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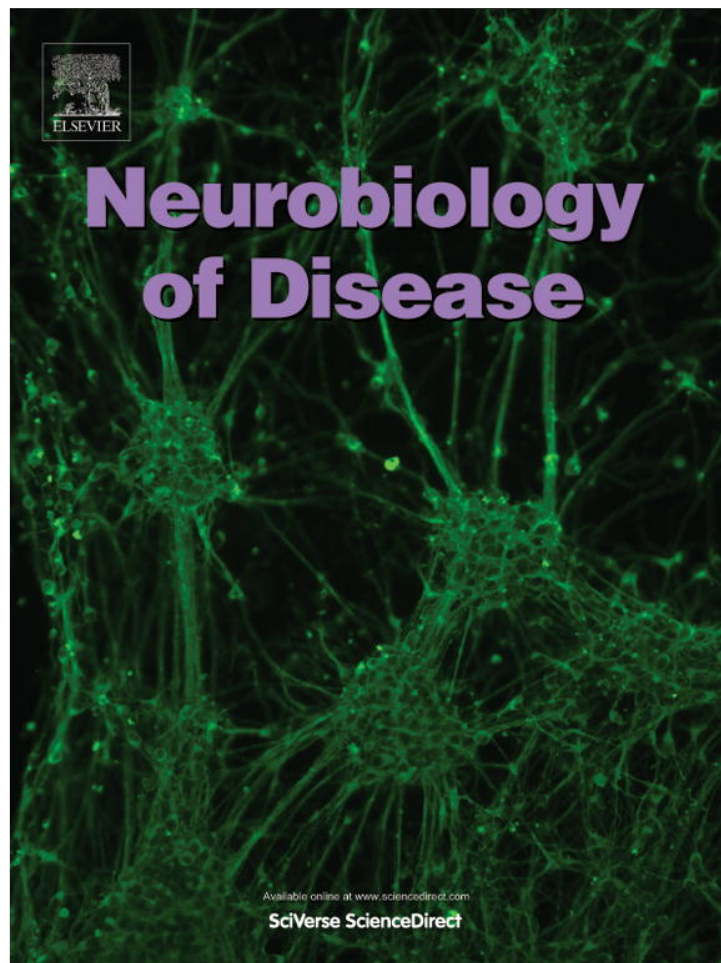
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The 70 kDa heat shock protein protects against experimental traumatic brain injury

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A B S T R A C T

Traumatic brain injury (TBI) causes disruption of the blood brain barrier (BBB) leading to hemorrhage which can complicate an already catastrophic illness. Matrix metalloproteinases (MMPs) involved in the breakdown of the extracellular matrix may lead to brain hemorrhage. We explore the contribution of the 70 kDa heat shock protein (Hsp70) to outcome and brain hemorrhage in a model of TBI. Male, wildtype (Wt), Hsp70 knockout (Ko) and transgenic (Tg) mice were subjected to TBI using controlled cortical impact (CCI). Motor function, brain hemorrhage and lesion size were assessed at 3, 7 and 14 days. Brains were evaluated for the effects of Hsp70 on MMPs.

In Hsp70 Tg mice, CCI led to smaller brain lesions, decreased hemorrhage and reduced expression and activation of MMPs compared to Wt. CCI also significantly decreased right-biased swings and corner turns in the Hsp70 Tg mice. Conversely, Hsp70 Ko mice had significantly increased lesion size, worsened brain hemorrhage and increased expression and activation of MMPs with worsened behavioral outcomes compared to Wt. Hsp70 is protective in experimental TBI. To our knowledge, this is the direct demonstration of brain protection by Hsp70 in a TBI model. Our data demonstrate a new mechanism linking TBI-induced hemorrhage and neuronal injury to the suppression of MMPs by Hsp70, and support the development of Hsp70 enhancing strategies for the treatment of TBI.

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Introduction

Traumatic brain injury (TBI) remains a significant cause of lifelong cognitive, physical, behavioral and emotional impairments globally (Kiraly and Kiraly, 2007; Langlois et al., 2006). TBI frequently leads to brain edema and hemorrhage due to disruption of the blood brain barrier (BBB). Furthermore, hemorrhage can complicate brain tissue damage by the release of excitotoxic substances, free radical damage from blood breakdown products, and tissue ischemia due to loss of microvessels (Kurland et al., 2012). It is one cause of the inflammatory response, involving microglial activation, leukocyte recruitment, and upregulation of cytokine secretion after TBI (Atkins et al., 2012; Namas et al., 2009; Oehmichen et al., 2003).

Recent work in the field has implicated matrix metalloproteinases (MMPs), a family of Zn-dependent endopeptidases, in the breakdown of the extracellular matrix and BBB leading to brain edema and hemorrhage (Lo et al., 2002; Suehiro et al., 2004). MMPs are proteases normally found in the cytosol in an inactivated form, but in pathologic states, are cleaved to an active form. This activated form contributes to BBB disruption by disrupting tight junction and basal lamina proteins, and may facilitate the immune response after brain injury (Gurney et al., 2006; Wang et al., 2007). MMP-2 and -9 are two isoforms which are increased in the brain and spinal cord following ischemia and trauma (Noble et al., 2002; Rosenberg, 1995).

Heat shock proteins (Hsps) are induced by many stressful stimuli, including a variety of central nervous system insults, such as cerebral ischemia, neurotoxin exposure and other metabolic stresses (Kelly et al., 2002; Yenari et al., 2005). The 70 kDa inducible Hsp (Hsp70) functions as a molecular chaperone, thus preventing abnormal protein folding and facilitating protein translocation. Prior work has shown that Hsp70 is increased in brain vessels following experimental TBI (DeGracia et al., 2007). Our group and others have shown the salutary effects of Hsp70 overexpression in brain ischemia models, and that Hsp70 may have multiple mechanisms of protection (Giffard and Yenari, 2004). Overexpression of Hsp70 or its induction by heat stress reduced expression of MMPs in cultured astrocytes at the transcriptional and translational level (Lee et al., 2001). Here we propose to further explore whether Hsp70 has the potential to protect the brain against TBI.

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Materials and methods

Three month old male Hsp70 transgenic (Hsp70 Tg) and Hsp70 deficient (Hsp70 Ko) mice (25–30 g) were established from breeder mice originally generated by the Dillmann (UCSD) and Pandita (Southwestern University) labs (Hunt et al., 2004; Zheng et al., 2008). The Hsp70 Tg and Ko mice were made a C57/B6 background, and have been backcrossed for over 10 generations in order to generate hemizygotic (Hsp70 Tg) or homozygotic (Hsp70 Ko) and wildtype (Wt) littermates. All animal housing and procedures were carried out according to a protocol approved by the local Institutional Animal Care and Use Committee (IACUC) in accordance with the NIH guidelines.

Controlled cortical impact (CCI)

The CCI model of TBI was carried out according to a previously established protocol (Chang et al., 2003; Potts et al., 2009). Briefly, mice were anesthetized with isoflurane (5% for induction and 2% for maintenance via a nosecone) in a mixture of medical air/oxygen (3:1). Rectal temperature was monitored throughout the procedure and maintained in the normal range. For induction of CCI, anesthetized mice were fixed in a stereotaxic frame and a midline scalp incision was made, followed by a circular craniotomy (5 mm in diameter) in the left parietal plate immediately posterior to the bregma. The dura was not disrupted. CCI was performed with an automated impactor (Pinpoint Precision Cortical impactor, Hatteras Instruments, Cary, NC) with a tip size of 3 mm (in diameter) at 1.5 m/s velocity to generate 2 mm penetration with a 100 ms dwell time. The excised cranial bone was replaced immediately and the incision was then closed with suture. At the end of the experiments, animals were euthanized with an overdose of isoflurane followed by decapitation.

Analysis of brain samples

Brains were collected 14 days after CCI and sectioned (50 μm in thickness) on a cryostat. Brain sections taken within the lesion were stained with hematoxylin and eosin (H & E, Sigma, MO). Brain sections were photographed using a Zeiss Axio Imager, and the lesion size was quantified using NIH Image J 1.45 s computer software from 9 sequential levels (every third section) and multiplied by distance between sections (150 μm) to estimate the lesion and/or cavity volume.

In a separate cohort of animals, brains were harvested 3 days post-CCI and gross sections measuring 2 mm thick were prepared and photographed to measure hemorrhage volume using a previously published Image J based technique (Tang et al., 2010). The volume was quantified by digitally measuring the volume of hemorrhage (in μl) and using the "Integrated Density" measurement function in ImageJ to account for variation in hemorrhage intensity.

Expression of Hsp70, MMP-2 and -9 proteins were determined from brains 3 days post injury (Kim et al., 2011). Briefly, mice were anesthetized and transcardially perfused with saline and then paraformaldehyde (PFA, 4%). Brains were dissected, post-fixed in 4% PFA, incubated in 20% sucrose at 4 °C for 24 hours, frozen, and cryosectioned. Brain sections (10 μm in thickness) from mice 3 days after CCI ($n = 3$/group) were subjected to immunostaining. Brain sections were incubated sequentially with 0.1% hydrogen peroxidase (3 min), a blocking buffer (0.5% Triton X-100, 0.1% BSA, 1.5% normal horse serum in PBS) for 30 min, and mouse anti-Hsp70 (1:100; Stressgen, NY) or rabbit anti-MMP-2 and -9 (1:100; Abcam, MA) antibodies overnight at 4 °C. Immunoreactivity was amplified and detected with biotinylated anti-mouse IgG and anti-rabbit IgG (1:200; Vector Laboratories, CA), peroxidase-conjugated avidin (ABC Elite, Vector Laboratories, CA) and DAB substrate. Sections were counterstained with hematoxylin (Sigma, MO). For immunoblots, ipsilateral hemispheres were homogenized and solubilized in Laemmli's lysis buffer containing the following protease inhibitors: Leupeptin 1 μg/ml, Aprotinin 5 μg/ml, Pepstatin 1 μg/ml, phenylmethyl-sulfonylfluoride 1 mM. Aliquots containing 30–60 μg of protein were subjected to 10% SDS-polyacrylamide gel electrophoresis, then transferred to polyvinylidinene fluoride (PVDF) membranes (Millipore, MA), and probed for the protein of interest by incubating in the mouse anti-Hsp70 (1:500; Stressgen, NY) or rabbit anti-MMP-2 and -9 (1:500; Abcam, MA) antibody of interest followed by a horseradish peroxidase (HRP) conjugated secondary antibody. Blots were visualized using the ECL system (Amersham, NJ) according to the manufacturer's directions, and exposed to X-ray film for 5 min.
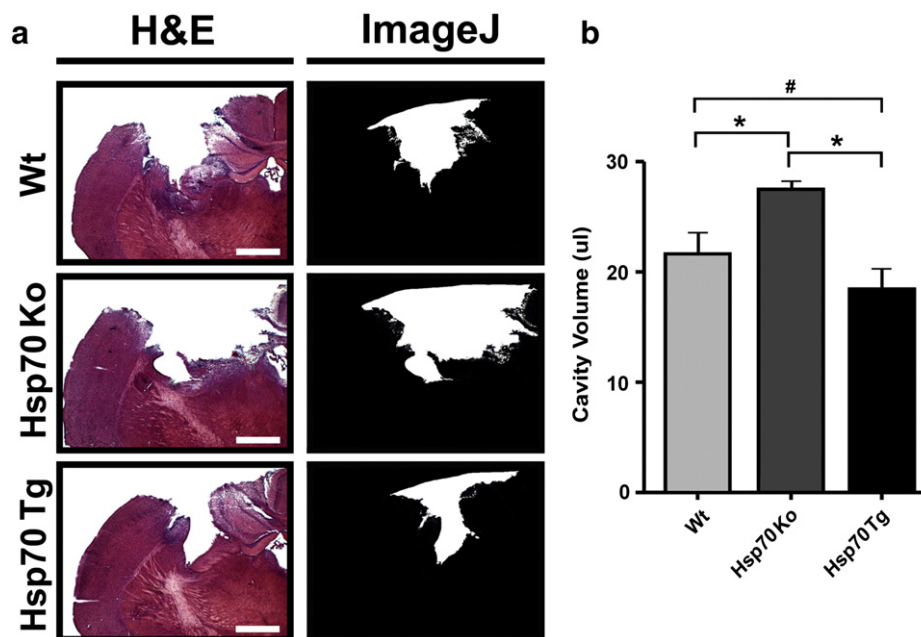
Fig. 1. Hsp70 reduces cortical lesion size following CCI in mice. a) Left panels show hematoxylin & eosin (H&E) stained brain sections from wildtype (Wt), Hsp70 knockout (Ko) and Hsp70 Transgenic (Tg) mice 14 days after CCI. Right panels show delineation of cortical lesion area (white area) from the corresponding images on the left by using the NIH Image J software. Bar = 100 μm. b) Cortical cavity volumes were quantified using H&E stained sections as described in Materials and methods. Lesion volumes were smallest in Hsp70 Tg mice, while lesions were the largest among Hsp70 Ko mice. *$p < 0.01$, #$p < 0.05$; $n = 6$–8 mice/group.

Equal protein loading was confirmed by stripping the membrane, then re-probing for mouse anti-β-actin (Sigma, MO). Densitometric measurements were made from the film using a BioRad Multianalyst scanner and densitometer (Hercules, CA). To detect of MMP-2 and -9 activation, similarly prepared protein samples as for Western blot analysis were subjected to gelatin zymography with Gelatin-zymography Kit (Cosmo Bio Co., Japan) according to the manufacturer's instructions. In brief, each sample was mixed with sample buffer and incubated for 15 min at RT. The samples and markers were loaded to gelatin-gel plate for electrophoresis. The gel was washed and incubated for 40 hours in reaction buffer at 37 °C. After the enzymatic reaction, the gel was stained with Coomassie blue and incubated for 30 min at RT.

Behavior tests

A panel of behavioral assays was carried out to assess neurological function as previously described (Tang et al., 2008; Wang et al., 2009). For the swing test, mice were suspended vertically by the tail with their heads lifted 3 in. above the test bench. A lateral swing
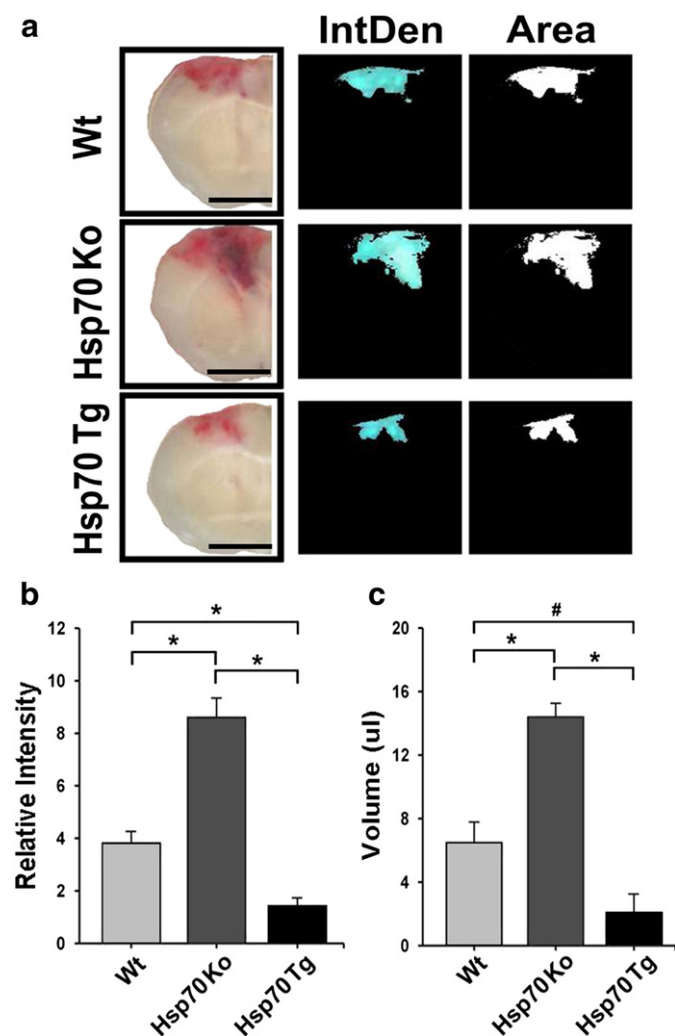
Fig. 2. Hsp70 reduces brain hemorrhage after CCI in mice. a) Each column of images shows coronal brain sections. The left hand columns are gross brain sections, while the center and right hand columns are NIH ImageJ derived maps of intensity (IntDen) and area (Area) of Wt, Hsp70 Ko and Hsp70 Tg mice 3 days after CCI. Bar = 25 mm. b) Average relative intensity and c) volume of hemorrhage is quantified from the brain sections. Statistical analysis showed a significant reduction in the intensity and volume of hemorrhage in Hsp70 Tg mice vs. Wt and Hsp70 Ko mice groups. *$p < 0.01$, #$p < 0.05$; $n = 6$–8 mice/group.
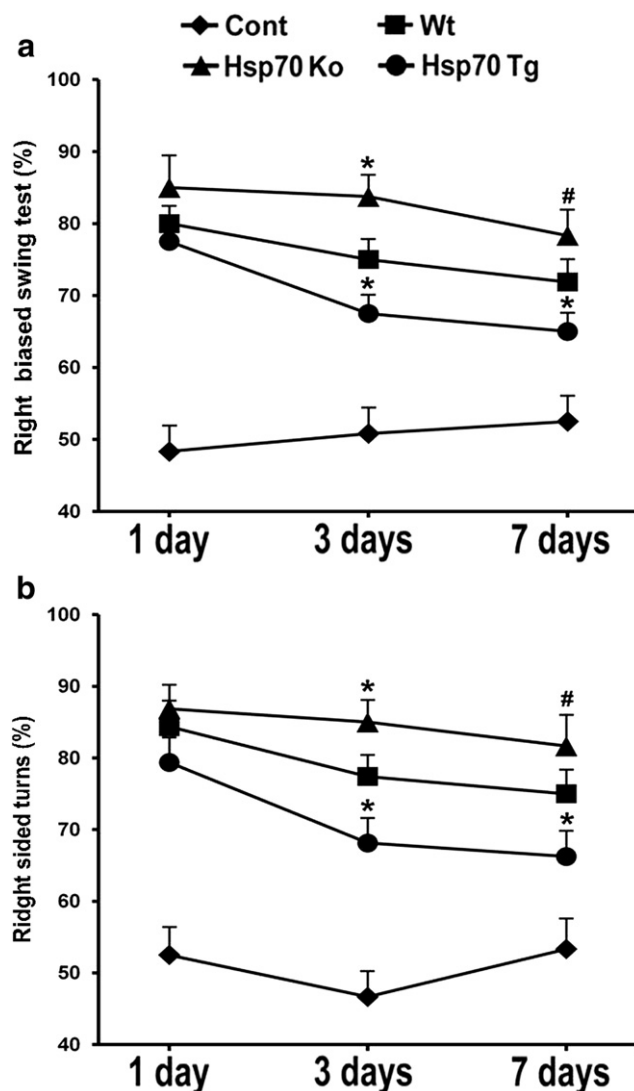

Fig. 3. Hsp70 overexpression improves motor function after CCI. a) Elevated body swing and b) corner tests were performed following CCI. The frequency of right-biased swing and corner turn was calculated after 20 trials for each test on each animal. Hsp70 Tg mice performed better than wildtype (Wt) and Hsp70 Ko mice, whereas Hsp70 Ko mice performed worse than Wt and Hsp70 Tg. *$p < 0.05$ (Wt mice vs. Hsp70 Ko and Hsp70 Tg mice), #$p < 0.1$ (Wt mice vs. Hsp70 Ko mice); $n = 6$–8 mice/group.

was counted if the animal moved its head >10° away from the vertical axis (Borlongan and Sanberg, 1995). A total of 20 swings were counted in each trial and frequency of right-biased swing was calculated for each animal. The corner test was performed by placing two sheets of cardboard in the animal's home cage and positioned to create a 30° corner with a 0.5–1 cm gap, which permitted light to shine through. Mice were placed between the boards and encouraged to enter the corner. Once the animal reached the corner, vibrissae were stimulated by the cardboard, causing the animal to rear and turn away from the corner. The direction of turns was recorded. Each animal was tested 20 times, and the number of turns toward the ipsilateral injured hemisphere (right) was counted.

Statistical analysis

All data analyses were carried out by investigators blinded to the experimental conditions. Experiments were carried out in a randomized fashion to minimize bias. Standard statistical tests were applied
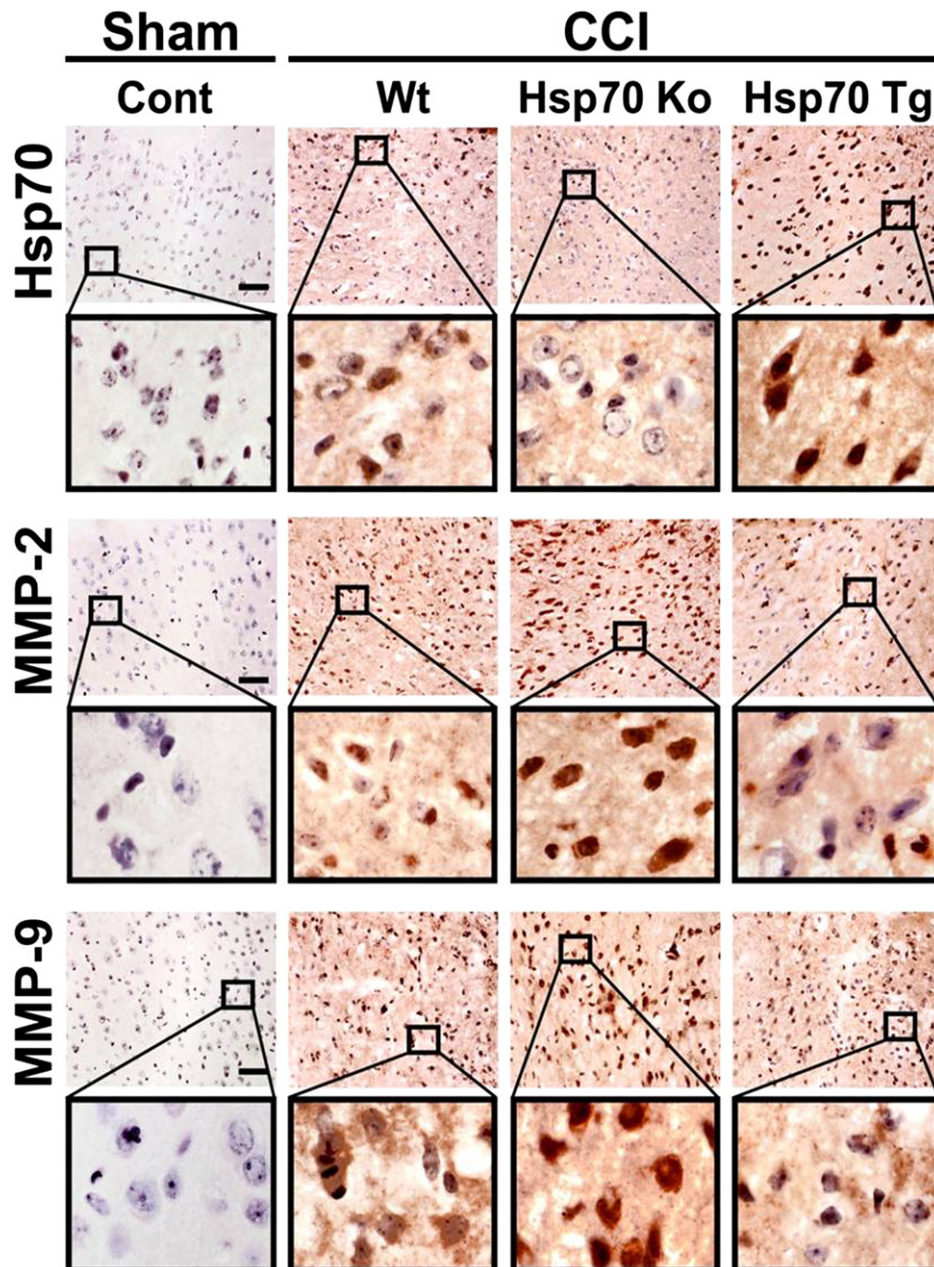
Fig. 4. Hsp70, MMP-2 and MMP-9 expression. Immunohistological detection of Hsp70, MMP-2 and MMP-9 in cortical neurons in Wt, Hsp70 Ko and Hsp70 Tg mice subjected to CCI vs. sham control, followed by 3 day recovery. Black boxes contain images of higher-power view of the corresponding brain regions. Brown DAB stain depicts immunoreactivity in the brain sections counterstained with hematoxylin. Hsp70 expression was highest among Hsp70 Tg mice, and nearly absent in the Hsp70 Ko mice. Some immunoreactivity in brains of Hsp70 Ko likely represent cross reactivity with other Hsp70 isoforms. Immunostains of MMP-2 and -9 show upregulation by CCI in Wt, but less reactivity among Hsp70 Tg and more reactivity among Hsp70 Ko brains. Bar = 25 μm.

depending on the condition (ANOVA or *T*-test for continuous data, and non-parametric tests for non-continuous data, repeated measures where appropriate) and analyzed (Systat Software, Inc., CA). *P*-values <0.05 were considered significant. Data are presented $x \pm$ SE.

Results

Hsp 70 reduced lesion size and brain hemorrhage following TBI

To study the neuroprotective effect of Hsp70 against TBI, we compared the degree of injury in mouse brains subjected to CCI in Wt, Hsp70 Ko and Hsp70 Tg mice. After a 14-day recovery period, the size of cortical brain lesion from H&E-stained sections was quantified

as described above. Hsp70 Tg mice had significantly decreased brain lesion size compared to Wt, whereas Hsp70 Ko mice had increased lesion size (Fig. 1). The extent of brain hemorrhage was similarly less among Hsp70 Tg mice, and significantly greater in Hsp70 Ko mice brains 3 days post CCI (Fig. 2).

Hsp70 improves motor function following experimental TBI

Motor function following CCI was also improved among Hsp70 Tg mice, and worsened in Hsp70 Ko mice. Both elevated body swing and corner tests showed less asymmetry among Hsp70 Tg mice, and worsened asymmetry among Hsp70 Ko mice following CCI (Fig. 3), observations that indicate better neurological function among Hsp70 Tg mice.
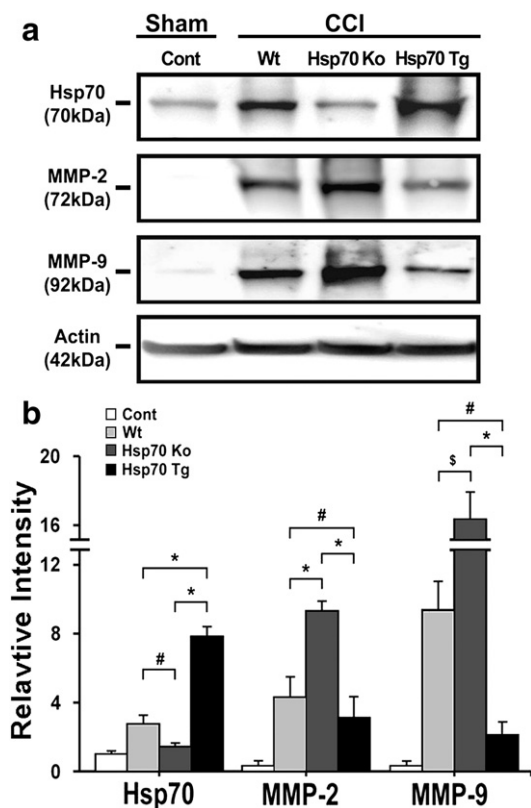
Fig. 5. Western blots of Hsp70, MMP-2 and MMP-9 after CCI. a) Immunoblots show expression of Hsp70, MMP-2 and MMP-9 proteins from Wt, Hsp70 Ko and Hsp70 Tg mouse brains 1 day after CCI. β-actin is shown as a housekeeping control. b) Relative intensities of Hsp70, MMP-2 and MMP-9 protein were quantified by NIH Image J software, and normalized to the intensity of β-actin. MMP-2 and -9 were decreased in Hsp70 Tg brains compared to Wt and Hsp70 Ko, whereas MMP-2 and -9 were increased in Hsp70 Ko brains compared to Wt and Hsp70 Tg. $*p < 0.01$, $#p < 0.05$, $$p < 0.1$; $n = 3$ mice/group.

Behavioral indices among sham controls did not reveal any baseline differences due to gene manipulation.

Hsp70 blunts TBI-induced MMP-2 and MMP-9 expression and activation

We previously showed in an in vitro stroke model that Hsp70 can suppress expression and activation of MMPs (Lee et al., 2004). We next explored whether similar patterns might be observed in our
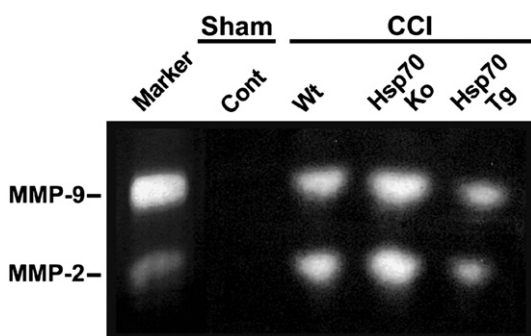
Fig. 6. Gelatin zymograms show relative activities of MMP-2 and MMP-9 after CCI. MMP-2 and MMP-9 gelatinolytic activity 1 d post CCI are shown for injured brains of Wt, Hsp70 Ko and Hsp70 Tg mice. Similar to MMP expression patterns observed on immunostains and immunoblots, zymography shows that MMP-2 and MMP-9 activity are upregulated in Hsp70 Ko mice relative to that of Wt and Hsp70 Tg mice, whereas gelatinolytic activity is decreased in Hsp70 Tg mice.

TBI model. Immunostains of mice subjected CCI are shown in Fig. 4. We first demonstrated that Hsp70 is increased following CCI in Wt and Hsp70 Tg brains, but is absent in brains from Hsp70 Ko mice. Both MMP-2 and MMP-9 increased in brains from Wt and Hsp70 Ko mice, but was notably decreased in brains of Hsp70 Tg mice (Fig. 4). Similar patterns were observed on Western blots (Fig. 5). While MMP expression was decreased in the brains of Hsp70 Tg mice, the Ko mice had 3-fold higher MMP-2 expression, and 10-fold higher MMP-9 expression (Fig. 5). To estimate Hsp's influence on the proteolytic properties of the MMPs, gelatin zymography showed that MMP-2 and MMP-9 activity was decreased by Hsp70 overexpression, but increased when Hsp70 was deficient (Fig. 6).

Discussion

Studies of the events involved in brain injury are essential to developing effective therapies for TBI. We and others previously showed that Hsp70 overexpression was protective in stroke models (Hoehn et al., 2001; Kelly et al., 2002; Lee et al., 2001; Rajdev et al., 2000; Yenari et al., 1998; Zheng et al., 2008). We now show that overexpression of Hsp70 is protective in a model of TBI which, to our knowledge, is the first demonstration in experimental brain trauma. This protection also led to decreased brain hemorrhage and improvement in neurological function. We further showed that MMP expression and its proteolytic activity were decreased in Hsp70 Tg mice. Hsp70 deficiency resulted in the converse, or increased injury, worsened neurological outcome and more brain hemorrhage and MMP expression and activity. These data suggest that the Hsp70 may be an appropriate therapeutic target to limit damage due to TBI.

Brain injury due to TBI, especially when complicated by disruption of the BBB leading to hemorrhage can lead to permanent neurological deficits or death (Vajtr et al., 2009). Recent work in TBI and related brain injuries has implicated, among other processes, matrix metalloproteinases (MMPs) in the breakdown of the extracellular matrix (ECM) and BBB, leading to brain edema and hemorrhage (Higashida et al., 2011; Lescot et al., 2010). MMPs are first expressed as pro-proteins, which are cleaved by other activated MMPs and proteases in the extracellular space (Cunningham et al., 2005). MMPs are activated plasmin as well as other MMPs. Processed, or active MMPs then cleave a variety of extracellular substrates including the basal lamina and lead to disruption of the BBB. MMP-2, MMP-9 and MMP-3 have been shown to increase in the central nervous system following TBI in experimental models (Truettner et al., 2005; Grossetete and Rosenberg, 2008; Jia et al., 2010) and in patients (Grossetete et al., 2009; Vilalta et al., 2008a; Vilalta et al., 2008b). Several studies have shown that at least during the acute phases of TBI, MMPs exacerbate damage. For instance, the elevation of MMP-9 is associated with inflammatory events following TBI, in part, by degrading components of the basal lamina disrupting the BBB (Suehiro et al., 2004). Other studies have shown that MMP-9 deletion (Wang et al., 2000) or pharmacologic inhibition of MMPs is protective (Homsi et al., 2009; Khan et al., 2009). These findings are also in line with a study from our labs which showed that therapeutic hypothermia, a robust neuroprotectant, is associated with decreased expression of both MMP-2 and MMP-9 in a model of stroke (Lee et al., 2005). MMPs may also play beneficial roles in brain injury, as they are important in several processes involved in recovery and repair (Cunningham et al., 2005), which occur during the more chronic phases of stroke. ECM disruption may pave the road to angiogenesis, for instance (Lo et al., 2002). Thus, MMPs play different roles during TBI, although the specific role may depend on the timing. In this study, we focused on the acute stages where we previously established protection by Hsp70 (Yenari et al., 1998; Zheng et al., 2008; Yenari et al., 1998; Zheng et al., 2008), expression of MMPs (Lee et al., 2005) and disruption of the BBB in a stroke model.

Hsp70 is one of several stress proteins upregulated following brain injury. It is expressed in a variety of cells in the brain in a time and spatially dependent pattern. Neurons are among the first cells to express Hsp70, followed by astrocytes and finally blood vessels and microglia (Sharp et al., 1999). While it is known that Hsp70 is protective, the mechanism of protection is only partially understood. Work by our lab and others have shown its beneficial effects against experimental brain injury (Kelly et al., 2002; Rajdev et al., 2000; Truettner et al., 2007; Yi et al., 2008). Hsp70's chaperone functions relate to its ability to bind to and assist in the conformational folding of nascent proteins, and its protective properties have been attributed to its chaperone functions, presumably leading to enhancement of nascent protein folding and preventing protein aggregation (Giffard et al., 2004). We and other groups have shown that Hsp70 may have multiple mechanisms of protection (Giffard and Yenari, 2004; Yenari et al., 2005). Hsp70 can prevent ischemic apoptotic cell death at multiple levels (Giffard et al., 2004; Matsumori et al., 2005), and Hsp70 is capable of interacting with and inhibiting the inflammatory transcription factor, nuclear factor kappa B (NFkB) (Ran et al., 2004; Zheng et al., 2008). There is very little published regarding the link between Hsp70 and MMPs, but we previously showed that overexpression of Hsp70 in cultured astrocytes inhibited MMP-2 and -9, and MMP-9 is one of the many genes regulated by NFkB.

To date, there are no satisfactory treatments for TBI or brain hemorrhage complicating TBI. Our data establish a protective role of Hsp70 overexpression which leads to improved neurological function and decreased brain hemorrhage. However, it should be noted that Hsp70 overexpression was already occurring prior to the injury, and this has obvious translational limitations. Our lab has previously reported that Hsp70 overexpression using gene transfer is also protective when given 2 hours following middle cerebral artery occlusion (Hoehn et al., 2001). Since these particular viral vectors do not begin to express transgene until 4 hours after the vector is administered, this suggests that Hsp70 overexpression may be protective with a temporal therapeutic window of at least 6 hours. Of late, it is now possible to pharmacologically induce Hsp70 in the brain (Kim et al., 2012), and pharmacologic induction of Hsp70 by geldanamycin was found to be protective against experimental stroke and brain hemorrhage (Kwon et al., 2008; Lu et al., 2002; Manaenko et al., 2011; Ouyang et al., 2005; Xu et al., 2003). Thus, the possibility of acute treatment of traumatic brain injury by Hsp70 induction should be explored, especially to establish whether its induction can be delayed. Other questions such as whether this effect is durable and positively influences long term outcome should also be addressed.

Acknowledgments

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