RESEARCH ARTICLE

Stress-induced cytokine changes in rats

Hubertus Himmerich[1,a], Johannes Fischer[2,a], Katrin Bauer[3], Kenneth C. Kirkby[4], Ulrich Sack[3], Ute Krügel[2]

[1] Department of Psychiatry and Psychotherapy, Medical Faculty, University of Leipzig, Semmelweisstraße 10, 04103 Leipzig, Germany
[2] Rudolf Boehm Institute of Pharmacology and Toxicology, Medical Faculty, University of Leipzig, Härtelstraße 16-18, 04107 Leipzig, Germany
[3] Department of Clinical Immunology, Medical Faculty, University of Leipzig, Johannisallee 30, 04103 Leipzig, Germany
[4] Department of Psychiatry, University of Tasmania, Hobart, Tasmania, Australia

Correspondence: Prof. Dr. Hubertus Himmerich, Department of Psychiatry and Psychotherapy, University of Leipzig, Semmelweisstr. 10, 04103 Leipzig, Germany
<Hubertus.Himmerich@medizin.uni-leipzig.de>

ABSTRACT. Stress-induced cytokine changes may be the link between stress and the pathogenesis of psychiatric disorders such as depression, and organic diseases such as infections, autoimmune diseases and cancer. We tested the effect of stress on interleukin (IL)-2, IL-4, IL-6, IL-10, IL-22, tumour necrosis factor (TNF)-α and interferon (IFN)-γ serum levels in male Wistar rats. Rats underwent either acute stress by forced swimming (N = 8), chronic restraint stress (N = 8), or were not subjected to any stress (N = 8). IL-2 serum levels were significantly higher in forced swimming, but not in restraint stress rats, compared to non-stressed rats. IL-4, IL-6, IL-10 and TNF-α levels were higher in both forced swimming and restraint stress compared to non-stressed rats. IFN-γ production was significantly decreased by restraint stress, but not by forced swimming. IL-22 was not affected significantly by either stress condition. Alterations in the pro-inflammatory cytokines IL-6 and TNF-α may indicate a pathophysiological pathway from acute and chronic stress to the development of depression. Changes in IL-4 and IL-10 may link acute and chronic stress to autoimmune disorders, allergies or cancer. The reported changes in IFN-γ could provide an explanation for the higher susceptibility to infection seen in life periods associated with sustained levels of stress.

Keywords: stress, cytokines, rats, IL-2, IL-4, IL-6, IL-10, IL-22, TNF-**α**, IFN-**γ**

Changes in the cytokine system have been repeatedly reported in the development of stress-induced psychiatric disorders such as depression [1-3]. Therefore, stress-induced cytokine changes may be an important link between psychologically perceived stress and the pathogenesis of depression.

The relationship between stress and cytokines has been investigated using a wide variety of experimental designs, in animal and human studies. Conclusions as to the precise mechanisms involved have been limited by some inconsistent results and the complexities of the approaches used. Indices of cytokine activity have included genetic abnormalities, mRNA expression, intracellular, serum or saliva cytokine levels, and skin or brain concentrations of cytokines [4-28].

With respect to individual cytokines, for interleukin (IL)-6, there are consistent findings that stress increases IL-6 levels in mice [4], rats [5-7] and humans [8-11]. The fewer studies of TNF-α show mainly an increase due to stress in rats [6, 7, 12, 13], mice [14] and humans [15], but some studies report no changes in TNF-α production during stress [16]. In contrast to IL-6 and TNF-α, interferon-γ (IFN-γ) is reported to decrease in the blood during stress. For

example, in stressed mice a decrease in IFN-γ production was found [17, 18]. Also in humans, IFN-γ decreased, in saliva [19] and blood [20] during stress. The latter result has been replicated in several independent studies. However, in the study of Trueba *et al.*, IFN-γ increased in the exhaled breath condensate of healthy individuals early during exams stress [19]. Chronic stress increased IFN-γ in the periodontal disease tissue of rats [21]. Hence, changes in IFN-γ production may depend on the kind of stress applied as well as the tissue where its concentration is measured.

For other cytokines such as IL-2, IL-4 and IL-6, the literature available shows a quite heterogeneous picture. IL-2 has been found to decrease [6, 22], or to increase [13] during stress. Similarly, IL-4 production increased [13, 23, 24], stayed unchanged [25] or even decreased [26, 27] in different experiments. IL-10 was found to be increased after stress in two different animal models [7, 18] and after surgical stress in humans [28]). However, to our knowledge, IL-10 blood concentrations have not yet been investigated in an experimental stress paradigm using rats. Although IL-22 plays a prominent mediator role in inflammation by stimulating the production of important antimicrobial peptides [29, 30], it has not yet been investigated with regard to stress.

Taken together, the literature on these cytokines indicates consistent findings as to how IL-6, TNF-α and IFN-γ

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[a]Both authors contributed equally to this work.

respond to stress: IL-6 and TNF-α production increases while IFN-γ production decreases during stress. Fewer, and partly contradictory, studies are available regarding IL-2, IL-4 and IL-10 levels; the expression of IL-22 in response to stress has not yet been measured despite its important role in inflammation.

Investigating the immunological consequences of stress is important for explaining, for example, the increased incidence of infectious diseases and increased viral activity during periods of stress [31], and also, for elucidating the pathophysiological pathway from stress to psychiatric disorders such as depression, whereby stress might lead to specific cytokine changes known to induce depressive symptoms [32]. It is advisable to test several cytokines within one experiment, because of their interdependence. For instance, on the basis of in vitro experiments, it is supposed that IL-10 might modulate IFN-γ production [33]. However, this relationship is not consistent across the literature.

To induce stress, we used two different approaches, an acute stress paradigm by means of forced swimming, and a chronic stress model using repeated restraint. Forced swimming is an inescapable stressor and thus a stress-inducing paradigm; further, the rat responds with immobility, which serves as an animal model of depression-like symptoms and which is reduced by antidepressant drugs [34-37]. In rats and mice, repeated restraint stress induces depression-like behavioural changes accompanied by working memory and learning deficits [38-40], We sought to investigate differences in cytokine production, comparing cytokine serum levels in rats stressed by forced swimming or chronic restraint stress, and non-stressed rats

METHODS

Animals

Adult male Wistar rats (outbreed, 12 to 14 weeks old, n = 24, Janvier, Le Genest Saint Isle, France) were housed in standard laboratory cages for two weeks for acclimatisation. The animals were allowed free access to food and water under a 12-hours light-dark schedule (lights on 7:00 a.m. to 7:00 p.m.). The experiments were approved by the Animal Welfare Office (Leipzig, Germany; TVV10/11) according to the German guidelines for the use of animals in biomedical research. All efforts were made to minimize the number of animals used and their suffering. Eight rats underwent no stress, eight were exposed to acute stress by forced swimming, and eight were subjected to a chronic restraint stress protocol.

Forced swimming stress

Acute stress was induced by forcing animals to swim according to the previously described protocol for the commonly used test to detect (anti-)depressant-like effects in rodents [40, 41]. Briefly, in a preceding trial the rats were placed in a cylindrical basin (0.5 m height × 0.25 m diameter) filled with water (25 ± 1°C) up to a height of 0.35 m for 15 minutes. After 24 h, an experimental trial of 7 min duration followed. The experiments were performed between 9:00 a.m. and 11:00 a.m.

Restraint stress protocol

Animals exposed to chronic, mild stress by repeated restraint were placed in perforated plexi glass tubes (6.5 cm inner diameter × 20 cm length) for four hours per day for 10 consecutive days [40]. The restraint allowed normal breathing and limited movements of head and limbs. The restraint occurred daily, but was otherwise imposed at random times, at various times of day and places, out of their home cages.

Cytokine measurement

Blood samples from animals that underwent the restraint stress protocol were taken under deep isoflurane anaesthesia between 9 and 11 a.m. by heart puncture 20 hours after the last restraint session. Blood samples from animals that underwent the forced swimming stress were taken within 15 min after the forced swimming trials using the same procedure. Plasma concentrations of IL-2, IL-4, IL-6, IL-10, TNF-α and IFN-γ were measured using a Cytometric Bead Array (CBA) for rats (Becton, Dickinson and Company [BD] Biosciences, San Jose, CA, USA). IL-22 was detected using the rat IL-22 Quantikine ELISA Kit (R&D Systems, Minneapolis, MN, USA). Duplicate measurements were performed according to the manufacture's protocols.

Data analysis

Statistical analysis was performed using SPSS (IBM SPSS Statistics 20). For all serum cytokine levels, the median and quartiles were calculated and Mann-Whitney-U tests for unpaired data were performed. A level of asymptotic 2-tailed significance of < 0.05 was considered to indicate a significant difference.

RESULTS

Plasma concentrations of IL-2 were significantly higher in rats who underwent forced swimming stress (p = 0.003) compared to non-stressed rats. However, restraint stress did not significantly increase IL-2 levels.

Plasma IL-4, IL-6, IL-10 and TNF-α concentrations were higher after forced swimming stress (IL-4: p = 0.006; IL-6: p = 0.001; IL-10: p = 0.016; TNF-α: p = 0.040) as well as after restraint stress (IL-4: p = 0.007; IL-6: p = 0.003; IL-10: p = 0.024; TNF-α: p = 0.035) compared to the unstressed rats.

IFN-γ production was significantly decreased by restraint stress (p = 0.012), but not by forced swimming.

Further, none of the stress paradigms applied affected plasma IL-22 significantly.

Table 1 shows the descriptive statistics of the cytokine plasma concentrations measured as median, 1st and 3rd quartile. As TNF-α might play a specific role in the pathophysiology of depression [2] and blockade of TNF-α having been reported to have antidepressant-like properties [42], and as IL-22 has never been tested in a stress paradigm, we also depict means and standard errors of the means (SEM) for these cytokines in *figure 1* and *figure 2*, respectively.

Table 1
Descriptive statistics for plasma cytokine concentrations in rats.

	IL-2 [pg/mL]	IL-4 [pg/mL]	IL-6 [pg/mL]	IL-10 [pg/mL]	TNF-α [pg/mL]	IFN-γ [pg/mL]	IL-22 [pg/mL]
No stress (N = 8)							
Median	0.24	1.16	0.00	10.51	15.06	29.63	34.00
1st quartile	0.02	0.29	0.00	5.21	10.67	15.05	19.68
3rd quartile	0.69	2.55	0.79	19.97	16.83	46.77	89.97
Forced swimmimg stress (N = 8)							
Median	1.28*	3.71*	86.07*	34.58*	25.01*	15.67	47.84
1st quartile	1.00	2.50	21.77	15.21	15.79	11.94	17.98
3rd quartile	2.12	5.11	119.49	46.20	28.86	48.04	83.69
Restraint stress (N = 8)							
Median	0.62	3.18*	53.68*	29.47*	20.58*	6.75*	22.92
1st quartile	0.20	2.52	8.79	17.79	14.77	0.77	13.99
3rd quartile	1.60	5.44	153.74	61.21	38.09	18.77	47.32

* indicates a significant difference for the values in a stress paradigm compared to non-stressed rats in the Mann-Whitney-U test for unpaired data.

Figure 1
Means and standard errors of the means (SEM) for TNF-α plasma concentrations in rats without stress, in rats after acute, forced swimming stress and after chronic restraint stress. * indicates a significant difference in the values in a stress paradigm compared to non-stressed rats in the Mann-Whitney-U test for unpaired data.
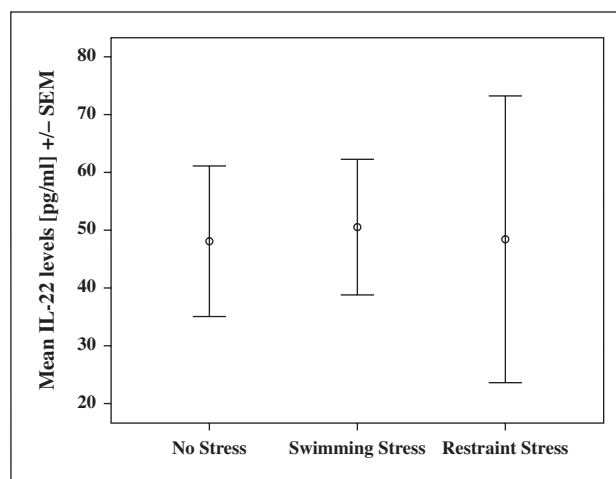

Figure 2
Means and standard errors of the means (SEM) for IL-22 serum concentrations in rats without stress, in rats after the forced swimming stress, and after restraint stress.

DISCUSSION

The main results of the present experiment show an increase in IL-2, IL-4, IL-6, IL-10 and TNF-α and a decrease in IFN-γ plasma concentrations after the application of two stress paradigms in rats. The decrease in IFN-γ was only significant in the chronic restraint stress model, and the increased IL-2 only after acute forced swimming stress. IL-22 was unaffected by both stress paradigms.

Forced swimming and repeated restraint are known to induce elevated corticosterone concentrations in rats, indicating that the experience of these aversive and threatening stimuli are stressful events [43, 44]. Our data confirm that experiencing an acute stressor or unpredictable, chronic, mild stress, impact on immune signalling as demonstrated by changes in plasma cytokine composition. In addition, the mode and duration of the stress have different effects on certain cytokines.

A consistent finding of the present study was the increased pro-inflammatory cytokines IL-6 and TNF-α levels. The increase in IL-6 under stress is in accordance with the available literature for IL-6 in rodents [4-6, 45] as well as in humans [8-11]. Prolonged exposure to circulating IL-6 is thought to blunt behavioural responsiveness including food intake, and is suggested to be a key contributor to depressive-like behaviours [46, 47]. Furthermore IL-6 has been repeatedly implicated in patients with major depression [48].

The increase in plasma TNF-α is in agreement with studies in which hyperproduction of TNF-α was induced by acute and chronic stress paradigms [6, 7, 12, 45]. This finding might be of particular interest for the pathophysiology of depression, because elevated levels of pro-inflammatory cytokines such as TNF-α have been found in depressed patients [2]. Additionally, experimental immune stimulation associated with an increase in TNF-α activity in humans [32] as well as in rodents [49-51], induced depression-like symptoms such as behavioural, emotional and cognitive disturbances [32, 51]. Peripheral pro-inflammatory cytokines such as TNF-α released from macrophages and monocytes in response to bacterial endotoxins, such as lipopolysaccharide (LPS), were

found to signal to the brain and to have profound effects on neuronal activity as indicated by electrophysiological data and by neurotransmitter recording *via* microdialysis. [52, 53]. The findings of a strong, time-dependent c-Fos expression and *de novo* synthesis of brain IL-6 and TNF-α with accompanying behavioural depression (sickness behaviour), indicates that immune-derived information is processed in the brain, e.g. in the amygdala.

Several pathways have been proposed as to how TNF-α and other cytokines might directly affect the brain [54]. They may exert their depressogenic effects by activation of the hypothalamic-pituitary-adrenal (HPA) axis, activation of neuronal serotonin transporters [55, 56], stimulation of indoleamine 2,3-dioxygenase [57], by the immunologically-mediated destruction of neurons [58-62], and/or the release of glutamate [63]. Therefore, stress, by inducing an increased production of pro-inflammatory cytokines, *inter alia*, might trigger neurobiological changes capable of inducing depression.

Anti-TNF-α-agents such as etanercept have been shown to exert antidepressant effects in patients with moderate to severe psoriasis [64]. Etanercept also reduces the depressive-like behaviour induced by chronic restraint stress in rats [42]. Based on our present findings, we hypothesize that inhibiting peripheral TNF-α would block the cascade from stress to depression that is mediated by increased pro-inflammatory plasma cytokines and their central signalling.

The increase in IL-2 after forced swimming and the decrease in IFN-γ after chronic restraint stress suggest specificity of action. IL-2 release is controlled by CD4+CD25+ regulatory T cells. In a chronic restraint stress model in mice, the inhibition of CD4+CD25+ regulatory T cells by anti-CD25 antibodies caused a marked increase in plasma IL-2, which was absent in non-treated, stressed animals, similar to our present data [65]. To our knowledge, no data are available on IL-2 in the early phase of acute stress; however, our data assume a fast release response, which possibly underlies adaptive regulatory mechanisms after repeated exposure to certain but not all stressors. Additional to the type of stressors and the pattern of their succession, the time point of observation within these experimental procedures impacts on the results. This may be relevant to the contradictory findings in pooled studies, for example increased plasma IL-2 is thought to be a key marker in major depression, but this was not supported by a recent meta-analysis [48].

In agreement with the present data on rat plasma IFN-γ after forced swimming stress is a report that single exposure to restraint stress suppressed innate IFN-γ production in mice following an *in vivo* lipopolysaccharide (LPS) challenge, whereas after restraint alone IFN-γ showed only a trend towards reduction [18]. Pre-treatment with mifepriston, a glucocorticoid receptor antagonist prevented the stress-related suppression of IFN-γ consistent with the immune suppressive effects of glucocorticoids. The decrease in IFN-γ production may contribute to the increased incidence of infections during stress periods such as examinations, and increased antibody titres to the Epstein-Barr virus (EBV) during examination periods [31]. A decrease in IFN-γ signalling may contribute to the reactivation of latent EBV due to weaker cellular immune control during periods of high stress levels. On the other hand, IFN-γ was found to be increased in very depressed subjects and in patients with an acute attack of multiple sclerosis and elevated Beck Depression Inventory scores (BDI) [66].

IL-10 is produced by T helper (Th)2 cells and monocytes, and induces proliferation and differentiation of autoreactive B cell clones, however, it also inhibits Th1 cell activation and IFN-γ production [33]. Therefore, an increase in IL-10 during stress might partly explain the decrease in IFN-γ serum levels that we found. However, this is only a speculative explanation, because the inverse relationship between IL-10 and IFN-γ is not systematically significant across the literature. IL-10 is involved in the development of autoimmune disorders, and, in turn, IL-10 antagonists have been shown to have a beneficial impact on autoimmune mechanisms, and may therefore be beneficial in the treatment of, for example, systemic lupus erythematosus [67]. Indeed, stress has been described to act as an exacerbating factor in this disease [68]. Moreover, IL-10 overexpression was also found in patients with certain tumours such as melanoma and several lymphomas, and is considered to promote further tumour development [69]. Given that a substantial body of research suggests a link between stress and cancer [70], IL-10 might be a promising research target for investigating a pathway from stress to cancer.

IL-4, which was increased by acute forced swimming and chronic restraint stress, has a well-established role in the pathology of allergies and other immunological diseases, where it induces B cell activation and immunoglobulin E (IgE) class switching [71]. Indeed, stress has been discussed as a causal or influential factor in the development and the disease course of allergic disorders such as asthma [72], food allergies [73] and other atopic disorders [74]. Children with asthma who had higher levels of chronic family stress showed increased production of IL-4 [75]. The combination of acute and chronic stress was associated with increased asthma symptoms. These cytokine changes could be an explanation for the higher risk of children experiencing life stressors to develop exacerbations of asthma [75].

IL-22 is a cytokine that has not attracted much attention in psychoimmunology to date, despite its importance in host defence against Gram-negative bacteria and in the development and pathogenesis of several autoimmune diseases [76]. However, no data are yet available for this cytokine under acute or chronic psychosocial stress or in depression in animals and humans; however, our study does not support the hypotheses of its responsiveness to stress.

Finally, it is important to note, that forced swimming, used to test novel antidepressant drugs or to assess depression-like behaviour in rodents in different stress or depression models, itself triggers activation of pro-inflammatory cytokines. Moreover, the regulation of plasma cytokines is dependent on the onset, duration and sequence of certain stimuli as well as the time point of observation, all of which are critical factors in interpreting snap-shots of cytokine responses and activity in experimental and clinical cytokine research.

The emerging picture is that changes in cytokine plasma concentrations may help to explain the association of stressful life events and stressful life cycle periods

with psychiatric or somatic diseases such as depression, autoimmune disorders, allergies, cancer and increased susceptibility to infection.

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