Chronic Hormonal Imbalance and Adipose Redistribution Is Associated with Hypothalamic Neuropathology following Blast Exposure

Pamela J. VandeVord,¹ Venkata Siva Sai Sujith Sajja,¹ Evon Ereifej,¹
Amy Hermundstad,¹ Shijie Mao,¹ and Timothy J. Hadden²

Abstract

Endocrine disorders have been shown to be a consequence of blast traumatic brain injury in soldiers returning from military conflicts. Hormone deficiency and adrenocorticotrophic hormone (ACTH) dysfunction can lead to symptoms such as fatigue, anxiety, irritability, insomnia, sexual dysfunction, and decreased quality of life. Given these changes following blast exposure, the current study focused on investigating chronic pathology within the hypothalamus following blast, in addition to systemic effects. An established rodent model of blast neurotrauma was used to induce mild blast-induced neurotrauma. Adipose tissue, blood, and brain samples were collected at one and three months following a single blast exposure. Adipose tissue and blood were evaluated for changes in ACTH, adiponectin, C-reactive protein, glial fibrillary acidic protein, interleukin (IL)-1 β , and leptin. The hypothalamus was evaluated for injury using immunohistochemical techniques. The results demonstrated that the weight of the blast animals was significantly less, compared with the sham group. The slower rate of increase in their weight was associated with changes in ACTH, IL-1 β , and leptin levels. Further, histological analysis indicated elevated levels of cleaved caspase-3 positive cells within the hypothalamus. The data suggest that long-term outcomes of brain injury occurring from blast exposure include dysfunction of the hypothalamus, which leads to compromised hormonal function, elevated biological stress-related hormones, and subsequent adipose tissue remodeling.

Key words: +6 adipose tissue; ACTH; adiponectin; blast; hypothalamus; leptin

Introduction

BLAST-RELATED TRAUMATIC BRAIN INJURY (bTBI) is a serious health concern for service members returning from combat and can have long-term effects on behavior, mood, and cognitive function.^{1–5} Recently, pituitary dysfunction has been shown to be a consequence of bTBI in soldiers returning from war,^{5–7} with evidence suggesting that bTBI can cause impairment of hypothalamic-pituitary pathways.⁸ Further, it has been hypothesized that altered pituitary function may lead to cognitive and neuropsychiatric dysfunction.^{8–10}

The anterior pituitary gland produces several hormones, including adrenocorticotrophic hormone (ACTH), prolactin, and growth hormone (GH), which act on various target organs, and the secretion of pituitary hormones is regulated by hypothalamus-derived release factors.⁷ Baxter and colleagues¹⁰ reported an increased prevalence of anterior pituitary dysfunction in a group of soldiers who suffered from moderate-to-severe bTBI, compared with matched civilians suffering from non-blast induced TBI. Soldiers with bTBI presented

with various manifestations of pituitary dysfunction, including GH deficiency, hyperprolactinemia (the presence of abnormally high levels of prolactin), ACTH deficiency, or a combination of multiple hormone deficiencies.⁹ However, the role of bTBI in pituitary dysfunction remains unclear.⁶

Hyperactivity of the hypothalamic-pituitary-adrenal (HPA) axis was determined to be a primary biological mechanism for depression, as feedback inhibition is weakened.^{11,12} GH deficiency and ACTH dysfunction have been related to cognitive deficiencies following TBI and can lead to symptoms such as fatigue, anxiety, irritability, insomnia, sexual dysfunction, and decreased quality of life.³ Tümer and colleagues¹⁴ reported on increased hypothalamic expression of oxidative stress and activation of the sympatho-adrenal medulla at an acute time-point of 6 h following blast-induced neurotrauma (BINT) in rats.¹³ This study demonstrated that exposure to blast was associated with increased biosynthesis of catecholamine in the brain and elevated levels of stress-related biomarkers in serum. To our knowledge, however, no animal studies have been conducted to investigate the long-term

¹School of Biomedical Engineering and Sciences, Virginia Tech, Blacksburg, Virginia.

²Research Services, John D. Dingell VA Medical Center, Detroit, Michigan.

hypothalamus pathology and associated pituitary dysfunction resulting from BINT.

Chronic dysfunction in the hypothalamus can lead to disruption of hormone levels on a long-term basis. Specifically, injury or dysfunction within neuronal circuits connecting the HPA axis may be associated with long-term anxiety-like behaviors and cognitive responses.^{14,15} Neuropathology in regions such as the prefrontal cortex has been observed following BINT.^{16,17} As such, injuries to networks that modulate activation and feedback inhibition of the HPA axis could result in hormonal dysfunction leading to chronic physiological stress. Given these physical changes observed following BINT (unpublished results), the current study focused on investigating chronic pathology within the hypothalamus following blast. Moreover, systemic changes were examined as adipose tissue was collected from lower jaw and abdomen for gene expression analysis, and blood serum was collected for measuring specific proteins reflective of hypothalamic signaling cascades affected by BINT. Overall, the data were found to be consistent with the hypothesis that chronic hormonal dysfunction following blast is associated with hypothalamus dysfunction.

Methods

Animals and testing parameters

Experiments were conducted according to the Guiding Principles in the Care and Use of Laboratory Animals, and procedures were approved by the Virginia Tech Institutional Animal Care and Use Committee. Prior to all experiments, male Sprague Dawley rats (250–300 g; Harlan Laboratories, San Diego, CA) were acclimated 12 h light/dark cycle with food and water provided ad libitum. Animal weights were recorded before blast and weekly following blast.

The shock front and dynamic overpressure were generated by a custom-built Advanced Blast Simulator (ABS) as described previously (ORA Inc., Fredericksburg, VA).¹⁸ The ABS, located at Center for Injury Biomechanics of Virginia Tech, consists of a driving compression chamber attached to a rectangular testing chamber with an end wave eliminator (EWE; Fig. 1). A passive EWE was installed at the venting end of ABS, which minimizes the shock wave outflow by means of a grill plate. The EWE was patterned to mirror reflected shocks and rarefactions, which tend to “cancel” each other and diminish unwanted effects within the test chamber. A calibrated peak static overpressure was produced with compressed helium and calibrated acetate sheets (Grafix Plastics, Cleveland, OH). Pressure measurements were collected at 250 kHz using a Dash 8HF data acquisition system (Astro-Med, Inc, West Warwick, RI).

The animals were briefly anesthetized with 4% isoflurane before being placed in a rostral cephalic orientation towards the shock front. Animals were randomly selected to be in a blast or sham group ($n=12$ /group). The blast group was exposed to a peak static overpressure of 117 kPa for 2.5 msec duration. The sham animals were

given the same treatment as the blast group with the exception that they did not experience the blast exposure. Animals were sacrificed at one or three months following blast exposure. Blood samples were taken via cardiac puncture at the time of euthanasia and centrifuged at room temperature in serum-separating tubes. Serum was collected and stored at -80°C until further analysis by enzyme-linked immunosorbent assays (ELISAs). Animals were anesthetized with 3% isoflurane prior to euthanasia and brains were collected from all animals. Half of the animals per group ($n=6$ /group) were perfused with 4% paraformaldehyde for immunohistochemistry while the other half was perfused with cold saline. Brains from paraformaldehyde-perfused animals ($n=6$ /group) were processed in 30% sucrose solution and fixed in Tissue-Tek[®] optimal cutting temperature embedding medium (Sakura Finetek USA, Inc., Torrance, CA), then frozen on solid CO_2 ; 40 μm tissue sections were prepared with a microtome. Brains from saline-perfused animals were stored at -80°C for gene expression analysis. Adipose tissue from the lower jaw and abdomen was collected from the saline-perfused animals at three months following blast and frozen at -80°C until analysis.

Immunohistochemistry of hypothalamus

Immunohistochemistry was performed on tissue sections from the hypothalamus (bregma -3.30 to bregma -4.30 , Paxinos and Watson) for glial fibrillary acidic protein (GFAP; an astrocyte specific cell activation indicator), Iba-1 (microglial marker), cleaved caspase-3 (apoptosis), neuronal nuclei staining (Neu N; neuronal marker), and CD31 (endothelial cell marker).

Hypothalamus sections (40 μm) were initially washed with phosphate-buffered saline (PBS), and then blocked with 3% bovine serum albumin (BSA) in PBS for 1 h at room temperature. Tissue sections were then incubated with a primary antibody (anti-GFAP (Invitrogen, Carlsbad, CA), anti-Iba-1 (Biocare Medical, Concord, CA), anti-cleaved caspase-3 (Invitrogen), anti-Neu N (Millipore, Billerica, MA) or anti-CD31 (BD Biosciences, San Jose, CA) at 1:200 overnight at 4°C . After PBS wash, the samples were incubated for 1.5 h with fluorescein isothiocyanate-tagged (FITC)-secondary anti-rat IgG antibodies (1:200; Vector Laboratories, Burlingame, CA) or Alexa flour-555 anti-rabbit IgG antibody (1:200; Cell Signaling, Danvers, MA). After a PBS wash, samples were mounted on slides, air dried, and cover-slipped with prolong antifade gold reagent containing 6-diamidino-2-phenylindole (DAPI; Invitrogen). Sections (five representative sections per stain across hypothalamus) were examined with a Zeiss fluorescence microscope (Carl Zeiss Microscopy, Peabody, MA) at $20\times$ magnification under appropriate filters. Fluorescence intensity of acquired digital images was quantified by ImageJ software (National Institutes of Health, Bethesda, MD).

ELISA for blood serum protein assessment

The protein abundance levels of hormone, pro-inflammatory mediators, and adipokines in serum were determined by using rat ELISA kits for GFAP, adiponectin, C-reactive protein (CRP,

FIG. 1. Blast overpressures are generated with an Advanced Blast Simulator located at Center for Injury Biomechanics, Virginia Tech, Blacksburg, VA. Color image is available online at www.liebertpub.com/neu

TABLE 1. PRIMER SEQUENCES FOR REAL-TIME PCR

Category	Gene	Primer sequence
Housekeeping gene	GAPDH	5'-TGGCCTTCCTGTTCCCTACC-3' (F)
		5'-AGCCCAGGATGCCCTTTAGTG-3' (R)
Hormone	Leptin	5'-TTCACACACGCAGTCGGTATC-3' (F)
		5'-CCCGGGAATGAAGTCCAAA-3' (R)
	Adiponectin	5'-AGGGCCAGGAGCTTTGGT-3' (F)
		5'-GCCATCCAACCTGCACAAG-3' (R)

GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

Millipore), leptin (Sigma-Aldrich, St. Louis, MO), IL-1 β (Alpco, Salem, NH), and ACTH (Phoenix Pharmaceuticals Inc., Burlingame, CA) following the manufacturer's protocols.

Real-time polymerase chain reaction for fat regulatory gene assessment

Real-time polymerase chain reaction (RT-PCR) was used to measure gene expression from blast and sham samples relative to the internal housekeeping gene control. Adipose tissue was collected from the lower jaw and abdomen of the saline-perfused animals ($n=6$ /group), rinsed with saline to remove residual blood, and frozen at -80°C . After thawing, the tissue was homogenized using a Branson Ultrasonicator (Fisher Scientific, Hampton, NH) and total RNA was extracted and purified from the tissues using the RNeasy Lipid Tissue Mini Kit (Qiagen, Valencia, CA) following the manufacturer's protocol. The RNA concentration and purity were estimated by measuring absorbance at 260 nm and 280 nm. Reverse transcriptase using random primers converted the messenger RNA (mRNA) to a complementary DNA (cDNA) template using a thermal cycler (Mastercycler Gradient; Eppendorf, Hauppauge, NY). For RT-PCR analysis, cDNA equivalent to 40 ng of total RNA was used. Specific primer pairs for adiponectin and leptin are shown in Table 1. As an internal control, the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase was used. The PCR reactions included 1 $\times$ SYBR[®] Green Master Mix (Applied Biosystems, Foster City, CA), as well as forward and reverse primers (0.4 μM each). RT-PCR was conducted in 96-well optical plates using a 7500 Fast Real-Time PCR System (Applied

Biosystems). The following profile was used: 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 sec, and 60°C for 1 min. Threshold cycle (Ct) values for each sample and primer pair were obtained and analyzed by the delta-delta ($\Delta\Delta$) Ct method in order to calculate the fold change in each target gene.^{19,20}

Statistical analysis

Statistical differences between sham and blast animals were assessed by analysis of variance (ANOVA) with $p<0.05$ considered significant using SPSS[™] statistical software (IBM, Armonk, NY) as two separate experiments at one month and three months time-points for any unknown residual effects. For the animal weights, a two-factorial repeated measures ANOVA was used for the behavioral testing, with $p<0.05$ considered statistically significant. Unless indicated otherwise, data are presented as mean $\pm$ standard error of the mean.

Results

Animal weight

Starting at Day 14 following blast, the weight of the blast animals was significantly less, compared with the sham group ($p<0.05$; Fig. 2). This slower rate of increase of their weight was maintained through the three month time-point.

Immunohistochemistry of hypothalamus

A significant decrease in CD31+ staining was seen one month post-blast ($p<0.05$) but not at three months following blast, compared with the sham group (Fig. 3). A significant increase in caspase-3+ staining was observed at three months ($p<0.05$) but not at one month following blast, compared with the sham group (Fig. 4). There were no significant changes in levels of GFAP, Iba-1, or Neu N at one or three months (data not shown).

Blood serum assessment

At one month following blast, a significant decrease in ACTH (Fig. 5A) and significant increase in leptin were found, compared with the sham group ($p<0.05$; Fig. 5C). At three months post-blast,

FIG. 2. Graph depicts a time-course analysis of body weight following blast. Starting at Day 14 following blast, the weight of the blast animals was significantly less, compared with the sham group ($*p<0.05$). Color image is available online at www.liebertpub.com/neu

FIG. 3. A significant reduction in CD31+ staining, a marker of endothelial cells, was observed at one month following blast, compared with the sham ($p < 0.05$). (A) Sham CD31, (B) blast CD31, (C) sham CD31 + 6-diamidino-2-phenylindole (DAPI), and (D) blast CD31 + DAPI. Color image is available online at www.liebertpub.com/neu

there was a significant increase in ACTH, compared with the sham group ($p < 0.05$; Fig. 5A) and significant decreases in IL-1 β (Fig. 5B) and leptin (Fig. 5C), compared with the sham group ($p < 0.05$). No significant changes were observed in adiponectin, GFAP, or CRP in the serum (data not shown).

RT-PCR assessment

Significant increases ($p < 0.05$) in the abundance of mRNA encoding adiponectin and leptin were found in abdominal adipose, while a significant decrease ($p < 0.05$) in adiponectin mRNA

FIG. 4. A significant reduction in cleaved caspase-3+ staining, a marker of early stage apoptosis, was observed at three months, compared with sham ($p < 0.05$). (A) Sham caspase-3, (B) blast caspase-3, (C) sham caspase-3 + 6-diamidino-2-phenylindole (DAPI), and (D) blast caspase-3 + DAPI. Color image is available online at www.liebertpub.com/neu

FIG. 5. Serum biomarkers were evaluated. (A) Adrenocorticotrophic hormone (ACTH) was found to be significantly decreased at one month, but was found to be increased at three months following blast ($*p < 0.05$). (B) Interleukin (IL)-1 β was significantly decreased at three months following blast ($*p < 0.05$). (C) Leptin levels were increased at one month but then decreased at three months following blast ($*p < 0.05$).

abundance was found in lower jaw adipose, compared with the sham at three months following blast (Fig. 6).

Discussion

Under control of the hypothalamus, the pituitary secretes hormones that participate in regulating a variety of systemic functions

and conditions including the stress response, glucose and lipid metabolism, adipose homeostasis, immune function, and feeding behavior. Endocrine abnormalities caused by hypothalamus dysfunction can result in altered regulation of these systemic functions. It is increasingly evident that hypothalamo-pituitary dysfunction commonly occurs among military personnel suffering from blast-related TBI. Further, clinical reports suggest that hormonal

FIG. 6. At three months following blast, significant increases in the messenger RNA levels of adiponectin and leptin were observed in the abdomen adipose tissue, compared with sham ($*p < 0.05$). However, a significant decrease of adiponectin was found at three months following blast in the lower jaw region, compared with sham ($*p < 0.05$).

dysregulation due to hypothalamus dysfunction may be associated with cognitive deficits after blast-related TBI.^{5,6,8} Indeed, previous studies have shown that corticosterone and ACTH play important roles in memory formation and in the regulation of neuronal regeneration via neurotrophins, such as brain derived neurotrophic factor.^{21,22}

Hormones released by the hypothalamus and pituitary play crucial roles in the systemic response to stress, inducing alterations in adipose metabolism, body weight regulation, and endocrine function. Under normal physiological conditions, systemic endocrine function acts as a feedback mechanism to return hypothalamo-pituitary function to baseline. However, injury that impacts hypothalamo-pituitary regions can disrupt this feedback mechanism, potentially leading to abnormal endocrine function. Recent studies have shown that blast-related TBI is often accompanied by hypothalamo-pituitary dysfunction, resulting in abnormal secretion of ACTH, cortisol, and GH.^{5, 6, 8} This, in turn, is speculated to mediate pathological changes in adipose deposition and function via altered glucose and lipid metabolism.^{23–26} Although pre-clinical studies are limited, one study of anterior pituitary function using the cortical contusion injury (CCI) model demonstrated that two months following brain injury in rats, there was an upregulation of IL-1 β and GFAP and decreased secretion of GH, possibly due to a persistent inflammatory reaction in the hypothalamus.²⁷ Another report showed that blast neurotrauma caused a disruption of hypothalamus function, resulting in increased circulating levels of IL-6, further supporting the role of BINT in inducing a proinflammatory state.²⁸

During the course of our ongoing TBI studies, we observed that animals subjected to blast developed enlarged adipose deposits in their lower jaw region, though their overall weight gain during the three months of observation lagged behind that of control animals (Fig. 2). This observation suggested that the blast may induce metabolic anomalies, including adipose remodeling, increased risk for obesity, insulin resistance, and diabetes. To understand the role, if any, of blast exposure on metabolic function, we initiated a study of key factors involved in adipose tissue deposition, development, and function.

The current study detected decreased levels of ACTH at one month and increased ACTH levels at three months in animals exposed to the blast. ACTH acts on the adrenal cortex to stimulate synthesis/secretion of glucocorticoids that are known to affect lipid and glucose metabolism and adipose distribution, among other activities. It is likely that the long-term (three-month) increase of ACTH, through glucocorticoid action, contributes to the altered pattern of adipose tissue deposition observed in these animals.

Adipocytes synthesize a number of adipokines that act on a variety of cell types and tissues to influence various conditions and behaviors that impact energy homeostasis. Leptin is secreted from adipocytes and its concentration in the circulation is proportional to adipose mass. Upon binding to its receptors in the hypothalamus, leptin promotes satiety. We detected increases in leptin at one month in serum and decreases at three months post-blast exposure. This finding is consistent with the observation that abdominal adiposity was decreased at three months in blast-exposed animals, compared with sham.

Adiponectin mRNA abundance increased approximately three-fold in abdominal adipose tissue but was significantly lower in adipose tissue from the lower jaw of animals exposed to blast. However, adiponectin protein abundance did not change with blast exposure, an observation that raises the possibility that adiponectin expression is differentially regulated in blast-exposed animals (i.e.,

at a level other than mRNA abundance). The findings regarding leptin and adiponectin may reflect altered adipose function in blast animals.

Hypothalamo-pituitary dysfunction associated with blast was reported recently by Tümer and colleagues¹⁶ who detected elevated levels of the catecholamine biosynthesizing enzymes, tyrosine hydroxylase, dopamine- β hydroxylase, and of neuropeptide Y, and plasma norepinephrine (NE) at 6 h following blast. Acute elevation of these proteins and NE indicates that somatosensory neuronal activity increases following blast exposure. Increased neuronal activity in the nucleus accumbens was reported by Sajja and colleagues,²⁹ who found elevated biosynthesis of monoamines, such as serotonin and dopamine, after blast.³⁰ In addition, Tümer and colleagues¹⁴ found increased levels of angiotensin II receptor type 1 (AT1), a vasoconstrictor. Potential damage to neurovasculature and vascular integrity could result in AT1 activation. In the current study, a significant decrease in the levels of the endothelial cell marker CD31 was observed at one month post-blast. This decrease in vascular integrity supports the increased AT1 data reported by Tümer and colleagues.¹⁴ AT1 upregulation can relate to protection of the blood brain barrier from toxic substances entering the brain under compromised vasculature. Altogether, the evidence suggests that monoamine biosynthesis may contribute to the elevated stress environment following BINT.

Conclusion

Overall, the data suggest that long-term outcomes of brain injury occurring from blast exposure include dysfunction of the hypothalamus, which leads to compromised hormonal function, elevated biological stress-related hormones (ACTH), and subsequent adipose tissue remodeling.

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Author Disclosure Statement

No competing financial interests exist.

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Address correspondence to:

Pamela J. VandeVord, PhD

Department of Biomedical Engineering and Mechanics

Virginia Tech

447 Kelly Hall, 325 Stanger Street

Blacksburg, VA 24061

E-mail: pvord@vt.edu