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Cardiac reactive oxygen species after traumatic brain injury

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Abstract

Background—Cardiovascular complications after traumatic brain injury (TBI) contribute to morbidity and mortality and may provide a target for therapy. We examined blood pressure and left ventricle contractility after TBI, and tested the hypothesis that beta-adrenergic blockade would decrease oxidative stress after TBI.

Material and Methods—Rodents received fluid-percussion injury or sham surgery, confirmed with magnetic resonance imaging (MRI) and histopathology. We followed recovery with sensorimotor coordination testing and blood pressure measurements. We assessed left ventricular ejection fraction using ECG-gated cardiac MRI and measured myocardial reactive oxygen species (ROS) with dihydroethidium. We randomized additional TBI and sham animals to post-operative treatment with propranolol or control, for measurement of ROS.

Results—Blood pressure and cardiac contractility were elevated 48 hours after TBI. Myocardial tissue sections showed increased ROS. Treatment with propranolol diminished ROS levels following TBI.

Conclusions—TBI is associated with increased cardiac contractility and myocardial ROS; decreased myocardial ROS after beta-blockade suggests that sympathetic stimulation is a mechanism of oxidative stress.

Keywords

Traumatic brain injury; reactive oxygen species; adrenergic receptor antagonist

Introduction

Approximately 1.7 million Americans sustain a traumatic brain injury (TBI) each year,¹ and cardiovascular complications, such as electrocardiogram (ECG) changes, arrhythmias, ischemia, and heart failure, may delay recovery or contribute to mortality.² Sympathetic stimulation following head injury causes not only hypertension, but also a marked

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hyperdynamic cardiac state.^{3–5} Catecholamines may induce generation of reactive oxygen species (ROS) in vascular and myocardial tissue;^{6, 7} however, a direct association between myocardial ROS and TBI has not been previously described.

The administration of adrenergic blockade after head trauma in human and animal models has been previously explored but not prospectively validated.^{8–10} Two retrospective studies suggested that mortality in trauma patients who received beta-adrenergic blockers was significantly decreased, but the mechanism of cardioprotective effects is unknown.^{11, 12}

We used a rodent survival model of TBI to examine the association between TBI and myocardial oxidative stress. We hypothesized that TBI would increase myocardial ROS, and furthermore, that beta-adrenergic blockade after TBI would mitigate this oxidative stress.

Methods

Animal model

The fluid-percussion injury method was used to produce TBI¹³. Adult male Sprague Dawley rats (Charles River, Saint Constant, Quebec, Canada; 300–350g) were anesthetized with 2–5% isoflurane. A 3mm diameter craniotomy was drilled and a 2mm internal diameter stainless steel hollow intracranial screw was placed in the skull. Once secured, the intracranial screw was filled with 0.9% normal saline and attached with tubing to the fluid percussion device. TBI was administered using controlled fluid percussion injury. Control animals received anesthesia and scalp incision only. Animals were allowed to recover for at least 36 hours with free access to food and water. Recovery was defined as ability to maintain upright posture, ambulate, and take oral hydration. Animals with hypotension, persistent seizure activity or severe neurological deficits were euthanized and excluded from further analysis. All animals received buprenorphine analgesia while under anesthesia and at 12 hours after surgery. Additional doses of buprenorphine were administered to animals with signs of pain or suffering until the completion of the experiment. All studies were conducted in accordance with the *Guidelines for the Care and Use of Laboratory Animals* (NIH) and approved by the Institutional Animal Care and Use Committee of the University of Vermont.

Propranolol administration

Animals were randomized to receive either propranolol (Sigma-Aldrich, St. Louis, MO) or control. Propranolol was dissolved daily in tap water (460mg/L) and taken ad lib by animals for 10 days after surgery. We assumed a mean intake of 35 mL/ day of water, and administered each animal 50mg/ kg/ day. Previous studies have shown adequate beta-adrenergic receptor blockade in rats with similar oral doses of 40¹⁴, 50¹⁵, or 80¹⁶ mg/kg/ day, based on measurements of plasma concentrations of the drug¹⁵, effects of the drug on adrenergic receptor binding parameters¹⁴, and effects on heart rate and arterial blood pressure response to isoproterenol infusion¹⁶.

Neurological testing

Sensorimotor coordination of our research animals was assessed using a RotaRod (Med Associates Inc., St. Albans, VT), which is a device that measures the time a rat is able to stay on a rod rotating at a constant acceleration¹⁷. Rather than measuring voluntary exercise, the elevation of the rod above the cage floor serves as motivation to perform the task until failure. The initial velocity of the rod was 4 rpm, and it accelerated every 10 seconds until it reached 40 rpm. The amount of time spent on the rod before falling (maximum: 5 minutes) was recorded for ten trials. Animals were acclimated to the device for five days prior to surgery, and then tested on post-operative day three.

Blood pressure measurement

Blood pressure was measured using both invasive and non-invasive techniques¹⁸. For monitoring of blood pressure during surgery, the caudal tail artery was cannulated by either percutaneous approach or a cutdown to place a 24 gauge angiocatheter. Blood pressure measurements using a tail cuff volumetric sensor (CODA NIBP system; Columbus Instruments, Columbus, OH) were obtained after seven days of acclimation¹⁸. Rats were warmed during measurements to ensure proper blood flow to the tail. Ten measurements were recorded and then averaged for each time point.

Magnetic resonance imaging

MRI was conducted with an Achieva 3.0 Tesla TX magnet (Philips Medical Systems International B.V., Best, Netherlands); this imaging was performed *in vivo* with the rats under isoflurane anesthesia. Heart rates were measured, and the level of anesthesia was titrated to achieve heart rates of 150–200 beats per minute for optimal cardiac imaging. A 16 channel Torso-XL receive coil (InVivo Corp., Gainesville, FL) was used in combination with a small single-channel e-Coil (Medtronic Inc., Minneapolis, MN) for image acquisition. Three axially-acquired pulse sequences were used to assess brain injury: T2-weighted (T2W) gradient and spin echo (GRaSE), T2W fluid attenuated inversion recovery (FLAIR), and diffusion-weighted imaging (DWI). The GRaSE acquisition was performed with TR=4266 ms, TE=80 ms, acquired matrix=200×190, field-of-view (FOV)=60 mm × 49 mm, slice thickness=0.8 mm, signal averages (NEX)=3. For the FLAIR sequence, images were acquired with TR=8000 ms, TE=125 ms, acquired matrix=200×171, FOV=60 mm × 49 mm, slice thickness=1 mm, NEX=2. The DWI images were acquired with a diffusion-weighted spin echo EPI (echo planar imaging) sequence using $b=1000 \text{ s/mm}^2$, TR=1201 ms, TE=81ms, acquired matrix=116×111, FOV=70 mm × 70 mm, slice thickness=1.3 mm, NEX=2. To assess left ventricular function, ECG gated short-axis MR images (8mm thick) of the beating heart were acquired with a balanced steady-state free precession (b-SSFP) acquisition at a matrix size of 144×144 with a FOV of 35–40mm and 2mm slice thickness with 20–30 phases per R-R interval. The TE is 1.3 ms and the TR is 3.0 ms with this sequence. Local B0 and B1 shimming were performed to minimize image artifacts. This provides a temporal resolution of each frame of 40–50 ms. Analysis was performed off-line using a IDL-based software program (Cine Tool). LVEF was estimated by determining the average ratio of biplane area of short-axis images of the ventricle in end-systole and end-diastole at three different LV sections (base, mid-LV, and apex) for each animal.

Immunohistochemistry for detection of glial fibrillary acidic protein (GFAP)

Reactive gliosis was measured in cortical and hippocampal tissue sections after incubation with a fluorescent antibody specific to GFAP, as previously described^{19, 20}. In preparation for histological studies, animals were euthanized by injection with sodium pentobarbital (130 mg/kg), and after resection the desired tissue sample was loaded into a biopsy tray well with Tissue-Tek Optimal Cutting Temperature (Sakura Finetek USA, Inc., Torrance, CA) (OCT) compound. Samples were flash frozen for 30 seconds in a bath of 2-methylbutane cooled by liquid nitrogen, and then immediately transferred to storage at -80°C . Slides were prepared by placing tissue blocks in a cryostat at -20°C and allowed to come to temperature, then removed from biopsy trays and adhered to sectioning chucks with additional OCT. Twenty micron sections were cut in the cryostat, placed on slides, and stored at -20°C . For immunohistochemistry, slides were fixed with 3% paraformaldehyde in phosphate buffered saline (PBS), and then blocked with 5% Donkey Serum (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA), rinsed with PBS-Tween, and then incubated in rabbit polyclonal IgG AntiGFAP (1:10,000; Dako North America, Inc., Carpinteria, CA) overnight at 4°C . Samples were then incubated in Anti-Rabbit Ig G – Cy3 (1:800 in 5% Donkey Serum; Invitrogen) for 3 hours at room temperature, followed by the

application of SlowFade Gold with DAPI (Invitrogen) immediately prior to mounting. Each experiment included negative controls with omission of primary antibody to test for nonspecificity. For each sample sixteen images in 0.5 micron steps were collected using a DeltaVision restoration microscopy system. Deconvolution was completed using SoftWoRx software (Applied Precision Inc., Issaquah, WA) and analysis was done using ImageJ software (National Institute of Health). At least 3 microscope fields per animal were normalized to background and averaged to determine total area of fluorescence per animal

Dihydroethidium staining

Reactive oxygen species were measured in myocardial tissue by confocal microscopy using dihydroethidium (EMD Biosciences, La Jolla, CA) (DHE), as previously described²¹. Slides were allowed to come to room temperature in a light-protected tray and then incubated for 30 minutes at 37°C with 4µM DHE, and then washed with PBS. Imaging was completed with a Zeiss LSM 510 META Confocal Laser Scanning Imaging System (Carl Zeiss Microimaging, Thornwood, NY) with excitation at 488nm. Negative control slides were incubated for 30 minutes at 37°C with 1000 Units/ mL Super Oxide Dismutase (Sigma) prior to incubation with DHE.

Statistical Analysis

Data are presented as mean $\pm$ standard error of the mean (SEM). Differences between experimental groups in experiments were determined by two-tailed t-test or analysis of variance (ANOVA). In all experiments, $P < 0.05$ was considered to indicate statistical significance.

Results

To obtain the images shown in Figure 1, we pioneered a novel technology for in vivo, rodent imaging using a 3.0 Tesla magnet. Other investigators have achieved similar resolution using specialized 7.0 or higher Tesla animal magnets, so we now demonstrated a technique that can work on the 3.0 Tesla human magnets found at most medical centers. The key innovation is an inexpensive, single loop disposable receive coil designed for single use prostate imaging that can be easily modified by removal of the balloon to image mice, rats, and very small regions of human anatomy. MRI reveals damage after fluid percussion injury, centered at the site of the craniotomy (Figure 1). Parenchymal hemorrhage is evident throughout the ipsilateral cortex, with a smaller area of ischemia. There is also edema involving both the ipsilateral and contralateral cortex.

We also measured reactive gliosis in cortical tissue after TBI. In response to insult, astrocytes undergo reactive gliosis, characterized by increased expression of GFAP^{19, 20}. We observed increased gliosis in cortical tissue ipsilateral to site of fluid percussion injury, as measured by total GFAP fluorescence in the brains of animals receiving TBI vs. control surgery (Figure 2).

Additionally, we were able to quantify a significant difference in our research animals' ability to perform a sensorimotor coordination task three days after injury. This task evaluated balance, coordination, and capacity for physical activity¹⁷. In our experiments, the mean duration of time a rat remained on the rod was 80 seconds vs. 145 seconds, for TBI (n=8) vs. control (n=10), respectively (Figure 1).

We measured the short term and long term blood pressure response to TBI. Using arterial monitoring of blood pressure during TBI surgery (n=3), we noted that blood pressure peaked within one minute (147mmHg $\pm$ 11 mmHg, from baseline 105 $\pm$ 18 mmHg) and normalized within 5 minutes. We acclimated 20 additional animals to tail cuff measurement

of blood pressure, and collected serial measurements of blood pressure before and after TBI (n=10) or sham surgery (n=10). Two TBI animals had hypotension after surgery, and did not survive. There were no differences in blood pressure between TBI and sham animals on the first day after surgery. At 48 hours post-injury, TBI animals showed a significant increase in systolic blood pressure (Figure 3). Differences between TBI and sham were no longer significant by 5 days post-injury; some TBI animals had persistent hypertension but others had normalized their blood pressure. At 2 days after surgery, cardiac MRI demonstrated that TBI animals were hypercontractile with significantly elevated ejection fraction (Figure 4). Heart rates during left ventricle ejection fraction (LVEF) measurement were 162 $\pm$ 12 bpm, with no significant differences between TBI and sham animals.

We measured cardiac ROS in 24 TBI animals who survived surgery and received two weeks of treatment with either propranolol (n=12) or no propranolol (n=12), and compared these groups to animals receiving the sham surgery (n=12). ROS in myocardial tissue was recorded as fluorescence of DHE (total counts/ area) and revealed a significant increase in TBI as compared with control (Figure 5a). We found that the addition of propranolol to the drinking water of TBI animals caused normalization of ROS levels to that of control animals (Figure 5b). As a control of the DHE assay, we added of superoxide dismutase to TBI tissue (n=9) and demonstrated that the signal drops, which confirms that the fluorescence is due to ROS.

Discussion

We provide new insight into the patterns of traumatic brain injury in an animal model visualized on MRI *in vivo*, with demonstration of reactive gliosis by immunohistochemistry *in vitro*. We have pioneered a novel technology for collecting images in rodent models using a 3.0 tesla magnet and an inexpensive, single loop disposable receive coil. We were surprised by the extent of injury seen on MRI, because animals appeared generally normal and were able to recover from surgery to an extent it would not be expected in a human with a similar pattern of injury; however, when we tested sensorimotor coordination, we measured significant deficits in neurological function in TBI compared to sham surgery animals.

Our observations of hypertension and increased cardiac contractility after TBI, are consistent with reports of a hyperdynamic cardiac state during recovery from TBI in both animal models and humans.³⁻⁵ The hyperadrenergic state observed in moderate to severe TBI patients contributes to increased mortality and may guide surgical therapy²².

In our study, animals with hypotension immediately after surgery did not survive (<20%). Cardiovascular responses to TBI have been well described, and sympathetic hyperactivity following TBI in its most severe form, paroxysmal sympathetic storm, is characterized by tachycardia, hypertension, tachypnea, mydriasis, and diaphoresis. Blood pressure elevation occurs not only when intracranial pressure is elevated (Cushing response)²³ but also occurs in models of brain trauma, with or without elevation in intracranial pressure²⁴. The hypertension of a Cushing response is due sympathetic neuron activation, including both direct cardiac stimulation and adrenal release of circulating catecholamines^{25, 26}. Severe head injury has been associated with a greater than 500 fold increase in plasma epinephrine and a 100 fold increase in plasma norepinephrine in experimental models.²⁷ The increase in catecholamines correlates with the severity of brain injury and has a direct correlation with the increase in systolic blood pressure. Elevations in plasma catecholamines following human head injury occur immediately but may peak at 10–15 days.²⁸ The increased ejection fraction we observed is consistent with the known effects of elevated catecholamines, and with prior research showing that head injury causes a hyperdynamic cardiac state.³⁻⁵

Prior research has also shown damage to the myocardium after severe brain injury²⁹. Pathologic studies showed that the myofibrillar degeneration associated with head injury was similar to the histological findings in patients with pheochromocytoma or in experimental animals following exogenous catecholamine administration, suggesting that high levels of catecholamines exerted a direct toxic effect on the myocardium³⁰. The pattern of injury associated with catecholamine toxicity, distinct from the inflammatory pattern characteristic of infarction, has been called contraction band necrosis³⁰. In traumatic brain injury, signs of cardiac dysfunction including ECG changes, arrhythmias, or necrosis, develop in 20% of patients, and cardiac complications have been identified in one third of TBI cases that result in mortality.¹² The ECG abnormalities that have been described following TBI include ST-T changes, ventricular arrhythmias, and reduced heart rate variability.^{31, 32} Elevated creatinine kinase and troponin I levels are found in trauma patients with brain injury, even without mechanical chest injury.^{33–35} Transient, reversible left ventricular dysfunction has been described in patients with acute brain injury³⁶.

One mechanism that might explain this cardiac damage would be oxidative stress. Recent studies have shown oxidative damage in the central nervous system after traumatic brain injury³⁷. There is evidence that catecholamines generate free radicals leading to oxidative stress, and altered redox balance reduces nitric oxide bioavailability and impairs endothelial reactivity^{6, 7}. We now show that myocardial oxidative stress after TBI can be mitigated by administration of a beta-adrenergic receptor antagonist.

Cardioprotective strategies including beta-adrenergic blockade are common in the surgical setting and there is evidence to support beta-adrenergic blockade in trauma, even in those patients with cardiac risk factors.^{38, 11, 128–10} It may be that the increased contractility in TBI is not pathologic, per se, but rather a physiologic response to the increased catecholamines after TBI. This response may be necessary for homeostasis, in the short term, but it may also become deleterious particularly in patients with pre-existing cardiovascular risk factors, or possibly even in otherwise healthy individuals who experience a prolonged or extended catecholaminergic response. Otherwise healthy individuals with adequate cardiac reserve may be able to tolerate the cardiac stimulation and oxidative stress without complications; however, in those patients with pre-existing cardiac disease or decreased cardiac reserve due to their injury, the stress of TBI may result in cardiac complications through the mechanisms we have described. Alternatively, the mechanism by which beta-adrenergic blockade may be cardioprotective in trauma may be multifactorial and could involve central effects such as inhibition of the Bezold-Jarish reflex³⁹. Further research is required before our results can be extrapolated to a clinical scenario. Our results suggest that prevention of deleterious cardiovascular ROS may be a mechanism of the beneficial effect of beta-adrenergic blockade in trauma. The implications of these findings are that adrenergic blockade or antioxidant administration following TBI may protect against cardiovascular stress.

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B.

Figure 1.

A. Neuroimaging with Achieva 3.0T TX magnet after TBI surgery (trauma) or sham surgery (control). Imaging obtained from animals under isofluorane anesthesia 36–48 hours following surgery. Representative sequences are shown with enhancement indicating structural injury (T2-weighted gradient and spin echo [GRaSE]), surrounding edema (fluid-

attenuated inversion recovery [FLAIR]) and larger ischemic penumbra (diffusion weighted images [DWI]). B. Neurological function assessment after TBI surgery or sham surgery. Animals were pre-acclimated to the rotarod test, subjected to surgery, and allowed to recover. On the third day after surgery, animals were tested in five consecutive trials to measure length of time on the rotating rod. Values are expressed as mean $\pm$ SEM (control, n = 17; trauma, n=16). *Different from control, two-tailed t test ($P < 0.05$).

A. **Control** **Trauma****B.** **Figure 2.**

A. Immunohistochemistry for reactive gliosis after TBI surgery (trauma) or sham surgery (control). Rats were euthanized 7–10 days after surgery for acquisition of ipsilateral cortical tissue adjacent to the area of injury. Confocal microscopy was used to detect fluorescence targeted by an antibody specific to glial fibrillary acidic protein (GFAP) in frozen tissue. Representative slides are shown. B. Graph depicts GFAP fluorescence from cortical tissue sections. At least 3 microscope fields per animal were normalized to background and averaged to determine total area of fluorescence per animal. Values are expressed as mean $\pm$ SEM (control, $n = 9$; trauma, $n=9$). *Different from control, two-tailed t test ($P < 0.05$).

Figure 3.

A. Fluid percussion injury measured using high frequency pressure transducer in line with fluid column during procedure. The maximum pressure is shown (49 PSI) over the 500 msec time period. B. Representative tracing of systolic blood pressure after fluid percussion injury (60 second tracing). Intra-operative blood pressure was transduced from the tail artery using an indwelling line placed under anesthesia. The blood pressure increased from 100 mmHg to 153 mmHg over 3 seconds, and then returned to baseline within 30 seconds.

B. Blood pressure after TBI surgery (trauma) or sham surgery (control) measured with a non-invasive tail cuff. Representative cuff pressure tracings are shown for control and trauma animals. B. Summary blood pressure data at 2 days after injury. Five to ten readings were taken and averaged for each animal. Values are expressed as mean $\pm$ SEM (control, n=8; trauma, n=8). *Different from control, two-tailed t test ($P < 0.05$).

Figure 4.

A. Cardiac MRI to measure left ventricular ejection fraction (LVEF) after TBI surgery (trauma) or sham surgery (control). Rats recovered from surgery for 2 days and were then placed under isoflurane anesthesia for respiratory and ECG-gated cardiac MRI. LVEF was estimated by determining the average ratio of biplane area of short-axis images of the ventricle in end-systole and end-diastole at three different LV sections (base, mid-LV, and apex) for each animal. Representative images from diastole and systole are shown with the LV cavity highlighted. B. Summary LVEF data. Values are expressed as mean $\pm$ SEM (control, n=4; trauma, n=6). *Different from control, two-tailed t test ($P < 0.05$).

Figure 5.

A. Reactive oxygen species (ROS) in LV myocardium after sham surgery (control, n=12), TBI surgery (trauma, n=12), or TBI surgery followed by treatment with propranolol (trauma + propranolol, n=12). Rats were euthanized 7– 10 days after surgery for acquisition of myocardial tissue and fresh frozen sections were incubated with dihydroethidium (DHE) to determine levels of ROS detected by fluorescence using Zeiss LSM 510 META Laser Scanning Microscope. Negative control slides for each animal were incubated with superoxide dismutase (SOD) immediately prior to addition of DHE (trauma + SOD, n=9). Representative images are shown. B. Graph depicts DHE fluorescence from myocardial tissue sections. Values are expressed as mean $\pm$ SEM *Different from control, ANOVA ($P < 0.05$).