. KT, NHA conducted experimental surgical procedures. KT performed brain water analysis. HFY reviewed and provided guidance for manuscript preparation. ASF and CMB assisted in data analysis and the writing of the final manuscript.

(n=6/group). In CCI-vehicle, sham and CCI-SR49059 groups, GFAP was 1.58 ± 0.04 , 0.47 ± 0.02 , and 0.81 ± 0.03 , respectively; V1aR was 1.00 ± 0.06 , 0.45 ± 0.05 , and 0.46 ± 0.09 ; and AQP4 was 2.03 ± 0.34 , 0.49 ± 0.04 , and 0.92 ± 0.22 . Confocal immunohistochemistry gave analogous results. In CCI-vehicle, sham and CCI-SR49059 groups, fluorescence intensity of GFAP was 349 ± 38 , 56 ± 5 , and 244 ± 30 , respectively, V1aR was 601 ± 71 , 117.8 ± 14 , and 390 ± 76 , and AQP4 was 818 ± 117 , 158 ± 5 , and 458 ± 55 (n=3/group). The results support that edema was predominantly cellular following CCI and documented that V1aR inhibition with SR49059 suppressed injury-induced up regulation of GFAP, V1aR and AQP4, blunting edematous changes. Our findings suggest V1aR inhibitors may be potential therapeutic tools to prevent cellular swelling and provide treatment for post-traumatic brain edema.

Keywords

Traumatic brain injury; Edema; Aquaporin 4; Vasopressin; Astrocyte; SR49059

1. Introduction

Traumatic brain injury (TBI) is the leading cause of death and disability in children and adults and results in more than 52,000 deaths per year (Coronado et al., 2011, Faul et al., 2007, Langlois et al., 2006). In TBI, post traumatic brain edema results from increased brain volume due to water uptake, and concomitant elevated intracranial pressure (ICP) leads to brain herniation and death or poor prognosis in survivors (Marmarou et al., 1994, 2006, Masson et al., 2001, Thurman and Guerrero, 1999). Current treatment is limited to either hyperosmotic therapy or surgical decompression (Diaz-Arrastia et al., 2014, Marmarou, 2007, Stein et al., 2010), as few specific pharmacological therapies are available.

Our previous work supports the hypothesis that brain edema is predominantly cellular and that astrocytic swelling, a characteristic response to injury, may be a target for drug treatment designed to reduce edema or improve edema clearance at the cellular level (Amorini et al., 2003, Fazzina et al., 2010, Ito et al., 1996, Kleindienst, et al., 2006b, 2013, Marmarou et al., 2006, Okuno et al., 2008, Taya et al., 2008, 2010). Astrocytic swelling following TBI was initially described by Klatzo as cytotoxic edema (Klatzo, 1987). Astrocytes are key players in cerebral water homeostasis and respond to injury with molecular and morphological changes described as astrogliosis. Up regulation of glial fibrillary acidic protein, (GFAP), a cytoskeletal structural intermediate protein expressed only in mature astrocytes, reflects a rapid response (<1 h) of astrocytes to injury and cellular edema (Eng et al., 2000, Eng and Ghirnikar, 1994, Vijayan et al., 1990). Following TBI, injury-induced astrogliosis measured by GFAP protein (Guo et al., 2006, Vijayan et al., 1990) or mRNA (Dietrich et al., 1999) analysis is correlated to the severity of experimental injury and clinically provides a cellular index of injury classified as mild, moderate or severe with scar formation (Bullock et al., 1991, Hamby and Sofroniew, 2010). Subsequent evidence highlighted the link between increased GFAP and concomitant elevation of AQP4 and V1a receptors (V1aR) (Verkman, 2009) that may respond to injury-induced discharge of the hormone arginine vasopressin (AVP). V1aR are the predominant AVP receptor subtype in the CNS and are widely distributed in neurons, cortical astrocytes, and endothelial cells

(Hernando et al., 2001, Landgraf, 1992, Ostrowski et al., 1994, Szmydynger-Chodobska et al., 2011, Szot et al., 1994). AVP is elevated in serum and cerebrospinal fluid (CSF) in humans following TBI (Huang et al., 2008, Kleindienst et al., 2010, Sorensen et al., 1985), and centrally released AVP may be instrumental in the development of brain edema (Cserr and Latzkovits, 1992, Landgraf, 1992). V1aR on astrocytes potentially facilitate the transport of water across astrocytic cell membranes providing a mechanism for cellular swelling and brain edema formation in TBI (Pascale et al., 2006, Szmydynger-Chodobska et al., 2004) (Latzkovits et al., 1993, Pascale et al., 2006, Szmydynger-Chodobska et al., 2011). Taken together, these data implicate AVP and the V1aR as potential mediators of cell swelling and subsequent fulminating edematous change following cerebral injury (Dickinson and Betz, 1992, Doczi et al., 1982, 1984, Latzkovits et al., 1993, Niermann et al., 2001, Raichle and Grubb, Jr., 1978, Vajda et al., 2001).

Experimental models of intracerebral hemorrhage, middle cerebral artery occlusion, ischemia, cold injury and TBI provide strong evidence that V1aR inhibition reduces brain edema (Bemana and Nagao, 1999, Kagawa et al., 1996, Kleindienst, et al., 2006b, Liu et al., 2010, Manaenko, A. et al., 2011a, Manaenko, A. et al., 2011b, Okuno et al., 2008, Rosenberg et al., 1992, Shuaib et al., 2002, Taya et al., 2008, Taya et al., 2010, Vakili et al., 2005), but the underlying mechanisms remain elusive. There is growing evidence that brain V1aR modulate the expression and function of AQP4 (Kleindienst, A. et al., 2006b, Liu et al., 2010, Niermann et al., 2001, Okuno et al., 2008, Taya et al., 2008, 2010). AQP4 is the predominant water channel in brain and plays a significant role in the pathophysiology of edema post-TBI (Amiry-Moghaddam and Ottersen, 2003, Amorini et al., 2003, Manley et al., 2000, Sun et al., 2003, Verkman, 2009).

Our laboratory has explored the hypothesis that selective V1aR inhibition by the non-peptide antagonist SR49059 (Serradeil-Le et al., 1993) can reduce brain edema. Our previous work demonstrated the efficacy of SR49059 to reduce brain edema in animal models of middle cerebral artery occlusion (MCA-O) and focal TBI (Filippidis et al., 2014, Kleindienst, et al., 2006b, Kleindienst et al., 2013, Okuno et al., 2008, Taya et al., 2008). The present study is the first to examine the pleiotropic effects of SR49059 on astrocytic swelling, GFAP, V1aR and AQP4 expression in focal TBI using biochemical and histological quantification. We found that infusion of SR49059 post-TBI reduced expression of V1aR, AQP4, and GFAP and reduced both astrocyte swelling and brain edema. These data suggest that V1aR inhibition by SR49059 may prove to be a promising therapeutic approach to prevent fulminating edematous change following TBI.

2. Results

2.1. Intraoperative physiology parameters

There were no significant differences in temperature, mean atrial blood pressure (mABP), arterial blood pH, pO₂, pCO₂, and plasma Na⁺ concentration among sham-vehicle, CCI-vehicle, and CCI-SR49059 groups. pH decreased slightly in the CCI-SR49059 group between baseline and 30 min post-injury (pH 7.34 ± 0.02 vs. 7.24 ± 0.02, pH = 0.10; P 0.05), but the difference was not sustained (Table 1).

2.2. Percent water content/brain edema

The regions analyzed in the injured and non-injured hemispheres for percent brain water content/brain edema and protein expression following CCI are illustrated in Figs. 1A and 1B. Percent brain water content (Fig. 2) of the right hemisphere (injured) was significantly higher in the CCI-vehicle treated group ($80.5 \pm 0.3\%$) than the sham operated group ($78.3 \pm 0.1\%$). Treatment with SR49059 significantly reduced water within the injured hemisphere ($79.0 \pm 0.2\%$; $P < 0.05$) to sham levels. In the left non-injured hemisphere, there were no significant brain water content differences between sham ($78.4 \pm 0.2\%$) compared to CCI-vehicle ($78.7 \pm 0.2\%$) and CCI-SR49059 groups ($78.0 \pm 0.2\%$). However, brain water content was significantly greater in the CCI-vehicle ($78.7 \pm 0.2\%$) than CCI-SR49059 group ($78.0 \pm 0.2\%$; $P < 0.05$) (Fig. 2).

2.3. Protein expression

GFAP, V1aR and AQP4 protein expression levels were assessed by Western blot in sham, CCI-vehicle and CCI-SR49059 groups and normalized to the expression of cyclophilin-A, which also served as a loading control in the non-injured (left, L) and injured (right, R) hemisphere (Figs. 3 A–F). Quantitative densitometry analyses confirmed an injury-induced up regulation of GFAP (Figs. 3 A and B), V1aR (Figs. 3C and D) and AQP4 (Figs. 3E and D) expression that was significantly reduced by administration of SR49059. GFAP was significantly higher in the injured hemisphere in the CCI-vehicle (1.58 ± 0.04) and CCI-SR49059 treated groups (0.81 ± 0.03) compared to the sham group (0.47 ± 0.02 ; $P < 0.05$), indicating that SR49059 treatment significantly reduced GFAP expression after CCI ($P < 0.05$). These data indicate a significant reduction in astrogliosis when a selective V1aR inhibitor is administered after trauma. In the non-injured hemisphere, GFAP levels were also significantly higher in the CCI-vehicle (0.65 ± 0.01) and CCI-SR49059 groups (0.60 ± 0.03) compared to the sham group (0.46 ± 0.01 ; $P < 0.05$). However, GFAP expression after CCI was not significantly altered on the uninjured side by SR49059 (Figs. 3A and B).

V1aR levels were significantly higher than sham (0.45 ± 0.05) only in the injured hemisphere of the CCI-vehicle group (1.00 ± 0.06 ; $P < 0.05$), and SR49059 treatment significantly suppressed injury-induced up regulation of V1aR (0.46 ± 0.09) restoring expression back to sham levels. In contrast, on the contralateral side, neither CCI-vehicle nor CCI-SR49059 groups were different than sham (Figs. 3C and D).

AQP4 expression also was higher in the injured hemisphere in the CCI-vehicle group (2.03 ± 0.34) than in sham (0.49 ± 0.04 ; $P < 0.05$), and AQP4 up regulation was significantly reduced in the CCI SR49059 group (0.92 ± 0.22 ; $P < 0.05$). This indicates that selective V1a receptor inhibition reduced AQP4 protein expression after trauma. On the contralateral hemisphere, AQP4 expression in CCI-vehicle and CCI-SR49059 groups were not distinguishable from sham (Figs. 3E and F).

2.4. Immunohistochemistry

2.4.1 Mean astrocytic area—As we illustrate in Figs. 1C, D and E, mean astrocytic area and relative fluorescence intensity was determined using images acquired from tissue sections from sham (Fig. 1C), CCI-vehicle (Fig. 1D) and CCI-SR49059 (Fig. 1E) using

methods similar to those reported by others (Bagheri et al., 2013, Carrasco et al., 2007, Fattore et al., 2002, Girardi et al., 2004). Compared to sham groups (Fig. 4A1), GFAP staining became darker and astrocytes became larger following CCI (Fig. 4A2). CCI caused a significant increase in astrocytic area ($18.0 \pm 1.5 \mu\text{m}^2$) compared to the sham group ($9.5 \pm 0.9 \mu\text{m}^2$; $P < 0.05$), and SR49059 treatment blunted the increase in astrocytic area ($9.4 \pm 0.8 \mu\text{m}^2$; $P < 0.05$; Figs. 4A3 and 4B).

2.4.2 Relative fluorescence intensity analysis of GFAP, V1aR and AQP4—The relative fluorescence intensity of GFAP, V1aR and AQP4 was quantitatively evaluated in the injured hemisphere sham (Figs. 4C1–4), CCI-vehicle (Figs. 4C5–8) and CCI-SR49059 treated (Figs. 4C9–12) animals that have undergone sham, CCI-vehicle or CCI-SR49059 (Figs. 4C–F). In the injured hemisphere, a significantly higher fluorescence intensity for GFAP was observed in the CCI-vehicle (348.5 ± 38.4) and CCI-SR4905 (244.3 ± 30.3) groups compared to sham group (55.7 ± 4.8 ; $P < 0.05$; Fig. 4D). Between the CCI groups, GFAP labeling intensity was significantly reduced in SR49059 versus vehicle-treated groups ($P < 0.05$, Fig. 4D) which confirmed quantitatively the observed increase in GFAP positive cortical astrocytes following CCI that was reduced following SR49059 treatment (Figs. 4C1–4). On the non-injured hemisphere, GFAP fluorescence intensity was lowest in the sham group (51 ± 4.3), highest in the CCI-vehicle group (98.7 ± 3.2), and the CCI-induced response was significantly diminished by SR49059 treatment (85.6 ± 1.7 , $P < 0.05$, Fig. 2C). Thus, as judged by GFAP labeling intensity, treatment with SR 49059 reduced cellular swelling on both the non-injured and injured sides of the brain after TBI.

The spatial distribution of V1aR fluorescence intensity showed a parallel up-regulation of V1aR which was reduced following treatment with SR49059 (Figs. 2C2, 6, 10 & 2E). There appeared to be a marked up-regulation of V1aR expression in brain cortex in CCI injured animals (Fig. 4C6) compared to sham animals (Fig. 4C1) which was reduced following treatment with SR49049 (Fig. 4C10). These observations were confirmed quantitatively, where in both the CCI-vehicle group (601.2 ± 70.6) and CCI-SR49059 treated group (390.3 ± 75.7) there was a significant higher fluorescence intensity of V1aR labeling comparing to sham animals (117.6 ± 13.5 , $P < 0.05$; Fig. 4E). V1aR labeling intensity in the injured hemisphere of CCI-SR49059 group compared to CCI vehicle treated group was significantly reduced ($P < 0.05$; Fig. 4E). On the contralateral side, CCI also significantly increased V1aR intensity compared to sham (101.1 ± 14.4). Moreover, V1aR up regulation was reduced by SR49059 treatment (161.6 ± 6.4) of CCI animals as compared to vehicle treatment (205.6 ± 9.5 , $P < 0.05$). However, the V1aR labeling was lower on the uninjured hemisphere than in the injured hemisphere ($P < 0.05$, Fig. 4E).

The spatial distribution of AQP4 fluorescence intensity appeared to increase in the CCI-vehicle group compared to sham, which was down regulated with SR49059 administration (Fig. 4C 3, 7, 11). Quantitative fluorescence intensity analysis of AQP4 expression confirmed these observations (Fig. 4F). In the injured hemisphere, there was elevated AQP4 intensity in the CCI-vehicle (818.4 ± 117.3) and CCI-SR49059 (457.8 ± 55.1) groups compared to sham (158.3 ± 5.5 , $P < 0.05$). Between the CCI groups, there was a significant reduction of AQP4 labeling intensity in the injured hemisphere of the animals that were treated with SR49059 compared to CCI-vehicle animals ($P < 0.05$). In the un-injured

hemisphere, AQP4 fluorescence intensity in the CCI vehicle treated group (221.1 ± 13.2) was significantly higher than in sham animals (127.2 ± 0.9 , $P < 0.05$) but indistinguishable from the CCI-SR49059 group (150.3 ± 14.2).

2.4.3 Evaluation of distribution and co-localization of GFAP, V1A and AQP4—

Parallel, qualitative evaluation of triple label labeling immunofluorescence for GFAP, V1aR and AQP4 in sham (Figs. 5 A–D), CCI-vehicle (Figs. 5 E–H) and CCI-SR49059-treated animals (Figs. 5 I–L) demonstrate the co-localization of V1aR and AQP4 in GFAP positive astrocytic cell processes and cell bodies within the cortex adjacent to a blood vessel (Figs. 5 D, H, L). Compared to sham (Figs. 5 A–D), CCI appeared to increase GFAP (Fig. 5 A and E), V1aR (Figs. 5 B and F) and AQP4 (Figs. 5 C and G). The astrocytic processes and cell bodies of GFAP positive astrocytes were seen to co-localize with V1aR and AQP4 (Figs. 5 D, H, L). Compared to sham, astrocytic cell bodies are enlarged following CCI and astrocytic end-feet embracing the blood vessel become thicker and more prominent (Figs. 5 A and E) while a greater, more punctate appearance of V1aR and AQP4 distribution was observed at the astrocytic end-foot, endothelium interface adjacent to the blood vessel (Fig. 5 D and H). Compared to CCI (Fig. 5H) inhibition of V1aR with SR49059 seemed to reduce the pronounced pattern of GFAP positive astrocytic swelling, decrease GFAP, V1aR and AQP4 co-localization as the injury-induced punctate association of AQP4 and V1A within the GFAP positive astrocytic end-feet was more dispersed with SR49059 treatment (Fig. 5 L).

Discussion

The results from the current communication extend our previous observations (Amorini et al., 2003, Fazzina et al., 2010, Filippidis et al., 2014, Kleindienst, A. et al., 2006a, Kleindienst, A. et al., 2006b, Kleindienst et al., 2010, Kleindienst et al., 2013, Okuno et al., 2008, Taya et al., 2008, Taya et al., 2010) as well as others (Bemana and Nagao, 1999, Doczi et al., 1982, Doczi et al., 1984, Fazzina et al., 2010, Filippidis et al., 2014, Guo et al., 2006, Liu et al., 2010, Manaenko, et al., 2011b, Okuno et al., 2008, Rauen et al., 2013, Serradeil-Le et al., 1993, Shuaib et al., 2002, Szmydynger-Chodobska et al., 2004, Trabold et al., 2008, Vakili et al., 2005) as we continue to identify the mechanisms of post-traumatic cellular swelling and explore potential therapies to blunt post-traumatic edema. Based on the hypothesis that edema in TBI is predominately cellular in origin, the goal of this work was to elucidate injury-induced changes in astrocytes that we posit are the underlying mechanisms of cellular swelling and subsequent post-traumatic edema. Our study investigates the efficacy of a selective vasopressin (ADH) receptor, V1aR SR49059, to reduce brain edema in a rat model of focal TBI at 5 h post-injury (Fig. 1). Our results demonstrate that SR49059 decreased brain edema (Fig. 2) and diminished TBI-induced up regulation of key astrocytic proteins, GFAP, V1aR and AQP4 (Figs. 3 & 4). Co-localization of V1aR and AQP4 were noted within astrocytic cell bodies and at their interface with blood vessels within their end-feet (Figs. 4 & 5). In the uninjured hemisphere, TBI elicited smaller but significant increases in GFAP, V1aR, AQP4 and astrocyte area that also were suppressed by SR49059. Taken together, these data suggest that the activation of V1aR in injured brain by AVP following CCI is instrumental in triggering the molecular and cellular

changes in astrocytes that lead to the evolution of brain edema. The results of this study support V1a inhibition with SR49059, as a promising treatment for targeting astrocytic swelling and post-traumatic edema.

Our work confirms the notion that edema in TBI is predominantly cellular in origin (Ito et al., 1996, Marmarou et al., 2006, 2007), and astrocytic cell swelling is a key component (Bullock et al., 1991, Floyd and Lyeth, 2007). The observed injury-induced increase in GFAP protein (Fig. 3), astrocytic area (Figs. 4 A & B) and fluorescence intensity (Fig. 4 C–F) are similar to reports in analogous TBI models (Dietrich et al., 1999, Guo et al., 2006, Vijayan et al., 1990). Further, as we show a significant increase in mean astrocytic area (Fig 3A, B), coupled with an increase in brain water (Fig. 2), we argue that CCI produces a robust effect in astrocytes that is related to up regulation of AQP4 and V1aR channels in response to AVP discharge and V1aR activation following TBI. The proposed scenario is schematically depicted in Fig. 6.

AVP is a hormone that regulates cerebral water homeostasis and ionic movement (Cserr and Latzkovits, 1992, Landgraf, 1992, Thibonnier et al., 1998). AVP modifies brain water permeability and induces edema and water flux after central administration (Doczi et al., 1982, Raichle and Grubb, Jr., 1978, Vajda et al., 2001), in models of subarachnoid hemorrhage (Doczi et al., 1982) and ischemia (Dickinson and Betz, 1992), and in brain slice preparations (Niermann et al., 2001). Moreover, AVP is elevated in serum and CSF of TBI patients (Huang et al., 2008, Kleindienst et al., 2010, Sorensen et al., 1985) and animal models (Pascale et al., 2006, Szmydynger-Chodobska et al., 2004, 2011). AVP may influence brain water and brain volume homeostasis by increasing the permeability of the BBB via modulation of V1a receptors thereby regulating the cell volume of neurons and astrocytes (Vakili et al., 2005). AVP administration increases glial cell volume in cultured astrocytes which can be blocked by V1aR antagonists as well as the Na-K-Cl co-transporter inhibitor bumetanide (Latzkovits et al., 1993). Further, OPC-21268, a non-peptide V1aR antagonist, reduced brain tissue water and electrolytes following cold lesion brain injury (Bemana and Nagao, 1999). Recent novel studies using V1aR deficient rats (Szmydynger-Chodobska et al., 2010) and mice (Rauen et al., 2013) demonstrate the importance of this receptor following CCI; in V1aR deficient animals there was no astrocytic production of inflammatory chemokines or edematous change. These findings add an additional mechanistic context to our results showing a decrease in V1aR following treatment with SR49059 (Figs. 2, 3C, 4C & E).

A dramatic TBI-induced increase in expression and co-localization of V1aR and GFAP in astrocytes along with up regulation of V1aR mRNA was previously reported (Pascale et al., 2006, Szmydynger-Chodobska et al., 2004) and confirmed in our work using similar multiple label immunohistochemistry (IHC) (Figs. 4C, D, E & 5) to identify the cellular localization and injury-induced up regulation of GFAP and V1aR within astrocytes and at the BBB interface (Fig 5) that were suppressed by V1aR inhibition (Fig 4s C, D, E). Blockade of AVP using SR49059 may prove to be a viable clinical strategy for reducing brain edema, possibly by limiting the pool of receptors activated by AVP, as seen in Fig. 4C, 2, 6, 10, or alternatively, by re-establishing the ionic homeostasis and balance of intra and extracellular cations.

In this respect, AVP may mediate the expression, cellular reorganizing or allosteric modulation of AQP4 proteins (Lo et al., 2013) that work synergistically with V1aR (Szmydynger-Chodobska et al., 2004). AQP4 is the predominant water channel in the brain, mainly located at the astrocytic end-feet surrounding the BBB at the glia limitans, the ependyma and osmosensory areas (Amiry-Moghaddam and Ottersen, 2003, Nielsen et al., 1997).

The presence of AQP4 in brain or astrocytic cultures is important for the evolution of cellular edema, whereas AQP4 absence confers protection (Haj-Yasein et al., 2011, Manley et al., 2000, Solenov et al., 2004). Importantly, AQP4-deficient astrocytic cell lines exhibit a sevenfold reduction in osmotic water permeability (Haj-Yasein et al., 2011, Solenov et al., 2004).

It is well known that AQP4 expression increases after TBI in animal (Amorini et al., 2003, Neal et al., 2007, Taya et al., 2008, 2010), in cell culture models (Rao et al., 2011), and in humans (Suzuki et al., 2006), and these observations were confirmed in the present study (Figs. 3 & 4). AQP4 distribution in cortex can either be associated with the astrocytic end-feet intimately located within the glial limitans, at the interface of the vasculature, or dispersed within the cell membrane of the cell body (Nielsen et al., 1997). In our study, post-traumatic AQP4 expression was pronounced at the astrocytic end-feet surrounding cortical vessels, at the blood-brain-barrier interfacing blood vessels as well as within the cell bodies (Fig. 5). SR49059 reduced both AQP4 up-regulation and brain water content back to baseline levels (Figs. 2, 3 & 4) suggesting that the reduction of brain water content could be also attributed, at least in part, to less water crossing the BBB of the injured hemisphere through the endothelium and the AQP4 bearing astrocytic end-feet. As a result, we posit treatment with SR49059 reduces water flux and therefore suppresses astrocytic swelling identified by increases in GFAP and astrocytic area (Fig. 2A).

The interaction between vasopressin, V1aR inhibition and AQP4 has been studied in models of ischemia (Kleindienst, A. et al., 2006b, Liu et al., 2010, Szmydynger-Chodobska et al., 2004), intracerebral hemorrhage (Manaenko, A. et al., 2011a, Manaenko, A. et al., 2011b), brain slices (Niermann et al., 2001), and TBI (Kleindienst et al., 2013, Taya et al., 2008). The results indicate that V1aR inhibition reduces AQP4 expression and water flux in the brain (Fazzina et al., 2010, Kleindienst, et al., 2006b, Okuno et al., 2008, Zelenina et al., 2002), but the detailed mechanism of this interplay remains elusive.

Obviously, cellular swelling requires an osmotic gradient, and AQP4 expression and/or inhibition of V1aR may alter the normal gradient thus creating conditions favoring increased intracellular water. This hypothesis was recently supported by our work using ion selective electrodes to measure, in-situ, changes in brain extracellular Na^+ and K^+ and the neuroprotection afforded by SR49059 (Filippidis et al., 2014). Our observations suggest that CCI results in an increase in cellular Na^+ uptake relative to K^+ loss which creates an osmotic gradient and results in increased intracranial pressure (ICP). In this study, ICP remained elevated for 5 h despite the recovery of extracellular cations, which may suggest early cation changes create a favorable condition for the evolution of edema that, when addressed early, can blunt the increase in edema. This premise is supported by our current work, where an

injury induced change in astrocytic cell area using morphometric analysis of GFAP (Figs. 4A & B) as well as protein and cellular up-regulation of GFAP, AQP4 and V1aR (Fig 3, Figs 4C–F) would indicate water influx into astrocytes consistent with the observed increased brain water content (Fig. 2). Although water flux into astrocytes was not directly measured, work by Nierman and colleagues suggest that the rapid onset and high capacity of water flux is indeed mediated through AQP4-containing astrocytes (Niermann et al., 2001) lending support to our conclusion that the increase in brain edema was due to an influx of water into the astrocyte. Although, this premise would warrant additional studies, what is clear is that the synergistic interplay of V1aR, GFAP and AQP4 are the mechanistic underpinnings of increased cellular edema.

In sum, the present study confirms that selective V1aR inhibition via SR49059, ameliorates astrocytic swelling and reduces the expression of GFAP, V1aR and AQP4 after focal brain injury. The present data support the concept that manipulation of glial cell properties may emerge as a viable strategy for the prevention of edema following TBI and highlights the potential of V1aR inhibitors as tools for reducing astrocytic swelling and concomitant brain edema in future clinical studies.

4. Experimental procedures

4.1 Animal preparation

Experimental, surgical and post-operative procedures were approved by the Virginia Commonwealth University Institutional Animal Care and Use Committee (IACUC) in compliance with National Institute of Health (NIH) Guide for the Care and Use of Laboratory Animals, 8th Ed. (National Academies Press, Washington, DC, 2011).

4.2. Controlled cortical impact injury and surgical procedures

A well-established controlled cortical impact injury model (Dixon et al., 1987) was used to produce TBI as previously described by our laboratory (Taya et al., 2008, Taya et al., 2010). Briefly, rats were initially anesthetized with isoflurane (4.5%), intubated, and mechanically ventilated with a gas mixture of N₂O (66%), O₂ (32%) and isoflurane (1.5–2.0%). Rectal temperature was maintained at 37.0 ± 0.5 °C using a self-adjusting heating pad. Catheters were placed into the femoral artery and vein. Mean arterial blood pressure (mABP) was monitored continuously using a data acquisition system (ADInstruments, Colorado Springs, CO), and arterial blood gas parameters (pH, pO₂, pCO₂ and plasma Na⁺ concentration) were obtained at 15-minute intervals (ABL800 Flex, Radiometer America, OH). The femoral vein was used to administer vehicle, dimethyl sulfoxide (DMSO), or drug (SR49059) treatment.

Animals were secured in a stereotactic frame (David Kopf, Tujunga, CA), a midline scalp incision was made, and the skin and periosteum retracted from the skull surface. A 10-mm-diameter craniotomy was made midway between Bregma and Lambda on the right side, 1-mm lateral to the midline. Injury was produced using a pneumatic impactor (5 mm diam.) mounted at an angle of 10° from the vertical plane. A single impact (velocity, 6 m/s; deformation depth, 3.0 mm; dwell time, 0.3 s) was delivered to the right parietal cortex. After injury, the excised skull section was replaced, sealed with bone wax and the skin

incision was closed. Sham operated animals were exposed to identical surgical procedures without CCI.

4.3. Experimental protocol and drug delivery

A total of 54 adult male Sprague-Dawley rats (350 to 400 g) were randomly assigned to the sham-operated (n=18), CCI-vehicle (n=18), or CCI-SR49059 (n=18) group and arbitrarily designated for brain water analysis (n=6/per group), protein analysis (n=6/per group), or IHC studies (n=6/per group). SR49059 (Sanofi Recherche, Montpellier, France), 2.76 mg/kg dissolved in 1.5% DMSO (Sigma-Aldrich, St Louis, MO) in normal saline (volume, 4.8 ml; concentration, 82.6 mM), was delivered IV by an infusion pump immediately after CCI at 960 μ L/h for 5 h. In the vehicle group, only 1.5% DMSO in normal saline was infused using the same protocol. Five hours post CCI or sham surgery, animals were sacrificed by an overdose of isoflurane (5%) and either decapitated for brain water content and protein analysis or subsequently perfused with 4% paraformaldehyde for IHC studies.

4.4. Brain water content measurement

For brain water content (n=6/group), brains were harvested, the most rostral and caudal areas and cerebellum were discarded, and the injured and uninjured hemispheres were separated. Percent brain water content was determined by the wet-to-dry weight method and was calculated as: % *brain water content* = $[(\text{wet weight} - \text{dry weight}) / \text{wet weight}] \times 100$, as previously described by our laboratory using the same TBI model (Taya et al., 2008, Taya et al., 2010).

4.5 Protein analysis

4.5.1 Tissue preparation—For GFAP, V1aR and AQP4 protein analysis (n=6/group), brains were harvested excluding the most rostral and caudal areas and cerebellum, and the injured and uninjured hemispheres were separated, homogenized on ice in radioimmunoprecipitation (RIPA) buffer containing proteolysis inhibitors, centrifuged at 13,500 g (30 min, 4°C), and the supernatants were frozen at –80 °C until use. Protein concentrations were assayed (Bio-Rad, Hercules, CA), whole-lysate samples were adjusted to 0.6 μ g protein/ μ L in sample buffer (Invitrogen, Carlsbad, CA) and then heated at 70°C for 10 min.

4.5.2. Immunoblotting and densitometric analysis—Immunoblotting of AQP4 (total protein, 15 μ g), V1aR (30 μ g), GFAP (10 μ g), and cyclophilin A (loading control) and densitometry followed procedures previously described (Taya et al., 2008, Taya et al., 2010). Briefly, total protein from each sample underwent electrophoresis into 4–12% Bis-Tris polyacrylamide gels (Invitrogen), then was transferred to a nitrocellulose membrane (Invitrogen), and blocked for 2 h in Tris-buffered saline plus Tween-20 (TBS-T) with 10% instant non-fat dried milk. The membrane was then incubated overnight at 4°C in the same buffer with primary antibody using either: (1) mouse monoclonal anti-glial fibrillary acidic protein (GFAP) antibody (1:5,000; Sigma-Aldrich); (2) rabbit polyclonal anti-V1a receptor antibody (1:500; Alpha Diagnostics, San Antonio, TX); or (3) mouse monoclonal anti-AQP4 antibody (1:500; Abcam, Cambridge, MA). The following day, membranes were washed, blocked for 30 min, and incubated for 1 h in appropriate secondary antibodies: (1) goat anti-

mouse IgG-HRP (1:10,000; Santa Cruz Biotechnology, Santa Cruz, CA) for GFAP; (2) goat anti-rabbit IgG-HRP (1:500; Santa Cruz Biotechnology) for V1aR; or (3) goat anti-mouse IgG-HRP (1:5,000; Rockland Biotechnology, Rockland, MA) for AQP4. Immunodetection utilized an ECL system exposed to X-ray film for 5 min. The following day, membranes were stripped, blocked for 30 min at room temperature in Tris-buffered saline plus TBS-T with 3% instant non-fat dried milk, and incubated for 2.5 h with rabbit polyclonal anti-cyclophilin A antibody (1:5,000, Millipore, Billerica, MA). Membranes were then washed in TBS (3-times, 10 min) and incubated for 1 h in secondary antibodies using goat anti-rabbit IgG-HRP (1:5,000; Rockland). Following extensive washing, immunodetection of cyclophilin-A was accomplished using the same ECL system described above.

GFAP, V1aR or AQP4 protein levels relative to those of cyclophilin-A (loading control) were quantified by densitometry (Quantity One software and VersaDoc Imaging System; BioRad) as previously described (Taya et al., 2010).

4.6. Immunohistochemistry studies—IHC was used to assess astrocytic area via light microscopy or distribution, co-localization and fluorescence intensity of GFAP, V1aR and AQP4 by confocal microscopy (n=3 animals/group for each method). For both approaches, 40- μ m coronal sections were collected from -0.36 mm to -2.40 mm posterior to Bregma (Paxinos and Watson, 2007), and processed for routine IHC using single label GFAP staining for light microscopy or triple label fluorescence IHC using GFAP, V1aR and AQP4.

4.6.1 Mean astrocytic area analysis—Astrocytes were identified with GFAP, and their area was assessed as an index of cellular swelling. After blocking endogenous peroxidase activity (0.3% H_2O_2 in PBS, 30 min), sections were pre-incubated (1% normal goat serum (NGS)/PBS, 0.2% Triton X, 60 min) and then incubated overnight using mouse anti-GFAP antibody (1:1000; Sigma-Aldrich) in 1% NGS/PBS. The next day, sections were washed, incubated with biotinylated anti-mouse IgG (1:100 in 1% NGS/PBS, 60 min), treated with avidin-biotin peroxidase complex (1:200, 60 min; Vector, Burlingame, CA), processed for visualization using 0.05% diaminobenzidine (DAB; Sigma-Aldrich), 0.01% hydrogen peroxide, 0.3% imidazole in 0.1 M PBS, dehydrated and mounted.

Sections were viewed at 20 $\times$ using a Nikon Eclipse 800 microscope, and images were digitally captured by a Spot-RT camera (Diagnostic Instruments, Sterling Heights, MI) and saved (pixel, 0.365 μ m square). For quantitative analysis, 3 sections/animal were selected, corresponding to -0.36 mm Bregma (level of anterior commissure), -1.08 mm Bregma (level of lateral and dorsal 3rd ventricle; foramen Monro), and -2.40 mm Bregma (level of ventricles). Within each section, 6 images were acquired within the peri-contusional cortex, (3 within the uninjured hemisphere, and 3 within the injured hemisphere); this yielded 54 images per group a sum total of 162 images. After conversion of the images to an 8-bit grey scale, the outlines of 10 astrocytes per image were traced (540 astrocytes/group), by an investigator blinded to group designation, and their area (μ m²) was calculated using ImageJ (NIH, Bethesda, MD) and reported as the mean astrocytic area per hemisphere/per group.

4.6.2 Triple-label fluorescence immunohistochemistry—Triple-label fluorescence IHC studies were conducted from tissue harvested from a separate set of animals (n=3/group). After preparation and sectioning as described above, sections were collected in semi-sequential order into 6 wells, ensuring homologous subsets. Briefly, free floating sections were washed and blocked for 60 min. To determine co-localization of GFAP, V1aR and AQP4, sections were incubated in combinations of polyclonal chicken anti-GFAP (1:7,000; Millipore), polyclonal rabbit anti-V1aR (1:150 Santa Cruz Biotechnology) and monoclonal mouse anti-AQP4 (1:200, Santa Cruz Biotechnology) antibodies at 4°C overnight. The next day, the sections were washed and incubated with the appropriate secondary antibodies for 2 h [Alexa Fluor 488 goat anti-chicken (1:400), Alexa Fluor 568 goat anti-rabbit (1:400), and Alexa Fluor 647 goat anti-mouse (1:400); Invitrogen], and mounted after extensive washings. Multiple controls were performed to confirm the specificity of the primary GFAP, V1aR and AQP4 antibodies. First, to obviate auto-fluorescence, sections were collected, mounted and examined without any further processing. Second, to determine the affinity of the antibody for its target antigen, sections from each animal were processed without primary antibody (GFAP, V1aR, AQP4) or with a pre-absorbed antigen/antibody solution. Finally, to rule out cross-talk between fluorophores and identify any spectral bleed-over, sections were prepared using each label individually and compared to dual- and triple-labeled scenarios.

Sections were examined using a Zeiss LSM 510 NLO META confocal microscope. The 488, 543 and 633 nm laser lines were used for illumination of Alexa Fluor 488 goat anti-chicken IgG (GFAP), Alexa Fluor 568 goat anti-rabbit IgG (V1aR), and Alexa Fluor 647 goat anti-mouse IgG (AQP4), respectively. To avoid signal cross-talk, images were collected by sequential illumination (i.e., one laser line per channel) using a 63x/1.4 n.a. oil immersion lens and were generated with the scan resolution set to meet Nyquist sampling criteria. The emission filters were BP 505–530 for Alexa Fluor 488 goat anti-chicken IgG, BP 560–615 for Alexa Fluor 568 goat anti-rabbit IgG, and META 645–752 for Alexa Fluor 647 goat anti-mouse IgG. Appropriate detector gain and offset settings were established on control tissues stained with secondary antibodies alone to account for any possible auto-fluorescence and endogenous back-ground staining. Detector and acousto-optic tunable filter settings were normalized with uniform intensity fluorescent beads (Invitrogen) to account for system variation between experiments.

For quantitative analysis, 3 sections/animal were selected, corresponding to –0.36 mm Bregma, –1.08 mm Bregma, and –2.40 mm Bregma and 6 images were acquired within the peri-contusional cortex (3 within the uninjured hemisphere, and 3 within the injured hemisphere), yielded a total of 164 images a total of 54 images per group. Qualitative analysis of fluorescence intensity was accomplished with ImageJ by and investigator blinded to the designated experimental group. Average fluorescence intensity levels for each channel were measured (12-bit scale) and averaged for each series of images and the mean fluorescence intensity level of GFAP, V1aR or AQP4 was reported for each experimental group.

4.7. Statistical analysis

Data are expressed as means $\pm$ SEM. Physiological assessments were analyzed using one-way ANOVA comparing baselines with post-trauma measurements in different groups during the predesigned monitoring period (baseline to 1 h post-trauma; see Table 1). Percent brain water, western blot densitometry, astrocytic cell volume and fluorescence intensity results were assessed using one-way ANOVA followed by Tukey's post hoc test. Differences were regarded as statistically significant at $P < 0.05$. Analysis was performed using SPSS (Chicago, IL), SAS (Cary, NC) and Graphpad Prism (La Jolla, CA) statistical software.

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Highlights

- Post traumatic brain edema is predominantly cellular following rat focal injury.
- TBI causes astrocytic swelling and concomitantly increases AQP4 expression.
- Edema is modulated by vasopressin V1aR signaling and AQP4 expression.
- Treatment with SR49059 reduces AQP4 and V1aR and blunts edematous brain swelling.
- We conclude that post traumatic edema can be modulated pharmacologically.

Fig. 1.

Superior (A) and lateral (B) views of the gross brain demonstrating the regions of non-injured and contused, injured hemispheres analyzed following CCI at 5 h post-injury for percent water content/brain edema using wet/dry methods or AQP4, GFAP and V1aR protein expression using western blot (A, B). For quantitative microscopy, within each brain, three sections corresponding to stereotactic co-ordinates -0.36 , 1.08 and 2.40 mm Bregma (Paxinos and Watson, 2007) were harvested from sham operated (C), CCI-vehicle (D), or CCI-SR49059 (E) groups. Note that -0.36 mm Bregma was used to illustrate that

within each section, six images per section were acquired within the non-injured and injured cortical regions (C, D, E, boxes). For astrocytic cell area measurements, 10 astrocytes within each image were measured by tracing the outline of the GFAP positive cell to determine the area within each cell using Image J software. A Zeiss LSM 510 confocal/multiphoton laser scanning microscope was used to acquire and collect triple label fluorescent images and fluorescent intensity for AQP4, GFAP and V1A reactivity was assessed using Image J software.

Fig. 2.

Selective V1aR inhibition significantly reduced percent water content after CCI at 5 h post-injury. Brain water content, which reflects brain edema, was assessed in sham-operated, CCI-vehicle and CCI-SR49059 treated experimental groups. Brain water content in the injured hemispheres of CCI-vehicle and CCI-SR49059 groups was significantly greater than in sham ($P < 0.05$), and SR49059 significantly reduced brain water content after CCI ($P < 0.05$). In the uninjured hemispheres, there was a significant increase in percent brain water content in the CCI-vehicle than the CCI-SR49059 group ($P < 0.05$). Data are expressed as mean $\pm$ standard error of the mean (SEM) and were analyzed by one-way ANOVA followed by Tukey's post hoc tests.

Fig. 3.

Western blot analysis of protein expression of GFAP (A, B), AQP4 (C, D) and V1aR (E, F) in non-injured (L) and injured right (R) hemispheres in sham, CCI-vehicle and CCI-SR49059 treated groups at 5 h post-injury. Cyclophilin-A was an internal control for lane loading and densitometry. Compared to sham, GFAP protein levels (A, B) were significantly increased in the CCI-vehicle groups in the injured hemispheres, and SR49059 significantly reduced GFAP expression in the CCI-vehicle treated group compared to the CCI-SR49059 group (B, $P < 0.05$). On the non-injured hemispheres, GFAP levels were significantly higher in the CCI-vehicle than sham group (B, $P < 0.05$). V1aR expression (C, D) was significantly increased in the CCI-vehicle group compared to sham and CCI-SR49059 groups (D, $P < 0.05$). V1aR protein expression was not significantly affected in the uninjured hemispheres (D). AQP4 protein expression (E, F) in the injured hemisphere was significantly increased in the CCI-vehicle group compared to both sham and SR49059-treated groups (F, $P < 0.05$). Although SR49059 treatment reduced AQP4 expression, it remained greater than in sham (F, $P < 0.05$). On the contralateral side, AQP4 expression was unaffected (F).

Fig. 4. SR49059 reduces astrocytic area (A, B) and fluorescence intensity of GFAP, V1aR and AQP4 (C–F) following CCI at 5 h post-injury. Compared to sham (A1), GFAP staining following CCI (A2) was markedly darker, and astrocytes became visible larger; both effects were attenuated by SR49059 treatment (A3, B). Mean astrocyte area was 9.52 ± 0.87 , 18.03 ± 1.47 , and $9.40 \pm 0.40 \mu\text{m}^2$ in sham, CCI-vehicle (CCI-veh), and CCI-SR49059 (CCI-SR) groups, respectively, in the injured hemisphere ($P < 0.05$; B, black bar), and analogous responses were observed in the uninjured hemisphere ($P < 0.05$; B, gray bar). GFAP (C1, C5, C9; green; D), V1aR (C2, C6, C10; red; E) and AQP4 (C3, C7, C11; blue, F) fluorescence intensity in representative sections from sham (C1–C4), CCI-vehicle (C5–C8) and CCI-SR49059 (C9–C12) groups showed co-localization of AQP4 and V1aR within GFAP positive astrocytes (merged; C4, C8, C12), and quantitative summary data are shown (D–F). In the injured hemisphere, GFAP was markedly increased following CCI (C5; D, 348.5 ± 38.4) compared to sham (C1; D, 55.7 ± 4.8), and CCI-induced increase in GFAP was reduced after treatment with SR49059 (C9; D, 244.3 ± 30.3) ($P < 0.05$). V1aR fluorescence intensity also was markedly increased following CCI (C6, E, 601.2 ± 70.6) compared to sham (C2, E, 117.6 ± 13.5) and V1aR up regulation was suppressed in the CCI-SR49059

group (C10, E, 390.3 ± 75.7) ($P < 0.05$). AQP4 intensity displayed a similar pattern; AQP4 fluorescence was significantly augmented in the CCI-vehicle group (C7, F, 818.4 ± 117.3) compared to sham (C3, F, 158.3 ± 5.5) and was significantly reduced in the CCI-SR49059 group (C11, F, 457.8 ± 55.1) ($P < 0.05$). Very modest but statistically significant increases in GFAP, V1aR, and AQP4 fluorescence also were detectable in the contralateral hemisphere after CCI (D-F; $P < 0.05$).

Fig. 5.

Triple labeling immunofluorescence for GFAP, V1aR and AQP4 at 5 h post-CCI within the rat cortex adjacent to a blood vessel (BV) in sham (A–D), CCI-vehicle (E–H) and CCI-SR49059-treated (I–L) animal and merged fluorescence (D, H, L; GFAP, green; V1aR, red; AQP4, blue). Compared to sham (A–D), CCI (E–H) increased GFAP (A, E), V1aR (B, F) and AQP4 (C, G). The astrocytic processes (downward arrows) and cell bodies (upward arrows) of GFAP positive astrocytes (A, E, I), co-localize with V1aR (B, F, J) and AQP4 (C, G, K) as demonstrated in merged panels (D, H, L). Compared to sham (A–D), astrocytic cell bodies are enlarged (E, upward arrow) following CCI (E–H), and astrocytic end-feet embracing the BV become thicker and more prominent (E, downward arrow) while a greater, more punctate appearance of V1aR (F) and AQP4 (G) distribution was observed at the endothelium interface (H, downward arrows) and within the cell bodies (H, upward arrows). Compared to CCI (E–H), inhibition of V1aR with SR49059 (I–L), decreased GFAP (E, I), V1aR (F, J) and AQP4 (G, K) expression. SR49059 reduced the pronounced pattern of GFAP positive astrocytic swelling (I, upward arrow) and V1aR interfacing at BV (J, downward arrow). The injury-induced punctate association of AQP4 (G, downward arrow)

within the GFAP positive astrocytic end-feet (H, downward arrow) was more dispersed with SR49059 treatment (K, downward arrow).

Fig. 6.

Schematic representation of astrocytic response to TBI. In normal conditions astrocytes interface with blood vessels at the blood brain barrier (BBB) transporting normal levels of arginine vasopressin (AVP), balancing fluid and electrolytes to maintain cerebral homeostasis. Note that astrocytes have multiple processes containing AQP4 and V1aR within the terminal processes termed astrocytic end feet that are positioned within the cortical parenchyma or intimately associated with the endothelium of the basal membrane of blood vessels; collectively referred to as BBB (A). Following TBI, we posit that an increase in edema is associated with increased AVP levels, and increased expression of V1aR and AQP4. These factors, in concert, cause increased permeability of water (H₂O) resulting in an increase in astrocyte area that ultimately leads to brain swelling and fulminating post-traumatic brain edema.

Physiological monitoring and blood gas analysis at baseline, 15 min., 30 min. and 1 h post-CCI in sham-vehicle, CCI-vehicle and CCI-SR49059 treated experimental groups.

Table 1

Group	Temperature (C°)	mABP (mmHg)	pH	P _a CO ₂ (mmHg)	P _a O ₂ (mmHg)	Plasma Na ⁺ (mEq/L)
Sham-vehicle (n=6)						
Baseline	37.13 ± 0.12	88.17 ± 2.61	7.300 ± 0.03	38.70 ± 1.41	150.25 ± 6.29	142.20 ± 1.85
15 min	37.15 ± 0.10	82.00 ± 3.94	7.308 ± 0.02	36.42 ± 2.27	147.33 ± 4.17	142.67 ± 1.69
30 min	37.18 ± 0.10	81.60 ± 2.98	7.284 ± 0.02	39.02 ± 2.20	146.00 ± 4.53	143.00 ± 1.41
1h	37.17 ± 0.05	79.17 ± 2.73	7.266 ± 0.02	41.17 ± 3.20	144.00 ± 4.02	144.33 ± 1.84
CCI-vehicle (n=11)						
Baseline	37.04 ± 0.06	90.36 ± 4.31	7.309 ± 0.02	35.71 ± 1.59	153.55 ± 3.79	142.64 ± 1.42
15 min	36.96 ± 0.02	83.00 ± 3.28	7.284 ± 0.02	37.17 ± 1.74	151.27 ± 3.25	143.36 ± 1.45
30 min	37.08 ± 0.03	84.30 ± 2.82	7.266 ± 0.02	40.18 ± 1.25	147.64 ± 3.24	143.55 ± 1.25
1h	37.13 ± 0.05	84.80 ± 2.83	7.264 ± 0.02	38.35 ± 1.31	147.09 ± 2.82	144.09 ± 1.64
CCI-SR49059 (n=12)						
Baseline	36.96 ± 0.06	87.42 ± 2.36	7.339 ± 0.02	33.72 ± 1.91	154.07 ± 3.91	142.74 ± 0.84
15 min	36.97 ± 0.03	77.83 ± 3.95	7.318 ± 0.02	36.75 ± 1.55	151.84 ± 3.05	143.23 ± 1.03
30 min	37.12 ± 0.04	76.75 ± 3.36	7.243 ± 0.02**	38.90 ± 2.12	150.84 ± 4.59	143.43 ± 2.42
1h	37.18 ± 0.05	77.25 ± 3.05	7.308 ± 0.02	37.30 ± 1.44	150.63 ± 2.78	143.30 ± 1.49

** P<.01 versus baseline.