Neurobiology of Disease 49 (2013) 22–28

Contents lists available at SciVerse ScienceDirect

Neurobiology of Disease

journal homepage: www.elsevier.com/locate/ynbdi

Susceptibility to intracerebral hemorrhage-induced brain injury segregates with low aerobic capacity in rats

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ARTICLE INFO

Article history:
Received 8 May 2012
Revised 19 July 2012
Accepted 16 August 2012
Available online 24 August 2012

Keywords:
Metabolic syndrome
Intracerebral hemorrhage
Brain injury
PAR-1

ABSTRACT

Although low exercise capacity is a risk factor for stroke, the exact mechanisms that underlie this connection are not known. As a model system for exploring the association between aerobic capacity and disease risks we applied two-way artificial selection over numerous generations in rats to produce low capacity runners (LCR) and high capacity runners (HCR). Here we compared intracerebral hemorrhage (ICH)-induced brain injury in both genders of these rat lines. HCR and LCR rats had 100 μl blood injected into the right caudate and were killed at *days 1, 3, 7* and *28* for brain water content determination, immunohistochemistry, histology, Western blot, and behavioral tests. Compared to male HCRs, male LCRs had more severe ICH-induced brain injury including worse brain edema, necroptosis, brain atrophy, and neurological deficits, but not increased numbers of Fluoro-Jade C positive cells or elevated cleaved caspase-3 levels. This was associated with greater microglial activation, and heme oxygenase-1 and protease activated receptor (PAR)-1 upregulation. In females, edema was also greater in LCRs than in HCRs, although it was less severe in females than in males for both LCRs and HCRs. Thus, ICH-induced brain injury was more severe in LCRs, a model of low exercise capacity, than in HCRs. Increased activation of microglia and PAR-1 may participate mechanistically in increased ICH-susceptibility. Females were protected against ICH-induced brain edema formation in both HCRs and LCRs.

Introduction

Large-scale clinical studies show that low exercise capacity is a stronger predictor of morbidity and mortality relative to other commonly reported risk factors including hypertension, type II diabetes, obesity, or smoking (Blair et al., 1996; Kavanagh et al., 2003; Kokkinos et al., 2008; Myers et al., 2002). These clinical association studies led to us formulate the idea that variation in capacity for oxygen metabolism is a central mechanistic determinant of the divide between complex disease and health that we termed the Aerobic Hypothesis (Koch and Britton, 2008). Starting in 1996, we prospectively tested this hypothesis by applying divergent (two-way) artificial selection for low and high aerobic treadmill running capacity using the genetically heterogeneous N/NIH rats as the founder population (Koch and Britton, 2001). As expected, selection produced low capacity runners (LCRs) and high capacity runners (HCRs) that differ markedly for running performance. Consistent with the aerobic hypothesis numerous disease risks segregated with selection in the LCR and resistance to disease risks segregated with selection for HCR. The LCR scored higher than the HCR for risk factors including diminished aging and longevity (Koch et al., 2011), the metabolic syndrome (Wisloff et al., 2005), hepatic steatosis (Thyfault et al., 2009), and disordered capacity to oxidize lipids (Lessard et al., 2009; Rivas et al., 2011).

While low exercise capacity is a risk factor for stroke (Kurl et al., 2009) as well as other forms of cardiovascular disease (Kavanagh et al., 2003; Kokkinos et al., 2008; Myers et al., 2002), the mechanistic connection has not been defined. Here we examined differences in brain injury in an experimental model of intracerebral hemorrhage (ICH) using LCR and HCR rats. Spontaneous ICH is a common and often fatal stroke subtype accounting for 10–15% of all strokes. Mortality rates for ICH are more than 40% and many survivors have significant neurological deficits (Mendelow et al., 2005).

We compared ICH-induced brain injury in LCRs and HCRs by measuring brain edema, brain atrophy, and behavioral deficits. We also examined a potential novel marker of ICH-induced injury, receptor-interacting protein 1 (RIP1), because this protein plays a critical role in necroptosis and contributes to renal ischemia/reperfusion injury (Linkermann et al., 2012). The mechanisms of brain injury after ICH also include coagulation cascade activation with thrombin production (Lee et al., 1997; Xi et al., 1998), inflammation (Aronowski and Zhao, 2011), and hemoglobin- and iron-induced toxicity (Huang et al., 2002; Nakamura et al., 2004). Therefore, we compared ICH-induced changes in LCRs and HCRs for: 1) protease activated receptor (PAR)-1, a thrombin receptor that is involved in thrombin-induced brain injury after hemorrhagic and ischemic

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Available online on ScienceDirect (www.sciencedirect.com).

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http://dx.doi.org/10.1016/j.nbd.2012.08.014

stroke (Junge et al., 2003; Xue et al., 2009), 2) microglial activation that can exacerbate ICH-induced brain injury (Wu et al., 2008), and 3) heme oxygenase (HO)-1, an enzyme involved in the breakdown of heme and the release of iron (Gong et al., 2006).

There is evidence that low exercise capacity is a risk factor for cardiovascular disease in men and women (Kavanagh et al., 2003; Kokkinos et al., 2008; Myers et al., 2002) but gender affects ischemia- and hemorrhage-induced brain injury (Nakamura et al., 2005; Pelligrino et al., 1998) and there is evidence that the relationship between insulin resistance and age-associated coronary and cerebrovascular diseases could be mediated by estrogen receptors (Cardona-Gomez et al., 2002). Thus, the current study also compared ICH-induced brain injury in both male and female LCRs and HCRs. Our results demonstrate more severe ICH-induced brain injury in LCRs and that females were provided relative protection against ICH-induced brain edema formation for both LCRs and HCRs.

Materials and methods

Animals

A previous report (Wisloff et al., 2005) provides a detailed description on the development of the rat models for aerobic exercise capacity. In brief, divergent selected lines were generated from a founder population of 80 male and 88 female N-NIH stock rats based on intrinsic aerobic treadmill running capacity. Thirteen families for each line were set up for a within-family rotational breeding paradigm. This schedule permits <1% inbreeding per generation to maintain a heterogeneous genetic substrate within each selected line. At each generation young adult rats (11 weeks of age) were tested for their intrinsic (untrained) ability to perform forced speed-ramped treadmill running until exhausted. This test was performed daily over five consecutive days. The greatest distance in meters (m) achieved out of the five trials was considered the best estimate of an individual's exercise capacity. The highest scored female and male from each of the thirteen families were selected as breeders for the next generation of HCRs. The same process was used with lowest scored females and males to generate LCRs. Rats were phenotyped for running capacity in the Koch/Britton laboratory and then transferred to the Hua/Keep laboratory for subsequent study. Rats were studied for response to ICH at least 20 weeks after their last run test. Comparison data between LCR and HCR groups are presented for male rats from generation (G) 25 (n = 6 per group), G26 (n = 14 per group), and G27 (n = 12 per group) and for females from G 28 (n = 18 per group). Rats were studied at 5–6 months of age.

Animal preparation and intracerebral infusion

All animal procedures were approved by the University Committee on Use and Care of Animals, University of Michigan. Rats were anesthetized with pentobarbital (50 mg/kg, i.p.) and the right femoral artery catheterized to monitor arterial blood pressure and to obtain blood for analysis of blood gases, pH, glucose, and hematocrit. Body temperature was maintained at 37.5 °C using a feedback-controlled heating pad. Rats were positioned in a stereotactic frame (Kopf Instrument, Tujunga, CA) and a cranial burr hole drilled near the right coronal suture 4.0 mm lateral to the midline. A 26-gauge needle was inserted stereotaxically into the right basal ganglia (coordinates for rats: 0.2 mm anterior, 5.5 mm ventral, and 4.0 mm lateral to the bregma) and blood infused into right basal ganglia at a rate of 10 μl/min using a microinfusion pump. After injection, the needle was removed and the skin incisions closed.

Experimental groups

This study was divided into two parts. First, male HCR and LCR rats had 100 μl blood injected into the right basal ganglia. Rats were euthanized at (1) *day 3* (n = 6) for brain water content determination, (2) at *day 1* and *day 3* (n = 4) for immunohistochemistry, (3) at *days 3 and 7* (n = 4) for Western blot and (4) at *day 28* (n = 6) for histology. Rats had behavioral testing at days 3, 7, 14 and 28 after ICH. In the second part of the study, female HCR and LCR rats had 100 μl blood injected into the right basal ganglia and were killed at *day 3*. The brains were used for water content measurement (n = 6) and Western blot (n = 4).

Brain water content

Animals were reanesthetized with pentobarbital (60 mg/ kg, i.p.) and decapitated three days after ICH (Xi et al., 1999). Brains were removed and a 3-mm thick coronal brain slice cut 4-mm from the frontal pole, and dissected into ipsi- and contralateral cortex, and ipsi- and contralateral basal ganglia. The cerebellum was taken as a control. Samples were immediately weighed to obtain the wet weight, and then dried at 100 °C for 24 h to obtain the dry weight. Percent water content was determined as: ((wet weight − dry weight)/wet weight) * 100%. In measurements of the basal ganglia contralateral to the ICH, the LCRs had significantly lower water content than HCRs (76.2 ± 0.5 vs. 77.5 ± 0.6%; $p<0.01$), a phenomenon that we have found previously in comparing aged and young rats (Gong et al., 2004). We also found that the water content of the cerebellum (a site distant from the ICH) was slightly lower in LCRs (76.9 ± 0.1%) than in HCRs (77.3 ± 0.4%), $p<0.05$. Therefore, edema was assessed as the difference in water content between ipsi- and contralateral hemispheres.

Immunohistochemistry

Immunohistochemistry was performed as described previously (Xi et al., 1999). Briefly, rats were anesthetized and perfused with 4% paraformaldehyde in 0.1 M phosphate-buffered saline (pH7.4). Brains were removed, kept in 4% paraformaldehyde for 6 h and then immersed in 25% sucrose for 3–4 days at 4 °C. After embedding in a mixture of 25% sucrose and OCT (SAKURA Finetek, USA), 18-μm sections were taken on a cryostat. The avidin–biotin complex technique was used for staining with hematoxylin as counter stain. Primary antibodies were mouse anti-rat OX6 (1:400 dilution, AbD Serotec) and rabbit anti-HO-1 (1:400 dilution, Stress Gen). Normal rabbit or mouse serum and the absence of primary antibody were negative controls.

Eighteen-micrometer thick coronal sections from both 1 mm anterior and 1 mm posterior to the blood injection site were used for cell counting. Three high-power images (×40 magnification) were taken adjacent to the hematoma and positive cells counted manually.

Western blot analysis

Western blot analysis was performed as previously described (Xi et al., 1999). Briefly, brain tissue was immersed in Western sample buffer and sonicated. Protein concentration was determined by the Bio-Rad protein assay kit and 50-μg protein from each sample was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a Hybond-C pure nitrocellulose membrane (Amersham). Membranes were probed with the following primary antibodies: rabbit anti-RIP1 (1:1000, Abcam), rabbit anti-PAR-1 (1:1000), rabbit anti-HO-1 (1:2000), rabbit anti-LC3 (1:1000, Abgent) and rabbit anti-cleaved caspase-3 (1:1000, Cell Signaling). Antigen–antibody complexes were visualized with the ECL chemi-luminescence system (Amersham) and exposed to a Kodak X-OMAT film. The relative densities of bands were analyzed with NIH ImageJ.