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The role of extracellular heat shock proteins in posttraumatic stress disorder-related inflammation

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Uloga vanćelijskih proteina toplotnog stresa u inflamaciji povezanoj sa posttraumatskim stresnim poremećajem

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The role of extracellular heat shock proteins in posttraumatic stress disorder-related inflammation

Abstract

Post-traumatic stress disorder (PTSD) is a debilitating psychiatric disorder that may develop following exposure to life-threatening traumatic event. PTSD represents a serious medical and economic burden for the society due to its relatively high life-time prevalence rate and comorbidity with variety of other mental disorders and somatic illnesses. General opinion is that the best results in understanding of PTSD pathogenesis, diagnosis and treatment could be achieved by integrating psychological, biological and pharmacotherapeutical approaches.

Current research has suggested that PTSD is associated with neuroendocrinological disturbances and immune function alterations. The hypersensitivity of hypothalamic-pituitary-adrenocortical (HPA) axis to cortisol feedback inhibition is considered the neuroendocrine hallmark of PTSD, while the nature of PTSD-related immune alterations is not well understood. Most studies report that PTSD is associated with excessive inflammation, but unaltered inflammatory state and even decreased circulatory levels of inflammatory markers were also reported. It has been hypothesized that excessive inflammation in individuals with PTSD might be a consequence of insufficient immunosuppression by cortisol. However, the data on cortisol levels and cortisol receptor functioning in PTSD patients are inconsistent.

Exposure to a variety of stressors will induce intracellular heat shock proteins (HSPs) with cellular functions beneficial for cell survival. For many years HSPs have been viewed as intracellular proteins, but it is now known that they can be released from various mammalian cells into the peripheral circulation, where they are capable of activating pro-inflammatory responses. Taking that PTSD-associated excessive inflammation is not likely to be caused by impaired anti-inflammatory action of cortisol, we hypothesize that it might be a consequence of Hsp60 and Hsp70 induction and release by psychological trauma.

The aim of the present study was to relate the parameters of inflammatory status with circulatory levels of HSPs and cortisol, and to uncover possible associations between inflammatory markers and extracellular HSPs with trauma-exposure, PTSD symptoms, vulnerability and resilience to PTSD. Toward that end the relevant parameters were determined in patients with current PTSD (N=118) and life-time PTSD (N=63), in trauma-exposed individuals without PTSD (N=94) and in non-traumatized healthy subjects (N=95). The measured parameters were: circulatory levels of a number of cytokines and C-reactive protein, as inflammatory markers; intracellular levels of HSPs (Hsp90, Hsp70, Hsp72 and Hsp60); plasma levels of HSPs (Hsp70, Hsp72 and Hsp60); plasma level of cortisol before and after suppression of HPA axis by dexamethasone and the level of corticosteroid receptors, glucocorticoid receptor (GR) and mineralocorticoid (MR), in peripheral blood mononuclear cells (PBMCs).

Plasma levels of pro-inflammatory cytokines, markers of inflammation and extracellular HSPs (eHsp60 and eHsp70) were determined by ELISA, while semi-quantitative Western blot was performed for determination of lymphocyte level of corticosteroid receptors and HSPs.

The results demonstrate that the alterations in plasma levels of cortisol, Hsp70s and Hsp60, as well as the MR level and MR/GR balance in peripheral lymphocytes are not associated with trauma exposure, current or life-time PTSD symptoms, resilience to PTSD, vulnerability to PTSD and/or with remission of the disorder. However, vulnerability to PTSD is characterized by hypersensitivity of HPA axis to cortisol and by increased level of GR expression in PBMCs. The levels of expression of HSPs (Hsp90, Hsp70, Hsp72 and Hsp60) in the lymphocytes do not vary between current PTSD, life-time PTSD, trauma control and healthy control groups of subjects. Interestingly, MR level in peripheral lymphocytes is correlated with the levels of both Hsp90 and Hsp70, while the GR level is correlated only with that of Hsp90. The strength of correlation between the lymphocyte levels of MR and Hsp70 is related to current PTSD, while war trauma-exposure may improve correlation between the levels of GR and Hsp90. Judging by the levels of general inflammatory markers (CRP, erythrocytes sedimentation rate, leukocyte count) and cytokines (TNF-α, IL-6, IL-12p70, IL-2, IL-8, IFN-γ, IL-5, IL-4, IL-10), trauma-exposure and PTSD could rather be linked to immunosuppression than to enhanced inflammation. The

levels of some cytokines (TNF-α, IL-6, IL-12p70, IL-2 and IL-8) might be connected with trauma-exposure rather than with the presence of current or life-time PTSD symptoms, while the levels of others (IFN-γ, IL-5, IL-4 and IL-10) could be related to vulnerability to PTSD rather than to trauma-exposure.

The results of the present PhD project constitute an original scientific contribution to better understanding of the molecular mechanisms underlying pathophysiology of PTSD. Specifically, they add to unraveling the relationship between inflammation and trauma or vulnerability/resilience to PTSD, and shed a new light on the role of extracellular HSPs in PTSD-related inflammatory actions of the immune system.

Key words: posttraumatic stress disorder; heat shock proteins; extracellular heat shock proteins; cytokines; glucocorticoid receptor; cortisol.

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Uloga vanćelijskih proteina toplotnog stresa u inflamaciji povezanoj sa posttraumatskim stresnim poremećajem

Rezime

Posttraumatski stresni poremećaj (PTSP) je psihijatrijska bolest koja se može razviti posle izlaganja traumatskom događaju. PTSP predstavlja veliko medicinsko i ekonomsko opterećenje za društvo, zbog relativno velike učestalosti i komorbiditeta sa mnogim drugim psihijatrijskim i somatskim bolestima. Smatra se da se najbolji rezultati u razumevanju patogeneze PTSP-a i njegovoj dijagnostici i terapiji mogu postići integrisanjem psiholoških, bioloških i farmakoterapeutskih pristupa.

Aktuelna istraživanja sugerišu da je PTSP povezan sa neuroendokrinim poremećajima i promenama imunskih funkcija. Smatra se da je hiperosetljivost hipotalamo-hipofizno-adrenokortikalne (HHA) ose na kortizol neuroendokrini „pečat" PTSP-a, dok je priroda imunskih poremećaja povezanih sa PTSP-om još uvek nejasna. Većina studija pokazala je da je PTSP povezan sa pojačanom inflamacijom, ali su objavljeni i podaci o nepromenjenom inflamatornom statusu, pa čak i smanjenom nivou inflamatornih markera u cirkulaciji PTSP pacijenata. Pretpostavlja se da pojačana inflamacija kod osoba obolelih od PTSP-a može biti posledica nedovoljne imunosupresije kortizolom. Međutim, podaci o nivou kortizola i funkcionisanju kortizolskih receptora kod pacijenata sa PTSP-om nisu konzistentni.

Izlaganje različitim stresorima indukuje sintezu unutarćelijskih proteina toplotnog stresa (HSP) čije funkcije potpomažu preživljavanje ćelija. Tokom mnogo godina HSP su posmatrani kao unutarćelijski proteini, ali sada se zna da ih mnoge sisarske ćelije oslobađaju u cirkulaciju, gde mogu da aktiviraju pro-inflamatorne odgovore. Uzimajući da pojačana inflamacija koja je povezana sa PTSP-om verovatno nije posledica umanjenog anti-inflamatornog delovanja kortizola, mi smo pretpostavili da ona može biti posledica indukcije i oslobađanja Hsp60 i Hsp70 psihološkom traumom.

Cilj ove studije bio je da se povežu parametri inflamatornog statusa sa nivoima kortizola i HSP u cirkulaciji, kao i da se otkriju moguće veze između inflamatornih markera i vanćelijskih HSP sa izlaganjem traumi, simptomima PTSP-a, osetljivošću i otpornošću na

PTSP. U tom cilju u studiju su bili uključeni pacijenia sa aktuelnim PTSP-om (N=118), osobe koje su prebolele PTSP (N=63), osobe izložene traumi koje nisu razvile PTSP (N=94) i zdrave, netraumatizovane kontrole (N=95). Izmereni parametri bili su: unutarćelijski nivoi HSP (Hsp90, Hsp70, Hsp72 i Hsp60), nivoi HSP (Hsp70, Hsp72 i Hsp60) u krvnoj plazmi, nivo kortizola u plazmi pre i posle supresije HHA ose deksametazonom i nivoi kortikosteroidnih receptora, glukokortikoidnog receptora (GR) i mineralokortikoidnog receptora (MR), u perifernim limfocitima.

Nivoi proinflamatornih citokina, markera inflamacije i vanćelijskih HSP (eHsp60 i eHsp70) u krvnoj plazmi određivani su metodom ELISA, dok je semi-kvantitativni „Western blot" korišćen za određivanje nivoa kortikosteroidnih receptora i HSP u limfocitima.

Rezultati su pokazali da se promene nivoa kortizola, Hsp70 i Hsp60 u plazmi, kao i nivo MR i ravnoteža između MR i GR u limfocitima ne mogu povezati sa izlaganjem traumi, aktuelnim ili prebolovanim PTSP-om, osetljivošću i otpornošću na PTSP, niti sa oporavkom od PTSP-a. Međutim, osetljivost na PTSP karakteriše se hiperosetljivošću HHA ose na kortizol i povećanim nivoom ekspresije GR-a u perifernim limfocitima. Nivo ekspresije HSP (Hsp90, Hsp70, Hsp72 i Hsp60) u limfocitima ne razlikuje se između osoba sa aktuelnim PTSP-om, prebolovanim PTSP-om, traumatizovanih osoba bez PTSP-s i zdravih, netraumatizovanih kontrola. Interesantno je da postoji korelacija nivoa MR-a sa nivoima Hsp90 i Hsp70 u limfocitima, dok nivo GR-a koreliše samo sa nivoom Hsp90. Jačina korelacije između nivoa MR i Hsp70 u limfocitima može se povezati sa aktuelnim simptomima PTSP-a, dok izlaganje traumi pojačava korelaciju između nivoa GR-a i Hsp90. Sudeći prema opštim inflamatornim markerima (CRP, sedimentacija eritrocita, broj leukocita) i nivoima citokina (TNF-α, IL-6, IL-12p70, IL-2, IL-8, IFN-γ, IL-5, IL-4, IL-10), izlaganje traumi i PTSP se mogu povezati sa imunosupresijom, pre nego sa pojačanom inflamacijom. Nivoi nekih citokina (TNF-α, IL-6, IL-12p70, IL-2, IL-8) mogli bi se povezati sa izlaganjem traumi, pre nego sa prisustvom aktuelnih ili prošlih simptoma PTSP-a, dok se nivoi drugih citokina (IFN-γ, IL-5, IL-4, IL-10) mogu povezati sa osetljivošću na PTSP, pre nego sa izlaganjem traumi.

Rezultati ovog doktorskog projekta predstavljaju originalan naučni doprinos boljem razumevanju molekularnih mehanizama koji se nalaze u osnovi patofiziologije PTSP-a.

Posebno, oni doprinose razotkrivanju veze između inflamacije i traume, odnosno osetljivosti/otpornosti na PTSP, i rasvetljavanju uloge vanćelijskih HSP u inflamatornim funkcijama imunskog sistema povezanim sa PTSP-om.

Ključne reči: posttraumatski stresni poremećaj; proteini toplotnog stresa; vanćelijski proteini toplotnog stresa; citokini; glukokortikoidni receptor; kortizol

Naučna oblast: Biologija

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Abbreviations

ACTH – adrenocorticotropic hormone
AHA1 – Hsp90 co-chaperone
ANCOVA – analysis of covariance
ANOVA – one-way analysis of variance
AP-1 – activating protein-1
BDI-II – Beck's Depression Inventory II
BSA – bovine serum albumine
CAPS-DX – Clinician Administered PTSD Scale
CBP – cyclic AMP-responsive element-binding protein
CD14 – TLR co-receptor for bacterial lipopolysaccharide
CD40 – receptor on the surface of antigen presenting cells
CD91 – receptor for Grp96
CDC37 – Hsp90 co-chaperone
CRH – corticotrophin-releasing hormone
CRP – C-reactive protein
Cyp40 – cyclosporin A-binding immunophilin
DEX – dexamethasone
DSM-IV – Diagnostic and Statistical Manual of Mental Disorders-IV
DST – dexamethasone suppression test
eHsp60 – extracellular Hsp60
eHsp70 – extracellular Hsp70
eHSPs – extracellular HSPs
ELISA – enzyme-linked immunosorbent assay
FKBP51/52 – FK506-binding protein 51/52
GR – glucocorticoid receptor
GRE – glucocorticoid-responsive element
GroEL/GroES complex – prokaryotic homolog of mitochondrial Hsp60 chaperone system
Grp96 – 96 kDa glucose-regulated protein
HAT – histone acetyltransferase
HDAC2 – histone deacetylase-2

HDL – high density lipoproteins

HIP – Hsp70-interacting protein

HOMA – Homeostasis Model Assessment

HOP – Hsp70/Hsp90 organizing protein

HPA axis – hypothalamic-pituitary-adrenocortical axis

HRP – horse-radish peroxidase

HSBP1 – heat shock protein binding factor 1

HSE – heat shock element

HSF – heat shock factor

Hsp100 – 100kDa heat shock protein family

Hsp40 – 40kDa heat shock protein family

Hsp60 – 60kDa heat shock protein family

Hsp70 – 70kDa heat shock protein family

Hsp90 – 90kDa heat shock protein family

HSPs – heat shock proteins

ICAM-1 – intracellular adhesion molecule

IFN-γ – interferon γ

IL-10 – interleukin-10

IL-12p70 – interleukin-12p70

IL-1β – interleukin-1 beta(

IL-2 – interleukin-2

IL-4 – interleukin-4

IL-6 – interleukin-6

IL-8 – interleukin-8

K_D – equilibrium dissociation constant

LDL – low density lipoproteins

MR – mineralocorticoid receptor

NFκB – nuclear factor κB

nGREs – negative GREs

p23 – Hsp90 co-chaperone

PBMCs – peripheral blood mononuclear cells

PBS – phosphate buffered saline

POMC – proopiomelanocortin

PTSD – posttraumatic stress disorder

SAM system – sympathetic-adrenal medullary system

SCID-I – Structured Clinical Interview for DSM Disorders

SD – standard deviation

SDS-PAGE – SDS-polyacrylamide gels electrophoresis

sHSPs – small heat shock proteins

SPSS – Statistical Programme for Social Sciences

SUMO – small ubiquitine-like modifier

TLRs – Toll-like receptors

TNFα – tumor necrosis factor alpha

TRIC (or CCT) – cytoplasmic form of eukaryotic Hsp60

VCAM-1 – vascular cell adhesion molecule

Contents

1. Introduction

1.1 Posttraumatic stress disorder

Since the Vietnam War, posttraumatic stress disorder (PTSD) has become one of the most discussed psychiatric conditions in the United States. The recent spates of natural disasters worldwide have brought PTSD to the forefront again. Trauma is an integral part of human existence, but only recently have the biological and psychological aspects of exposure to different traumatic conditions been scientifically studied. By definition, PTSD is a debilitating psychiatric disorder that may develop following exposure to life threatening traumatic event. The traumatic event that may lead to development of PTSD symptoms is defined as experiencing, witnessing or being confronted with one or several events that involve actual or threatened death or serious injury or threat to the physical integrity of self or others (Blake *et al.*, 1996). The person exposed to such a traumatic event responds with intense fear, helplessness, or horror. As a consequence of trauma-exposure, PTSD symptoms may emerge immediately or be delayed in the months after the exposure. The major PTSD symptoms include re-experiencing of the trauma (intrusions), avoidance of trauma-related stimuli, emotional numbing and hyperarousal, and they significantly impair the person's social, occupational and other important areas of functioning (criteria B to F).

PTSD is frequently comorbid not only with a wide variety of mental disorders, primarily major depression, but also with physical illness, particularly cardiovascular disease (Boscarino, 2004; Schnurr & Green, 2004). These data, together with relatively high life-time prevalence rates, show that PTSD represents a serious medical and economic burden for the society.

1.1.1 Current definition of PTSD

PTSD may develop in individuals who experience horror, helplessness, and fear after threat or death. In addition, it is represented by presence of three unequivocal, but co-occurring, symptom clusters: 1) Individuals must have been exposed to a traumatic event; 2) The event involved a perceived or actual threat to the person's own life or physical integrity, or witnessing the threat to life or physical integrity of another person, such as a physical or sexual assault, rape, a serious accident, a natural disaster, combat, being taken hostage, torture, displacement as a refugee, sudden unexpected death of a loved one, and witnessing a traumatic event; 3) The traumatic memory can be insuppressible in the form of nightmares or images which are chaperoned by violent physiological agony. The reminders of the event-related revocation symptoms include impose restrictions on thoughts and distancing, as well as more generalized emotional and social withdrawal. PTSD can be defined clearly when an individual was exposed to a traumatic event and this is required for diagnosis of PTSD, making PTSD a psychiatric disorder which by definition is related to and occurs as a consequence of a stressful or traumatic event.

1.1.2 Prevalence of posttraumatic stress disorder

Nearly 50-70% of the U.S.A. population is exposed to traumatic event some times during the lifetime. In a nationally representative study of 5877 people aged 15-45 in the U.S.A. (Kessler *et al.*, 1995), a lifetime prevalence of exposure to trauma was found to be 60.7% in men and 51.2% in women, while the prevalence of PTSD is about 5% in men and 10% in women. In the U.S.A., the National women`s study (NWS) (Resnick *et al.*, 1993) found that 69.0% of women were exposed to a traumatic event sometimes in their lives. In 2005, an estimated 162 million of people worldwide were affected by disasters. Earthquakes illustrate the burden of natural disasters. Worldwide, there are over 20000 earthquakes and over 1300 per year with magnitude 5 or greater (National Earthquakes information center).

Exposure to a variety of stressors that most often give rise to PTSD include assault, combat and rape, whereas natural disasters or man-made accidents result in PTSD far less frequently. Disaster workers, who combat with fires, earthquakes, hurricanes, plane crashes and other disasters, are at high risk of PTSD as well (Fullerton *et al.*, 2004; Benedek *et al.*, 2007). In addition, across the world most of soldiers deployed to fight and civilians in modern cities face the large number of traumatic stressors, including terrorism, rape, war, assault, accidents, childhood abuse and other acute psychological traumas.

1.1.3 Symptoms of posttraumatic stress disorder

The symptoms of PTSD include intrusive thoughts (e.g. nightmares, unwanted thoughts), hyperarousal, such as startle responses or physiological arousal, avoidance of reminders of traumatic event and numbing of emotional responses. Avoidance symptoms involve restricting thoughts and distancing oneself from reminders of the event, as well as more generalized emotional and social withdrawal. Hyperarousal symptoms explain more overt physiological manifestations, for example impaired concentration, hypervigilance, insomnia, irritability, and increased distaste responses. The diagnosis of PTSD can be put forward one month after exposure to a traumatic event if the symptoms last at least one month and are severe enough to weaken social skills or interpersonal functioning. Vulnerability to PTSD is enhanced by poor coping strategies, substance abuse, co-occurring mood and anxiety disorders, lack of social support, and the accelerated development of stress related medical conditions (Yehuda, 2002b). Actually, the expected response to traumatic events is resilience. However, minority of trauma-exposed men and women suffer the psychological stress of the trauma exposure and develop distress, psychiatric illness, and exhibit health risk behaviors. Indeed, concern for future, increased fear and arousal, and altered sense of safety after exposure to trauma, can affect not only those who develop mental health problems, but also those who keep on working and care for their families and loved ones (Ursano & Shaw, 2007).

1.1.4 Comorbid disorders

PTSD is frequently comorbid with major depression and a wide variety of other mental disorders, and also with physical illness. Chronic sympathetic activation in PTSD may have important implications for the individual risk to develop cardiovascular disease as well as other chronic medical illnesses. Indeed, patients with PTSD have increased rates of comorbidity with cardiovascular diseases and other chronic somatic conditions (Boscarino, 2004; Boscarino, 2008; Kibler, 2009). Recently Heppner and colleagues reported that PTSD severity predicted the presence of metabolic syndrome (Heppner *et al.*, 2009). However, PTSD does not appear to be associated with diabetes mellitus (Qureshi *et al.*, 2009) or increased waist-to-hip ratio (Heppner *et al.*, 2009). This suggests that PTSD may enhance the risk for insulin resistance, especially in the context of obesity. PTSD has also been linked to rheumatoid arthritis (Qureshi *et al.*, 2009), independent of genetic and familial factors (Boscarino *et al.*, 2010). Other chronic medical conditions that have been associated with PTSD include psoriasis and thyroid disease (Boscarino, 2004). Interestingly, each of these chronic somatic disorders has inflammatory or autoimmune underpinnings. It is therefore reasonable to suspect that patients with PTSD may display immune alterations in addition to neuroendocrine changes.

1.1.5 Vulnerability and resilience to and recovery from PTSD

The findings that only a minority of trauma-exposed individuals develop or, even more importantly, maintain PTSD led to a hypothesis that PTSD involves a failure of mechanisms involved in recovery and restitution of physiological homeostasis. These findings have also prompted an interest in identifying vulnerability (risk) factors for this disorder. It has been supposed that individual variations in biological phenotypes of PTSD originate from pretraumatic vulnerability factors. Further research revealed that PTSD vulnerability factors include event characteristics (e.g., severity of trauma) and individual differences (e.g., preexisting traits, pre- or posttraumatic life events). For example, it has been

shown that prevalence of PTSD is greater after exposure to interpersonal violence than after accidents, raising the possibility that distinct biological subtypes of PTSD are based on trauma type. However, the line delineating stressor severity based on objective characteristics of the traumatic event *versus* the subjective response of the victim has never been clear. The risk factors for PTSD also include a family history of psychopathology, cognitive factors (such as low intelligence quotient), childhood adversity, preexisting avoidant personality or behavioral problems, and poor social support (Yehuda *et al.*, 2007).

It has been clear for a long time that response of different individuals to the same traumatic event is not uniform (Yehuda & LeDoux, 2007). A multitude of responses to adversity has raised an interest not only in vulnerability (risk) factors, but also in resilience factors. Resilience may be a mediator that may explain why psychopathology does not always develop. If biomarkers of resilience could be identified, these could be used to either predict (if they are trait-related *i.e.* genetically determined) or track (if they are state-related *i.e.* reflect adaptability to stress) recovery from traumatic events. Identifying true correlates of resilience could certainly provide important insights into the mechanisms promoting resilience or recovery from traumatic stress. Moreover, if resilience is an enduring characteristic or trait that is identifiable even before trauma exposure, it could be used to predict responses to adversity in those who may be at high risk for occupational or other hazards (Yehuda & Flory, 2007).

Up to date little is known about the biological basis of individual differences in response to trauma and specific questions regarding associations among risk and resilience factors, trauma exposure, development of psychopathology and recovery from the disorder still remain to be answered. To that end, choosing an appropriate research design that can lead to distinguishing factors associated with risk, symptom severity, recovery, and stress resistance is of an utmost importance. Prospective studies examining psychological aspects of vulnerability, resilience and recovery over time, may provide information on biological features that can predict outcome in response to adversity. In cross-sectional studies, it is important to carefully differentiate between trauma-exposed and non-exposed persons. If

putative resilience-related measures are preexisting traits that are unaffected by trauma exposure, these would presumptively be measurable in at least some nonexposed persons, perhaps making exposed and nonexposed groups indistinguishable on this measure. Conversely, if resilience markers reflect trauma exposure, because they are correlated with exposure or activated by exposure, group differences may not be discernible if a nonexposed group is excluded from the study. These factors suggest that cross-sectional studies should include nonexposed and exposed individuals, although only a prospective, longitudinal approach can evaluate the impact of trauma exposure, as well as assess resilience-related factors, since they may develop and change over time (Yehuda & Flory, 2007).

An interesting question is whether resilience is the opposite of vulnerability, or in other words, whether psychopathology will occur in the absence of resilience. The approach to answering this question should include differentiating trauma-exposed persons on the basis of presence or absence of (current and/or lifetime) PTSD, and then making a second distinction based on the presence or absence of a known risk factor for PTSD. This strategy can distinguish between measures reflecting resilience, risk, and psychopathology. It is also very important to distinguish between resistance to PTSD and recovery from this condition. Toward that end, special attention should be paid on how participants are classified, and how the group comparisons are made.

1.2 Neuroendocrine alterations in PTSD

The symptoms of PTSD are believed to reflect stress-induced changes in neurobiological systems and/or an inadequate adaptation of neurobiological systems to exposure to severe stressors. Over the last three decades, biological research on PTSD has focused on two major hormone systems that form the backbone of the physiological response to stress: the hypothalamic-pituitary-adrenocortical (HPA) axis and the sympathetic-adrenal-medullary (SAM) system. Focus on these systems stem from the facts that PTSD develops subsequent to exposure

to a traumatic event and that glucocorticoids and catecholamines are ”stress hormones” that may regulate responses to trauma. In the 1990’s, it became clear that PTSD is not a normative response to extreme stressors, as only a subset of people exposed to trauma will develop the disorder. Yehuda therefore reconceptualized PTSD as a disorder of stress response systems, leading to maladaptive responses and a failure to cope with the stressor (Yehuda, 2009). A search of the literature reveals multiple papers documenting changes of the HPA axis and the SAM system in PTSD patients.

1.2.1 Hypothalamic-pituitary-adrenocortical (HPA) axis

The HPA axis is activated when an organism is confronted with challenge and it acts to re-establish the homeostasis of the body. Therefore, the HPA axis functions as a feedback loop, which results in a cascade of associated processes to down-regulate the bodily responses to stress. The HPA axis is a complex set of interactions between the hypothalamus, the pituitary gland and the adrenal glands. The primary structure of the central nervous system involved in the regulation of HPA axis is the paraventricular nucleus of the hypothalamus. The paraventricular nucleus is the principal source of the corticotrophin-releasing hormone (CRH), which is the major physiological regulator of pituitary adrenocorticotropic hormone (ACTH) secretion. The CRH hypophysiotropic neurons from the paraventricular nucleus project to the external zone of the median eminence and release CRH into a specialized capillary network. Within the anterior pituitary, CRH interacts with a specific G protein-coupled receptor on the corticotrope cell surface, resulting in the stimulation of the synthesis of the ACTH precursor peptide proopiomelanocortin (POMC) and the secretion of ACTH and other POMC-derived peptides. ACTH potently induces the secretion of glucocorticoids from the zona fasciculata of the adrenal cortex. In a classical endocrine feedback manner, these steroids inhibit the synthesis and secretion of CRH within the hypothalamus and POMC-derived peptides in the pituitary (**Figure 1.1**).

HPA axis function is frequently investigated by measuring changes in cortisol, ACTH, or CRH release. The dexamethasone suppression test (DST) is a sensitive clinical measure of the functional integrity of the negative feedback mechanism mediated by glucocorticoid receptor (GR): the cortisol-suppressing activity of the synthetic glucocorticoid, DEX, is an indicator of GR status (Carroll *et al.*, 1981). A newer test is the combined dexamethasone/CRH (DEX/CRH) challenge test, in which the HPA axis is both stimulated by the administration of CRH and inhibited with dexamethasone. This test is said to be more sensitive for detecting HPA axis abnormalities in patients with psychiatric disorders (Heuser *et al.*, 1994).

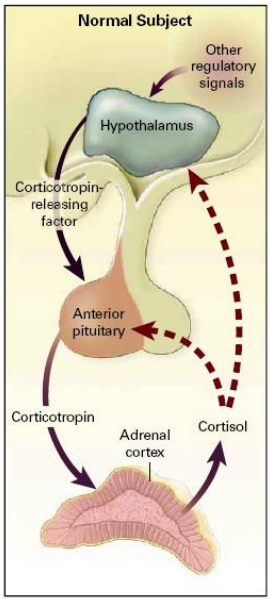

Figure 1.1 – The hypothalamic-pituitary-adrenocortical (HPA) axis. The HPA axis functions as a feedback loop. The paraventricular nucleus of the hypothalamus is the principal source of the corticotrophin-releasing factor, which is the major physiological regulator of pituitary adrenocorticotropic hormone secretion. Adrenocorticotropin potently induces the secretion of cortisol from the zona fasciculata of the adrenal cortex. In a classical endocrine feedback manner, cortisol inhibits the synthesis and secretion of corticotrophin-releasing factor within the hypothalamus and adrenocorticotropin in the pituitary *(modified from Yehuda, R., N. Engl. J. Med., 2002, 346:108-114)*.

1.2.2 Corticosteroid receptors

Activation of the HPA system results in secretion of glucocorticoids. In humans, the major glucocorticoid is cortisol, but in the rat and mouse,

corticosterone is the main steroid product of the zona fasciculata. Corticosteroids (cortisol and aldosterone) exert their actions through specific intracellular receptors, mineralocorticoid (or Type I) and glucocorticoid (or Type II) receptors (MR and GR, respectively). More recently, evidence has been presented for the existence of cell surface steroid receptors, and second messengers inside cells that may result in steroid-induced non-genomic actions (Christ et al., 1999). Historically, glucocorticoids were thought to bind exclusively to GR and aldosterone to MR, and regulate carbohydrate and sodium homeostasis respectively. However, following in vitro observations that both receptors bind to glucocorticoids with high affinity, they were classified as Type 1 „high affinity" receptor (corresponding to the MR) and Type 2 „low affinity" receptor (corresponding to GR). Separate receptors for glucocorticoids and mineralocorticoids appear to have occurred via gene duplication late in evolution, explaining why they behave in a similar fashion in some circumstances. The MR is classified as „high affinity" since it binds cortisol with a high affinity (Kd ~ 1nM) (Arriza et al., 1987). The GR binds cortisol with equilibrium dissociation constant (K_D) between 20 and 40 nM and therefore is classified as „low affinity" receptor (Reul & de Kloet, 1985). The MR is expressed in target tissues such as epithelia of renal distal tubules, salivary glands and distal colon, as well as within the central nervous system, in the placenta and fetal tissues, and in bone cells. The GR is widely expressed in tissues involved in glucose homeostasis, such as liver, adipose tissue and muscle, as well as bone cells and cells in the immune system.

Cytosolic GR is found in its target cells in the form of multiprotein heterocomplexes the components of which are molecular chaperones (Hsp90 and Hsp70), co-chaperones, immunophilins (FKBP51/52) and some other proteins. Steroid binding to the cytosolic GR results in the activation of the receptor complex, involving the dissociation of Hsp90 and Hsp70 (Hutchison *et al.*, 1993). Nuclear localization signal on the receptor is masked by chaperone proteins, but are exposed upon hormone binding and chaperone dissociation. The two unmasked receptor's nuclear localization signals allow protein interaction with the nuclear pores, enabling transport into the nuclear compartment. Once the GR has

been translocated to the nucleus, there is stimulation or repression of gene transcription by its binding to glucocorticoid response elements (GRE) in the promoter regions of target genes (Beato *et al.*, 1996).

The similar mechanism of activation and transcriptional activation/repression is characteristic for MR (or Type I receptor). However, its regulation and properties are still a matter of investigation. The high affinity MR is substantially occupied even at basal levels of HPA axis activity, suggesting that this receptor is implicated in the maintenance of basal activity of the stress system by *proactive* feedback. High concentrations of corticosteroids progressively saturate GR, implying that the suppression of stress-induced HPA activity occurs, in particular, through GR by *reactive* feedback in a coordinated manner with MRs. Consequently, the balance between MR- and GR-mediated actions on the stress system is of critical importance to the set point of HPA activity and proper response to stress.

1.2.3 HPA axis alterations in PTSD

While cerebrospinal fluid levels of CRH are increased in patients with PTSD (Bremner *et al.*, 1997), the data on peripheral cortisol concentrations are inconsistent. Many studies have found that peripheral concentrations of cortisol are decreased (Rohleder *et al.*, 2004), whereas other studies found no differences between PTSD patients and controls (Young & Breslau, 2004), or even higher cortisol levels in PTSD (Lemieux & Coe, 1995).

With respect to HPA axis function, several studies suggest that PTSD patients exhibit enhanced glucocorticoid sensitivity. In studies using DST with a low dose of dexamethasone, a potent synthetic glucocorticoid with five times higher affinity for the GR, HPA axis functioning was suppressed and enhanced negative feedback inhibition was observed in PTSD patients as compared to controls, reflecting in hypersuppression of cortisol secretion in blood or saliva (Kosten *et al.*, 1990; Griffin *et al.*, 2005). Yehuda and colleagues were the first to demonstrate that patients with PTSD showed enhanced sensitivity to glucocorticoids during the DST, especially when a low dose of dexamethasone was used (Yehuda, 2009) (**Figure**

1.2). This "sensitization" of the HPA axis is in line with the main PTSD symptoms like an unusually heightened response to stress, hypervigilance and especially physiological hyperarousal (Boscarino & Chang, 1999; Yehuda, 2001; Wessa *et al.*, 2006). However, later studies using the DST have found little or no differences between PTSD patients and trauma exposed controls without PTSD (Wessa & Rohleder, 2007). Thus, it is somewhat unclear if PTSD patients consistently display enhanced glucocorticoid sensitivity during the DST. Heim and colleagues found lower cortisol levels during the DEX–CRH test in men with abuse-related PTSD, which is consistent with the idea of enhanced feedback suppression under conditions of additional challenge (Heim *et al.*, 2008).

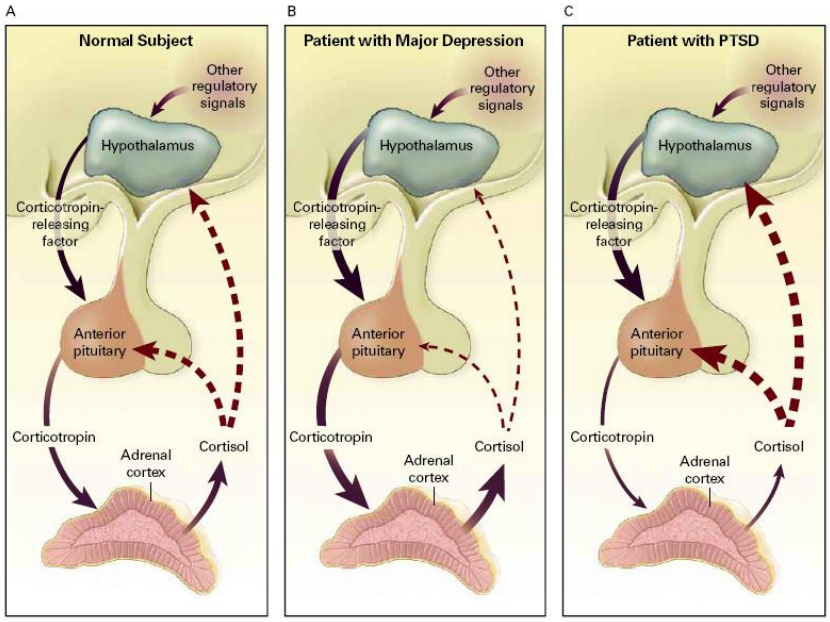

Figure 1.2 – Response to stress in a normal subject, a patient with major depressive disorder and a patient with PTSD. In normal subjects (**panel A**) and in patients with major depression (**panel B**) brief or sustained periods of stress are typically associated with increased levels of both cortisol and corticotrophin-releasing factor. In each panel the thickness of the interconnecting arrows denotes the magnitude of the biological response. Corticotrophin-releasing factor stimulates the production of corticotrophin, which in turn stimulates the production of cortisol. Cortisol inhibits the release of corticotrophin from the pituitary and the release of corticotrophin-releasing factor from the hypothalamus. It is also responsible for the containment of many biological reactions. In patients with PTSD (**panel C**), levels of cortisol are low and levels of corticotrophin-releasing factor are high. In addition, the sensitivity of the negative feedback system of the HPA axis is increased in patients with PTSD, rather than decreased, as often occurs in patients with major depression *(reproduced from Yehuda, R., N. Engl. J. Med., 2002, 346:108-114)*.

Glucocorticoid sensitivity can also be measured in circulating immune cells. Several original reports suggest that circulating immune cells from patients with PTSD may be more sensitive to glucocorticoids compared to immune cells from healthy individuals (Wolf *et al.*, 2009; Rohleder *et al.*, 2010). For example, lysozyme activity has been found to be more sensitive to dexamethasone in bulk leukocytes collected from PTSD patients compared to cells obtained from healthy controls. In addition, lipopolysaccharide-induced cytokine production from whole blood, a measure of monocyte responsiveness, has been reported to be more sensitive to dexamethasone in samples collected from PTSD patients compared to samples from controls (Wolf *et al.*, 2009; Rohleder *et al.*, 2010). However, not all findings point to enhanced glucocorticoid sensitivity in immune tissues from PTSD patients. De Kloet and colleagues compared the sensitivity of various immune parameters to dexamethasone in cell samples from patients with PTSD, trauma exposed controls, and controls not exposed to trauma (de Kloet *et al.*, 2007). While they found that the sensitivity of lipopolysaccharide-induced cytokine production to dexamethasone did not vary as a function of PTSD or trauma exposure, the sensitivity of phytohemagglutinin-induced T cell proliferation to dexamethasone was actually reduced in samples collected from PTSD patients *versus* those obtained from controls with or without trauma exposure (de Kloet *et al.*, 2007). The disagreement between measures of glucocorticoid sensitivity when using lipopolysaccharide-induced cytokines as an endpoint may be explained by differences in assay protocols or control conditions (de Kloet *et al.*, 2007). The finding of decreased sensitivity of T cell proliferation to glucocorticoids may not necessarily conflict with studies using lipopolysaccharide-induced cytokines or lysozyme activity endpoints, as each explores different aspects of immune function. In other words, there is the intriguing possibility that PTSD involves decreased glucocorticoid sensitivity in some immune tissues and increased glucocorticoid sensitivity in others.

There is also a lack of clarity with respect to changes of GR density in immune cells as a correlate of PTSD. Several studies investigated the number of GR on lymphocyte or leukocyte cells (Yehuda *et al.*, 1991; Boscarino & Chang, 1999).

They demonstrated that GR number correlated with the severity of combat-related PTSD symptoms while no relationship with plasma cortisol levels could be observed (Yehuda *et al.*, 1991). In addition, higher lymphocyte counts in PTSD patients were found as well as positive correlations of total lymphocyte GR expression with the number of years after trauma (PTSD patients) and with serum cortisol concentration (Vidovic *et al.*, 2007). While a number of studies have reported increased expression of GR in circulating immune cells, other reports suggest that GR density may actually be decreased (Gill *et al.*, 2009). As with measures of glucocorticoid sensitivity, this disagreement in GR expression in patients with PTSD could involve clinical differences between studies regarding patient groups. Methodological difference could also be important, including different techniques to assess GR density or expression levels, as well as assessments of these parameters in different subpopulations of immune cells.

Collectively, the findings of the summarized prospective studies indicate that individuals vulnerable to PTSD have deregulations on various levels of the glucocorticoid signaling cascade (**Figure 1.3**): low levels of circulating cortisol shortly after trauma (McFarlane *et al.*; Delahanty *et al.*, 2000; Aardal-Eriksson *et al.*, 2001; Ehring *et al.*, 2008), high GR number in peripheral blood mononuclear cells (PBMCs) (van Zuiden *et al.*, 2011a; van Zuiden *et al.*, 2012), high GILZ mRNA expression and low FKBP5 expression in PBMCs prior to trauma (van Zuiden *et al.*, 2012), and high sensitivity of immune cells for regulation by glucocorticoids prior to trauma (van Zuiden *et al.*, 2012). In addition, single nucleotide polymorphisms in the GR and *fkbp5* genes have been found to be associated with PTSD (Binder *et al.*, 2008; Xie *et al.*, 2010; Boscarino *et al.*, 2011; Hauer *et al.*, 2011; Boscarino *et al.*, 2012) and the non-genetic vulnerability factors for PTSD (van Zuiden *et al.*, 2011b). The results of these studies tentatively suggest that the development of PTSD may be preceded by a high sensitivity of various cells to regulation by glucocorticoid hormones.

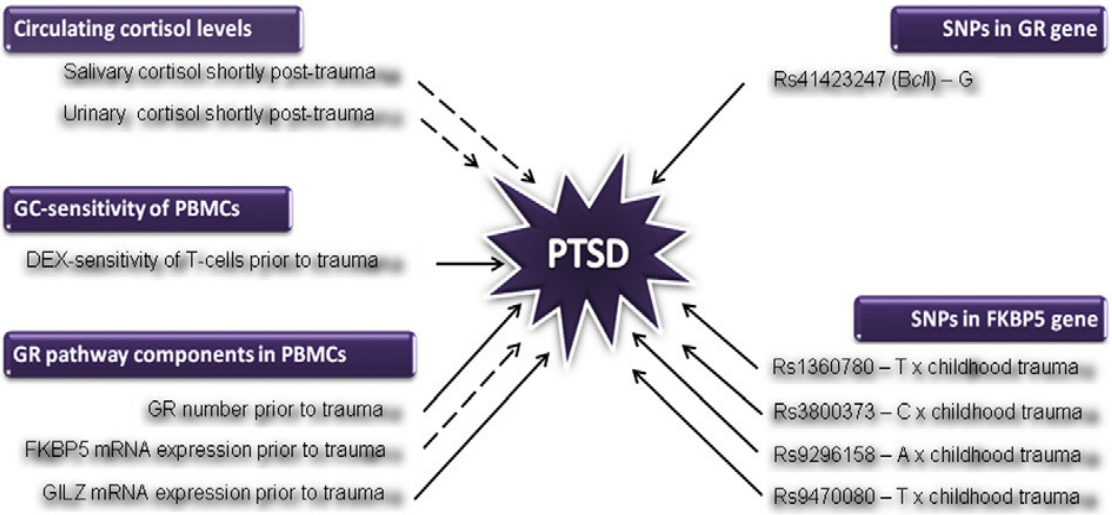

Figure 1.3 – Overview of currently identified vulnerability factors for PTSD in the glucocorticoid signaling pathway. Solid arrows between the vulnerability factors and PTSD indicate that presence/high levels of the vulnerability factors are associated with the development of PTSD. Striped arrows indicate that absence/low levels of the vulnerability factors are associated with development of PTSD. For the single nucleotide polymorphisms, the allele associated with increased risk for PTSD is presented. Abbreviations: GC: glucocorticoid; DEX: dexamethasone; PBMCs: peripheral blood mononuclear cells; GR: glucocorticoid receptor; SNPs: single nucleotide polymorphisms *(reproduced from van Zuiden, M. et al., Biol. Psychiatry, 2012, 71:309-316).*

1.2.4 SAM system alterations in PTSD

A cardinal feature of patients with PTSD is sustained hyperactivity of the sympathetic branch of autonomic nervous system, as evidenced by heart rate, blood pressure, skin conductance level, and other physiological measures. Moreover, central and peripheral concentrations of noradrenaline appear to be elevated in patients with PTSD. One study reported increased CSF concentrations of noradrenaline in veterans with the disorder (Geracioti *et al.*, 2001). Decreased platelet α2 receptor binding further suggests noradrenaline hyperactivity in PTSD (Vermetten & Bremner, 2002; Strawn & Geracioti, 2008). Accordingly, increased urinary excretion of noradrenaline and adrenaline, and their metabolites, has been documented in combat veterans, abused women, and children with PTSD. There is also evidence for a role of altered central noradrenaline function in PTSD. Administration of the α2 receptor antagonist yohimbine, which increases NE release, induces symptoms of flashbacks and increased autonomic responses in

patients with PTSD (Southwick *et al.*, 1999). Serial sampling revealed sustained increases in noradrenaline concentrations in cerebrospinal fluid and increased noradrenaline responses to psychological stressors in PTSD (Geracioti *et al.*, 2001; Geracioti *et al.*, 2008). Taken together, increased noradrenaline reactivity plausibly contributes to features of PTSD, including hyperarousal, increased startle, and encoded fear memories (Strawn & Geracioti, 2008). Some studies have found increased urine noradrenaline levels in PTSD patients exposed to various types of trauma including domestic abuse, childhood abuse, combat, automobile accidents, and exposure to war as a refugee (reviewed in Wessa & Rohleder, 2007). Urinary adrenaline concentrations are also increased in patients with PTSD (Wessa & Rohleder, 2007). Only one study has attempted to measure noradrenaline levels in blood (Yehuda *et al.*, 1998). Beyond catecholamines, studies investigating cardiovascular variables in PTSD patients support the notion of increased SAM reactivity. Thus, patients with PTSD exhibit increased heart rate, blood pressure, and tremor responses when presented with reminders of traumatic events (Bedi & Arora, 2007). The 5-HT system interacts with the CRH and noradrenaline systems in coordinating affective and stress responses (Ressler & Nemeroff, 2000; Vermetten & Bremner, 2002). Indirect evidence suggests a role of 5-hydroxytryptamine in the pathophysiology of PTSD, including symptoms of impulsivity, hostility, aggression, depression, and suicidality. Most important role of 5-hydroxytryptamine circuits in PTSD is the demonstrated efficacy of the selective serotonin reuptake inhibitors. Other evidence for altered 5-hydroxytryptamine neurotransmission in PTSD includes decreased serum concentrations of 5- hydroxytryptamine, decreased density of platelet 5-hydroxytryptamine uptake sites, and altered responsiveness to central serotonergic challenge (Ressler & Nemeroff, 2000; Vermetten & Bremner, 2002). However, no differences in 5- hydroxytryptamine 1A receptor binding were detected in patients with PTSD compared to controls using positron emission tomography imaging (Bonne *et al.*, 2005).

1.2.5 Is neuroendocrine deregulation a risk factor for the development of PTSD?

While most studies conceptualized neuroendocrine changes as correlates of the disease state of PTSD, several studies now suggest that neuroendocrine deregulation may in fact be a risk factor that predicts the development of the disorder upon exposure to extreme stress. Some studies have evaluated whether altered neuroendocrine responses observed in the immediate outcome of a trauma predict PTSD. Indeed, SAM and HPA axis responses to traumatic events have been shown to predict later development of PTSD. According to Yehuda's model, PTSD develops because key aspects of the biological response to trauma interfere with recovery after the trauma (Yehuda, 2009). Indeed, low plasma concentrations of cortisol and elevated plasma catecholamines in the period of time immediately after trauma have been found to predict PTSD development (Yehuda, 2009). If cortisol responses to trauma normally facilitate suppression of catecholamine responses to the same event, then reduced cortisol following a trauma may promote an excessive catecholamine response. This enhanced sympathetic activity may amplify encoding of traumatic memories, which would encourage expression of key PTSD features (Yehuda, 2009). These results raised the intriguing possibility that neuroendocrine deregulation may be a pre-existing risk factor that is present well before and independent of the traumatic event that elicits PTSD. Several prospective studies have now assessed neuroendocrine changes prior to trauma exposure. Indeed, increased glucocorticoid binding in peripheral blood mononuclear cells before deployment has been found to predict the development of PTSD 6 months after deployment in army personnel (van Zuiden *et al.*, 2009). In addition, exaggerated startle responses in police academy cadets, measured prospectively, have also been found to predict the development of PTSD (Pole *et al.*, 2009). This suggests that healthy people who show enhanced startle responses are at increased risk for developing PTSD. Risk for PTSD may also be transmitted across generations. Lower plasma concentrations of cortisol have been found in children of holocaust survivors (who also displayed enhanced HPA axis sensitivity

at DST), and in babies from mothers who developed PTSD after the World Trade Center attack in New York City (Yehuda, 2009).

1.3 PTSD and immunity/inflammation

1.3.1 Central and peripheral inflammatory markers

In 1997, Spivak and colleagues were the first to measure plasma cytokine levels in PTSD patients. They discovered that, compared to healthy, non-trauma exposed controls, veterans with PTSD displayed increased circulating concentrations of interleukin-1 beta (IL-1β) that were also correlated with the duration of PTSD symptoms (Spivak *et al.*, 1997). This finding has been bolstered by subsequent studies. Higher tumor necrosis factor alpha (TNFα) was found in PTSD patients with mixed trauma experience *versus* non-PTSD controls (von Kanel *et al.*, 2007). In addition, higher plasma IL-6 levels and soluble interleukin-6 (IL-6) receptors were found in victims of an automobile accident or a hotel fire compared to healthy controls (Maes *et al.*, 1999). Higher IL-6 levels have also been reported in patients with PTSD subsequent to myocardial infarction as compared to patients with myocardial infarction who did not develop PTSD, when controlling for depressive symptoms (von Kanel *et al.*, 2010). Refugees with PTSD following hurricane Katrina exhibited higher circulating IL-6 concentrations compared to refugees without PTSD (Tucker *et al.*, 2010). Moreover, the analysis of serum IL-2, IL-4, IL-6, IL-8, IL-10 and TNF-α level in PTSD patients has revealed significantly elevated peripheral cytokine levels for all cytokines compared to age- and gender-matched healthy controls (Guo *et al.*, 2012). Alterations in inflammatory markers are evident not just in the peripheral circulation, but in the central nervous system as well. Hence, elevated IL-6 concentrations have been measured in cerebrospinal fluid of PTSD patients with combat trauma exposure compared to healthy controls (Baker *et al.*, 2001). Given that IL-6 is thought to drive C-reactive protein (CRP) production from the liver, several recent studies have also examined CRP levels in patients with PTSD. In one of these studies, PTSD patients were found to have

nearly two-fold higher chance of having elevated CRP levels, even after controlling for possible confounds including sex, age, and alcohol use (Spitzer *et al.*, 2010). Despite multiple reports of increased circulating and central nervous system cytokines in PTSD patients, two studies have found decreased inflammatory markers. Most recently, von Känel and colleagues found lower CRP in patients with PTSD (von Kanel *et al.*, 2007). Lower CRP was also found in Iraqi refugees with PTSD (Sondergaard *et al.*, 2004). The lack of agreement between these studies may involve unique participant characteristics. For example, Sondergaard and colleagues noted that control participants in their study may have infections more likely than PTSD patients (Sondergaard *et al.*, 2004), leading to higher circulating CRP concentrations in the control group. Results from studies on patients with myocardial infarction should be interpreted carefully as well, given the complicated medical status of these individuals. Finally, a recent report suggests that trauma exposure and PTSD may instill epigenetic changes that impact long-term inflammatory function (Uddin *et al.*, 2010). In this study, patients with PTSD were found to exhibit a greater number of unmethylated genes related to innate immune and inflammatory function compared to healthy controls, which could eventually encourage the expression of altered immune function and enhanced inflammatory activity.

1.3.2 Neuroendocrine–immune interactions in PTSD

Neuroendocrine studies suggest that PTSD is not exclusively dependent on exposure to trauma. Instead, the onset and course of the disorder may involve a number of biological variables including altered cortisol levels, enhanced glucocorticoid sensitivity, altered GR numbers on immune cells, altered startle responses, and increased SAM system activity. Thus, atypical interactions between immune and neuroendocrine systems may help to explain the inflammatory excess found in a portion of patients with PTSD. Because glucocorticoids are well known for their anti-inflammatory effects, it is reasonable to theorize that reduced circulating concentrations of cortisol seen in some patients with PTSD may be responsible for increased inflammation in the same individuals. While we are

unaware of studies directly relating cortisol and inflammatory markers in PTSD, attenuated cortisol responses have been associated with enhanced IL-6 and IL-1β responses to stress in healthy individuals (Kunz-Ebrecht *et al.*, 2003). This suggests that low circulating concentrations of cortisol may foster a hyperinflammatory state, especially in the context of stress. However, the actions of hormones are not determined solely as a function of circulating concentrations. Instead, hormone effects also depend on tissue sensitivity, which is determined by receptor density and receptor function. As discussed above, PTSD patients have been found to exhibit altered glucocorticoid sensitivity in endocrine tissues and immune cells. In addition, many studies suggest that GR number may be increased in immune cells. Assuming that patients with PTSD exhibit enhanced glucocorticoid sensitivity and higher GR number, we can propose that patients with the disorder should have ‘‘normal” concentrations of circulating inflammatory markers despite low plasma cortisol level (Pace & Heim). One may even predict that patients with PTSD would have reduced inflammatory markers. Yet, most of the studies suggest that PTSD involves enhanced inflammatory activation.

1.3.3 From PTSD to medical comorbidities via immune dysfunction

As discussed in Section 2.1.4, PTSD is often comorbid with a number of chronic medical illnesses that have immune underpinnings. Could inflammatory and autoimmune changes that appear with the onset of PTSD encourage later development of the same comorbid medical illnesses? Although short-lived inflammation may be beneficial for combating pathogen exposure or tissue damage, chronic inflammation is well known to increase the risk for chronic medical illnesses. In this section, we will briefly illustrate a psycho-neuroimmunological model of PTSD, by focusing on two disease states that are often comorbid with the disorder: cardiovascular disease and insulin resistance.

Connections between PTSD, immune function and cardiovascular disease may begin with increased concentrations of proinflammatory cytokines in the circulation that appear with the onset of PTSD. Circulating cytokine changes can persist for months, and perhaps years, along with ongoing PTSD symptoms. Although prospective studies are yet to demonstrate that the onset of PTSD symptoms is associated with increased circulating inflammatory markers, as noted above, increased plasma levels of IL-6 at the time of trauma have been associated with later development of PTSD (Pervanidou *et al.*, 2007). In addition, acute and chronic stressors are well known to increase inflammatory markers (Segerstrom & Miller, 2004). Of note, PTSD has already been prospectively associated with the development of heart disease 15 years later in veterans who were free of heart disease at baseline (Boscarino, 2008). Along with increased lipoproteins, inflammation within the wall of blood vessels fosters the atherosclerotic process (Sprague & Khalil, 2009). Increased circulating concentrations of proinflammatory cytokine associated with PTSD may eventually activate inflammatory signaling pathways (e.g. NFκB) in vascular cells of vessel wall. Inflammatory pathway activation in these cells encourages increased cell adhesion, increased vessel permeability, and apoptosis. Monocytes are then able to attach more readily to vessel walls, and eventually mature into macrophages. These macrophages then amplify the proinflammatory message in the vascular wall by secreting additional IL-1β and TNF-α, as well as other factors, which further increase expression of adhesion molecules, such as vascular cell adhesion molecule-1. Macrophages eventually become large foam cells, forming “fatty streaks” inside vessels that can develop into atherosclerotic lesions. Proinflammatory cytokines would also draw smooth muscle cells to the lesion, forming fibrous capsules over these lesions. Finally, circulating proinflammatory cytokines have been shown to potentiate thrombosis, or rupture of atherosclerotic lesions, leading to myocardial infarction or ischemic stroke (Tousoulis *et al.*, 2006). Although cardiovascular disease depends on a number of factors, including dietary habits, smoking, and exercise, it is conceivable that increased inflammation begun by trauma and PTSD may eventually push PTSD patients into comorbid cardiovascular disease.

Inflammation has also been implicated in the pathophysiology of insulin resistance and metabolic syndrome. Multiple studies have found that chronic activation of inflammatory signaling pathways can lead to obesity-related insulin resistance. In the psycho-neuroimmunological model of PTSD, elevated circulating concentrations of proinflammatory cytokines associated with PTSD would potentiate activation of macrophages in adipose tissue and the liver, the tissues that are central to insulin resistance. These activated macrophages are the main generators of inflammatory products, such as TNF-α, that go on to induce inflammatory signaling pathways within insulin target cells. Increased inflammatory signaling activity within insulin target cells is then able to antagonize normal insulin receptor signaling (Olefsky & Glass, 2010). Although increased inflammation as a result of trauma and PTSD may not be sufficient to induce insulin resistance alone, the model predicts that inflammatory changes induced by PTSD would synergize with other factors, including visceral adiposity and exercise habits, to encourage development of insulin resistance and metabolic syndrome. Although work to date supports an immune component in mechanisms linking PTSD and chronic medical illness, more information is needed. Prospective studies will need to demonstrate that immune changes subsequent to trauma and the onset of PTSD can predict disease development.

1.4 Heat Shock Proteins (HSPs)

The first evidence for a cellular stress response is generally associated with a set of experiments in fruit flies (Ritossa, 1962). Larvae of *Drosophila melanogaster* were accidentally kept overnight at an elevated temperature and upon inspection the next day, an unusual puffing pattern was noticed on salivary gland chromosomes, indicating that the "heat shock" episode had caused marked changes in the gene expression pattern of the larvae. However, the term "heat shock protein" was not coined until 1974, when specific protein products associated with these genes were identified in fruit flies (Tissieres *et al.*, 1974). Over the next three decades, the heat shock response, also named cellular stress

response, has been extensively studied, particularly with regard to stress proteins and their ever expanding array of functions (Lindquist & Craig, 1988; Hightower, 1991; Welch, 1992). Cellular stress response represents adaptive response which helps to maintain cellular homeostasis under stress and is based on macromolecular damage recognition without regard to the type of stress that causes such damage. Elevated expression of heat shock proteins (HSPs), also named stress proteins, is sufficient to protect cells from various cytotoxic agents, while at the same time downregulation of most other genes, responsible for "normal" cellular functions was noticed (Prahlad & Morimoto, 2009). The molecular meshanisms underlying cellular stress response are conserved from archaebacteria to mammals.

Up to date HSPs are classified on the basis of their apparent molecular masses into the following main distinct families: Hsp100, Hsp90, Hsp70, Hsp60, Hsp40 and small HSPs (sHsp). Some members of these Hsp families are present constitutively in cells, while some are expressed only after stress. Each family is comprised of multiple members that share sequence similarity and have common main functional domains, such as ATP-binding domain, substrate-binding domain and cofactor-binding domain. Most of HSPs are molecular chaperones, typically bound to nonnative conformation of proteins that are accumulated in the cells exposed to stressors. These interactions protect proteins from aggregation and enable refolding and the restoration of their biological active conformations (Hartl *et al.*, 1994; Morano & Thiele, 1999; Bukau *et al.*, 2006). Molecular chaperones have extremely conserved sequences, some of them sharing 50% homology between mammals and bacteria (Henderson & Henderson, 2009). However, it has become very apparent over the past two decades that molecular chaperones have additional functions besides their protein refolding actions. Namely, HSPs have a dual function depending on their intracellular or extracellular location. In the cell, these proteins play an essential role as molecular chaperones by assisting the correct folding of nascent and refolding of stress-accumulated misfolded proteins, prevent their aggregation, or promote degradation of irreparably damaged proteins. On the other hand, extracellularly localized or membrane-bound HSPs

mediate immunological responses. These proteins in contact with immune cells can elicit an immune response modulated either by the adaptive or innate immune system (Schmitt *et al.*, 2007). Therefore, HSPs have moonlighting functions and the nature of these functions depends on where these proteins exist in the organism.

The most important function of molecular chaperones in homeostasis is to prevent protein aggregation during the synthesis by trapping the hydrophobic surfaces of nascent polypeptide chains and to facilitating correct folding. If the functions of chaperons are decreased, misfolded proteins accumulate and aggregation rises in the cell. Thus, molecular chaperones are essential for protecting cells against the toxicity of misfolded proteins and theirs aggregates (Kubota, 2009).

On the other hand, the role of HSPs is inevitable in the response to variety of stressors, including extremes of temperature, cellular energy depletion, and extreme concentrations of ions, other osmolytes, gases, and various toxic substances (Feder & Hofmann, 1999). All known stressors, if sufficiently intense, induce HSPs expression. Accordingly, HSPs are equally well termed stress proteins, and their expression is termed the stress response. A common aspect of these stresses is that they result in intracellular accumulation of proteins having non-native conformations and HSPs assist partially denaturated proteins in restoring their native states.

1.4.1 Hsp70 chaperone system

Molecular chaperones form functional complexes with other molecules of the same or another chaperone group, or with co-chaperones and cofactors. These multiprotein complexes represent chaperone mashines, which usually involve more than one chaperoning team (Macario & Conway de Macario, 2007). In several cases chaperones interact with a specific target protein, named substrate or client, and become obligatory for its folding, as well as for its assembly with other proteins in specific protein complexes. These specific interactions make chaperones important part of the core of cellular networks, such as the protein

network, the signaling network, the membranous and organellar network, as well as the transcriptional network. One of the most important molecules in the entire protein folding network is Hsp70. The prokaryotic version, called DnaK, shares about 60% sequence identity with eukaryotic Hsp70 proteins, which are found in the cytosol and in organelles, such as the endoplasmic reticulum, mitochondria, and chloroplasts. Under physiological conditions, Hsp70s are involved in *de novo* folding of newly formed proteins, in uncoating of clathrin coated vesicles, in reorganization of cytoskeleton systems, translation initiation, transport of proteins through membranes, nuclear protein import and export, ribosome assembly, protection of nucleolar structure and in protein degradation (Nover & Scharf, 1997). Under stress they prevent the aggregation of misfolded proteins and can even refold them (Mayer & Bukau, 2005). The basic function of Hsp70 in all cases is evidently binding and subsequent release of partially unfolded proteins in an ATP-dependent cycle.

Members of the Hsp70 family consist of a single polypeptide with two functional domains, an ATPase domain and a protein binding domain. The N-terminal domain is able to bind and hydrolyze ATP, whereas the C-terminal protein-binding domain contains four hydrophobic pockets for binding unfolded target proteins. After binding of ATP, Hsp70 is able to interact with non-native proteins characterized by exposition of hydrophobic peptide motifs at their surface. Subsequent ATP hydrolysis generates an Hsp70-ADP complex with tightly bound substrate proteins. After ADP—ATP exchange, the target polypeptide is released to undergo folding in solution (**Figure 1.4**).

The activity of Hsp70s is regulated by cochaperones. The largest class of Hsp70 cochaperones is the group of Hsp40/J-domain-containing proteins (Kampinga & Craig). They bind the nonnative protein and deliver it to Hsp70. The J domains of these proteins interact with the ATPase domain of Hsp70 and stimulate the hydrolysis of bound ATP. The release of nucleotide and substrate is further accelerated by nucleotide-exchange factors.

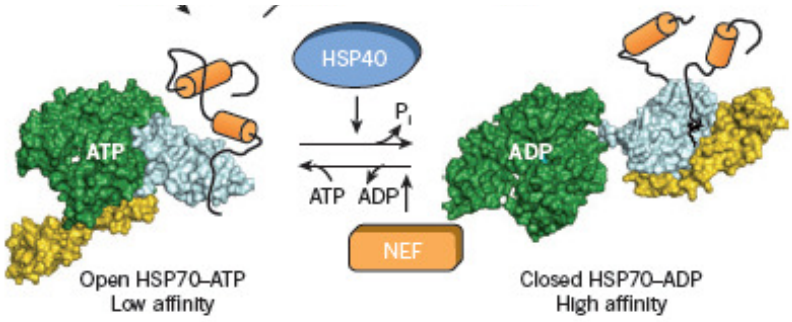

Figure 1.4 – The Hsp90 chaperone cycle. Hsp70 is switched between high- and low-affinity states for unfolded and partially folded protein by ATP binding and hydrolysis. Unfolded and partially folded substrate (nascent chain or stress-denatured protein), exposing hydrophobic peptide segments, is delivered to ATP-bound Hsp70 (open, low affinity conformation) by one of several Hsp40 cofactors. The hydrolysis of ATP, which is accelerated by Hsp40, results in closing of the α-helical lid of the peptide-binding domain (yellow) and tight binding of substrate by Hsp70 (closed, high affinity conformation). Dissociation of ADP catalyzed by one of several nucleotide-exchange factors (NEFs) is required for recycling. Opening of the α-helical lid, induced by ATP binding, results in substrate release (reproduced from Hartl, U.F. et al., Nature, 2011, 475:324-332).

1.4.2 Hsp60 chaperone system

Eukaryotes possess Hsp60s both in the cytoplasm and in the organelles, mitochondria and chloroplasts. Cytoplasmic forms are designated TRIC or CCT and are responsible, in cooperation with Hsp70, for *de novo* folding of nascent polypeptides and refolding of misfolded proteins. Mitochondrial and chloroplast Hsp60 chaperonin is typically held responsible for folding of nascent proteins and refolding of misfolded proteins in the mitochondrial matrix. The homolog of mitochondrial Hsp60 chaperone system is the *E. coli* GroEL/GroES complex (Hayer-Hartl *et al.*, 1996) that was structurally analyzed by Hartl (Hartl, 1996; Hayer-Hartl *et al.*, 1996; Hunt *et al.*, 1996) and Xu et al. (Xu et al., 1997). Under normal physiological conditions, Hsp60/GroEL is a 60 kDa oligomer composed of monomers that form a complex arranged as two stacked heptameric rings. This double ring structure forms a large central cavity in which the unfolded protein binds via hydrophobic interactions. Each Hsp60/GroEL monomer consist of three different domains: (i) an apical domain interacting with the cochaperone (Hsp10/GroEL), (ii) an intermediate domain which binds the equatorial domain

and the apical domain together and is required for ATP hydrolysis and (iii) an equatorial domain interacting with the second ring and harboring an ATP binding side. The apical domains expose a set of hydrophobic amino acid residues towards the inner surface of the cavity thus offering binding sites for partially unfolded proteins. Target proteins bound to the hydrophobic surfaces may therefore undergo further mechanical unfolding. The unfolded substrates remain encapsulated, protected from unwanted interactions and free to spontaneously fold into the native conformations, for the time needed for ATP hydrolysis. Once the ATP is hydrolyzed, the co-chaperone dissociates and folded/refolded substrate protein is released (**Figure 1.5**).

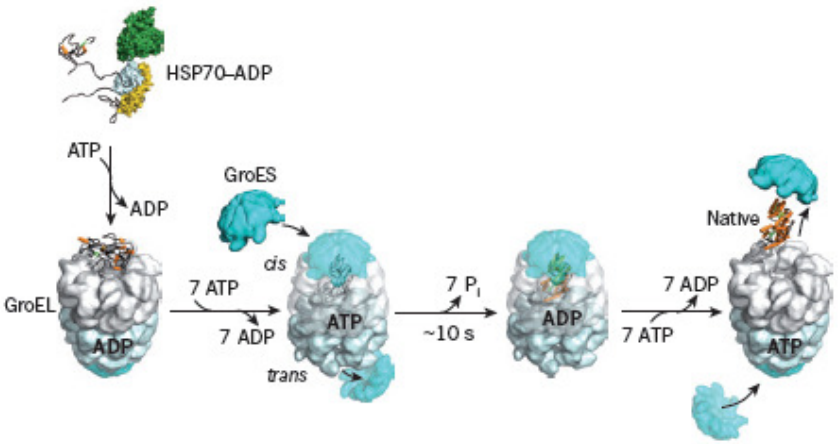

Figure 1.5 – Reaction cycle of the GroEL-GroES chaperonine system. Substrate protein is transferred to GroEL from Hsp70. ATP binding then triggers a conformational rearrangement of the GroEL apical domains. This is followed by the binding of GroES and substrate encapsulation for folding. The enclosed substrate protein experiences confinement in the cavity, as well as shielding from the crowded environment of the bacterial cytosol. At the same time, ADP and GroES dissociate from the opposite (*trans*) GroEL ring, allowing the release of substrate that had been enclosed in the former *cis* complex (omitted from the figure for simplicity). The new substrate remains encapsulated, free to fold, for the time needed to hydrolyze the seven ATP molecules in the newly formed *cis* complex (~10 s). Binding of ATP and GroES to the *trans* ring causes the opening of the *cis* complex (reproduced from Hartl, U.F. et al., Nature, 2011, 475:324-332).

Hsp60 are amongst the most evolutionary conserved proteins. The significant functional, structural, and sequential homology between Hsp60 and its prokaryotic homolog, GroEL, demonstrates this level of conservation. Hsp60 are primarily

responsible for maintaining the integrity of cellular proteins, particularly in response to environmental stresses such as temperature, concentration imbalance, pH change, and toxins. Hsp60 aids in the folding and conformation maintenance of approximately 15-30% of all cellular proteins.

1.4.3 Hsp90 chaperone system

Hsp90 belongs to the most abundant constitutively expressed cytosolic chaperones in eukaryotic cells (Hendrick & Hartl, 1993; Jakob & Buchner, 1994; Buchner, 1996; Nemoto & Sato, 1998). Its prokaryotic analogue is HtpG (high-temperature protein G), which share 40% sequence identity with the human protein (Chen *et al.*, 2006). The cytosolic protein in vertebrates exists in two isoforms (α and β) which demonstrate 85% sequence homology. The α- and β-forms are thought to be the result of a gene duplication event that occurred millions of years ago. Hsp90 functions as a dimer of subunits that are assembled by their C-terminal domains. An N-terminal domain binds and hydrolyses ATP and is joined to the C-terminal domain by a middle domain (**Figure 1.6**). The middle domain participates in substrate binding and interacts with co-chaperones. Similar to other chaperones, the Hsp90 dimer undergoes an ATP-driven reaction cycle that is accompanied by considerable structural rearrangement. ATP binding leads to the dimerization of the N-terminal domains, forming the Hsp90 'molecular clamp'. This results in a compaction of the Hsp90 dimer, in which the individual monomers twist around each other. After hydrolysis, the ATPase domains dissociate, and the Hsp90 monomers separate N-terminally (**Figure 1.6**). Various cofactors regulate this cycle: CDC37, which delivers certain kinase substrates to Hsp90, inhibits the ATPase activity, and HOP inhibits N-terminal dimerization. AHA1 stimulates ATP hydrolysis, whereas p23 stabilizes the dimerized form of Hsp90 before ATP hydrolysis. These factors are thought to adjust the kinetic properties of the cycle to achieve certain conformational transitions in Hsp90-bound substrates, as well as their release from Hsp90 (Hartl *et al.*, 2011).

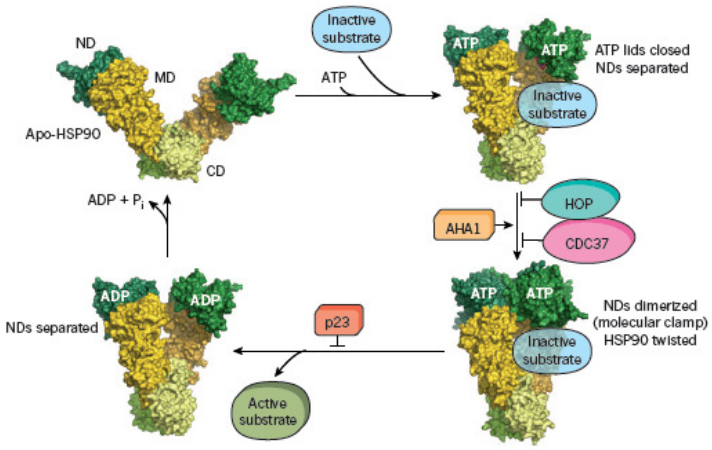

Figure 1.6 – Hsp90 chaperone cycle. Clockwise from top left, ATP binding to the N-terminal ATPase domain (ND) of apo-Hsp90 induces a conformational change and the closure of the ATP lid in the ND. After lid closure, the NDs dimerize, forming the closed Hsp90 dimer (molecular clamp) with twisted subunits. This metastable conformation is committed for ATP hydrolysis. After hydrolysis, the NDs dissociate. The inactive substrate molecule interacts mostly with the middle domain (MD) and is conformationally activated as Hsp90 proceeds through the ATPase cycle. The cofactors CDC37, HOP, AHA1 and p23 accelerate or slow certain steps of the cycle (reproduced from Hartl, U.F. et al., Nature, 2011, 475:324-332).

Hsp90 is reported to play an important role in signal transduction pathways, since its clients include most of the signaling proteins. For example, it has been demonstrated that Hsp90 participates in maturation and activation of Ser/Thr- and Tyr-specific protein kinases (Cutforth & Rubin, 1994; Dey *et al.*, 1996; Nair *et al.*, 1996; Schulte *et al.*, 1996; Stepanova *et al.*, 1996; Chang *et al.*, 1997; Nair *et al.*, 1997). The other well-known role of Hsp90 is chaperoning of steroid hormone receptors, including the GR and MR. Unliganded steroid receptors exist in cytosols in heterooligomeric complexes with Hsp90 and Hsp70 (Pratt & Toft, 1997b). The interaction between the Hsp70 and Hsp90 chaperone systems is mediated by cochaperones HOP (Hsp70/Hsp90 Organizing Protein) (Johnson *et al.*, 1998) and Hip (Hsp70-interacting protein) (Smith *et al.*, 1993; Johnson *et al.*, 1998). In addition, Hsp90 complex interacts with three other proteins, FKBP52, Cyp40 and a small acidic protein, p23. These proteins also act as co-chaperones that increase the overall efficiency of GR-Hsp90 heterocomplex assembly. The role of Hsp70 is to recognize newly synthesized receptors, and it is required for their initial folding, while Hsp90 regulates maturation of the receptors' high-affinity hormone-binding

conformation and maintains unliganded receptors in transcriptionally inactive state by keeping them in a form incapable of DNA binding. Hsp90 also controls intracellular trafficking, nuclear retention, transcriptional activity (Grad & Picard, 2007), as well as proteolytic degradation of the receptors (Siriani et al., 2005). Final receptor activation and translocation to the nucleus is achieved upon dissociation of the hormone-receptor complex from Hsp90. Therefore, the interaction of steroid hormone receptors with Hsp90 and Hsp70 represents an important determinant of target tissue sensitivity to these hormones (Pratt & Toft, 1997a; Grad & Picard, 2007).

1.4.4 Induction and regulation of heat shock protein expression

Transcriptional regulation of Hsp genes is mediated by the interaction of heat shock factor (HSF) with heat shock elements (HSE) located in the promoter regions of Hsp genes (Voellmy, 1994; Srivastava, 2002). In vertebrates, four HSFs have been identified, of which HSF1 and HSF2 are ubiquitously expressed and conserved (Hightower & Guidon, 1989; Theriault *et al.*, 2005). The main heat shock factor, participating in the response of vertebrates to physiological and environmental stress, is HSF1 (Guzhova *et al.*, 2001). The activity of HSF2 is more selective, and is mostly induced during differentiation and early development (Pockley *et al.*, 2003) Usually, HSF1 is present in the cytoplasm as a latent monomeric molecule in a complex with Hsp70 or Hsp90 and their cofactors, in which it is unable to bind to DNA. When the cell is exposed to stress, the accumulation of non-native proteins leads to HSF1 activation (Morimoto *et al.*, 1994), by sequestration of Hsp70 and release of HSF1 from the complexes with chaperones. HSF1 is then converted to trimeric form that have a capacity to translocate from the cytoplasm to the nucleus (**Figure 1.7**). In the nucleus, it becomes hyperphosphorylated in a Ras-dependent manner by mitogen-actived protein kinases (Knauf *et al.*, 1996; Kim *et al.*, 1997) and sumoylated, acquiring the ability to bind to DNA. The induction of HSPs has to be tightly controlled, since their presence would adversely affect protein homeostasis and intracellular functions, leading to inappropriate growth control and possibly cell death. Once

the cell returns to normal function, the level of misfolded proteins decline and the free chaperones, now present in excess, capture HSF1 again, leading to cessation of Hsp genes expression (Shi *et al.*, 1998). A second mechanism regulating HSPs synthesis is the interaction between heat shock protein binding factor 1 (HSBP1), the active trimeric form of HSF1 and Hsp70, resulting in inhibition of the capacity of HSF1 to bind to DNA (Satyal *et al.*, 1998). HSBP1 is mainly localized in the nucleus, and HSBP1 mRNA is present at high concentrations in various cell lines and animal tissues that are unaffected by heat chock.

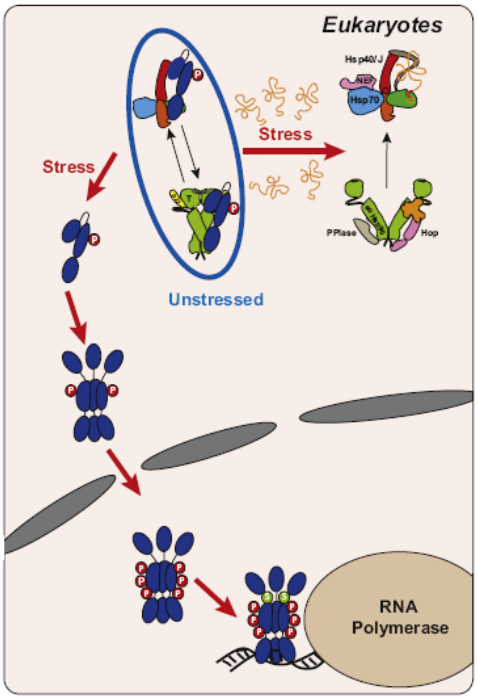

Figure 1.7 – Regulation of the Heat Shock Response. In eukaryotes, a storage form of the heat shock specific transcription factor HSF1 is maintained in an inactive monomeric form in complexes with the chaperones Hsp70 and Hsp90. As in the case of the prokaryotic system, the titration of chaperones by massive protein unfolding upon proteotoxic stress will result in the release of HSF1 from chaperone inactivation. Monomeric HSF1 then trimerizes, is transported into the nucleus, hyperphosphorylated, sumoylated, and activates heat shock gene transcription *(modified from Richter, K. et al., Molec. Cell, 2010, 40:253-266).*

1.5 Extracellular HSPs

To date, extracellular HSPs (eHSPs), have mainly been studied in the context of intracellular chaperones, and their release from cells has typically been believed to occur only after lysis. Now it is known that HSPs can be secreted from a variety of cell types, even when cells are completely viable. Release of such proteins from cells is triggered by physical trauma and psychological stress, as well as by exposure to immunological "danger signals". Extracellular stress protein release occurs through both physiological secretion mechanisms and cell death by necrosis. After release into the extracellular fluid, HSPs may bind to the surfaces of adjacent cells and initiate signal transduction cascades, as well as the transport of cargo molecules such as antigenic peptides. In addition, some HSPs are able to enter the bloodstream and may possess the ability to act at distant sites in the body (Calderwood *et al.*, 2007). It is now known that these proteins can be released from a variety of viable (non-necrotic) mammalian cells including neuronal cells, monocytes, macrophages, B cells, tumor cells of epithelial origin, endothelial cells and hepatosplanchnic tissue (Child *et al.*, 1995; Bassan *et al.*, 1998; Liao *et al.*, 2000; Febbraio *et al.*, 2002). The release of HSPs into circulation was found to be connected to behavioral stress or trauma, extreme exercise (Pockley, 2002; Campisi & Fleshner, 2003) and physical or psychological acute stressors (Fleshner & Johnson, 2005). However, some of these proteins (e.g. Hsp60 and Hsp70) were found to be present also in the peripheral circulation of normal individuals (Pockley *et al.*, 1998; Pockley *et al.*, 1999), with, as yet, unclear impact on pathophysiological processes.

1.5.1 Immune and inflammatory effects of extracellular HSPs

Extracellular stress proteins of the Hsp and Grp families have powerful effects on the immune response (Srivastava, 2000; Calderwood *et al.*, 2005). These stress proteins interact with the immune response in a number of contexts. Mammalian cells express HSPs at high levels after trauma or exposure to bacterial proteins (Hunter-Lavin *et al.*, 2004). Released into the extracellular space, HSPs

can act pro-inflammatory and lead to increased cytokine synthesis and release (Asea *et al.*, 2000b; Asea *et al.*, 2002). In addition, HSPs can stimulate adaptive immune response through their ability to bind antigenic peptides during antigen processing (Noessner *et al.*, 2002). When Hsp-peptide complexes are released from dead and dying cells, they bind to receptors on antigen processing cells. Antigens can then be delivered to MHC class I molecules on the surfaces of antigen processing cells through a process known as antigen cross-presentation (Arnold-Schild *et al.*, 1999; Singh-Jasuja *et al.*, 2000). However, HSPs can also act anti-inflammatory and their anti-inflammatory actions are noticed specifically in inflammatory diseases. Diseases such as rheumatoid arthritis can be triggered by cross reactive T cells which recognize common epitopes in mammalian and highly immunogenic prokaryotic HSPs (van Eden *et al.*, 2005; Hauet-Broere *et al.*, 2006). It was speculated that high degree of sequence homology between prokaryotic and mammalian HSPs may be a factor triggering an autoimmune response to mammalian HSPs. Interestingly, however, application of some mammalian Hsp can suppress pro-inflammatory responses to bacterial Hsp epitopes and leads to remission of inflammatory diseases (Kingston *et al.*, 1996). Thus, depending on context, extracellular HSPs can act both profoundly immunostimulatory and immunosuppressively, implying their dual immunoregulatory role (Daniels *et al.*, 2004; van Eden *et al.*, 2005). In line with this observation, there is growing evidence that two extracellular HSPs (eHsp60 and eHsp70) are capable of activating pro-inflammatory responses, which can exacerbate diseases, such as atherosclerosis (Camici, 2002; Pockley *et al.*, 2003) and coronary heart disease (Pockley *et al.*, 2000; Zebrack & Anderson, 2002), but can also act beneficially, to facilitate recovery from bacterial infection (Campisi *et al.*, 2002).

As yet, there are no data in the literature on human HSPs induction and extracellular release related to trauma-exposure or to PTSD. However, given that psychological stressors have been shown to increase both intracellular and circulating levels of HSPs (Lewthwaite *et al.*, 2002; Fleshner *et al.*, 2004) thus contributing to development of coronary heart disease (Lewthwaite *et al.*, 2002), it

is tempting to assume that trauma-exposure and/or PTSD might be associated with alterations in HSPs levels and consequent changes of inflammatory status.

1.5.2 Mechanisms of Hsp70 release

Hsp70 is not secreted by the classical pathway, since its sequence encodes no secretion leader signal. However, a number of non-canonical pathways for release of "leaderless" proteins exist. In some cases release of leaderless proteins involves cell lysis and this may occur both in pathological conditions that give rise to necrosis or in physiological conditions (Wewers, 2004). For instance, it has been suggested that Hsp70 may be released into the blood stream under a number of pathological conditions that lead to widespread cell death (Pockley, 2002). A second pathway involves release of intracellular proteins by extracellular membrane vesicles (De Maio, 2011). Stress proteins such as Hsp27, Hsp70, Hsc70 and Hsp90 can apparently be released within the lumen of exosomes through such pathway when B cells are exposed to heat shock (Clayton *et al.*, 2005). The postulation of vesicles or exosomes as a source of extracellular Hsp70 requires that these structures should rupture or lyse on entering the extracellular microenvironment (MacKenzie *et al.*, 2001). A third secretion pathway involves the entry of the leaderless protein into secretory lysosomal endosomes, migration of these organelles to the cell surface and release of the contents of the endolysosome into the extracellular space (Baraldi *et al.*, 2004). Indeed, Hsp70 has recently been shown to be secreted from tumor cells and macrophages by this pathway (Mambula & Calderwood, 2006). Study of these processes is still in its infancy and further studies are required to determine the favored pathways for Hsp70 release by neuronal and immune cells.

1.5.3 Hsp70 uptake mechanisms

HSPs interact with a range of cell surface receptors on target cells, most notably CD14, CD40 and Toll-like receptors (TLRs). It was proposed that CD91 receptor, found on antigen presenting cells and other cell types, could be the

common receptor for all immunogenic HSPs, including Hsp60 and Hsp70 (Binder *et al.*, 2000; Basu *et al.*, 2001). However, its role as a direct high/medium affinity Hsp binder is still not clear. In addition, CD40 can also function as an Hsp70 receptor (Wang *et al.*, 2001). CD40 is a member of the tumor necrosis factor receptor family and plays a major role in antigen presenting cell maturation (Baraldi *et al.*, 2004). However, the exact role of CD40 as a direct binder for mammalian HSPs is still a matter of debate.

TLRs are subject of current investigations as potential Hsp receptors. The TLR couples exposure to prokaryotic "danger signals" (bacterial lipopolysaccharide, lipopeptides, CpG DNA) with intracellular signal transduction pathways that include the NF-κB and interferon signaling pathways (Takeda *et al.*, 2003). There are now at least 11 members of the TLR family, most of which have not been tested for Hsp binding. However, at least two TLR family members function as Hsp receptors and can couple the binding of Hsp60, Hsp70 and Grp96 to NF-κB activity (Asea *et al.*, 2000a; Asea *et al.*, 2002; Quintana & Cohen, 2005). In addition, the cell surface protein CD14 is also required for induction of cytokines TNF-α, IL-1β and IL-6 by Hsp70 (Asea *et al.*, 2000a). However, some studies suggest that the interactions between Hsp70 and TRL probably do not involve direct binding of Hsp70 to CD14, TLR2 or TLR4, since null cell that stably expresses CD14, TLR2 or TLR4 doesn't bind avidly to Hsp70 (Theriault *et al.*, 2005).

1.5.4 Hsp60 as intercellular signaling molecule

The usual view of eukaryotic HSPs is that they are intracellular molecules that are released from necrotic, but not apoptotic cells, and that their release into (and presence in) the extracellular environment reflects pathophysiological state. For example, the circulating level of Hsp60 is significantly elevated in subjects who show signs of atherosclerosis. However, human Hsp60 induces the expression of the adhesion molecules E-selectin, intracellular adhesion molecule (ICAM-1) and vascular cell adhesion molecule (VCAM-1) in vascular endothelial cells, as well as

the secretion of IL-6 from vascular endothelial cells, smooth muscle cells and macrophages (Kol *et al.*, 1999; Kol *et al.*, 2000). Furthermore, bacterial and mycobacterial HSPs induce proinflammatory cytokine expression (Peetermans *et al.*, 1994; Galdiero *et al.*, 1997) and bacterial Hsp60 (termed GroEL) induces expression of ICAM-1 and VCAM-1 in vascular endothelial cells (Galdiero *et al.*, 1997). The findings showing that proteins of the Hsp60 family could induce adhesion molecule expression and cytokine secretion from various cell types prompted the search for Hsp60 receptor. The CD14 antigen has been identified as the receptor for this protein on human peripheral blood mononuclear cells and coenocytes. Therefore, it seems that Hsp60 uses the signaling pathway also used by lipopolysaccharide (Kol *et al.*, 2000). It was also shown that TLR4 (Ohashi *et al.*, 2000), which is an important mediator of innate immunity and lipopolysaccharide signaling in mouse cells, is needed for Hsp60 signaling (Hoshino *et al.*, 1999). Data from another study (Vabulas *et al.*, 2001) suggest that Toll-like receptor 2 plays a part in human Hsp60 signaling. More recent studies of human Hsp60 have revealed that this protein has unexpected role in immunological response.

2. The aims of the research

The aim of the present study was to relate the parameters of inflammatory status with circulatory levels of HSPs and cortisol, and to uncover possible associations between inflammatory markers and extracellular HSPs with trauma-exposure, PTSD symptoms, vulnerability and resilience to PTSD. Toward that end the relevant parameters were determined in patients with current PTSD and life-time PTSD, in trauma-exposed individuals without PTSD and in non-traumatized healthy subjects. The measured parameters were: circulatory levels of a number of cytokines and CRP, as inflammatory markers; intracellular levels of HSPs Hsp90, Hsp70, Hsp72 and Hsp60; plasma levels of HSPs Hsp70, Hsp72 and Hsp60; plasma levels of cortisol before and after suppression of HPA axis by dexamethasone and the level of corticosteroid receptor proteins (GR and MR) in peripheral blood mononuclear cells.

Specific aims of the study were:

1. To learn whether excessive inflammation is associated with PTSD or with trauma, as an essential component of the disorder;
2. To determine whether the intensity of inflammation correlates with PTSD symptoms, trauma exposure, vulnerability to PTSD, resilience to the disorder and/or with its remission;
3. To reveal the relationship between extracellular HSPs and inflammatory status in all study groups.
4. To examine the relation of plasma cortisol level and the lymphocyte level of corticosteroid receptors with inflammatory markers, trauma exposure, and vulnerability/resilience to PTSD.

The results of the present study are expected to provide new insight into relationship of inflammation with PTSD pathology, trauma and vulnerability/resilience to PTSD, as well as to unravel possible causal role of extracellular HSPs in PTSD-related inflammatory actions of the immune system.

3. Materials and Methods

3.1 Subjects

3.1.1. Study groups

The study was performed on four groups of subjects: war veterans with current PTSD (current PTSD group, n = 133), veterans with life-time PTSD (life-time PTSD group, n = 66), those with war-related traumatic experiences but without PTSD, herein referred to as "trauma controls" (n = 102) and healthy controls (n = 99). All the participants in the study were male and were recruited in Serbia in the period from March 2005 to June 2008. PTSD patients were recruited from the Association of Veterans of Wars after 1990, the Association of ex-detained persons and war victims assessed or treated in the International Aid Network non-governmental organization. Trauma control subjects were selected from the Association of Veterans of Wars after 1990 and from the Special Forces via the Military Medical Academy. Healthy subjects were found through available social networks, national trade unions and from the general population with the assistance of the Strategic Marketing Agency. The groups were matched by age and educational level.

The inclusion criteria for subjects with current or life-time PTSD included: (1) exposure to war traumatic event(s); (2) current PTSD or life-time PTSD as defined by the DSM-IV criteria and assessed by the SCID-I and CAPS-DX and (3) CAPS Criteria A-F satisfied, with the score of B+C+D subtotals above 50. Trauma controls had no diagnosis of current or life-time PTSD, and did not fulfill CAPS Criteria A-F, the score of B+C+D subtotals being below 30. For the healthy control group the inclusion in the study required: no exposure to traumatic war event(s) and no diagnosis of current or life-time PTSD.

The Clinician Administered PTSD Scale CAPS-DX (Paunovic & Ost, 2005) was used for diagnosing PTSD. Subjects with satisfied CAPS criteria A-F and a total

CAPS score higher or equal to 50 were classified in current or lifetime PTSD group, depending on whether the PTSD symptoms were current or lifetime. Those scoring less than 30 were classified in the trauma control group. The latter group differed from the healthy control group by fulfilling CAPS-DX criterion A. The subjects belonging to trauma control and healthy control groups had no diagnosis of PTSD.

The exclusion criteria for all the groups included: (1) serious medical illness, (2) current psychotic disorder, as defined by DSM-IV criteria (except major depression), (3) current psychoorganic syndrome, as defined by DSM-IV criteria, (4) alcohol/substance dependence or abuse within 6 months prior to the entry procedure and (5) endocrinological or neurological illnesses likely to interfere with HPA axis function.

Since PTSD is frequently co-morbid with depression, the severity of depression was assessed by the Beck's Depression Inventory II (BDI-II), a 21-item multiple-choice self-report instrument (Beck, 1996).

The washout period for medications, such as benzodiazepines, antidepressants, neuroleptics and antipsychotics, was at least 4 weeks prior to the entry procedure.

Cigarette smoking and former alcohol consumption were assessed extensively in a clinical interview by the physician. These variables (the number of years of smoking, the number of cigarettes per day; the number of years, average quantity and frequency of drinking), introduced as covariates, did not influence any correlations of cortisol measures with other variables of interest and were not included in the analyses presented.

3.1.2. Procedure

This study is a part of a multidisciplinary FP6 research project "Psychobiology of Post-Traumatic Stress Disorder" performed by the international consortium "SPIN". Only the data relevant to the title of this thesis are presented

herein. For the purpose of the whole study, the participants were hospitalized for 2.5 days at the Institute for Endocrinology, Diabetes and Metabolic Diseases, University Clinical Centre of Serbia, Belgrade, and were subjected to simultaneous psychological and biological investigations. On admission to the hospital, the participants were first submitted to a comprehensive medical check-up, including a detailed anamnestic interview by a physician. Subsequently the blood sample was drawn for the analyses done within this part of the study.

The study was approved by the Ethics Committee of the University Clinical Center of Serbia, Belgrade. All the participants signed a written informed consent after they were given a detailed written and verbal description of the study and the procedure they were subjected to.

3.2 Reagents and antibodies

Bovine serum albumine (BSA) and polyxyethylene-sorbitan monolaurate (Tween 20) were from SERVA (Germany), and tetramethylbenzidine was a product of BioLegend (USA). Human standard proteins, Hsp60 (200 μg) (ADI-NSP-540-E) and Hsp70/Hsp72 (200 μg) (ADI-NSP-555-D), were obtained from Enzo Life Sciences (Victoria, Canada).

The primary antibodies used in this study are: rabbit polyclonal antibody against GR (PA1-511A), mouse monoclonal antibody against mineralocorticoid receptor (MR) (MA1620), both purchased from Affinity BioReagents; mouse monoclonal primary anti-Hsp60 antibody (ADI-SPA-806-F, clone LK-1), rabbit polyclonal anti-Hsp60 antibody (ADI-SPA-805-F), mouse monoclonal anti-Hsp70/Hsp72 antibody (ADI-SPA-810-F, clone C92F3A-5), rabbit polyclonal anti-Hsp70/Hsp72 antibody (ADI-SPA-812-F), mouse monoclonal antibody against Hsc70/Hsp70 (ADI-SPA-820-F, clone N27F3-4), mouse monoclonal antibody against Hsp90 (ADI-SPA-830-F, clone AC88), all from Stressgene. Secondary antibodies used in this thesis are goat anti-rabbit IgG polyclonal antibody horse-radish peroxidase (HRP) conjugate (ADI-SAB-300-J), Enzo Life Sciences (Victoria,

Canada) and mouse and rabbit alkaline phosphatase (AP)-conjugated secondary antibody (Amersham Pharmacia Biotech).

3.3 Preparation of blood plasma samples

Samples of peripheral blood (40 ml) for most of the analyses described herein were obtained by venipuncture into heparin anticoagulant between 8:30 and 9:30 a.m. on the day of admission to the hospital. For plasma cortisol measurements before and after dexamethasone administration, the blood samples were drawn at 9:00 a.m. on the second and third day of hospitalization, respectively.

3.4 Isolation of peripheral blood mononuclear cells

PBMCs were isolated from whole blood by Ficoll-Paque PLUS (Amersham) density gradient centrifugation. The blood was first diluted with PBS buffer (1.5 mM KH_2PO_4, 6,5 mM Na_2HPO_4, 2,7 mM KCl, 0,14 M NaCl, pH 7,2) in 2:1 ratio, in order to avoid aggregation of eritrocytes. Diluted blood was layered over the Ficcol, and the samples were centrifuged at 380 x g for 30 min at 20°C. After centrifugation the distinct layers are obtained: upper layer that contains blood plasma, interface with lymphocytes and ficoll layer with the pellet consisting of granulocytes and erythrocytes. The blood plasma was isolated and stored at -70ºC for subsequent processing. A portion of mononuclear cells, recovered from the plasma/Ficoll interface was immediately frozen liquid nitrogen and stored until use.

3.5 Preparation of whole cell extracts

For the whole cell extract preparation, PBMCs, stored in liquid nitrogen, were resuspended and incubated (15 min, 0°C) in ice-cold TEDG buffer (10 mM Tris, 1 mM ethylenediaminetetraaceticacid, 2.5 mM dithiothreitol, 10% glycerol, 0.1 mM

phenylmethylsulphonylfluoride, pH 7.4), sonicated (2 x 15 s, 1A, 50/60 Hz, 30% amplitude, Hielscher Ultrasound Processor) and centrifuged (14000 x g, 20 min, 4°C). The supernatants, referred to as whole cell extracts, were analyzed for total protein content by the method of Spector (1978).

3.6 Plasma cortisol measurements

Plasma cortisol concentrations were determined by RIA (CORT-CT2, CIS biointernational, Gif-Sur-Yvette Cedex, France). Minimal detectable concentration was 4.6 nmol/L, while intra- and inter-assay variations were below 5.4% and 7.3%, respectively.

We chose nocturnal cortisol levels as better representation of the resting state than the day values, because of the reduced external stimuli during the night. Basal cortisol is represented by individual mean values obtained from 13 night/morning time points.

Dexamethasone suppression test (DST) is used to test the self-regulation of HPA axis by cortisol via GR. The usual dexamethasone doses applied are 1 mg (considered high) and 0.5 mg (considered low). In this study, the DST was performed with the low dose (0.5 mg), which was applied at midnight. For assessment of HPA axis suppression, plasma cortisol values determined at 9:00 a.m. on two consecutive days were compared. The first value was determined prior to dexamethasone administration (preDEX cortisol) and the second after dexamethasone administration (postDEX cortisol). Cortisol suppression is expressed as percent of suppression and was calculated as follows: % suppression = [(pre-DEX cortisol – postDEX cortisol)/preDEX cortisol] x 100.

3.7 Determination of eHsp70 concentration by ELISA

The level of extracellular inducible Hsp70 (Hsp72) in blood plasma was determined by commercial sandwich ELISA (enzyme-linked immunosorbent

assay) according to the manufacturer's instructions (Enzo Life Sciences). Briefly, precoated plates were blocked with 250 µl of blocking buffer (PBS, pH 7.3, containing 1% BSA) for 2 h at 25°C on a shaker. After washing with PBST, Hsp70 standards (100 µl; 0–2500 ng/ml in PBS) or samples (plasma) were added and incubation proceeded for 2 h at 25°C on with shaking. Plates were washed four times and 100 µl of rabbit polyclonal anti-Hsp70 antibody was added. After 1 h at 25°C on a shaker, plates were washed and incubated with 100 µl of anti-rabbit IgG polyclonal antibody HRP-conjugate for 1 h at 25°C on a shaker. The wells were washed six times and 100 µl of the substrate solution (TMB) was added and incubated at room temperature in the dark for 15 min. The reaction was stopped using 50 µl of 1 M H_3PO_4. Absorbance was read at 450 nm and 650 nm on the Multiscan Spectrum (Thermo Electron). Results were expressed as ng of eHsp72 per ml of plasma.

In parallel, the level of constitutive Hsp70 (Hsc70) in blood plasma was determined by the sandwich ELISA procedure reported by Njemini et al. (2005), with slight modification. In this protocol plates were coated with the primary antibody, anti-Hsp70/Hsc70 (SPA-820) (50 µl; 2 µg/ml), diluted in 0.1 M carbonate buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, 3 mM NaN_3, pH 9.6). After overnight incubation on 4°C, the coated plates were washed four times with PBST and non-specific binding sites were blocked with 300 µl of blocking buffer (PBS, pH 7.3, containing 1% BSA) for 2 h at 25°C on a shaker. After washing with PBST, Hsp70 standards (50 µl; 0–2500 ng/ml in PBS) or samples (plasma) were added and incubation proceeded for 2 h at 25°C on a shaker. Plates were washed four times and 50 µl of rabbit polyclonal anti-Hsp70 antibody (ADI-SPA-812) (2 µg/ml) diluted in PBST was added. After 1 h at 25°C on a shaker, plates were washed and incubated with 50 µl of anti-rabbit IgG polyclonal antibody HRP conjugate in PBS/T (50 ng/ml) (ADI-SAB-300-J) for 1 h at 25°C on a shaker. The wells were washed six times and 50 µl of the substrate solution TMB was added and incubated at room temperature in the dark for 15 min. The reaction was stopped using 30 µl of 1 M H_3PO_4. Absorbance was read at 450 nm on the Multiskan Spectrum (Termo

Electron Corporation), subtracted from values at 650 nm and results were expressed as ng of eHsp70 per ml of plasma.

3.8 Determination of eHsp60 concentration by ELISA

The level of extracellular Hsp60 in blood plasma from the four groups of patients was determined by sandwich ELISA developed in our laboratory. Briefly, the 96-well plates were coated with the mouse primary anti-Hsp60 antibody (ADI-SPA-806) (50 µl; 4 µg/ml) diluted in 0.1 M carbonate buffer (15 mM Na_2CO_3, 15 mM $NaHCO_3$, 3 mM NaN_3, pH 9.6). After overnight incubation at 4ºC, the coated plates were washed four times with phosphate-buffered saline (PBS) (135 mM NaCl, 40 mM $Na_2HPO4 \times 2H_2O$, 1 mM KH_2PO_4, 3 mM KCl) containing 0.1% Tween-20 (PBST). Non-specific binding sites were blocked with 300 µl of blocking buffer (PBS, pH 7.3, containing 1% BSA) for 2 h at 37ºC on a shaker. After washing with PBST, Hsp60 standards (50 µl; 0–40 µg/ml in PBS) or samples (plasma) were added and incubation proceeded for 2 h at 37ºC on a shaker. Plates were washed four times and 50 µl of rabbit polyclonal anti-Hsp60 antibody (ADI-SPA-805) (2 µg/ml) diluted in PBST was added. After 1 h at 37ºC on a shaker, plates were washed and incubated with 50 µl of anti-rabbit IgG polyclonal HRP conjugate antibody in PBST (200 ng/ml) (ADI-SAB-300-J) for 1 h at 37ºC with gentle shaking. The wells were washed six times and 50 µl of the substrate solution (tetramethylbenzidine) was added and incubated at room temperature in the dark for 15 min. The reaction was stopped using 30 µl of 1 M H_3PO_4. Absorbance was read at 450 nm on the Multiskan Spectrum (Termo Electron Corporation), subtracted from values at 650 nm and results were expressed as µg of Hsp60 per ml of plasma.

3.9 Determination of plasma cytokine levels

FlowCytomix™ Multiplex Kit developed by Bender MedSystems (BMS810FF, eBioscience) was used in this study in order to simultaneously quantify 11

cytokines (IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12 (p70), TNF-α, TNF-β) in the blood plasma from the study participants. A FlowCytomix™ Multiplex assay represents a unique sandwich immunoassay. Namely, the kit contains fluorescent bead sets, each pre-coated with an antibody unique for one cytokine. Within two bead size populations, A and B (4 μm and 5 μm, respectively), there are multiple bead subsets, differentiated by varying intensities of an internal fluorescent dye. The dye can be excited by an Argon or He-Ne laser, and emits at 690 nm (the far red spectrum). The combination of the two different bead sizes and different internal dye intensities makes it possible to distinguish 11 bead sets in one fluorescent channel.

In our experiments, antibody-coated beads were added and incubated with plasma sample. Target analytes in plasma sample were captured by specific antibodies on each bead set. After 1 h of incubation and subsequent washing, a biotin-conjugated secondary antibodies specific for bound analytes were added. Following 2 h of incubation and washing, streptavidin-PE was added for detection of biotin-conjugated antibody. Streptavidin-PE, which binds to the biotin conjugate, emits at 578 nm, allowing the quantification of the analyte. Detection was performed by flow cytometry, which differentiates bead populations according to bead size and fluorescent signature. Concentration of each cytokine was determined using 5PL curve and the results were expressed as pg of specific cytokine per ml of blood plasma.

In most blood plasma samples, irrespectively of the study group, the levels of IL-1β and TNF-β were under the limits of the detection. Therefore, the results for these two cytokines are not presented herein.

3.10 SDS-Polyacrylamide gel electrophoresis (SDS-PAGE)

After boiling in 2x Laemmli's buffer (Laemmli, 1970), 40 μg of PBMC whole cell extracts were separated on 10 % gels by SDS-polyacrylamide gels electrophoresis (SDS-PAGE) overnight, at 120 V and at 4°C. Myosin (205 kDa), b-galactosidase (116 kDa), phosphorylase b (97 kDa), bovine serum albumin

(66 kDa) and carbonic anhydrase (29 kDa) were simultaneously run as molecular mass references.

3.11 Western blotting

Western transfer of proteins from the gels to polyvinylidene fluoride membranes was carried out overnight at 135 mA and 4°C in 25 mM Tris buffer, pH 8.3, containing 192 mM glycine and 20% (v/v) methanol. Unbound sites on the membranes were blocked by 1 h incubation at room temperature in PBS containing 0.5% nonfat dry milk. The membranes were then respectively probed with antibody against GR (PA1-511A; 1:1000), MR (MA1-620; 1:1000), inducible Hsp70/Hsp72 (SPA-810, 1:500), constitutive Hsp70/Hsc70 (SPA-820; 1:100), Hsp60 (SPA-805; 1:500); Hsp90 (ADI-SPA-830; 1:1000) by overnight incubation at 4ºC. Subsequently, the membranes were incubated 1.5 h at room temperature with antibody against β-actin (AC-15, 1:20000), which was used as equal loading control. After washing with PBS containing 0.1% Tween 20 the membranes were incubated with appropriate AP-conjugated secondary antibody (1:20000) under the same conditions. Probing for each protein was followed by stripping with 0.2 M NaOH and blocking. Immunopositive bands were visualized by an enhanced chemifluorescent method using STORM scanner (Amersham). Relative optical density of immunoreactive bands was determined by ImageQuant software (GE Healthcare). In order to make quantitative comparisons between multiple immunoblots reliable, an internal reference sample, obtained from a healthy blood donor, was run on each gel in triplicate: in the middle and both ending lanes. The intensity of each analyzed immuno-specific band was normalized to the intensity of the nearest internal reference band on the same blot and also to the intensity of β-actin band in the same lane.

3.12 Depletion of albumin from blood plasma

For the purpose of validation of in-house ELISAs by Western blot, it was necessary to remove albumin from plasma beforehand. In order to deplete albumin from plasma samples we performed a fractionation of the plasma proteins with increased concentrations of ammonium sulfate according to Jiang et al. (2004). Aliquot of plasma, containing 2 mg of total protein, was diluted with PBS to 500 µl and ammonium sulfate was added to reach 30% saturation. After gentle vortexing for 10 min, the sample was left at room temperature for 1 h and centrifuged at 10 000 xg, 20°C, for 30 min. The supernatant was transferred to another tube and ammonium sulfate was added to reach 50% saturation. The mixture was treated as above. The proteins in the supernatant were further fractionated with 70% and 90% ammonium sulfate saturation. Each of the four pellets was washed with 300 µl of ice cold 90% acetone, dried in air and suspended in 200 µl of 2x Laemmli's sample buffer. Protein concentration in the blood plasma samples was determined by a Coommassie staining procedure as described by Spector (1978).

3.13 Validation of ELISA by Western blotting

In order to test specificity of the antibody used in in-house ELISAs for eHsp60 and eHsp70, we performed Western blotting with plasma samples depleted of albumin. After each ammonium sulfate-fractionation step 50 µl of supernatant was collected, mixed 1:1 with 2x Laemmli's sample buffer and boiled for 5 min. All samples were subjected to SDS-PAGE and Western blot analysis using rabbit polyclonal anti-Hsp60 antibody (ADI-SPA-805-F) and mouse monoclonal antibody against Hsc70/Hsp70 (ADI-SPA-820-F, clone N27F3-4). These experiments demonstrated that the best immunoreactive bands corresponding to Hsp60 or Hsp70, respectively, were observed in plasma fractions obtained after 70% ammonium sulfate saturation.

3.14 Determination of cytokines by multiplex ELISA

FlowCytomix™ Multiplex Kit developed by Bender MedSystems (BMS810FF, eBioscience) is used in this study in order to simultaneously quantify 11 cytokines (IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12 (p70), TNF-α and TNF-β) in the human plasma of the four studied groups. A FlowCytomix™ Multiplex assay represent a unique sandwich immunoassay. Namely, the kits contain fluorescent bead sets, each pre-coated with an antibody unique for one cytokine. Within two bead size populations, A and B (4 μm and 5 μm, respectively), there are multiple bead subsets, differentiated by varying intensities of an internal fluorescent dye. The dye can be excited by an Argon or He-Ne laser, and emits at 690 nm (the far red spectrum). The combination of the two different bead sizes and different internal dye intensities makes it possible to distinguish 11 bead sets in one fluorescent channel.

In our experiments, antibody-coated beads are added and incubated with plasma sample. Target analytes in plasma sample are captured by specific antibodies on each bead set. After 1 h of incubation and subsequent washing, a biotin-conjugated secondary antibodies specific for bound analytes are added. Following 2 h of incubation and washing, streptavidin-PE is added for detection of biotin-conjugated antibody. Streptavidin-PE, which binds to the biotin conjugate, emits at 578 nm, allowing the quantification of the analyte. For detection was performed by flow cytometry, which differentiate bead populations according to bead size and fluorescent signature. Concentration of all cytokines is determined using 5PL curve and the results are expressed as pg of specific cytokine per ml of plasma. In most of the plasma samples, irrespective of the study groups, the levels of two cytokines, IL-1β and TNFβ, were below the detection limit of the assay and the results are not presented.

3.15 Statistical analyses

Data analyses were done using SPSS, version 17.0. Variables were tested for normality by Kolmogorov-Smirnov test, which confirmed normal distribution. The values were considered outliers and were excluded if standardized z-scores fell outside the range of ±3.29. Between-group differences were assessed by one-way analysis of variance (ANOVA) and p values were considered statistically significant at the level of less than 0.05 (two-tailed). When ANOVA test revealed a significant main effect, Bonferroni *post hoc* test was used for multiple between-group comparisons. The group means are expressed as mean ± standard deviation (SD).

Possible influence of co-morbid depression on group differences in the tested variables was assessed by analysis of covariance (ANCOVA), or by partial correlation analysis, taking BDI-II score as a covariate.

The Pearson's correlation coefficients were compared between groups by converting correlation coefficients to z_r, calculating z-score and determining corresponding probability from The Table of the Standard Normal Distribution.

4. Results

4.1. Demographic characteristics of the study groups

Demographic characteristics of the study groups are summarized in **Table 4.1**. The groups were matched by age (43.6 ± 9.3, mean ± SD) and educational level (11.7 ± 2.4 years of education, mean ± SD). All traumatic experiences were related to the recent wars in the Balkan region. The average number of early traumatic events and the mean body mass index did not differ between the groups. All the groups had a similar average number of smokers *vs.* nonsmokers (data not shown), but the life-time PTSD group differed from the healthy control group in the number of cigarettes smoked per day. Therefore, this variable was analyzed as a covariate whenever it was necessary.

As expected, the total score on CAPS diagnostic tool for PTSD was significantly higher in all three traumatized groups in comparison to healthy control group, and in two PTSD groups in comparison to trauma controls. Among the three traumatized groups of participants, the highest number of traumatic experiences was registered in current PTSD group. The average score on Beck's Depression Inventory II was significantly higher in current and life-time PTSD groups than in trauma control group or healthy control group, while the two control groups did not differ in respect to BDI II score. These data showed that in our sample depression was frequently comorbid with current PTSD and even with life-time PTSD, so that it should be taken into consideration as a covariate.

Table 4.1. Demographic characteristics of the study groups.

	N	Current PTSD	Life-time PTSD	Trauma controls	Healthy controls	ANOVA	
		Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	F	p
Age (years)	132	44.1 ± 8.7	42.9 ± 10.3	42.9 ± 8.9	42.6 ± 10.2	0.16	0.930
BMI (kg/m^2)	132	27.5 ± 3.9	26.6 ± 4.4	27.5 ± 3.9	27.6 ± 3.0	0.45	0.720
Cigarettes/day	97	30 ± 16	34 ± 18*	26 ± 11	22 ± 8	3.20	0.025
$CAPS_{TOT}$	132	61.8 ± 10.1*#	25.6 ± 6.9*#	13.8 ± 9.4*	5.3 ± 6.8	286.50	0.001
$uPRSa_{TOT}$	130	65.8 ± 30.8*#	48.9 ± 31.9*	44.0 ± 23.4*	0.1 ± 0.5	41.30	0.001
BDI_{TOT}	132	26.5 ± 12.8*#	17.4 ±11.5*#	10.7 ± 9.1	4.8 ± 5.1	28.40	0.001

Group comparisons were done by one-way ANOVA followed by post hoc Bonferroni test.

N, number of subjects per group; BMI, body mass index; $CAPS_{TOT}$, total CAPS score; $uPRSa_{TOT}$, number of traumatic events; BDI_{TOT}, total BDI II score;

**, significantly different from healthy controls;*

 # significantly different from trauma controls.

4.2. Plasma cortisol level and HPA axis sensitivity

Cortisol, as the marker of HPA axis activity, was assessed in the blood plasma samples from all the participants in the study. Cortisol was measured in the clinical setting every hour during night, starting from 10 p.m. until 9 a.m. the next day, plus an additional sample at 7:30 a.m. to follow the morning rise of HPA axis activity. We chose nocturnal cortisol levels as a better representation of the resting state than daily values, because of the reduced external stimuli during night. The group means were calculated from the individual mean values of cortisol level derived from 13 measurements and taken as basal cortisol level.

Between group differences in basal cortisol level were not observed (one-way ANOVA, F= 2.39, p= 0.072) (**Figure 4.1**).

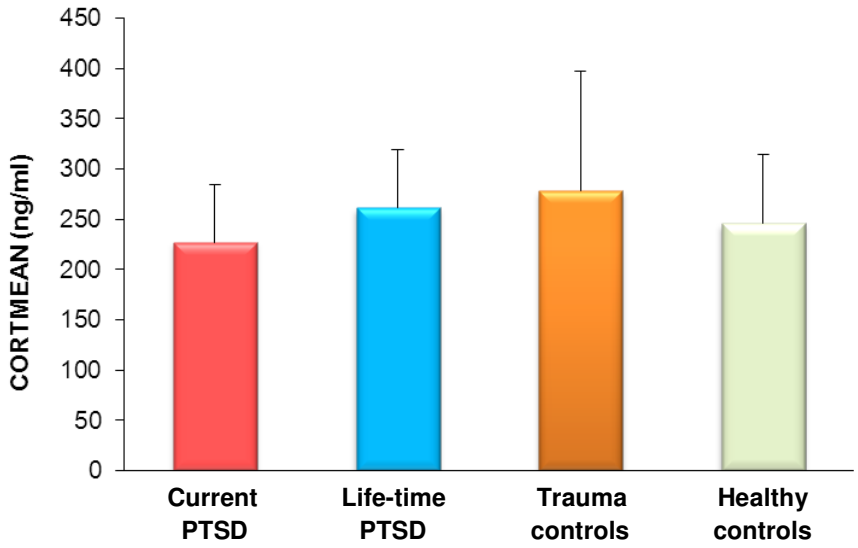

Figure 4.1. Basal plasma level of cortisol. *Plasma level of cortisol was measured hourly during night. The individual means calculated from 13 measurements were used to obtain group means (CORTMEAN), which were taken as average basal cortisol levels. The results are expressed as means ± SD.*

The release of cortisol from the adrenal gland is regulated by the hormone ACTH, so that its level is considered as an additional marker of HPA axis activity. ACTH level was assessed during night, at the same time points as cortisol, every

hour starting from 10 p.m. to 9 a.m. the next day. The individual mean values of ACTH were calculated on the basis of 13 nocturnal/morning measurements.

Group means of ACTH level, calculated from individual values, were not significantly different among the study groups (one-way ANOVA, F= 0.67, p= 0.573) (**Figure 4.2**).

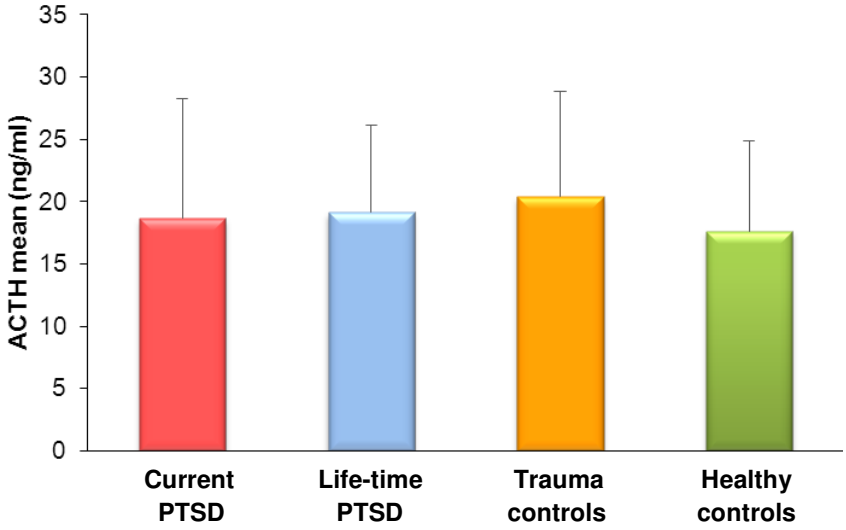

Figure 4.2. Plasma level of ACTH. *Plasma level of ACTH was measured hourly during night. The individual means calculated from 13 measurements were used to obtain group means (ACTH mean). The results are expressed as means ± SD.*

In order to test regulation of HPA axis activity *i.e.* to assess its sensitivity to feed-back inhibition by cortisol, we performed dexamethasone suppression test (DST) in all study groups. The plasma level of cortisol was assessed at 9 a.m. and designated as preDEX cortisol value. Dexamethasone was administered orally at the dose of 0.5 mg, at midnight. The plasma cortisol level after dexamethasone administration (postDEX cortisol value) was measured at 9 a.m. the next morning.

The percent of suppression of HPA axis by dexamethasone was calculated according to the formula [(preDEX cortisol – postDEX cortisol)/preDEX cortisol] × 100. The statistical analysis revealed that study groups were significantly different in respect to the degree of suppression (one-way ANOVA, F= 6.011, $p < 0.01$). Bonferoni post hoc test indicated a significant differences between

current PTSD and healthy control groups ($p < 0.0001$), as well as between the life-time PTSD and healthy control groups ($p = 0.038$) (**Figure 4.3**).

The results of DST show that HPA axis in patients with PTSD displays increased sensitivity to dexamethasone in comparison to healthy controls, which reflects increased HPA axis responsiveness to endogenous cortisol. Moreover, increased efficiency of feed-back inhibition of HPA axis is also a characteristic of life-time PTSD patients. Since both current and life-time PTSD patients are obviously vulnerable to PTSD, as they developed PTSD symptoms after exposure to trauma, this finding suggests that hypersensitivity of HPA axis to cortisol might be a biological correlate of vulnerability to PTSD.

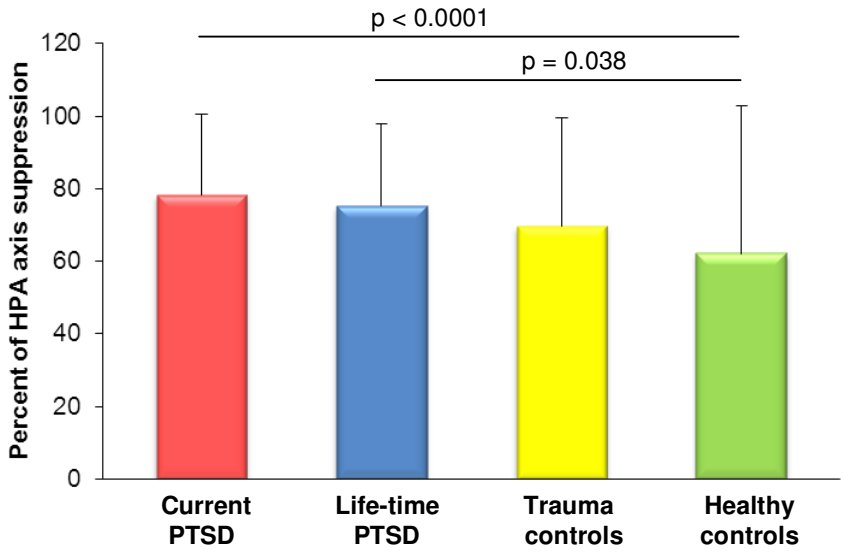

Figure 4.3. Suppression of HPA axis by dexamethasone. *Plasma levels of cortisol were measured at 9 a.m. before and after a midnight oral dexamethasone administration at the dose of 0.5 mg. Percent of suppression of HPA axis was calculated by the formula: [(preDEX cortisol – postDEX cortisol)/preDEX cortisol] × 100, and the results are expressed as means ± SD. The level of statistical significance of between-group differences is presented by p value.*

4.3 Corticosteroid receptors expression in the lymphocytes

Western blot analysis of the GR protein in the whole cell extract of PBMCs demonstrated presence of two immuno-specific bands: one migrating at 97 kDa and the other at 105 kDa. The higher molecular mass GR band could be detected only in 52-57% of the subjects in each group and in majority of participants was considerably less abundant than the lower molecular mass band. Considering all groups together, in the lymphocytes of the individuals presenting 105 kDa GR band, the intensity of the 97 kDa band was significantly lower in comparison to the individuals missing the higher band (t-test, $p < 0.001$). It was also valid for each of the four groups of subjects separately. The intensity of both bands together revealed statistically significant increase in the average total GR protein level in PBMCs from current PTSD patients ($p = 0.021$) and life-time PTSD subjects ($p = 0.011$) in comparison to trauma controls (**Figure 4.4**). Between-group differences in the average intensity of 105 kDa and 97 kDa bands measured separately were not observed.

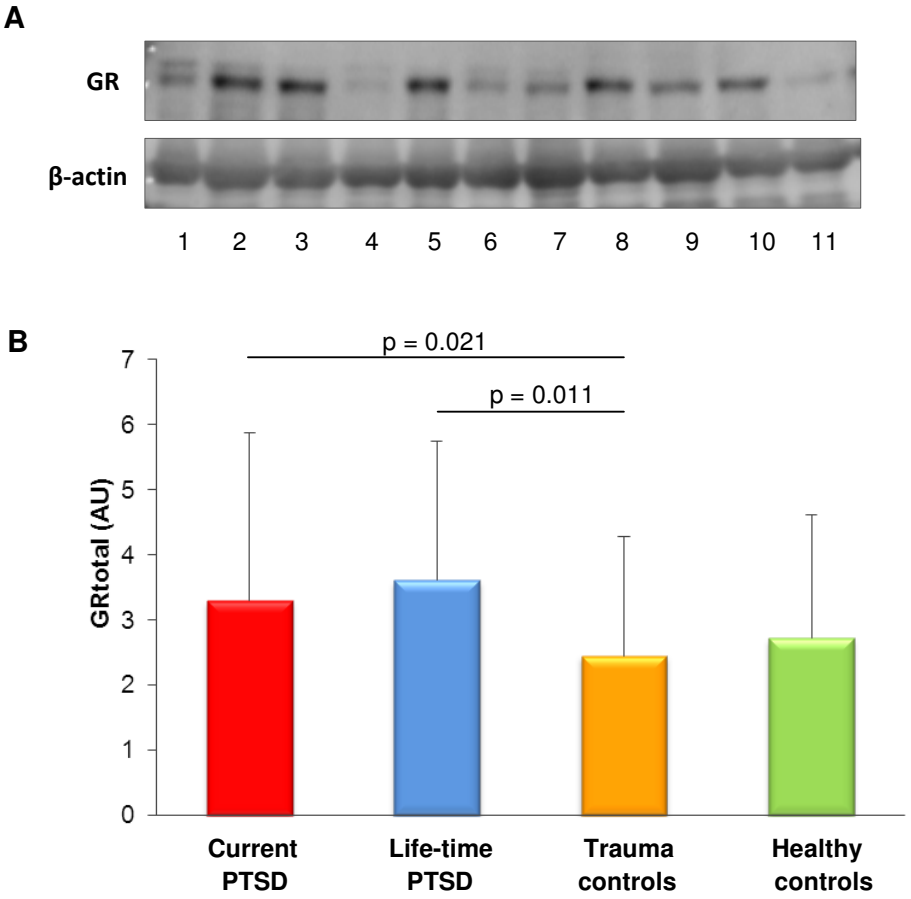

Figure 4.4. Relative level of GR in peripheral lymphocytes. (A) *The GR was detected by Western blot using rabbit polyclonal anti-GR antibody. A representative blot is shown, containing two sets of four participants and three internal reference samples. Lanes: 1, 6, 11 – internal reference samples; 2, 7 – patients with current PTSD; 3, 8 – life-time PTSD patients; 4, 9 – trauma controls; 5, 10 – healthy controls.* ***(B)*** *Quantitative analysis was done by the ImageQuant software. The intensity of each analyzed immuno-specific band was normalized to the intensity of the nearest internal reference band on the same blot and to the intensity of β-actin band in the same lane. Relative integrated optical densities of both GR bands together (GRtotal) are expressed in arbitrary units (AU) as means ± SD.*

A similar semi-quantitative immunoblot procedure was applied for determination of the relative level of MR in peripheral lymphocytes from the four groups of subjects. Measuring relative integrated optical densities of the immunoreactive bands corresponding to the MR, a rather uniform expression level of the MR protein was noticed throughout all study groups (**Figure 4.5**). Statistical

analysis did not reveal significant differences between the groups (ANOVA, F= 0.56, p= 0.640).

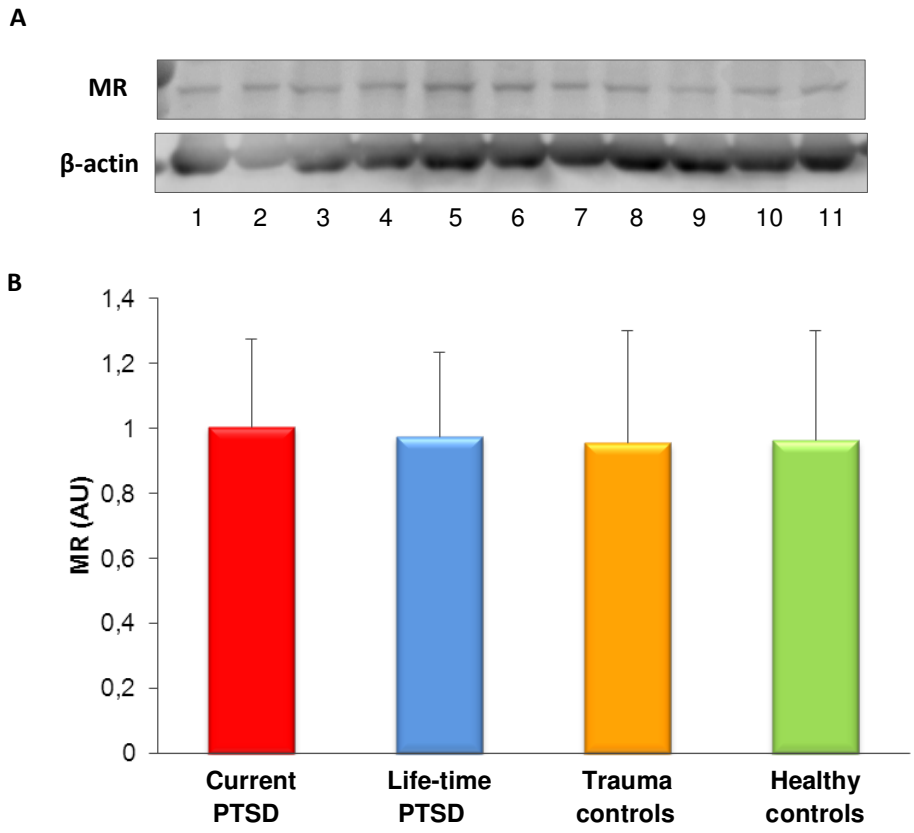

Figure 4.5. Relative level of MR in peripheral lymphocytes. (A) *MR was detected using rabbit polyclonal anti-MR antibody. A representative blot is shown, containing two sets of four participants and three internal reference samples. Lanes: 1, 6, 11 – internal reference samples; 2, 7 – patients with current PTSD; 3, 8 – life–time PTSD patients; 4, 9 – trauma controls; 5, 10 – healthy controls.* ***(B)*** *Quantitative analysis was done by the ImageQuant software. The intensity of each analyzed immuno-specific band was normalized to the intensity of the nearest internal reference band on the same blot and to the intensity of β-actin band in the same lane. Relative integrated optical densities of MR bands are expressed in arbitrary units (AU) as means ± SD.*

The balance between GR- and MR-promoted actions critically determines functioning of HPA axis. Analysis of protein levels of two corticosteroid receptors in the individual PBMC enabled us to calculate the MR/GR ratio in the lymphocytes. It was found that the ratio was not significantly different between the analyzed groups of subjects (ANOVA, F= 0.82, p= 0.487) (**Figure 4.6**).

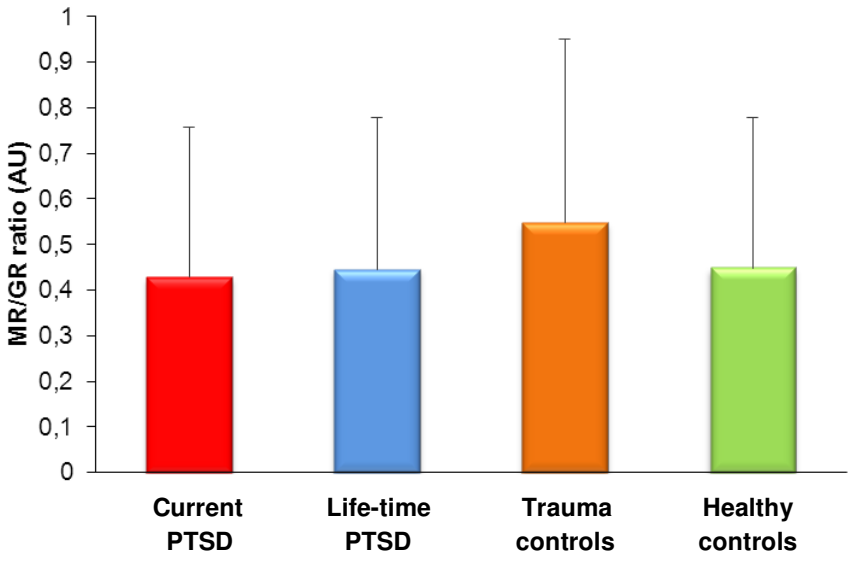

Figure 4.6. Relative ratio of MR and GR in peripheral lymphocytes. *The results are expressed as means ± SD.*

4.4 Heat shock protein expression levels

4.4.1 Heat shock protein levels in peripheral lymphocytes

The level of Hsp90 expression in the PBMC whole cell extracts was determined by a semi-quantitative Western blot procedure. The results show very similar levels of Hsp90 protein in all study groups (**Figure 4.7**). The ANOVA analysis revealed that there are no statistically significant differences in the level of Hsp90 expression between the study groups (ANOVA, F= 1.23; p = 0.29).

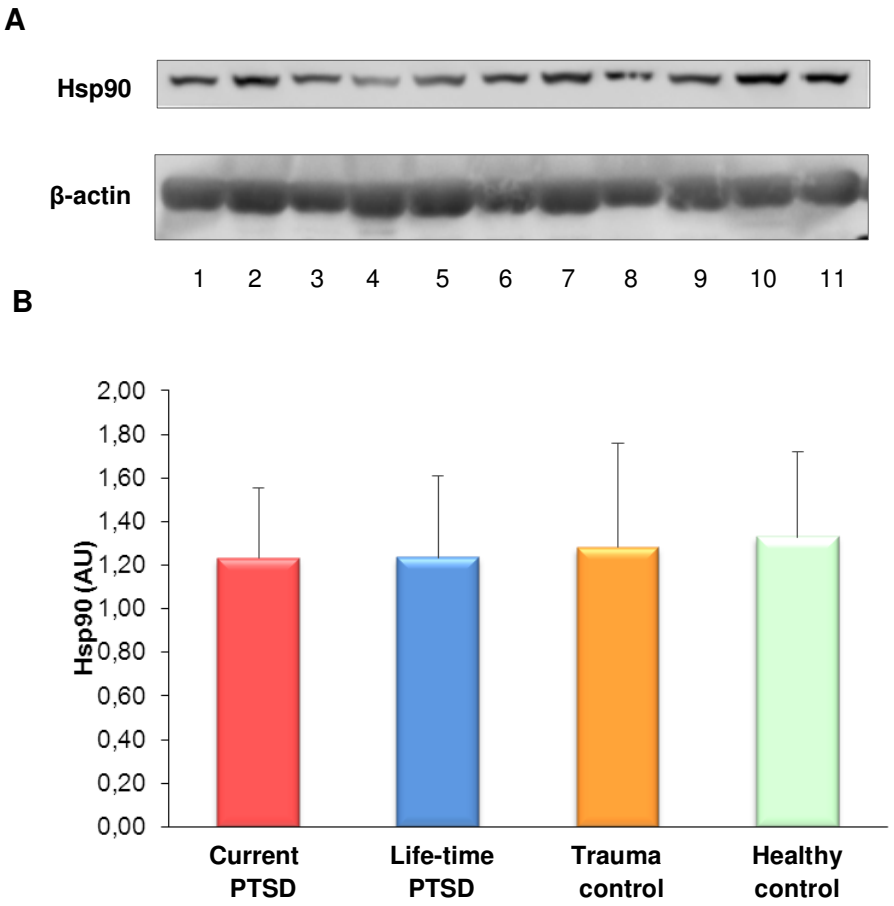

Figure 4.7. Relative level of Hsp90 in peripheral lymphocytes. (A) *Hsp90 was detected in the whole cell extract of PBMCs by a monoclonal anti-Hsp90 antibody. A representative blot is shown, containing two sets of four participants and three internal reference samples Lanes: 1, 6, 11 – internal reference samples; 2, 7 – patients with current PTSD; 3, 8 – life–time PTSD patients; 4, 9 – trauma controls; 5, 10 – healthy controls.* ***(B)*** *Quantitative analysis was done by the ImageQuant software. The intensity of each analyzed immuno-specific band was normalized to the intensity of the nearest internal reference band on the same blot and to the intensity of β-actin band in the same lane. Relative integrated optical densities of Hsp90 bands are expressed in arbitrary units (AU) as means ± SD.*

The level of Hsp70 expression in PBMCs was also determined by semi-quantitative Western blot. For detection of the protein two different antibodies were used: one recognizing both constitutive (Hsc70) and inducible

(Hsp72) isoforms of Hsp70 (herein designated as Hsp70) and the other recognizing only the inducible isoform, Hsp72. Measurement of relative optical densities of the immunospecific bands corresponding to Hsp70 (Hsc70+Hsp72) in the PBMC whole cell extracts showed that the intracellular levels of Hsp70, including both constitutive and inducible isoforms, was not significantly different between the study groups (ANOVA, F= 1.81; p = 0.14) (**Figure 4.8**).

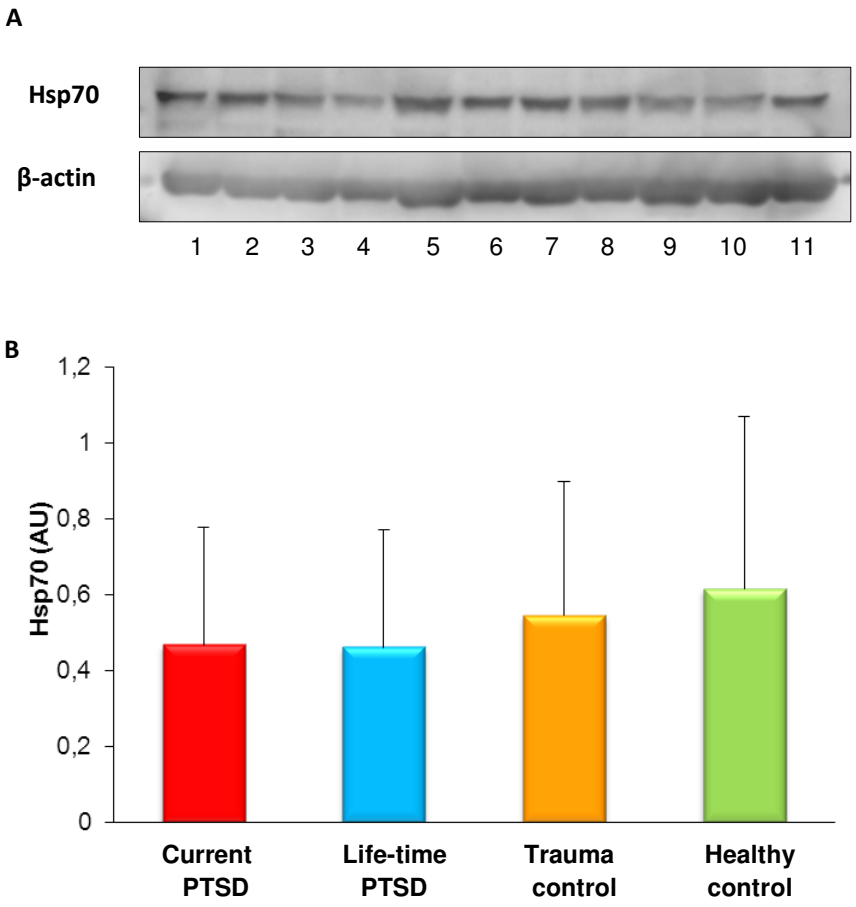

Figure 4.8. Relative level of Hsp70 in peripheral lymphocytes. (A) *The Hsp70 (Hsc70+Hsp72) was detected using rabbit polyclonal anti-Hsp70 antibody recognizing both constitutive and inducible isoforms of the protein. A representative blot is shown, containing two sets of four participants and three internal reference samples. Lanes: 1, 6, 11 – internal reference samples; 2, 7 – patients with current PTSD; 3, 8 – life–time PTSD patients; 4, 9 – trauma controls; 5, 10 – healthy controls.* ***(B)*** *Quantitative analysis was done by the ImageQuant software. The intensity of each analyzed immuno-specific band was normalized to the intensity of the nearest internal reference band on the same blot and to the intensity of β-actin band in the same lane. Relative integrated optical densities of Hsp70 bands are expressed in arbitrary units (AU) as means ± SD.*

When the expression of inducible Hsp70 (Hsp72) in the whole cell extracts of PMBCs was examined by immunoblotting, it was found that the intracellular level of Hsp72 was not significantly different between the study groups (ANOVA, F= 2.06, p= 0.11) (**Figure 4.9**).

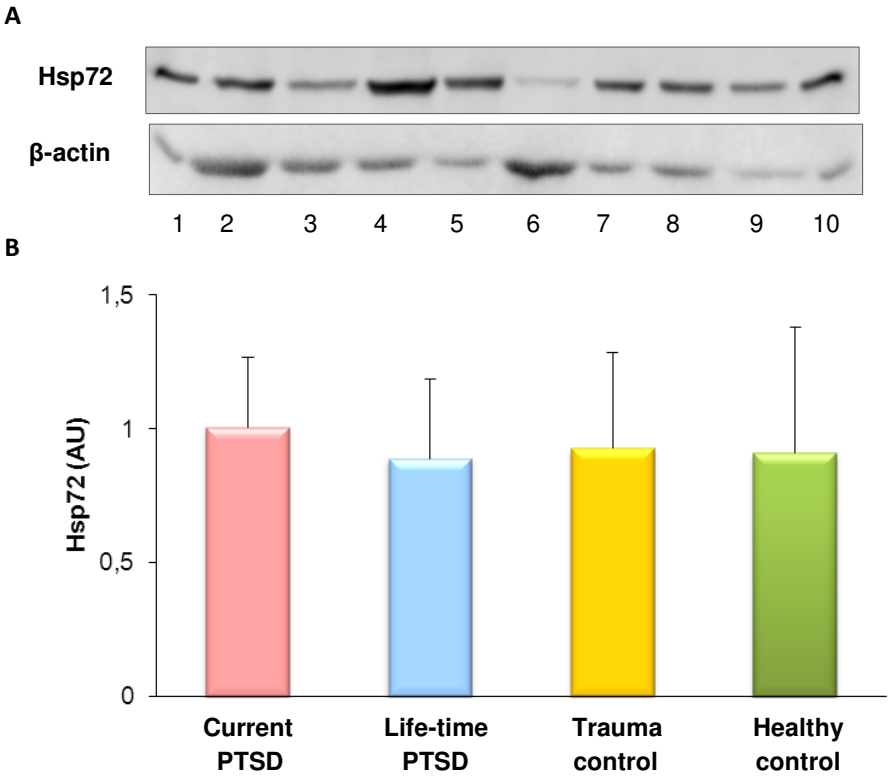

Figure 4.9. Relative level of Hsp72 in peripheral lymphocytes. (A) *Inducible isoform of Hsp70, Hsp72, was detected using rabbit polyclonal anti-Hsp72 antibody, recognizing only the inducible isoform of the protein. A representative blot is shown, containing two sets of four participants and two internal reference samples Lanes: 1, 10 – internal reference samples; 2, 6 – patients with current PTSD; 3, 7 – life–time PTSD patients; 4, 8 – trauma controls; 5, 9 – healthy controls.* ***(B)*** *Quantitative analysis was done by the ImageQuant software. The intensity of each analyzed immuno-specific band was normalized to the intensity of the nearest internal reference band on the same blot and to the intensity of β-actin band in the same lane. Relative integrated optical densities of Hsp72 bands are expressed in arbitrary units (AU) as means ± SD.*

Semi-quantitative immunoblot procedure was also applied to examine possible PTSD-and trauma-related alterations in the expression of Hsp60 in the whole cell extracts of peripheral blood mononuclear cells (PMBC). The

measurement of relative optical densities of the immunospecific bands corresponding to Hsp60 showed that the intracellular level of this HSP was not significantly different between the study groups (one-way ANOVA, F= 0.45, p= 0.72) (**Figure 4.10**).

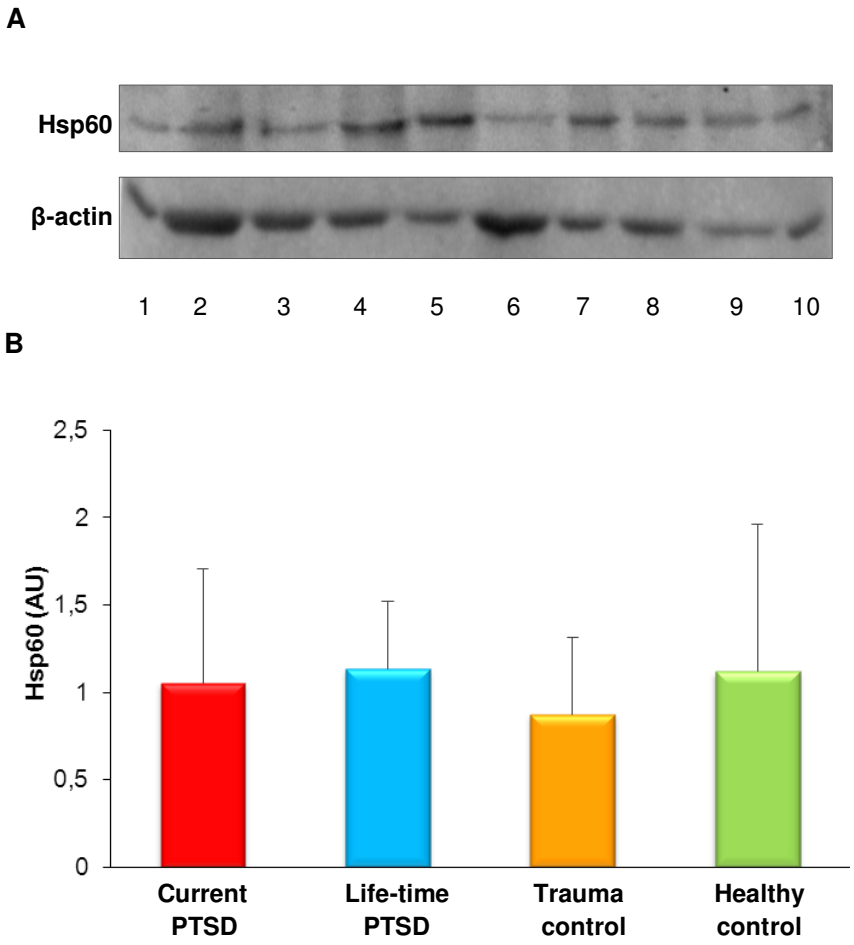

Figure 4.10. Relative level of Hsp60 in peripheral lymphocytes. (A) *Hsp60 was detected using rabbit polyclonal anti-Hsp60 antibody, followed by alkaline phosphatase-conjugated secondary antibody. Immunopositive bands were visualized by enhanced chemifluorescence. A representative blot is shown, containing two sets of four participants and two internal reference samples Lanes: 1, 10 – internal reference samples; 2, 6 – patients with current PTSD; 3, 7 – life–time PTSD patients; 4, 8 – trauma controls; 5, 9 – healthy controls.* ***(B)*** *Quantitative analysis was done by the ImageQuant software. The intensity of each analyzed immuno-specific band was normalized to the intensity of the nearest internal reference band on the same blot and also to the intensity of β-actin band in the same lane. Relative integrated optical densities of Hsp60 bands are expressed in arbitrary units (AU) as means ± SD.*

Correlation analyses of the data showed that Hsp90 concentration in PBMCs was significantly correlated with the concentrations of both MR ($r = 0.282$, $p < 0.0001$) and GR ($r = 0.362$, $p < 0.0001$), whereas Hsp70 concentration was correlated only with MR concentration ($r = 0.371$, $p < 0.0001$). Similar results were obtained when the analyses were performed on each of the four study groups separately (**Table 4.2**). Between-group comparison of the Pearson's correlation coefficients between Hsp90 and GR concentrations revealed a larger effect size in the trauma-exposed groups than in the healthy control group, as well as a smaller effect size of the correlation between Hsp70 and MR concentrations in the current PTSD group in comparison to trauma control group (**Table 4.2**). Additionally, it was found that the concentrations of the two examined HSPs were correlated ($r = 0.390$, $p < 0.0001$) without significant between-group differences in the effect size (**Table 4.2**), while the two corticosteroid receptor concentrations were not mutually related. Partial correlation analyses showed that co-morbid depression did not exert any influence on the relationship between the concentrations of corticosteroid receptors and HSPs.

Table 4.2. Statistically significant Pearson's correlation coefficients between the levels of HSPs and corticosteroid receptors

			Hsp70				MR				GR			
			Current PTSD	**Lifetime PTSD**	**Trauma controls**	**Healthy controls**	**Current PTSD**	**Lifetime PTSD**	**Trauma controls**	**Healthy controls**	**Current PTSD**	**Lifetime PTSD**	**Trauma controls**	**Healthy controls**
Hsp90	**Current PTSD**	r	0.397				0.209				0.463*			
		p	<0.0001				0.030				<0.0001			
	Lifetime PTSD	r		0.444				0.308				0.401*		
		p		<0.0001				0.016				0.019		
	Trauma controls	r			0.434				0.310				0.473*	
		p			<0.0001				0.003				<0.0001	
	Healthy controls	r				0.347				0.322				0.217
		p				0.001				0.002				0.048
Hsp70	**Current PTSD**	r					0.231#							
		p					0.014							
	Lifetime PTSD	r						0.277						
		p						0.029						
	Trauma controls	r							0.485					
		p							<0.0001					
	Healthy controls	r								0.405				
		p								<0.0001				

The number of participants per group: current PTSD, 113; life-time PTSD, 61; trauma controls, 88;healthy controls, 85.

** sifnificantly different from healthy controls (current PTSD: Z = 1.92, p = 0.027; life-time PTSD: Z = 1.91, p = 0.021; trauma controls: Z = 1.90, p = 0.029).*

 # significantly different from trauma controls (current PTSD: Z = 1.98, p = 0.024.

r, Pearson's correlation coefficient; p, the level of statistical significance.

4.4.2 Plasma levels of heat shock proteins

The level of extracellular HSPs (eHsp70, eHsp72 and eHsp60) were measured in blood plasma by commercial or home-made sandwich ELISA protocols, using the appropriate monoclonal and secondary antibodies. The level of extracellular Hsp70 (Hsc70+Hsp72) was assessed in 104 samples and ranged from 0.25 ng/ml to 80 ng/ml. The level of extracellular Hsp72 were determined in 118 plasma samples and ranged from 20 ng/ml to 90 ng/ml.

Figure 4.11 demonstrates the level of eHsp70 in the blood plasma from current PTSD patients, life-time PTSD patients, trauma controls and healthy controls. The statistical analyses revealed that there are no significant differences between groups (ANOVA, F= 0.95, p= 0.420).

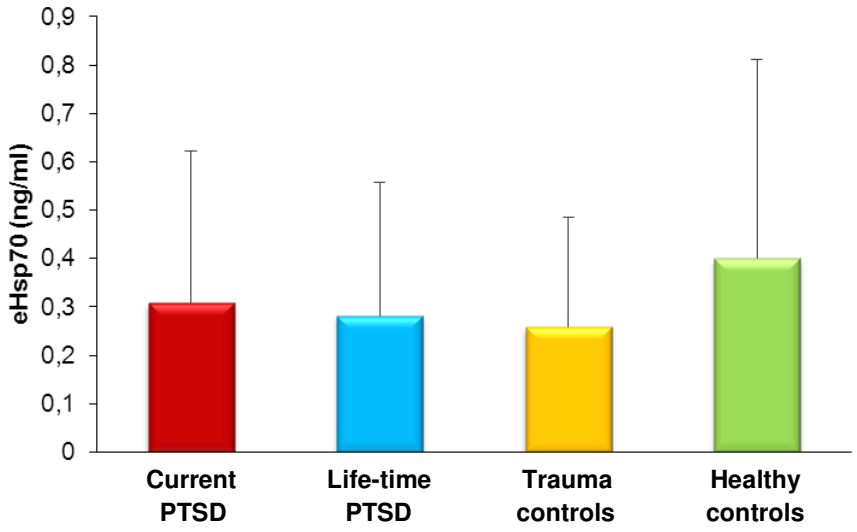

Figure 4.11. Plasma level of Hsp70. *The level of Hsp70 (Hsc70+Hsp72) in blood plasma was determined by an in-home sandwich ELISA. Plates were coated with the primary antibody, anti-Hsp70/Hsc70 (SPA-820), and the protein was detected by rabbit polyclonal anti-Hsp70 antibody (ADI-SPA-812). The results are expressed as means ± SD.*

The level of inducible isoform, eHsp72, in blood plasma from current PTSD patients, life-time PTSD patients, trauma controls and healthy controls are displayed in **Figure 4.12**. Statistical analysis revealed that there are no significant differences between groups (ANOVA, F= 1.95, p= 0.120).

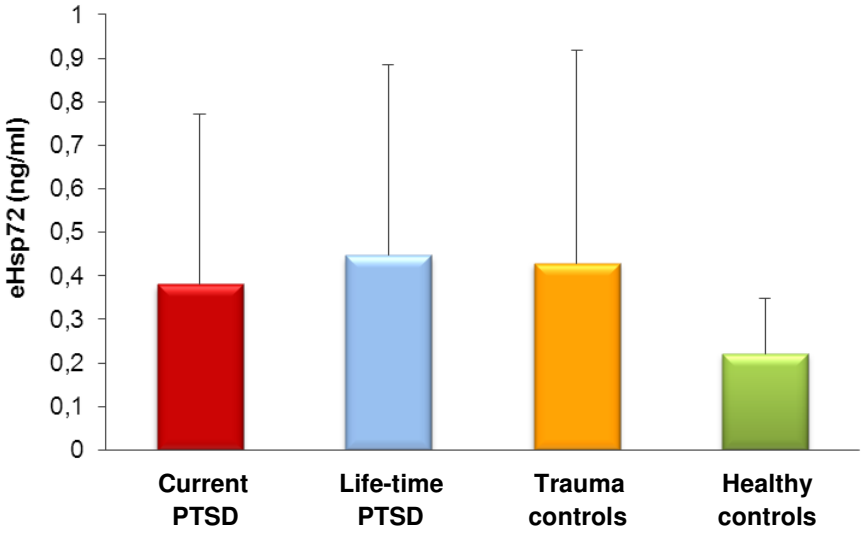

Figure 4.12. Plasma level of Hsp72. *The level of extracellular inducible Hsp70 (Hsp72) in blood plasma was determined by commercial sandwich ELISA according to the manufacturer's instructions (Enzo Life Sciences). The results are expressed as means ± SD.*

Interestingly, the correlation analyses have shown that there is a significant correlation ($r = 0.469$, $p = 0.032$) between the number of traumatic events and eHsp70 level only in the group of current PTSD patients.

A total of 250 plasma samples were assayed for extracellular Hsp60, with 89% (222 samples) being positive for the presence of this protein. Levels of Hsp60 ranged from 0.2 µg/ml to 50 µg/ml, with the median level of 20 µg/ml. This wide range of Hsp60 concentrations in the circulation is in accordance with previously reported concentrations of Hsp60 found in human blood. It has been found that 40-50% of the humans have no measurable eHsp60 in the blood plasma. The level of plasma Hsp60 in the remaining 50-60% men and women is measured in nanograms/ml or micrograms/ml, while in a small proportion of the population in milligrams/ml (Shamaei-Tousi et al., 2007).

Our results show that the mean level of eHsp60 in current PTSD patients was 20.31 ± 10.98 µg/ml, in life-time PTSD group 19.83 ± 10.76 µg/ml, in trauma controls 19.38 ± 11.22 µg/ml and in healthy controls 20.51 ± 11.84 µg/ml. No

significant differences in the level of extracellular Hsp60 were observed between the four study groups (ANOVA, F=0.11, p=0.95) (**Figure 4.13**).

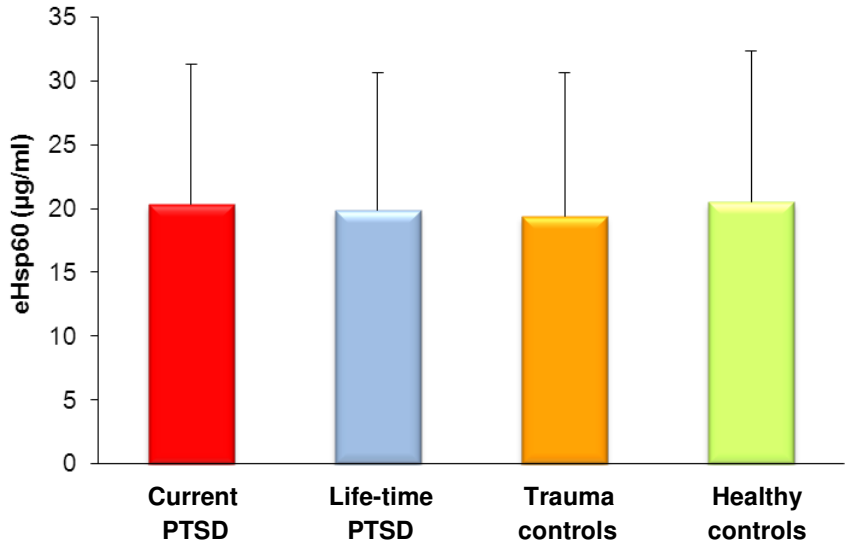

Figure 4.13. Plasma level of Hsp60. *The level of eHsp60 in blood plasma from the four groups of subjects was determined by sandwich ELISA developed in our laboratory. Mouse primary anti-Hsp60 antibody (ADI-SPA-806) was used as capture antibody and rabbit polyclonal anti-Hsp60 antibody (ADI-SPA-805) was used as a detection antibody. The results are expressed as means ± SD.*

4.5 Inflammatory markers

Current research has suggested immune function alterations in individuals with PTSD, but the nature of these alterations is not well understood. Most studies report that PTSD is associated with excessive inflammation, characterized by increased plasma levels of pro-inflammatory cytokines. However, unaltered inflammatory state and even decreased circulatory levels of inflammatory markers were also found in association with PTSD. There is also a proposal that excessive inflammation is, at least in part, due to insufficient immunosuppression by cortisol. In order to examine the inflammatory status of trauma-exposed individuals with current PTSD, with life-time PTSD and without PTSD, in this study we analyzed general inflammatory markers, such as plasma level of CRP, leukocyte count in peripheral circulation and erythrocytes sedimentation rate, as well as plasma

levels of multiple cytokines: TNF-α, IL-6, IL-12p70, IL-2, IL-8, IL-5, IL-4, IFN-γ and IL-10. One of the examined cytokines, IL-10, is anti-inflammatory cytokine, while the others are pro-inflammatory, or the cytokines with both pro- and anti-inflammatory actions.

Determination of general inflammatory markers demonstrated no significant differences between current PTSD, life-time PTSD, trauma control and healthy control groups, the statistical parameters being as follows: CRP – ANOVA, $F = 2.1$, $p = 0.105$; number of leukocytes – ANOVA, $F = 0.9$, $p = 0.420$; sedimentation – ANOVA, $F = 8.48$, $p = 0.978$ (**Table 4.3**). However, these results coincide with general suppression of proinflammatory cytokines observed in current PTSD, life-time PTSD and trauma controls, compared to healthy controls.

Table 4.3. The levels of inflammatory markers in the blood plasma.

	Current PTSD (n= 33)	Life-time PTSD (n= 33)	Trauma controls (n=33)	Healthy controls (n=33)		
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	F	P
CRP	1.69 ± 1.46	2.27 ± 2.20	3.57 ± 5.42	3.43 ± 3.94	2.09	0.105
Leukocytes	6.9 ± 1.8	7.2 ± 1.7	7.6 ± 2.2	7.1 ± 1.9	0.95	0.420
Sediment.	8.2 ± 4.7	7.9 ± 6.2	7.9 ± 7.2	8.5 ± 7.5	0.07	0.978
TNF-α	89.6 ± 78.0[a, b]	41.1 ± 44.8[a, c]	83.8 ± 64.7[a]	140.3 ± 67.6	12.47	<0.001
IL-6	115.3 ± 145.0[a]	95.9 ± 88.3[a]	113.1 ± 119.6[a]	221.7 ± 132.2	7.21	<0.001
IL-12p70	121.4 ± 98.8[a, b]	47.6 ± 70.7[a, c]	123.2 ± 100.7[a]	201.2 ± 115.5	12.82	<0.001
IL-2	344.2 ± 234.9[a, b]	202.5±211.9[a, c]	400.8 ± 226.7[a]	566.4 ± 131.5	16.04	<0.001
IL-8	84.8 ± 90.0[a, b]	20.1 ± 33.1[a]	63.1 ± 51.9[a]	128.8 ± 76.8	13.93	<0.001
IL-5	422.9 ± 393.7[a]	213 ±313.43[a, c]	493.2 ± 392.2	690.4 ± 353.5	9.44	<0.001
IL-4	302.6 ± 175.2[a]	188.1 ± 201.1[a]	334.1 ± 200.6	436.3 ± 196.6	8.90	<0.001
IFN-γ	89.4 ± 73.8	75.2 ± 66.5[a]	92.4 ± 57.6	126.5 ± 63.2	3.56	0.016
IL-10	96.6 ± 89.2[a]	100.2 ± 94.3[a]	126.2 ± 90.8	167.3 ± 95.0	4.06	0.009

[a] *significantly different from healthy controls, p< 0.05*

[b] *significantly different from life-time PTSD, p< 0.05*

[c] *significantly different from trauma controls, p< 0.05.*

TNF-α is a cytokine involved in systemic inflammation and its primary role is to partake in the regulation of immune cells. We found that the plasma levels of TNF-α significantly differ between the study groups (ANOVA, $F = 12.47$, $p < 0.0001$) (**Table 4.3**). As presented in **Figure 4.14**, significantly lower level of TNF-α was registered in the three groups of trauma-exposed subjects in comparison to healthy controls. Besides, the level of this cytokine was significantly lower in life-time PTSD group than in current PTSD and trauma control groups.

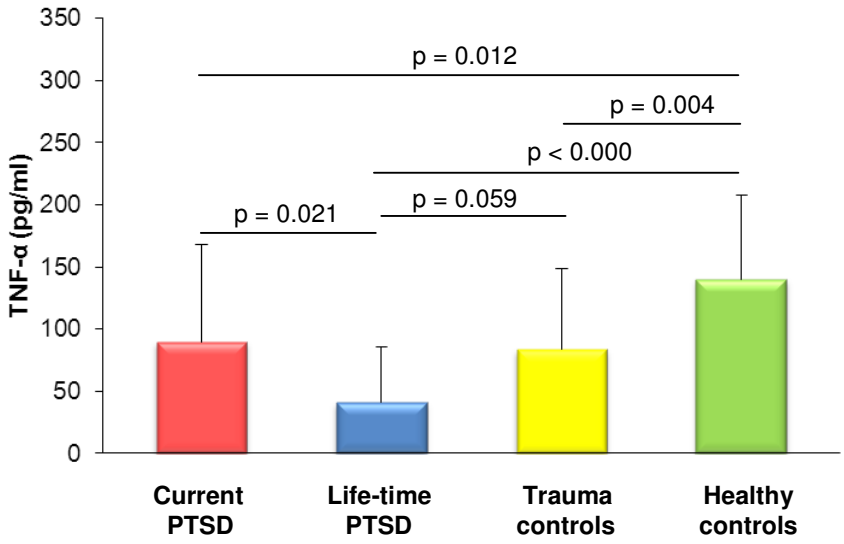

Figure 4.14. Plasma level of TNF-α. *The level of TNF-α was determined using multiplex ELISA kit according to the manufacturer's instructions. The results are expressed as the group means ± SD.*

One of the most important mediators of fever and acute phase response, IL-6 is also produced by T cells and macrophages in response to trauma and infection. Both pro- and anti-inflammatory actions can be attributed to this cytokine. In our study, it appeared that war trauma led to diminishment of IL-6 plasma level, as all three groups of traumatized subjects displayed significantly decreased IL-6 plasma levels than healthy controls (ANOVA, $F = 7.21$, $p < 0.0001$) (**Table 4.3**, **Figure 4.15**).

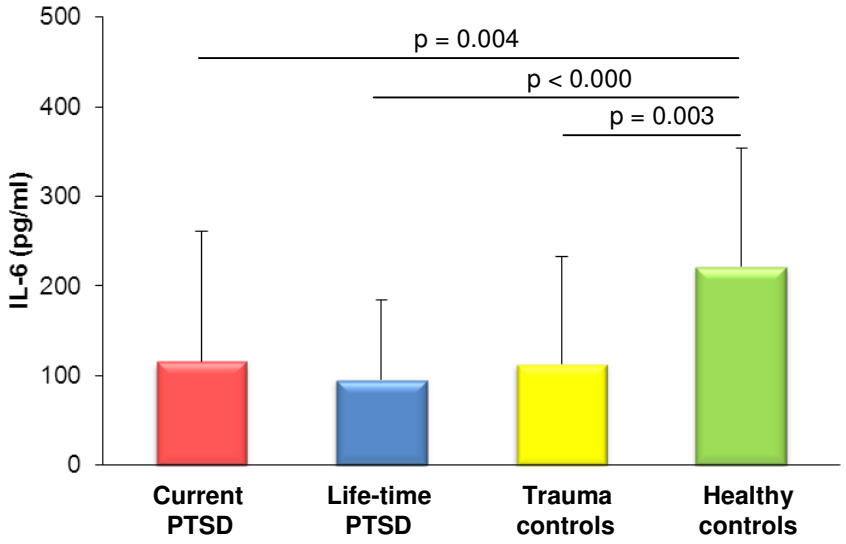

Figure 4.15. Plasma level of IL-6. *The level of IL-6 was determined using multiplex ELISA kit according to the manufacturer's instructions. The results are expressed as the group means ± SD.*

IL-12 or IL-12p70 serves to stimulate T cells growth and differentiation and is secreted from dendritic cells and macrophages in response to antigenic stimulation. Our results have shown that its blood plasma level was lower in war-traumatized individuals irrespective of the presence/absence of current or life-time PTSD symptoms in comparison to healthy controls, as well as in life-time PTSD subjects in comparison to both current PTSD patients and traumatized subjects without PTSD (ANOVA, $F = 12.82$, $p < 0.0001$) (**Table 4.3**, **Figure 4.16**). Therefore, the pattern of IL-12 plasma level between the study groups closely resembles that of TNFα.

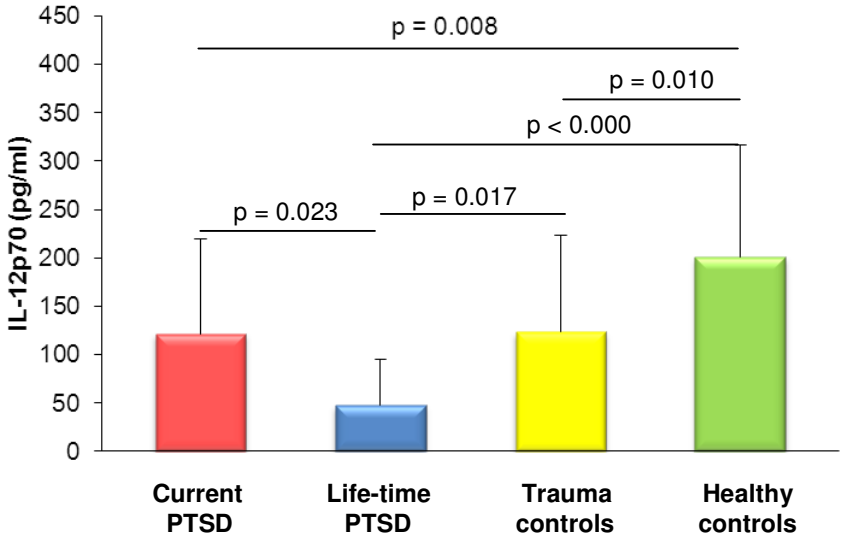

Figure 4.16. Plasma level of IL-12p70γ. *The level of IL-12p70 was determined using multiplex ELISA kit according to the manufacturer's instructions. The results are expressed as the group means ± SD.*

IL-2 regulates the immune functions of white blood cells in natural response to microbial infection and in discriminating between "self" and "non-self". It is also necessary for growth, proliferation and differentiation of T cells. The pattern of its plasma level between the groups in our study appeared to be very similar to those of TNF-α and IL-6. Its level was significantly lower in traumatized subjects with and without PTSD than in healthy controls, and among the three traumatized groups the life-time PTSD group displayed the lowest level of this cytokine (ANOVA, F = 16.04, p < 0.0001)(**Table 4.3**, **Figure 4.17**).

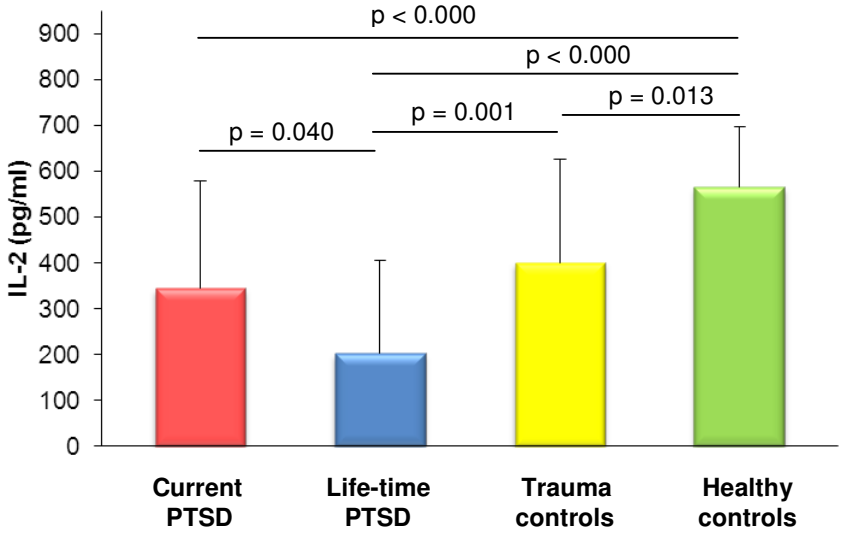

Figure 4.17. Plasma level of IL-2. *The level of IL-2 was determined using multiplex ELISA kit according to the manufacturer's instructions. The results are expressed as the group means ±SD.*

IL-8 is known as a neutrophil chemotactic factor and also as a potent promoter of angiogenesis. According to the result of our study, a decrees of the plasma level of this cytokine can be ascribed to traumatic experiences, since all traumatized groups (current PTSD, life-time PTSD and trauma controls) showed lower IL-8 level than healthy controls (ANOVA, $F = 13.93$, $p < 0.0001$). This cytokine is also less abundant in the blood plasma of life-time PTSD patients in comparison to both current PTSD patients and trauma controls, thus showing a pattern very similar to TNF-α, IL-6 and IL-2 (**Table 4.3**, **Figure 4.18**).

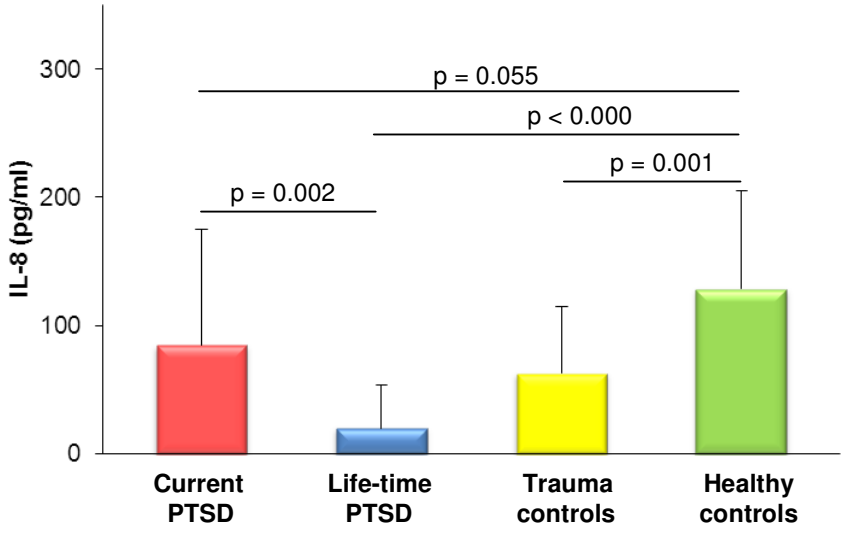

Figure 4.18. Plasma level of IL-8. *The level of IL-8 was determined using multiplex ELISA kit according to the manufacturer's instructions. The results are expressed as the group means ± SD.*

Produced by T helper cells and mast cells, IL-5 stimulates B cell growth, immunoglobulin secretion and eosinophil activation. In our study, its plasma level has been shown to depend on the presence of past or present PTSD symptoms, as both PTSD groups (current and life-time) express significantly lower plasma levels of this cytokine in comparison to healthy controls (ANOVA, $F = 9.44$, $p < 0.0001$). Of note, IL-5 level has also been significantly lesser in life-time PTSD patients than in traumatized non-PTSD subjects (**Table 4.3**, **Figure 4.19**).

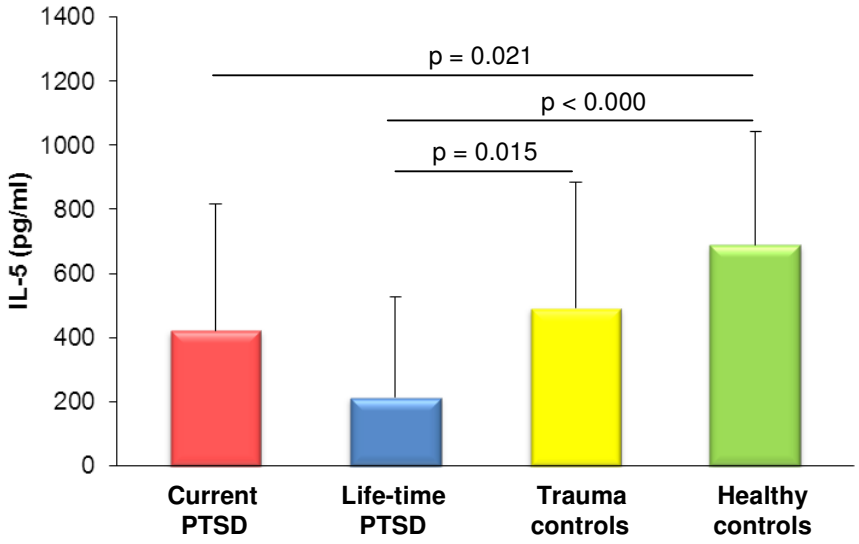

Figure 4.19. Plasma level of IL-5. *The level of IL-5 was determined using multiplex ELISA kit according to the manufacturer's instructions. The results are expressed as the group means ± SD.*

Among many roles of IL-4, the most prominent are stimulation of T helper cells and B cells differentiation, as well as T-cell and B-cell proliferation. Its pattern of abundance in the blood plasma between our study groups closely resembles that of IL-5: IL-4 plasma level reflects vulnerability to PTSD, as it is lower in both groups vulnerable to PTSD in comparison to the controls, and it is also lower in lifetime PTSD subjects than in trauma controls (ANOVA, $F = 8.90$, $p < 0.0001$)(**Table 4.3**, **Figure 4.20**).

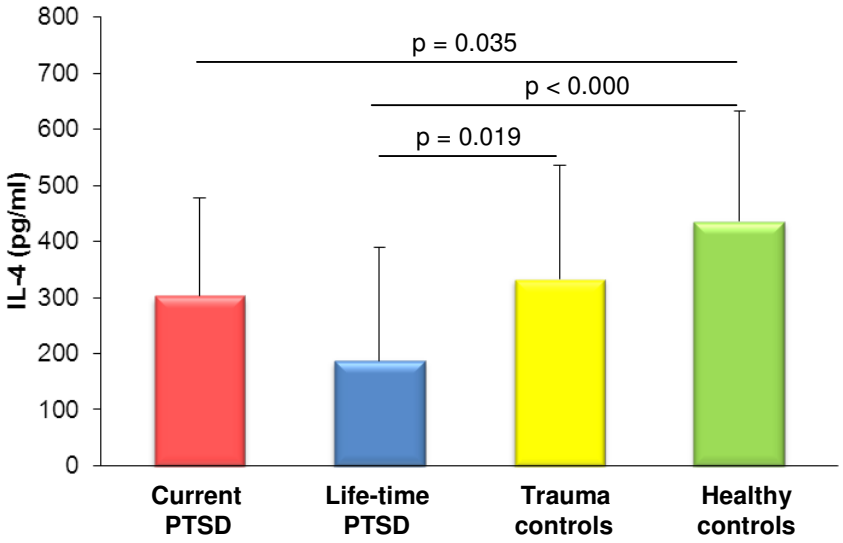

Figure 4.20. Plasma level of IL-4. *The level of IL-4 was determined using multiplex ELISA kit according to the manufacturer's instructions. The results are expressed as the group means ± SD.*

Immunostimulatory and immunomodulatory roles of IFN-γ are of an utmost importance for both innate and adaptive immunity. Its antiviral, immunoregulatory and anti-tumor actions are numerous. In our study, it has exhibited a unique pattern of plasma level between the study groups (**Table 4.3**, **Figure 4.21**): the only statistically significant between-group difference was a decrease of IFN-γ level in life-time PTSD subjects in comparison to healthy controls (ANOVA, F = 3.56, p = 0.016). This result implies that the level of IFN-γ might be associated with remission of PTSD.

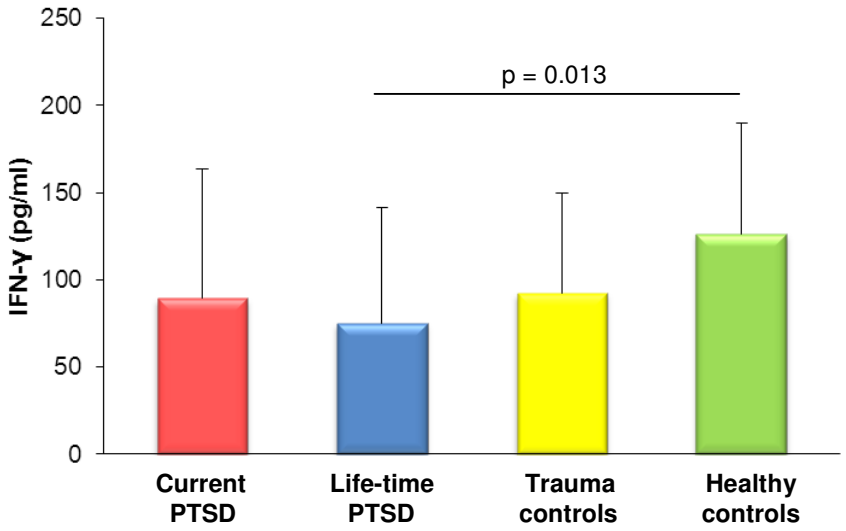

Figure 4.21. Plasma level of IFN-γ. *The level of IFN-γ was determined using multiplex ELISA kit according to the manufacturer's instructions. The results are expressed as the group means ±SD.*

Among the cytokines analyzed in this study, IL-10 was the only anti-inflammatory cytokine, counteracting the hyperactive immune response. This cytokine acts as human cytokine synthesis inhibitory factor and besides, exerts pleiotropic effects in immunoregulation and inflammation. Between-group differences in its blood plasma level, observed in our study, suggest that the level of this cytokine might be associated with vulnerability to PTSD. Namely, the individuals presenting both current or past PTSD symptoms had lower IL-10 plasma levels than healthy controls (ANOVA, $F = 4.06$, $p = 0.009$)(**Table 4.3**, **Figure 4.22**)

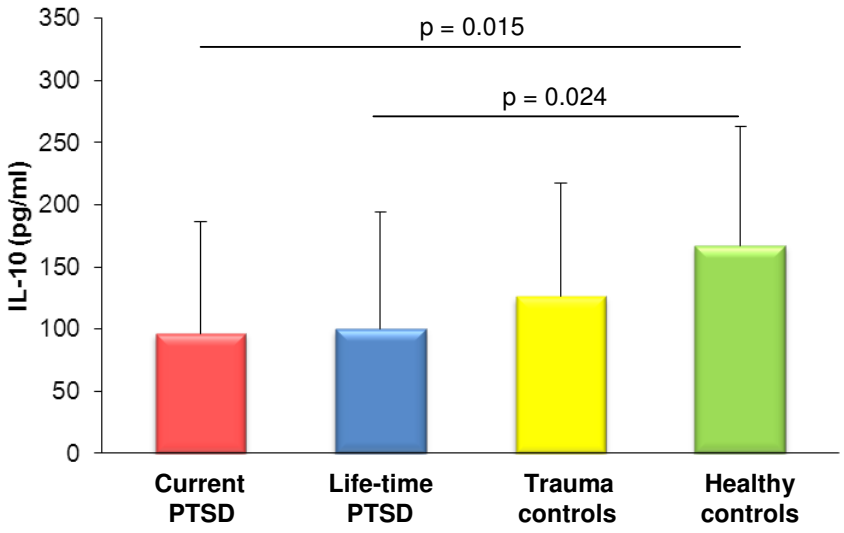

Figure 4.22. Plasma level of IL-10. *The level of IL-10 was determined using multiplex ELISA kit according to the manufacturer's instructions. The results are expressed as the group means ± SD.*

5. Discussion

5.1 Plasma cortisol level and HPA axis sensitivity

Proper negative feedback regulation of HPA axis exerted by cortisol appears to be critical for a healthy stress response. Profound alterations in the regulation of this system have been implicated in the pathogenesis of stress-related psychiatric disorders, such as PTSD (Yehuda, 2006). Numerous studies on cortisol signaling abnormalities in PTSD, however, yielded heterogeneous results, so that the exact role of cortisol and HPA axis in the pathogenesis and maintenance of this disorder is still a matter of debate. Some authors reported lower basal cortisol levels in the blood plasma, urine or saliva from PTSD patients as compared to control subjects (Yehuda *et al.*, 1990; Yehuda *et al.*, 1995b; Boscarino, 1996; Yehuda *et al.*, 1996; Heim *et al.*, 1998; Glover & Poland, 2002; Yehuda, 2002a; Wessa *et al.*, 2006), some reported the opposite data (Pitman & Orr, 1990; Lemieux & Coe, 1995; Liberzon *et al.*, 1999; Lindley *et al.*, 2004), while some found no differences (Baker *et al.*, 1999; Atmaca *et al.*, 2002; Eckart *et al.*, 2009). These discrepancies *"indicate that there is not a one-to-one correspondence between cortisol output and PTSD diagnosis"* as Rasmusson *et al.* (2003) conclude in their review on neuroendocrinology of PTSD. In an excellent meta-analysis, Meewisse *et al.* (2007) came to the same conclusion. Both these groups of researchers found that only a part of the variability of published results can be accounted for by methodological differences. Additional sources of variability may include various factors, such as comorbid depression, current *versus* life-time PTSD, severity of PTSD symptoms, time elapsed from the traumatic event, duration of trauma exposure, and whether or not control groups have a history of trauma (Meewisse *et al.*, 2007).

In our present study we measured plasma cortisol level in a relatively large cohort of only-male subjects divided into four groups in a way that allowed us to relate the cortisol level to trauma exposure, PTSD symptoms, resilience to PTSD, vulnerability to PTSD and remission of the disorder. We found no between-group

differences in the basal cortisol level, represented as the mean of thirteen nocturnal measurements encompassing the period from 22:00 p.m. to 9 a.m. and performed in the clinical setting. Considering the sample size, accuracy of subject classification, the applied methodology and precise matching of the study groups by a number of parameters (gender, age, type of trauma, educational level, body mass index, lifestyle habits, etc.), it can be concluded that basal cortisol level alterations cannot be associated with trauma exposure, current or life-time PTSD symptoms, resilience to PTSD, vulnerability to PTSD and remission of the disorder. A recent study by Savic et al. (2012) shows that the apparent connections between cortisol and PTSD are actually realized *via* personality traits, which might explain the heterogeneity of findings on basal cortisol levels in traumatized individuals with or without PTSD. This study demonstrated that the mediating role between cortisol level and PTSD can be ascribed to personality traits, since basal cortisol level was shown to be related to PTSD only indirectly, inasmuch as low Conscientiousness (one of the „Big Five" personality traits) is related to this psychopathology.

Patients with PTSD have been found to display blunted cortisol awakening response *i.e.* lesser cortisol "spike" upon awakening and higher circulating concentrations of cortisol in the evening (Wessa *et al.*, 2006). Interestingly, flattening of the daily cortisol rhythm has been associated with a number of chronic medical illnesses including cardiovascular disease and insulin resistance, as well as other psychiatric disorders including major depression and chronic fatigue syndrome (Nater *et al.*, 2008).

The cortisol level is thought to be a good measure of HPA axis activity, since cortisol is the final product of this axis. However, not only the activity, but also the regulation of HPA axis activity, should be taken into consideration as an important biological correlate of PTSD and any other psychiatric disorder related to stress. Hence, in a number of studies the challenge paradigms, such as DST and combined DEX/CRH test, were employed in order to evaluate the sensitivity of HPA axis to suppression by cortisol. Again, different studies provided rather inconsistent data. Some authors registered hyposuppression of HPA axis in PTSD patients compared

to healthy controls (Thaller et al., 1999; Atmaca et al., 2002), some reported no inter-group differences (Vythilingam et al., 2010), but most found that PTSD is characterized by hyperresponsiveness of HPA axis to cortisol feedback (Yehuda *et al.*, 1995a; Rinne *et al.*, 2002; Grossman *et al.*, 2003; Newport *et al.*, 2004; Yehuda *et al.*, 2004c; Lange *et al.*, 2005). A review of DST studies in PTSD is given in de Kloet et al. (2006). The key finding of these studies was that PTSD patients in comparison to control subjects display lower plasma cortisol level after administration of 0.5 mg dexamethasone dose, which was interpreted as a hypersensitive (or disease-sensitized) cortisol feedback (Yehuda *et al.*, 2004b) and afterwards considered as a hallmark of PTSD pathophysiology. The results of our study demonstrate that dexamethasone applied at a low dose of 0.5 mg leads to increased suppression of HPA axis activity in PTSD patients with current or past symptoms in comparison to healthy controls, which is reflected in the lower ratio between post-dexamethasone and pre-dexamethasone plasma cortisol levels in PTSD than in healthy subjects. These results confirm previous observations that PTSD is associated with hyperresponsiveness of HPA axis. Moreover, combined with the data that basal cortisol level is not changed in association with the disorder, our data on PTSD-sensitized HPA axis imply that there is a disagreement between indicators of HPA axis activity in central *versus* peripheral components of this axis. Namely, when DST indicates hyperresponsiveness of pituitary and hypothalamus to dexamethasone, one should expect decreased activity of HPA axis and, hence, a decreased level of plasma cortisol. Since it was not the case in our study, we suppose that adrenal glands are either downregulated by a so called "ultra short" glucocorticoid negative feedback, or insensitive to the increased activity of stress response systems in the brain. Therefore, our data open new questions regarding the regulation of cortisol secretion in adrenal glands in traumatized individuals with and without PTSD. These questions might be answered by detailed inspection of GR and ACTH receptors expression and activity in the cortex of adrenal glands.

In most previous studies on the relation between PTSD and HPA axis hypersuppression, the fact that subjects with psychiatric diagnosis had more

traumas than trauma controls was not taken into account (e.g. Yehuda *et al.*, 1995a; Newport *et al.*, 2004; Yehuda *et al.*, 2004a). Interestingly, the level of traumatization appeared to be an important confounding factor. A recent study of Savic et al. (2012) has shown that significant differences on the low dose DST between PTSD patients and trauma controls are not actually related to PTSD pathology, but to the level of war-related trauma exposure. Besides, Savic et al. (2012) suggested that even though war-related traumatic events are not of the same degree of distress, the factor influencing HPA axis sensitivity to cortisol feedback is the number of traumatic experiences rather than their intensity. This led to a conclusion that the repetition and not the degree of activation of the HPA axis plays a role in sensitizing its regulatory function.

5.2 Corticosteroid receptors expression in the lymphocytes

The observation that PTSD is associated with increased HPA axis sensitivity to cortisol has brought the GR into the focus of the research, since cortisol exerts its cellular effects through the GR. GR is a hormone-inducible transcription regulator belonging to the superfamily of nuclear receptors. It is a ubiquitous cytoplasmic protein, present in almost all human tissues, where it mediates various cellular responses to cortisol. It specifically binds cortisol and upon hormone binding translocates from the cytoplasm to the nucleus where it regulates transcription of glucocorticoid target genes. GR can activate gene transcription by direct binding to specific regulatory DNA sequences called glucocorticoid-responsive elements (GREs). A consensus GRE is the pentadecameric imperfect palindrome (Nordeen *et al.*, 1990) and the receptor binds to it in the form of dimer (La Baer & Yamamoto, 1994). The response of many genes to glucocorticoids depends not only on GR binding to the GRE, but additionally requires the binding of other transcription factors to adjacent binding sites (Schoneveld et al., 2004a), which is a way of integrating multiple signal inputs into one response. GREs that negatively influence transcription by direct binding of

GR are referred to as negative GREs (nGREs) (Truss & Beato, 1993). In the case of certain genes, including the genes encoding cytokines, GR does not bind directly to the DNA to exert its transcriptional effect, but is recruited to DNA-bound transcription factors, such as NFκB and AP-1 in a regulatory complex, a mechanism known as tethering (Schoneveld et al., 2004b).

The investigations of expression and functional properties of GR in PBMCs from PTSD patients have yielded inconsistent results. The number of glucocorticoid binding sites (B_{max}) in PBMCs from PTSD patients has been reported to be increased (Yehuda et al., 1991; Yehuda et al., 1995a), decreased (de Kloet et al., 2007) and unchanged (Wheler et al., 2006; Shalev et al., 2008) in comparison with control individuals. The level of the GR protein in different lymphocyte subpopulations, measured by flow cytometry, was found to be lower in PTSD patients than in the controls in one study (Gotovac *et al.*, 2003) and unchanged in the other (Vidovic *et al.*, 2007). In addition, the evidence has been provided for both decreased (Su et al., 2009) and unchanged (van Zuiden *et al.*, 2011a) PTSD-related level of GR gene expression evaluated by quantitative PCR.

In the present study we had at least two reasons to examine PTSD- and war trauma-related alterations in the GR level of expression in PBMCs. The first includes the inconsistency of the existing literature data, and the second derives from our result that basal cortisol level in the four study groups was rather uniform, while the responsiveness of HPA axis to cortisol was increased in the current PTSD group. One of the possible explanations for these results might be found in the level of expression of the GR, as an increased level of GR protein in PTSD patients compared to non-PTSD subjects might be responsible for increased responsiveness of HPA axis to cortisol, despite unchanged plasma level of the hormone. Therefore, we sought to determine relative GR protein level and for that purpose we applied a semi-quantitative Western blot approach and used PBMCs, among which the lymphocytes predominate. In order to learn whether GR protein level may be a biological correlate of PTSD pathology and war trauma exposure, or a factor of vulnerability or resilience to PTSD, these experiments included war

trauma survivors with current PTSD, with life-time PTSD and without PTSD, as well as non-traumatized healthy controls.

Examining the GR protein level in the lymphocytes, we detected two immuno-specific bands, one of which corresponded to molecular mass of the GRα isoform (97 kDa), while the other had higher molecular mass. It is known that GR is subject to mono- and polysumoylation (Le Drean *et al.*, 2002), which regulates the stability of the receptor protein and influences its transcriptional activity (Holmstrom *et al.*, 2003). GR has three attachment sites for SUMO-1, which does not form polymeric chains (Tian *et al.*, 2002). It seems that GR is also susceptible to monoubiquitination which also affects its transactivation activity (Ismaili *et al.*, 2005). Assuming that the slower migrating band detected in our experiments represents the GR monosumoylated by SUMO-1 or monoubiquitinated, and taking into account that the appearance and the abundance of both bands exhibited a rather uniform pattern among the study groups, it could be supposed that the rate of GR monosumoylation or monoubiquitination is not related to trauma exposure, PTSD symptoms or resilience to PTSD. Besides, comparison of the intensities of the two GR bands within the whole sample, as well as within the study groups separately, suggested a redistribution of the receptor between the two isoforms, which is an expectable consequence of the presumed modification(s).

An important result of these experiments was that current PTSD patients in comparison to trauma controls, displayed elevated GR protein level in PBMCs, suggesting that increased GR expression should be considered as a factor underpinning the observed increased sensitivity of HPA axis to cortisol. However, all human studies on HPA axis activity, including the ours, suffer from a limitation that, because of inaccessibility of human brain tissues and pituitary, the conclusions have to be drawn out of the experiments done on available human cells, most frequently PBMCs. These, easily accessible immune cells, express high levels of GR and represent targets for GR anti-inflammatory and immunosuppressive actions. As such, they can be exploited as a very useful model for basic studies concerning immunological aspects of PTSD pathophysiology, as well as for clinical studies on GR gene expression, polymorphism and GR functional

alterations as potential preexisting vulnerability/resilience factors, or correlates of PTSD or trauma. Besides, it has been shown that gene expression changes in PBMCs reflect various pathological states (Tang et al., 2001), including PTSD and other psychiatric disorders (Segman *et al.*, 2005; Tsuang *et al.*, 2005; Zieker *et al.*, 2007; Su *et al.*, 2009; Yehuda *et al.*, 2009), and in some instances are paralleled by changes in neural tissue (Sullivan et al., 2006; van Heerden et al., 2009). These are arguments in favor of the use of these cells even in basic research on neuroendocrine and cognitive aspects of PTSD pathophysiology (Gladkevich et al., 2004).

In the brain structures controlling HPA axis activity, cortisol operates via two types of receptors: mineralocorticoid receptor (MR) and GR. MR is predominantly expressed in the limbic structures, binds cortisol with high affinity and is substantially occupied throughout the ultradian and circadian cycle. The lower affinity GR is widely distributed and becomes occupied only at cortisol peaks and after stress. While the MR is primarily responsible for integrity and stability of the stress circuitry, the GR is implicated in termination of the stress response, recovery from stressful challenge and facilitation of behavioural adaptation in preparation for future challenges. Therefore, the balance between MR- and GR-mediated actions is considered crucial for proper processing of stressful information, while the imbalance may contribute to increased vulnerability to stress-related mental disorders (De Kloet *et al.*, 1998; Oitzl *et al.*, 2010; Harris *et al.*, 2012; ter Horst *et al.*, 2012). So far the studies on corticosteroid receptor alterations accompanying PTSD and trauma were focused mainly on GR (Yehuda, 2009) and, to our knowledge, there are only two papers examining MR function in PTSD patients (Kellner et al., 2002; Otte et al., 2006). Both papers reported no alterations in MR-mediated HPA axis regulation related to PTSD. However, Wellhoener et al. (2004) evidenced a rise of both basal and stress-induced cortisol levels in healthy humans upon MR blockade, and some animal studies demonstrated an increase in hippocampal MR density after psychological stress (Gesing et al., 2001; Muller et al., 2003; Ladd et al., 2004). These studies provided the basis for a proposal that, in addition to GR, MR could also contribute to HPA axis alterations in PTSD.

It is well known that cortisol exerts immunosuppressive and anti-inflammatory effects and there is a growing body of evidence that PTSD pathogenesis is connected with alterations in inflammatory functions of the immune system. Therefore, cortisol and GR became a focus of PTSD research not only because of the implication in HPA axis regulation, but also because of immunomodulatory actions. Measurements of the production of cytokines in the presence of GR or MR antagonists have led to the conclusion that cortisol exerts its immunomodulatory effects not only *via* GR, but *via* both corticosteroid receptors, GR and MR, which act synergistically to mediate suppression of proinflammatory responses (Sauer *et al.*, 1996; Dimitrov *et al.*, 2004). Since both GR and MR operate at neuroendocrine-immune interface, in order to better understand the role of these receptors in PTSD pathophysiology and to unravel their possible contribution to inter-individual differences in coping with stress, it is necessary to examine the level of expression of both the receptors, functional status and molecular interactions in the central nervous system, as well as in the peripheral tissues.

Since PBMCs express both GR and MR it might be expected that disbalance in the expression levels of the two receptors may affect the immunosuppressive actions of glucocorticoids in these cells. However, the data from the present study show that the level of MR protein was similar in all four groups of participants and, combined with the GR data, indicate that the lymphocyte MR/GR ratio was not altered regardless of traumatic experiences and presence of past or current PTSD symptoms. Knowing that the level of corticosteroid receptors expression is only one among numerous determinants of the sensitivity of a target cell to cortisol, this observation suggests that differences in cortisol signaling between traumatized individuals with or without PTSD and non-traumatized healthy controls should be searched for among other regulatory mechanisms, such as those regulating the receptors cytoplasmic/nuclear shuttling, covalent modifications and transactivation activity. A key role in regulating these important steps of cortisol action belongs to chaperones (Hsp90 and Hsp70) and co-chaperones bound to the corticosteroid receptors heterocomplexes.

5.3 Heat shock proteins

5.3.1 Intracellular heat shock proteins

GR and MR act as ligand-activated transcription regulators that, when unliganded, predominantly reside in the cytoplasm of target cells within Hsp90-based chaperone complexes, the components of which are HSPs Hsp90 and Hsp70, co-chaperones and immunophilins (Pratt & Toft, 1997a). Hsp70 recognizes newly synthesized receptors, and is required for their initial folding, while Hsp90 regulates maturation of the receptors' high-affinity hormone-binding conformation and maintains unliganded receptors in transcriptionally inactive state by keeping them in a form incapable of DNA binding (Galigniana *et al.*, 2004; Grad & Picard, 2007). Hsp90 also controls intracellular trafficking, nuclear retention, transcriptional activity (Galigniana *et al.*, 2010), as well as proteolytic degradation of the receptors (Siriani et al., 2005). Therefore, the interaction of corticosteroid receptors with Hsp90 and Hsp70 represents an important determinant of target tissue sensitivity to corticosteroid hormones (Pratt & Toft, 1997a; Galigniana *et al.*, 2004; Grad & Picard, 2007; Galigniana *et al.*, 2012).

HSPs are essential components of the cellular stress response. Their synthesis can be induced in virtually all cells by diverse physiological and environmental stressful factors (Richter et al., 2010). Even the pure psychological stressors have been shown to increase both intracellular and circulating levels of HSPs (Lewthwaite *et al.*, 2002; Fleshner *et al.*, 2004). These proteins are thought to serve functions beneficial for cell survival in various stress-related disorders, possibly including PTSD (Zhang et al., 2010) and considering their role in chaperoning corticosteroid receptors, it can be expected that changes in their intracellular concentrations may affect cellular responsiveness to cortisol. This was the reason why we examined the level of expression of Hsp90 and Hsp70 in PBMCs from war trauma-exposed individuals with current PTSD, life-time PTSD and without PTSD, as well as from non-traumatized healthy controls. Furtheron, we analyzed the relationship between the levels of the corticosteroid receptors

and the HSPs expression, as well as the relationship between the level of expression of the studied proteins to trauma exposure, PTSD symptoms, vulnerability or resilience to PTSD and remission of the disorder.

Current study provides evidence that the level of Hsp90 and Hsp70 expression in PBMCs do not differ between men with current PTSD, life-time PTSD and without PTSD as compared to non-traumatized healthy individuals. Besides, the data reveal that the two corticosteroid receptors (MR and GR) expression levels are differently correlated with the levels of Hsp90 and Hsp70. Some of these associations have been shown to be weakened when accompanied by PTSD symptoms, while some were influenced by war trauma exposure.

Induction of HSPs is known to be a part of the generalized stress response that serves to protect cells against cytotoxic stimuli (Richter et al., 2010), including cytokine toxicity (Bocci, 1988; Barone *et al.*, 1997). It is also known that trauma exposure and PTSD are accompanied by increased production of proinflammatory cytokines (Kawamura *et al.*, 2001; Rohleder *et al.*, 2004; Gill *et al.*, 2008; Gill *et al.*, 2010). Our data, however, show that lymphocyte Hsp90 and Hsp70 expression levels in war trauma-exposed men with and without PTSD are similar to the levels in non-traumatized healthy controls. We suppose that one of possible explanations of this finding might be the long time elapsed between trauma exposure and recruitment for the study. Namely, HSPs induction might serve to restrain the proinflammatory response and, thus, protect cells from cytokine toxicity only in the early aftermath of trauma. Recently, Sriram et al. (2012) have formulated a mathematical model of Hsp90 dynamics in animals exposed to acute and chronic stress, and hypothesized that the model can be extended to psychiatric disorders such as PTSD. The model predicts low levels of Hsp90 upon high levels of stress, and our data are probably the first experimental data corroborating this prediction.

Interestingly, our data suggest that the concentrations of corticosteroid receptors are correlated with that of HSPs, and that the two receptors differ in regard to their relationship with Hsp70. On the basis of the data showing that heat shock factor 1 (HSF1) has a role in potentiation of GR activity by stress (Jones et al.,

2004) and inversely, that glucocorticoids can attenuate the heat shock response by preventing HSF1 recruitment to the promoters of heat shock genes (Wadekar et al., 2004), Sanchez and coworkers proposed a reciprocal mechanism regulating GR and HSF1 signaling (Jones et al., 2004). It is tempting to speculate that the proposed complex functional relationship between GR and HSF1 may underlie the observed correlation between GR and Hsp90 expression levels.

The Hsp90-based chaperone heterocomplexes of corticosteroid receptors include one of the two Hsp90-binding immunophilin co-chaperones, FKBP51 or FKBP52, the functional exchange of which has been demonstrated for both GR (Davies et al., 2002) and MR (Gallo et al., 2007). FKBP52 is regarded as a positive regulator of GR, but does not affect the functions of MR, while FKBP51 impairs nuclear localization of both the receptors (Wochnik *et al.*, 2005; Gallo *et al.*, 2007). Besides, FKBP51 expression is induced by glucocorticoids as a part of an intracellular ultra-short feedback loop regulating GR activity (Vermeer et al., 2003). Functional and genetic variations of FKBP51 are thought to be a major risk factor for development of PTSD (Binder et al., 2008; Xie et al., 2010). The fact that FKBP51 and FKBP52 are Hsp90 co-chaperones associated with both corticosteroid receptors, may explain our finding that both GR and MR expression levels correlate with Hsp90 level.

On the other hand, the differences between GR and MR in respect to their correlation with Hsp70 may stem from a dissimilar interaction of the two receptors with Bag-1, a co-chaperone of Hsp70, which has been reported to inhibit DNA-binding and transactivation functions of GR in an isoform-specific manner, while leaving the activity of MR unaffected (Schneikert *et al.*, 1999; Crocoll *et al.*, 2000). Further investigation is necessary in order to learn whether different functional relationships of GR and MR with this Hsp70 co-chaperone may result in different relations between the two receptors expression levels with the level of Hsp70.

The results of the present study demonstrate that the association of GR and Hsp90 expression levels was affected by exposure to war trauma, since the correlation coefficients between GR and Hsp90 concentrations in the lymphocytes

from the three trauma-exposed groups of participants were significantly higher than in the group of non-traumatized healthy controls. The correlation between MR and Hsp70 concentrations, on the other hand, was found to depend on the presence of PTSD symptoms, since the correlation coefficient in the current PTSD group was significantly lower in comparison to that in the trauma control group. These results imply that war trauma and symptoms of PTSD may affect the relations between GR and MR expression levels, on one hand, and Hsp90 and Hsp70 levels, on the other, without affecting the expression levels of the studied proteins, the GR being the only exception (Matić et al., 2013). The changes in these relations may reflect the changes in the interactions of the corticosteroid receptors with the HSP chaperones. Knowing that corticosteroid receptor heterocomplexes are dynamic structures, the composition of which changes under different conditions (Edwards *et al.*, 1992; Čvoro *et al.*, 1998; Brkljačić *et al.*, 2004) and that chaperones are important factors controlling the activity of corticosteroid receptors (Pratt & Toft, 1997a; Galigniana *et al.*, 2004; Grad & Picard, 2007; Galigniana *et al.*, 2010), one can propose that changes of these interactions would, consequently, lead to alterations of the receptors functional activity.

The notable strengths of the current study include relatively large sample size, the uniformity of the study groups in respect to gender, age, ethnicity, types of experienced traumatic events and educational level, as well as the detailed assessment of PTSD and precise classification of the participants into the four groups by experienced specialists. These advantages, encourage us to propose that the results presented herein provide reliable arguments that further research regarding the role of corticosteroid receptors in pathogenesis of PTSD should be focused at the interaction of these receptors with their chaperones and co-chaperones.

5.3.2 Extracellular heat shock proteins

PTSD is not a normative response to extreme stressors, since only a subset of people exposed to severe trauma go onto develop the disorder. That was the

reason why PTSD has recently been reconceptualized as a disorder of stress response systems – HPA and SAM axes, leading to maladaptive responses and a failure to cope with the stressor (Yehuda, 2009). Based on such a concept and given that neuroendocrine systems are known to regulate immune functions, it is reasonable to predict that patients with PTSD may show immune changes. Namely, both glucocorticoids and catecholamines exert powerful effects on the immune system, explaining numerous findings that suggested changes in inflammatory function of the immune system in patients with PTSD. Moreover, these patients are known to have increased rates of comorbidity with somatic disorders that involve immune and inflammatory processes. Therefore, it is of interest to explore possible mechanisms by which changes of the neuroendocrine system may be related to immune alterations and medical comorbidities of PTSD.

On the basis of the data demonstrating low levels of cortisol and high levels of inflammatory markers in association with PTSD, it has been hypothesized that excessive inflammation in individuals with PTSD might be a consequence of insufficient immunosuppression by cortisol. However, the data on cortisol levels and cortisol receptor functioning in PTSD patients are inconsistent, and most studies, including our own, have not presented evidence for cortisol decline (Meewisse *et al.*, 2007) or for its receptor functional impairment (Yehuda *et al.*, 1995a; Yehuda *et al.*, 2004b; Vidovic *et al.*, 2007) associated with the disorder.

In the present study we investigated possible role of HSPs in the relationship between PTSD and inflammation. We hypothesized that alterations in inflammatory status related to trauma and/or PTSD might be a consequence of Hsp70 and Hsp60 induction and release by psychological trauma. To test this hypothesis, we examined circulatory levels of Hsp70 and Hsp60 in patients with current PTSD and with life-time PTSD, in traumatized individuals without PTSD and in healthy subjects.

Until recently, HSPs have mostly been regarded as intracellular molecules that mediate a range of essential housekeeping and cytoprotective functions. The usual view of eukaryotic HSPs is that they are intracellular molecules that are released from necrotic, but not apoptotic cells, and that their release into (and

presence in) the extracellular environment indicates non-physiological tissue damage and therefore induces a range of proinflammatory responses. Findings from several studies are consistent with this idea. Human Hsp60 induces the expression of the adhesion molecules E-selectin, ICAM-1 and VCAM-1 on vascular endothelial cells, and the secretion of interleukin-6 from vascular endothelial cells, smooth muscle cells and macrophages (Kol et al., 1999; Kol et al., 2000). The intracellular localization of eukaryotic HSPs in healthy circumstances, the typical release of HSPs from infectious agents and the capacity of both human and bacterial HSPs to elicit innate and adaptive proinflammatory responses seem to be consistent with the proposed role for these proteins as links between pathogenic processes involving necrotic cell death, and the induction of innate and adaptive immunity.

However, further evidence suggests an alternative to this point of view. Interest in the role of HSPs as intercellular signalling molecules has been fuelled by the observations that these molecules can be released and are present in the extracellular environment under physiological conditions. They can be released from some viable (non-necrotic) mammalian cell types, including cultured rat embryo cells (Hightower & Guidon, 1989), human islet cells (Child et al., 1995), rat glial cells and a human neuroblastoma cell line (Bassan et al., 1998) and cultured vascular smooth muscle cells exposed to oxidative stress (Liao et al., 2000). These findings have profound implications for the perceived role of these proteins as exclusively proinflammatory intercellular signalling molecules and danger signals. They can elicit cytokine production and adhesion molecule expression in a range of cell types, and they can deliver maturation signals and peptides to antigen presenting cells through receptor-mediated interactions. These functions suggest that HSPs could be immunoregulatory agents with potent and widely-applicable therapeutic uses.

The two HSPs examined in our study, Hsp60 and Hsp70, are present in the peripheral circulation of healthy individuals (Pockley et al., 1998; Pockley et al., 1999; Pockley et al., 2000; Xu et al., 2000; Rea et al., 2001; Lewthwaite et al., 2002; Pockley et al., 2002). There is growing evidence that these two extracellular HSPs

are capable of activating pro-inflammatory responses, which can exacerbate diseases, such as atherosclerosis (Camici, 2002; Pockley *et al.*, 2003) and coronary heart disease (Pockley *et al.*, 2000; Zebrack & Anderson, 2002), but can also act beneficially, to facilitate recovery from bacterial infection (Campisi *et al.*, 2002). Psychological stressors have been shown to increase both intracellular and circulating levels of HSPs (Lewthwaite *et al.*, 2002; Fleshner *et al.*, 2004), thus contributing to development of coronary heart disease. Therefore, there was a rationale for a belief that eHsp60 and eHsp70 levels might be increased in patients with PTSD.

When we determined plasma levels of constitutive and inducible isoforms of Hsp70, as well as the level of Hsp60 in current and life-time PTSD patients, traumatized non-PTSD subjects and healthy controls, it was found that the four study groups did not differ in regard to plasma levels of the examined HSPs. The lack of between-group differences in the plasma level of HSPs might be possibly explained by a long time period elapsed between trauma exposure and recruitment for the study (minimum 6, maximum 16 years), since HSPs induction and release might serve to modulate proinflammatory response only in the early aftermath of trauma. Furthermore, serum Hsp60 and Hsp70s levels were not correlated with a range of measures including age, number of traumatic events, score on CAPS PTSD scale, score on BDI depression scale and markers of inflammation, such as CRP, sedimentation and number of leukocytes per ml blood. In addition, no associations were found between eHsp60 or eHsp70s and cardiovascular risk factors, including body mass index, blood pressure, smoking status, body composition, triglycerides, HDL, LDL and total cholesterol, blood glucose and insulin concentration, HOMA index and plasma leptin level. Interestingly, we did not observe the correlation between the intracellular and extracellular levels of Hsp60 or Hsp70s, a result speaking in favor of the possibility that HSPs release from the cells is a regulated process.

5.4 Inflammatory markers

The immune system is well regulated; however, psychological stress can exert an excessive demand on regulatory functions, particularly if the stressor is excessive or prolonged, resulting in risk for excessive inflammation (Fries *et al.*, 2005). Indeed, current studies have reported immune function alterations in individuals with PTSD, but the data are inconclusive, leading to an insufficient understanding of the nature of immune function alterations in PTSD. Some studies provided evidence for association of chronic PTSD with excessive inflammation, but this finding is not universal and the nature of immune function alterations in individuals with PTSD remains to be determined.

The immune and neuroendocrine systems are closely related. Neuroendocrine systems are known to regulate immune function, while, on the other hand, immune alterations are thought to play a central role in the pathophysiology of neuropsychiatric disorders. Therefore, it is reasonable to predict that patients with PTSD may show immune changes and that immune system plays a role in the pathophysiology of PTSD and in medical illnesses found along with PTSD. Furthermore, because glucocorticoids are well known for their antiinflammatory effects, it is reasonable to theorize that reduced circulating concentrations of cortisol seen in some patients with PTSD may be responsible for increased inflammation in the same individuals. However, studies directly relating cortisol and inflammatory markers in PTSD are scarce. Attenuated cortisol responses have been associated with enhanced IL-6 and IL-1 beta responses to stress in healthy individuals (Kunz-Ebrecht *et al.*, 2003), suggesting that low circulating concentrations of cortisol may foster a hyperinflammatory state, especially in the context of stress.

In the present study no alterations in the plasma cortisol level were observed in association with trauma-exposure or PTSD, since the basal cortisol level, calculated as a mean from thirteen nocturnal measurements, did not differ between trauma-exposed men with or without PTSD and corresponding non-traumatized, healthy controls. Besides, in order to inspect direct relation

between cortisol level and inflammatory status in the four groups of participants, we performed determination of general inflammatory markers, such as plasma CRP level, erythrocyte sedimentation rate and leukocyte count. No between-group differences in these inflammatory parameters were observed, demonstrating that general inflammatory status of current and life-time PTSD patients and trauma-exposed non-PTSD subjects was indistinguishable from that of non-traumatized healthy participants. Furthermore, correlation analyses revealed no associations between cortisol level and any of the examined inflamatory markers. Since low grade inflammation might not be noticable at the level of general inflammatory markers, such as CRP, erythrocyte sedimentation rate and leukocyte count, we continued with more detailed studies on inflammatory functions of the immune system and more deeply examined the relations between cortisol signalling and inflammatory response to trauma. In the present study we measured plasma levels of multiple pro-inflammatory and anti-inflammatory cytokines in order to unravel possible PTSD- or trauma-related alterations in inflammatory functions of the immune system and to correlate various cytokine levels with cortisol signaling parameters.

The results of our study showed the highest plasma levels of all examined cytokines in the participants belonging to healthy control group in comparison to other study groups, implying that trauma-exposure and PTSD could rather be linked to immunosuppression than to enhanced inflammation. Precisely, cytokines such as TNF-α, IL-6, IL-12p70, IL-2 and IL-8 were present at significantly lower levels in the plasma from the three groups of trauma-exposed subjects (current PTSD, life-time PTSD and trauma controls) as compared to healthy controls, meaning that the level of these pro-inflammatory cytokines might be connected with trauma-exposure rather than with the presence of current or life-time PTSD symptoms. On the other hand, the levels of IFN-γ, IL-5, IL-4 and anti-inflammatory cytokine IL-10 were significantly lower in current PTSD and life-time PTSD groups than in trauma and/or healthy control groups, suggesting that the level of these cytokines could be related to PTSD pathology rather than to trauma-exposure. Interestingly, the correlation analyses revealed that, in general, plasma cortisol

level is not associated with the levels of the cytokines. The only exception in the healthy control group was IL-12p70, the level of which was significantly correlated with basal cortisol level. In the current PTSD group, basal cortisol level was related only to the levels of IL-4 and IL-5, and in the trauma control group it correlated with the levels of IL-12p70, IL-8 and IL-6.

The explanation for the lack of correlation between cytokine and cortisol levels might be found in the fact that the actions of hormones can not be determined solely as a function of circulating concentrations. Instead, hormone effects also depend on tissue sensitivity, which is determined by the corresponding receptor number and functional status. PTSD patients have been found to exhibit altered glucocorticoid sensitivity in both endocrine tissues and immune cells (Yehuda *et al.*, 2004b; Yehuda *et al.*, 2004c; de Kloet *et al.*, 2007). In addition, some studies suggest that GR number per cell may be increased in immune cells (Yehuda *et al.*, 1991; Yehuda, 2009) and our present study shows that current and life-time PTSD patients, in comparison to trauma controls, display an increased level of GR expression in peripheral lymphocytes. Assuming that patients with PTSD exhibit enhanced glucocorticoid sensitivity, both in the central nervous system and in peripheral (immune) tissues, it can be expected that they should have ‘‘normal” concentrations of circulating inflammatory markers, even when cortisol level is low. One may even predict that patients with PTSD would have even reduced inflammatory markers, as observed in our study.

Nevertheless, multiple studies suggest that PTSD involves enhanced inflammatory activation. How can this apparent paradox be explained? First, it is possible that enhanced inflammation in patients with PTSD may not be the direct result of low circulating concentrations of cortisol. Instead, increased inflammation may be driven by enhanced SAM system activity found in PTSD patients. Increased sympathetic activity in patients with PTSD could be secondary to reduced cortisol, especially in the context of stress challenge (Yehuda, 2009). Increased SAM system activity may then enhance the activity of inflammatory pathways, including NF-κB (Bierhaus *et al.*, 2003). With this possibility, low circulating concentrations of cortisol would indirectly influence inflammatory function via the SAM system.

Second, it also may be possible that the low circulating concentrations of cortisol found in a portion of patients with PTSD may fall below critical thresholds needed to maintain normal inflammatory function, even in the presence of enhanced glucocorticoid sensitivity. If this hypothesis is correct, inflammatory excess in these patients should respond rapidly to glucocorticoid therapy without altering SAM system activity. It is also important to point out that inflammatory alterations in patients with PTSD may develop independently of cortisol levels, a possibility corroborated by our findings that plasma levels of most cytokines are not correlated with cortisol levels in healthy, non-traumatized individuals as well as in trauma-exposed men with and without PTSD. An important issue connected with inflammatory status of trauma-exposed individuals with and without PTSD, which remains to be further explored, is that increased inflammation may be a pre-existing risk factor that results from personality traits or genetic factors. If reduced cortisol is not involved, or if patients with PTSD have normal circulating concentrations of cortisol (Meewisse *et al.*, 2007), it is possible that enhanced glucocorticoid sensitivity of immune cells may be unable to overcome this inflammation.

Corticosteroids are the most effective anti-inflammatory therapy for many chronic inflammatory diseases, such as asthma, rheumatoid arthritis and inflammatory bowel disease, but are relatively ineffective in other diseases, such as chronic obstructive pulmonary disease. Chronic inflammation involves the infiltration and activation of many inflammatory and immune cells, which release inflammatory mediators that interact and activate structural cells at the site of inflammation. Different inflammatory diseases involve different cells and mediators (Barnes et al., 1998), but common to all of them is increased expression of multiple inflammatory proteins, which are regulated at the level of gene transcription by proinflammatory transcription factors, such as NF-κB and AP-1. It is now believed that chromatin remodeling plays a critical role in the transcriptional control of inflammatory genes. Stimuli that switch these genes on act by changing the chromatin structure of the genes, whereas corticosteroids reverse this process mainly by binding of liganded GR to coactivators and

recruitment of histone deacetylase-2 (HDAC2) to the activated transcription complex (Barnes, 2006).

Anti-inflammatory actions of glucocorticoids are typically mediated by pro-inflammatory transcription factors, such as NF-κB and AP-1 (Adcock & Caramori, 2001). Several models exist to explain the glucocorticoid-induced repression of AP-1 and NF-κB signaling. In the direct-interaction model, GR and AP-1 interact and prevent binding of each other to their response elements (Wargnier et al., 1998). In the competition model, GR and AP-1 compete with one another for binding to their overlapping response elements, while in the co-activator competition model, GR competes with NF-κB and/or AP-1 for binding to cyclic AMP-responsive element-binding protein (CBP), which is present in the cell in limiting amounts (Kamei et al., 1996; Sheppard et al., 1998). Furthermore, the GR-mediated repression of trans-activation by the p65 subunit of NF-κB is increased in the presence of CBP, suggesting that CBP functions as an integrator of the NF-κB/GR cross-talk (McKay & Cidlowski, 2000). In the histone acetyltransferase (HAT) inhibition model, GR binding to CBP inhibits recruitment of CBP by DNA-bound p65, reducing the HAT activity of the complex (Adcock & Caramori, 2001). Yet another possibility is an up-regulation of the inhibitor of NF-κB action (I-κB) in response to glucocorticoids (Deroo & Archer, 2001). However, recent results showing that dimerization-impaired GR DNA-binding domain is capable of DNA-binding (Adams et al., 2003) and up-regulation of GRE-dependent genes (Rogatsky et al., 2003) have shed some doubts on whether the anti-inflammatory actions of glucocorticoids are indeed independent of DNA-binding.

An important observation in the present study is that life-time PTSD subjects demonstrated the lowest level of analyzed cytokines compared to all other groups, thus particularly underlying the role of attenuated inflammatory response in th e recovery from PTSD. It is noteworthy that IFN-γ is the only cytokine that is decreased in the life-time PTSD patients, compared to healthy controls, whereas it was not changed in other two groups (current PTSD and trauma control). The decrease of IFN-γ in the life-time PTSD group could be a consequence of the most prominent decrease of IL-12p70 observed in the same group compared to all other

groups, since this cytokine acts as a potent inducer of IFN-γ production. Besides, this finding points to IFN-γ and IL-12p70 as the cytokines with possibly important roles in recovery from PTSD.

Since net effects of inflammatory response are determined by the balance between proinflammatory and anti-inflammatory cytokines, we analyzed the level of anti-inflammatory cytokine IL-10. The level of IL-10 was decreased in the groups of current PTSD and life-time PTSD patients, compared to healthy controls, while trauma controls were not significantly different from healthy controls. This result, in the light of the observed decrement of proinflammatory cytokines TNF-α, IL-6, IL-12p70, IL-2 and IL-8 in the three groups of trauma-exposed subjects, makes a difference between patients with PTSD and trauma controls in the overall balance between pro- and anti-inflammatory cytokines.

As yet, there is insufficient information to provide answers about the immune system contribution to PTSD vulnerability or progression. Additional research is needed, which hopefully will uncover physiologic mechanisms, thus providing opportunities for new pharmaceuticals or novel treatment approaches (Silverman & Sternberg; von Kanel *et al.*, 2008; Alten *et al.*, 2010).

6. Conclusions

1. Basal plasma cortisol level alterations are not associated with trauma exposure, current or life-time PTSD symptoms, resilience to PTSD, vulnerability to PTSD or with remission of the disorder.
2. Hypersensitivity of HPA axis to cortisol might be a biological correlate of vulnerability to PTSD.
3. Vulnerability to PTSD is characterized by increased level of GR expression in PBMCs.
4. War trauma-exposure, symptoms of PTSD, vulnerability or resilience to PTSD and remission of the disorder are not associated with the changes in MR level or in MR/GR balance in PBMCs.
5. The levels of expression of HSPs (Hsp90, Hsp70, Hsp72 and Hsp60) do not vary between current PTSD, life-time PTSD, trauma control and healthy control groups of subjects.
6. MR level in peripheral lymphocytes is correlated with the levels of both Hsp90 and Hsp70, while the GR level is correlated only with that of Hsp90.
7. Current PTSD is related to the strength of correlation between the levels of MR and Hsp70, while war trauma-exposure may improve correlation between the levels of GR and Hsp90 in peripheral lymphocytes.
8. Subjects belonging to current PTSD, life-time PTSD, trauma control and healthy control groups do not differ in respect to blood plasma levels of Hsp60 and constitutive and inducible isoforms of Hsp70. Thus, the extracellular levels of these HSPs can not be related to war trauma-exposure, symptoms of PTSD, vulnerability or resilience to PTSD and remission of the disorder.
9. Judging by the level of general inflammatory markers (CRP, erythrocytes sedimentation rate, leukocyte count), inflammatory status of the patients with

current or life-time PTSD is similar to that of traumatized non-PTSD subjects and non-taumatized healthy controls.

10. The highest levels of pro-inflammatory cytokines (TNF-α, IL-6, IL-12p70, IL-2, IL-8, IFN-γ, IL-5, IL-4) and anti-inflammatory cytokine (IL-10) were observed in the blood plasma from participants belonging to healthy control group in comparison to other study groups, implying that trauma-exposure and PTSD could rather be linked to immunosuppression than to enhanced inflammation.

11. Cytokines such as TNF-α, IL-6, IL-12p70, IL-2 and IL-8 were present at significantly lower levels in the plasma from the three groups of trauma-exposed subjects (current PTSD, life-time PTSD and trauma controls) as compared to healthy controls, meaning that the level of these pro-inflammatory cytokines might be connected with trauma-exposure rather than with the presence of current or life-time PTSD symptoms.

12. The levels of IFN-γ, IL-5, IL-4 and IL-10 were significantly lower in current PTSD and life-time PTSD groups than in trauma and healthy control groups, suggesting that the level of these cytokines could be related to vulnerability to PTSD rather than to trauma-exposure.

13. In general, plasma cortisol level is not associated with the levels of the cytokines. The exceptions are IL-12p70 in the healthy control group, IL-4 and IL-5 in the current PTSD group, and IL-12p70, IL-8 and IL-6 in the trauma control group.

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Biography

Younis Mouftah Elzaedi was born in Benghazi, Libya, on July 6th, 1968. He started his Bachelor studies in 1986. at the Department of Animal Production, Faculty of Agriculture, University of Omar Almokhter, Albeda, Libya. He graduated in 1990. with an average score of 2.5 out of 4.

From 1990. to 1996. Younis Elzaedi worked in the laboratory at the milk factory, where from 1997. to 2000. he was appointed General Director.

From 1990. to 1998. he served as a Teaching Assistant at the School of Arts and Science, Almerghib University, Libya, where he organized mid-term and end-of-year testing and grading, graduation activities and was in charge of zoology laboratory.

In 2000. he started Master studies at the Department of Zoology, Faculty of Science, University of Almerghib, Khoms, Libya, and graduated in 2004.

From 2004 to2008 Younis Elzaedi worked as Head of Biology Department, School of Arts and Science, Elmerghib University, Alkhomes, Libya. He taught Cellular Biology, Genetics, Physiology and Invertebrates to 2nd, 3rd and 4th year students and supervised graduation research papers. Besides, he was responsible for creating curriculum, organizing faculty schedules, heading the Department meetings and fulfillment of administrative duties.

In the period from 2004 to 2008. Younis Mouftah Elzaedi also served as a part-time lecturer at Alfateh University in Tripoli, Nasser University in Tarhuna and Seventh of October University in Musrata, Libya. He taught the courses such as Foundations of Research Methods, Physiology, Genetics, Invertebrates and Molecular Biology; supervised graduation research papers and worked as an external examiner.

Прилог 1.

Изјава о ауторству

Потписани-а Younis M. Elzaedi

број индекса _____

Изјављујем

да је докторска дисертација под насловом

Улога ванћелијских протеина топлотног стреса у инфламацији повезаној са посттрауматским стресним поремећајем

- резултат сопственог истраживачког рада,
- да предложена дисертација у целини ни у деловима није била предложена за добијање било које дипломе према студијским програмима других високошколских установа,
- да су резултати коректно наведени и
- да нисам кршио/ла ауторска права и користио интелектуалну својину других лица.

Потпис докторанда

У Београду, 23.09.2013.

Прилог 2.

Изјава о истоветности штампане и електронске верзије докторског рада

Име и презиме аутора Younis M. Elzaedi

Број индекса

Студијски програм Молекуларна биологија

Наслов рада Улога ванћелијских протеина топлотног стреса у инфламацији повезаној са посттрауматским стресним поремећајем

Ментор др Наташа Величковић, научни сарадник, Институт за биолошка истраживања „Синиша Станковић", Универзитет у Београду и др Гордана Матић, редовни професор, Биолошки факултет, Универзитет у Београду

Потписани/а Younis M. Elzaedi

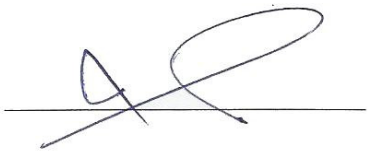
Изјављујем да је штампана верзија мог докторског рада истоветна електронској верзији коју сам предао/ла за објављивање на порталу **Дигиталног репозиторијума Универзитета у Београду.**

Дозвољавам да се објаве моји лични подаци везани за добијање академског звања доктора наука, као што су име и презиме, година и место рођења и датум одбране рада.

Ови лични подаци могу се објавити на мрежним страницама дигиталне библиотеке, у електронском каталогу и у публикацијама Универзитета у Београду.

Потпис докторанда

У Београду, 23.09.2013.



Прилог 3.

Изјава о коришћењу

Овлашћујем Универзитетску библиотеку „Светозар Марковић“ да у Дигитални репозиторијум Универзитета у Београду унесе моју докторску дисертацију под насловом:

Улога ванћелијских протеина топлотног стреса у инфламацији повезаној са посттрауматским стресним поремећајем

која је моје ауторско дело.

Дисертацију са свим прилозима предао/ла сам у електронском формату погодном за трајно архивирање.

Моју докторску дисертацију похрањену у Дигитални репозиторијум Универзитета у Београду могу да користе сви који поштују одредбе садржане у одабраном типу лиценце Креативне заједнице (Creative Commons) за коју сам се одлучио/ла.

1. Ауторство

2. Ауторство - некомерцијално

3. Ауторство – некомерцијално – без прераде

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5. Ауторство – без прераде

6. Ауторство – делити под истим условима

(Молимо да заокружите само једну од шест понуђених лиценци, кратак опис лиценци дат је на полеђини листа).

Потпис докторанда

У Београду, 23.09.2013.