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Altered hepatic mRNA expression of immune response and apoptosis-associated genes after acute and chronic psychological stress in mice

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ABSTRACT

Using a combination of transcriptional profiling and Ingenuity Pathway Analysis (IPA, www.ingenuity.com) we investigated acute and chronic psychological stress induced alterations of hepatic gene expression of BALB/c mice. Already after a 2-h single stress session, up-regulation of several LPS and glucocorticoid-sensitive immune response genes and markers related to oxidative stress and apoptotic processes were observed. Support for the existence of oxidative stress was gained by measuring increased protein carbonylation, but no alterations of immune responsiveness or cell death were measured in mice after acute stress compared to the control group.

When animals were repeatedly stressed during 4.5-days, we found reduced transcription of antigen presentation molecules, altered mRNA levels of immune cell signaling mediators and persisting high expression of apoptosis-related genes. These alterations were associated with a measurable immune suppression characterized by a reduced ability to clear experimental *Salmonella typhimurium* infection from the liver and a heightened hepatocyte apoptosis. Moreover, genes associated with anti-oxidative functions and regenerative processes were induced in the hepatic tissue of chronically stressed mice.

These findings indicate that modulation of the immune response and of apoptosis-related genes is initiated already during a single acute stress exposure. However, immune suppression will only manifest in repeatedly stressed mice which additionally show induction of protective and liver regenerative genes to prevent further hepatocyte damage.

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1. Introduction

Increasing demands and overwhelming environmental stimuli in our modern society continuously heighten the stress level of humans and escalate the pathogenesis of stress-associated illness such as the metabolic syndrome or depression and increase the risk of infections (Bartolomucci, 2007; Dallman, 2007; Leonard, 2006; Lundberg, 2005b; Swain, 2000). A physiological stress response is short lasting and adaptive processes are very rapidly mounted to reconstitute a balanced allostatic system in the stressed body which primarily include the neuroendocrine and immune system. However, if individuals are chronically stressed neuroendocrine dysregulation is prolonged and may cause malaise and disease (Bartolomucci, 2007; Dallman, 2007; Leonard, 2006; Lundberg, 2005b; Swain, 2000). Recently, we have described that BALB/c mice developed severe systemic immunosuppression, neuroendocrinological disturbances and depression-like behavior due to 4.5-days of intermittent combined acoustic and restraint stress which serves as a murine model of severe psychological stress (Kiank et al., 2006, 2007a; Depke et al., 2008).

To investigate primary causes for the severe loss of body mass in chronically stressed mice, we analyzed gene expression profiles of the liver which plays the major role in metabolism (Adams and Eksteen, 2006; Kmiec, 2001). These analyses revealed a chronic stress-induced hypermetabolic syndrome which was verified by *in vivo* analyses (Depke et al., 2008).

Importantly, animals suffered from the chronic stress-induced systemic immunosuppression shown by a heightened anti-inflammatory cytokine bias and an apoptotic loss of lymphocytes which increased the animals' susceptibility to experimental infection with *E. coli* (Kiank et al., 2006, 2007b). Repeatedly stressed mice actually developed spontaneous bacterial infiltrations into the lung measurable even 7 days after the last chronic stress cycle which was associated with a reduced inducibility of IFNγ, a cytokine that was shown to be important to prevent spreading of

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translocated commensals from the gut (Kiank et al., 2008). Because of the liver's important function as a gatekeeper between the intestinal tract and the general circulation (Adams and Eksteen, 2006; Dietrich et al., 2003) we now investigate how psychological stress may affect immune regulatory processes in the liver.

Veins drain the intestinal tract and then segment to secondary capillary beds in the liver where the removal and metabolism of absorbed nutrients or toxic substances takes place (Adams and Eksteen, 2006). Food antigens and products of commensal bacteria are abundantly detectable in the portal vein and are effectively cleared by Kupffer cells and sinusoidal endothelial cells (Kmiec, 2001; Limmer et al., 2000; Maemura et al., 2005; van Oosten et al., 2001).

Physiologically, because of a tolerogenic environment, there is no detectable intrahepatic immune activation in response to the low amount of microbial agent and food antigens derived from the gut (Kmiec, 2001; Limmer et al., 2000; Macpherson et al., 2002; Wiegard et al., 2005). However, when the antigenic challenge is increased an inflammatory response with a recruitment of neutrophils, monocytes/macrophages and lymphocytes may be induced (Adams and Eksteen, 2006; Kmiec, 2001). Then, an increased production of inflammatory mediators such as reactive oxygen species (ROS) may cause cell damage and loss of hepatocyte functions (Adams and Eksteen, 2006; Ott et al., 2007; Zangar et al., 2004).

It is important for the understanding of stress-induced immunoregulatory mechanisms in the liver that stress hormones like catecholamines and glucocorticoids (GCs) can modulate several liver functions including carbohydrate, protein and lipid metabolism or affect the immune response (Bartolomucci, 2007; Chida et al., 2006; Elenkov, 2004; Swain, 2000). Especially corticosteroids can suppress inflammatory processes by preventing the release of proinflammatory mediators, by diminishing immune cell trafficking, phagocytosis and radical production or by down-regulating antigen presentation, by inhibiting lymphocyte proliferation and by inducing apoptosis of immune cells (Bartolomucci, 2007; Elenkov, 2004; Lundberg, 2005a; Swain, 2000). Thus, prolonged increase of GC levels during chronic stress can activate hepatic catabolic pathways but also effectively suppress the local immune response.

To elucidate hepatic immune regulatory pathways that may contribute to chronic psychological stress-induced immune suppression, we now re-analyzed the hepatic gene expression profiling data (Depke et al., 2008) of stressed and non-stressed mice focusing on immune response and cell survival genes and verified hypotheses that were generated based on the expression data by *in vivo* experiments.

2. Materials and methods

2.1. Animal experiments

6–8 weeks old female BALB/c mice were randomly grouped into experimental and control groups starting 4 weeks before start of stress experiments. The group size differed from 6 to 9 mice per cage. Animals of the groups were not mixed before and during the experiments to avoid social stress. All animals were maintained with sterilized food and tap water *ad libitum* for adaptation under minimal stress conditions in animal rooms on 12:12 light/dark cycle. To avoid any additional stress and variation the handling of mice during the adaptation period and the experiments was restricted to one investigator. All animal procedures were carried out as approved by the Ethics Committee for Animal Care of Mecklenburg-Vorpommern, Germany.

2.2. Psychological stress model: combined acoustic and restraint stress

In the acute stress model, mice were exposed to a single 2 h combined acoustic and restraint stress cycle in the morning (8–10 AM). For immobilization mice were placed in 50-ml conical centrifuge tubes with multiple ventilation holes without penning the tail. Acoustic stress was induced by a randomized ultrasound emission device between 19 and 25 kHz with 0–35 dB waves in attacks (SiXiS; Pat. No. 109977, Taiwan) allowing the mice no adaptation to the stressor. For repeated/ chronic stress, mice were exposed to 2 h stress sessions on 4 successive days, twice a day (8–10 AM and 4–6 PM). On day 5 only one morning stress session was performed. Between the single stress sessions mice stayed in their home cages with free access to food and tap water. In all experiments, control mice were isolated from stressed animals during the whole period in the incubator where the animals were adapted. Stressed mice remained outside the incubator in the same animal laboratory during the whole period of the stress model to avoid any acoustic or olfactory communication between the groups. All further analyses were started immediately after the final stress exposure (10 AM) with $n = 6–9$ mice/group. For each analysis two independent stress experiments were performed to ensure reproducibility.

2.3. DNA array analysis

For this study, data generated for characterization of metabolic processes in the chronic and acute stress models were re-analyzed to further clarify immunological aspects of the models. The detailed methods for organ harvesting, RNA-preparation, DNA array hybridization and data analysis are described in our previous publication (Depke et al., 2008). In brief, RNA was prepared from flash-frozen liver tissue samples using a spin column based protocol. Subsequent Affymetrix GeneChip Mouse Expression 430A and 430A 2.0 Array expression analysis was performed according to standard protocols. Differential regulation of gene expression was assessed using a combined approach of detection (flag), signal difference and fold change cut-offs and the resulting sets of regulated genes were assigned to functional aspects with the aid of Ingenuity Pathway Analysis (IPA, www.ingenuity.com). Complete hepatic gene expression data of stressed and non-stressed mice are available at the NCBIs Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) database and are accessible through GEO Series accession number: GSE11126.

2.4. Functional analysis of gene expression data using Ingenuity Pathway Analysis

For functional analysis lists of differentially regulated probe sets were uploaded as Excel spreadsheets into the Ingenuity Pathway Analysis tool (IPA 6, www.ingenuity.com). In order to compare effects of acute and chronic stress two groups of genes were included: (1) genes displaying differential regulation after acute stress and (2) genes differentially regulated after chronic stress. IPA combined the uploaded Affymetrix probe set IDs and assigned annotations depending on the content of the so-called Ingenuity Pathway Knowledge Base (IPKB). The IPKB was used to get further insight into the relation of differentially regulated genes and immunological functions. Searches for relevant keywords and meaningful combinations of keywords resulted in lists linked to the functions in focus. IPA also offers the association of differentially expressed genes with canonical pathways, which can be rated by a corresponding p-value. This approach was used to depict functional aspects of differential gene expression.

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