Combat-training stress in soldiers increases S100B, a marker of increased blood-brain-barrier permeability, and induces immune activation

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Abstract

BACKGROUND: Experimental data suggest stress-related cognitive dysfunction may be associated with increased blood-brain-barrier (BBB) permeability secondary to immune activation.

METHODS: We investigated the relationship between prolonged and intense physical and psychological combat-training stress, immune activation and blood-brain-barrier permeability in 37 healthy male army rapid response troops.

RESULTS: Soldiers during intense combat training showed greater self-reported stress, anxiety and depression levels than at rest, as assessed by specific questionnaires. S100B, a marker of BBB permeability, as well as serum cortisol, IL-6 and TNF-α concentrations, were significantly increased in soldiers during combat training compared to rest (all \( p<0.05 \)). Serum S100B correlated negatively with morning serum cortisol in soldiers during combat training, but not during the rest period (\( r=-0.387, p<0.05 \)).

CONCLUSION: We conclude that combat training inducing significant levels of stress, depression and anxiety is accompanied by evidence of increased blood-brain barrier permeability and by increases in systemic pro-inflammatory mediators.

INTRODUCTION

Clinical data suggests that cognitive dysfunction, including emotional, memory and mental changes may be related to increased blood-brain-barrier (BBB) permeability in several human diseases, as well as in clinical conditions with increased immune activation (Davies & Hardy 1988; Huber 2008; Huber et al. 2001; Weiss et al. 2009). Increased ‘leakiness’ of this dynamic protective barrier of the brain has also been associated with several situations relevant to stressful combat settings, including psychological and physical stress, exercise in hot environments and inflammatory illnesses (Abbott et al. 2010; Baumgart & Dignass 2002; Esposito et al. 2001; Farrall & Wardlaw 2009; Huber et al. 2002; Trojano et al. 1992; Watson et al. 2006; Weiss et al. 2009; Wright...
and examination, or abnormal pre-training hematology and biochemistry tests. Participation in this study was voluntary, written informed consent was obtained from all the subjects and written parental consent was also obtained for those under the age of 21 years. The study protocol was approved by the Ethics Committees of the Singapore National Healthcare Group and the DSO National Laboratories Institutional Review boards.

**Combat-training course and study design**

The MRF combat-training conversion course is a 6-week period of all-day high-intensity stress and serves as a transition from the relatively stress-free classroom-based medical course to an environment with high physical and psychological demand, comprising combat-training missions, some in heavy anti-chemical suits. These identical activities for all soldiers are carried out at average ambient temperatures above 30°C and humidity above 80%. Blood sampling for stress and immune mediators was performed at identical times (see below) after 4 weeks of combat-training and on the twelfth full day of rest after the end of the combat-training. All subjects refrained from ingestion of alcohol for at least 5 days prior to test days and from smoking in the morning before testing, and fasted from 10 pm to 6 am before the tests.

**Questionnaires: Anxiety, Depression, Stress and Bowel function**

The Perceived Stress Scale-10 item (PSS-10) questionnaire was used for quantification of stress (Cohen et al. 1983), the Hospital Anxiety and Depression (HAD) scale for anxiety and depression (Zigmond & Snaith 1983). Ratings were performed independently by all subjects before blood sampling during the combat-training and rest periods.

**Stress markers and inflammatory mediators**

Fasting blood samples for cortisol quantification were collected between 6:00 and 6:15 AM. On the day of sampling the soldiers rose at 5:30 AM as usual and avoided any exercise before blood was drawn from a forearm vein by experienced phlebotomists. The collected blood was allowed to clot for 30 minutes at room temperature. The samples were then centrifuged at 1200xg for 15 minutes at 4°C and serum samples were stored in aliquots at –80°C. Serum cortisol was quantified by the Elecsys Cortisol Assay (Roche Diagnostics, USA) and IL-6, TNF-α, and IL-10 were measured using a commercial enzyme-linked immunosorbent assays (R&D Systems, USA). Inter- and intra-assay variation was below 8%.

**S100B measurement**

Serum S100B levels were measured using the fully-automated electro-chemo-luminometric immunoassay (Elecsys S100 Assay #03175243, Roche Diagnostics, Germany) by the Cobas e411 analyzer according to

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the manufacturer’s instructions. Inter- and intra-assay variation was below 5%.

**Statistics**

SPSS Statistics 17.0 software (SPSS Inc, Chicago, US) was used for statistical analysis. Data were expressed as medians and interquartile ranges and comparisons between the combat-training and rest periods were performed using the paired sample t-test. Simple correlations between parameters were evaluated by the Pearson’s rank correlation coefficient. A two-tailed p-value of <0.05 was considered statistically significant. Intra- and inter-assay variation coefficients were below 8% for all variables.

**RESULTS**

Thirty-nine non-smoker Asian male soldiers aged 19–23 years (mean: 20.7 years) from the same MRF troop were included in the study. Two soldiers were excluded because of fever on the day of blood sampling. No soldiers had clinical evidence of infections or acute illnesses during the course of the study and there were no further study drop-outs.

**Stress, anxiety and depression levels**

Combat training significantly increased anxiety, depression and stress scores in soldiers compared to the rest period (all p < 0.001) (Figure 1A). Serum cortisol was also significantly increased during combat training compared to at rest (p<0.05) (Figure 1B).

**S100B, and inflammatory markers**

Serum S100B was significantly greater during combat training than at rest (p<0.05) (Figure 2). Inflammatory cytokine serum levels were significantly higher during the combat-training period than at rest (p<0.01) (Figure 3). IL-10 concentrations were similar in both periods (4.12, 95%CI: 3.10–5.13, vs. 4.84, 95%CI: 2.68–6.99 pg/ml, p=0.56).

**Correlations between mediators**

There was a negative correlation between S100B and cortisol concentrations (r=-0.387, p<0.05) during stress, but not in the rest period (Figure 4). There were no correlations between the concentrations of immune mediators and S100B.
DISCUSSION

In the current human study serum S100B as a marker of BBB permeability, inflammatory mediator concentrations, as well as stress indices, were increased significantly in soldiers during combat training compared to rest, suggesting that stress modulates the immune milieu and BBB permeability. The underlying causes of the frequently observed stress-related cognitive dysfunction in soldiers are not clear, but increased permeability of the BBB secondary to immune activation may be one of the underlying mechanisms (Garvey et al. 2009; Hoge et al. 2004; Lieberman et al. 2005b; Lieberman et al. 2005a; Vasterling et al. 2010). The BBB is a dynamically-controlled and crucial element in actively regulating brain ingress and egress of a wide range of mediators (Weiss et al. 2009). Immune activation and stress of various origins in animals have been shown to modulate BBB function and permeability (Hawkins & Davis 2005).

Chronic stress can affect cognitive functions directly via stress hormones such as cortisol or by activating the secretion of proinflammatory and type-2 cytokines such as IL-6 and by promoting the immune responses driven by these cytokines (Anisman 2009; Kiecolt-Glaser et al. 2003; Li et al. 2008; Lutgendorf et al. 1999; Miller et al. 2002; O'Brien et al. 2004). Furthermore, inflammatory mediators, including IL-6 and TNFα, affect the integrity of the BBB in vitro, predominantly by increasing degradation and decreasing synthesis of tight junction (TJ) proteins (Argaw et al. 2006; Claudio et al. 1994; de Vries et al. 1996; Diebel et al. 2005). We recently reported increased intestinal permeability in the same soldiers under stress, very likely driven by the effect of immune activation on the very similarly structured intestinal barrier (Li et al. 2013). Increased brain access of pro-inflammatory mediators, especially IL-1, has in animal studies resulted in decreased cognitive performance (Banks et al. 2001). Increased BBB permeability as a cause for cognitive change has been implicated in a wide range of clinical conditions with proven immune activation, including pain, ageing, peripheral inflammation, closed head injury, early diabetes, multiple sclerosis, lacunar stroke, cardiac surgery and central nervous system tumours as well as athletic exercise in high ambient temperatures (Farrall & Wardlaw 2009; Hawkins & Davis 2005; Trojano et al. 1992; Weiss et al. 2009). These changes in cognition affect memory, concentration, learning, associative tasks and mood (Farrall & Wardlaw 2009; Yadavalli et al. 2008; Bell & Zlokovic 2009). The increased S100B concentrations demonstrated during combat training stress may therefore reflect increased BBB permeability due to greater stress-induced levels of the pro-inflammatory mediators IL-6 and TNFα. In the current study no direct correlations between the inflammatory mediator and S100b concentrations were confirmed, but this may be due to the choice of mediators as well as the sampling frequency and needs to be investigated further. The relationship between BBB changes, immune activation during stress and cognitive changes also need to be studied in a larger group of soldiers and using specific cognitive paradigms. The resting measurements were performed 12 days after cessation of the combat exercises. Although acute stressors generally have a physiologic effect lasting several hours, it is currently not known if the effects of a more prolonged
and intense stressor would have reverted to baseline levels after a period of 12 days and future studies should include several measurement time-points extending to a longer observation period.

Stress mediators have a dual action and can also protect against potential damage by proinflammatory cytokines by suppressing inflammatory-mediated activation (Elenkov & Chrousos 2002). Glucocorticoids have, for example, been reported to prevent BBB breakdown in response to TNF-α through tightening of the endothelial barrier and maintenance of adequate TJ protein levels (Forster et al. 2008). The negative correlation between serum S100B cortisol levels could therefore be explained by a counterregulatory protective effect of cortisol on BBB integrity. Serial determinations of cortisol concentrations in saliva would have yielded a more accurate estimation of the HPA-axis activation than serum levels, as up to 80–90% of total serum cortisol is bound to cortisol-binding globulin (CBG) or serum albumin (Gozansky et al. 2005).

S100B, being predominantly of astrocytic origin, has been widely used as a marker for neurometabolism, neuroinflammation and of increased BBB permeability (Marchi et al. 2004; van Munster et al. 2009; Vos et al. 2004). Validation of S100B as a marker of BBB opening has been performed with imaging techniques (Kanner et al. 2004). Although peripheral origins of S100B such as fat, muscle, and marrow, have been reported, a recent study has demonstrated that extracranial sources of S100B do not significantly affect serum levels in humans without traumatic brain or bodily injury from accident or surgery (Anderson et al. 2001; Pham et al. 2010). Animal research has also suggested the brain to be the main source of the increased release of S100B in BBB opening accompanying endotoxemia (Lipschey et al. 2010). Nonetheless, a comparison between S100B and more specific quantitative measures of early BBB disruption would be of interest in future studies.

CONCLUSIONS

In this prospective study, we found that real-life, prolonged and intense combat-training stress increased systemic levels of major pro-inflammatory mediators and of S100B as a marker of BBB permeability. Both of these changes may represent mechanisms underlying the cognitive compromise seen in soldiers under operational stress. These mechanisms require further study in larger cohorts, and with additional markers of BBB opening and immune activation. If confirmed, pre-emption of these changes may provide opportunities for preventing the cognitive compromise commonly seen during and following severe combat and operational action.

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