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SUMMARY

This report reviews a number of potential stress indicators of health status. The immunobiochemical stress markers are all related in some way to cortisol which may be regarded as the golden standard in stress research. Finally, the relation between the reviewed biological markers and the end points as determined via the psychological approach is discussed.
SAMENVATTING

Dit rapport geeft een overzicht van potentiële endocriene en immunologische stress indicatoren van de gezondheidsstatus. Nadruk is gelegd op de indicatoren die op een of andere wijze gerelateerd is aan cortisol, dat als de gouden standaard in stress onderzoek wordt beschouwd. Tot slot wordt nader ingegaan op de relatie tussen de biologische markers en de eindpunten zoals die middels de psychologische benadering worden bepaald.
1. INTRODUCTION

Society is experiencing increased chronic psychological stress that affects human psychological and physical health. Adequate coping strategies are required to resist to and to reverse the adverse effects of stressful events on human health. If stress is not adequately controlled, sustained activation leads to loss of the homeostasis which will not only affect the individual mentally but also physiologically. As such various diseases, notably immune-related diseases, may develop following prolonged exposure to psychological stress.

Both psychological and physiological stress markers have been developed to study the psychological and psychosomatic patterns and consequences of long lasting stress. Psychological stress markers are mainly assessed via questionnaires that characterize the amount of experienced stress as well as of distress, coping styles, mental and physical health problems. The physiological stress markers comprise a large array of endocrine, biochemical and immunological endogenous substances.

The present paper outlines that the HPA-axis (hypothalamus-pituitary-adrenals-axis) plays a prominent role in the psychobiological consequences of stressful events. Cortisol release is a hallmark of this pathway Though a large array of signaling molecules are involved in activation of the HPA-axis,. For this reason, various endocrine and immunological biomarkers related to cortisol are proposed to study the physiological effects of stress. Finally, the relation between psychological and physiological stress markers is discussed.
2. STRESS, STRESSORS AND DISEASE

Numerous definitions have been associated with prolonged psychological stress. Here we will use the term ‘stress’ to describe the consequences of the sources of stress or ‘stressors’ (1). Stressors include major life events, like death of a loved one, divorce, loosing a job, and unwished relocation. Other examples of stressors are: every day minor life events and daily hassles like a high work-load, traffic jams, conflicts, and many other situations that because of their chronicity often have a major impact on the mental and physical well-being. Stressors can lead to stress, with a consequential large array of psychological and psychosomatic complaints, finally developing to illness and disease (2,3). Associated with stress are mental diseases such as surmenage, burn-out syndrome, chronic fatigue syndrome, post-traumatic stress disorder, depression, and anxiety disorder. Despite certain differences, they share the aspect of negative emotional stress. Other diseases that have been associated with long-term negative stress are cardiovascular disease, rheumatic arthritis, diabetes, auto-immune disease, and an increased morbidity and mortality linked to e.g. infections and neoplasms (4-8).
3. ACUTE STRESS VERSUS CHRONIC STRESS

To adequately describe the psychophysiological consequences of stress, it is important to discriminate between acute and chronic stress (9). For instance, research in the interdisciplinary field of psychoneuroimmunology has shown that various forms of chronic stressors, such as marital problems, work-related stressors or bereavement, generally have a suppressive effect on parameters of the immune system (10-14). In the case of chronic stress, the down-regulation of components of the immune system may lead to increased susceptibility to infections (colds, herpes, HIV) and worsen existing diseases such as cancer (4,5). The impaired functioning of the immune system is likely to contribute to increased morbidity and mortality (6-8).

In contrast to the down-regulation of the immune system seen with the longer lasting chronic stress, acute stress, such as examination stress or experimentally induced stress, is rather associated with up-regulation of the immune functions (15-19). The related reactivity of cardiovascular variables and NK cell numbers to an acute stressor suggests that dynamic short-term changes in both systems are mediated by catecholamines (15). Considering that acute short-term stress does not result in severe health consequences, only markers of prolonged stress will be described here.
4. ENDOCRINE AND BIOCHEMICAL STRESS MARKERS

The biological effects of stress are generated via an integrated complex network of central-neural and endocrine processes. In addition to sympathetic nervous activation, numerous (hormonal) mediators like catecholamines, corticosteroids, endogenous opioids are released by this network (e.g. (3)).

Frequently applied endocrine markers in stress research are circulating cortisol, ACTH and CRH. While the hormones ACTH and CRH are associated with the initial steps of HPA-axis activation, cortisol, released from the adrenals, is a major final product of this route. Other routes activated by CRH, such as the thymus-pituitary-adrenal axis, and the thymus-pituitary-gonadal axis will not be considered here.

4.1. Cortisol

A key mediator of the systemic response to stressors is CRF, released by the hypothalamus. During the stress response CRF regulates ACTH secretion from the pituitary (20) and, via an endocrine action, ACTH controls the release of corticosteroids from the adrenal medulla. CRF also activates the SNS system resulting in catecholamine release into the circulation (21). CRH release after stress has been associated with reduced lymphocyte proliferation and NK-cytotoxicity (22). In addition, CRF slows IgG induction in response to the foreign protein KLH (keyhole limpet hemacyanin).

Negative life events increase plasma cortisol (23) and the level of cortisol is fully accepted in stress research as a valuable measure of stress. Measurement of cortisol in saliva is preferred because it is non-invasive, reliable, and elicits no stress. Salivary values compare well with the serological value. Like most hormones cortisol follows a circadian rhythm so that for proper measurement of cortisol levels the time of sampling should be carefully noticed. The flattening of this rhythm can also be used to characterize stress.

Cortisol (and ACTH) levels are also very frequently increased in severely depressed patients. In most depressive patients the dexamethasone suppression test (DST) is positive though false positive and negative results are regularly obtained. The combined dexamethasone-CRH test
is the best neuroendocrine tool currently available to identify HPA abnormalities in psychiatric patients. Surprisingly, the DST has never been applied in stress research. Cortisol can be easily assayed by commercial available immunoassays while HPLC-techniques have also been described.

4.2. Dehydroepiandrosterone (DHEA)

Another adrenal steroid hormone released by the adrenal cortex in response to ACTH is dehydroepiandrosterone (DHEA) that is further metabolized to its ester DHEA-sulfate (DHEA-S). Four week training in self management reduced cortisol by 23% and increased DHEA by 100% (24) and both effects were associated with decreased stress, anxiety, burn-out and hostility. This observation was recently confirmed in HIV-patients (25). In a cohort of senior male army officers Labbate et al. also showed that perceived stress was inversely related with DHEA-S levels (26) while Fava et al. (27) showed that Italian managers, who reported more cynicism and hostility, and less enjoyment in leisure activities than Americans managers, had significantly lower levels of serum DHEA.

Major depression is characterized by high evening cortisol and low morning DHEA level (28,29) and a raised cortisol/DHEA ratio (i.e. > 60th percentile) predicts delayed recovery (30,31). Barrett et al. (32) recently showed in a large cohort of older women (N=699) that mood and depression as scored by the Beck Depression Inventory was inversely associated with DHEA-S plasma level.

Dexamethasone and cortisol decrease DHEA level (29,33,34). DHEA can antagonize cortisol (35) and may thus be effective to treat depression (36). Indeed, a recent double blind study showed that treatment of depressive patients with DHEA induced a significant antidepressant effect as assessed via the Hamilton Depression Rating Scale (37).

Commercial immunoassays are available to measure DHEA and DHEA-S in saliva and serum.

4.3. Pteridines (THB, biopterine en neopterine)

Major depression is characterized by low levels of neurotransmitters (dopamine, serotonin and noradrenaline) in the CNS and pharmacotherapy addresses increased availability of these hormones in the synaptic cleft. The neurotransmitters are synthetized by different hydroxylases using tryptophan, tyrosine and phenylalanine as substrate. The essential co-factor
of these hydrolases is tetrahydro-biopterin (THB), that is synthetized from guanosine tri-phosphate (GTP) by GTP-cyclohydrolase. Both biopterin and neopterin are markers of this synthetic pathway.

THB-synthesis in CNS is decreased in depressive patients and increased by electro-shock therapy. Most studies report increased neopterin en biopterin level in plasma and urine of depressive patients (38,39), while plasma THB and ratio THB/biopterin in such patients is decreased. Finally, in patients with chronic fatigue syndrome increased neopterin levels have been reported (40,41).

In analogy with major depression, stressful stimuli affect the level of both pteridines. Two studies describe a decrease of neopterin in urine after examination stress (42,43). Serum neopterin was shown to be associated with high chronic professional stress (44). In rats cold stress (45), immobilisation stress and food deprivation (46) increased THB and adrenal GTP-cyclohydrolase. In adrenals of a genetic strain of depressive rats tyrosine hydroxylase, dopamine beta-hydroxylase and GTP cyclohydrolase I mRNA were markedly elevated compared to the control strain. Immobilization stress in the control strain but not the stressed strain, increased mRNAs of the enzymes (47). Serova et al. (48) recently described that incubation of PC12 cells in vitro with plasma of immobilized rats increased GTP-cyclohydrolase mRNA. Interestingly, incubation of the cells with cortisol induced the same effect. These findings confirm previous data of Abou-Donia et al. (49) showing that the stress hormones ACTH and adrenaline stimulate GTP-cyclohydrolase activity. In rat liver cells dexamethasone increased THB (50) and in the adrenals of the rat cortisol increased GTP-cyclohydrolase mRNA. On the other hand the synthetic glucocorticoid dexamethasone inhibited cytokine-induced stimulation of GTP-cyclohydrolase in cardiac endothelial cells (51), macrophages (52) and mesangial cells (53).

Kits, based on either radioimmuno assay or enzyme immunoassay are commercially available for neopterin, but not biopterin. Alternatively, HPLC determinations have been described for both pteridines.

### 4.4. Kynurenine

TDO (tryptophan dioxygenase, tryptophan pyrolase) resides in liver only and specifically degrades tryptophan. Interestingly, cortisol stimulates tryptophan degradation in vitro by TDO via enzyme induction 7-8 fold (54). In addition, cortisol stimulates in the liver the degradation
of tyrosine via induction of tyrosine aminotransferase (TAT). Tryptophan hydroxylase (TPH) is the rate-limiting enzyme in serotonin biosynthesis. Immobilization stress induced a 6-10 fold rise in TPH mRNA but did not affect GTP cyclohydrolase I mRNA (55). This is in apparent contrast with the decrease in TPH mRNA by dexamethasone observed by (56).

IDO (indole-dioxygenase) has a much broader substrate specificity and is localized in multiple tissues incl. macrophages. IDO metabolizes tryptophan, serotonin (5-HT) and melatonin. Cortisol does not induce IDO, but facilitates induction of IDO by γ-interferon (57). Like the formerly mentioned hydrolases, the enzyme IDO uses THB as co-factor for its metabolism of tryptophan to kynurenine. Kynurenine is further metabolized by enzymes which all are more or less dependent of vitamin B6. When the individual is deficient in vitamin B6, as depressive patients, increased tryptophan degradation will lead to a specific increase in the level of xanthurenic acid.

Indeed, immobilization-stress in the rat induces TDO en TAT via increased cortisol release. Stress increases the level of kynurenine and decreases the level of tryptophan and tyrosine. Cold-stress (48 h) decreases plasma tyrosine and tryptophan and increases kynurenine and xanthurenic acid in urine (58).

Mental depression coincides with decreased tryptophan level while a decreased kynurenine level was observed as well. The decreased kynurenine level was normalized by pharmacotherapy with antidepressives. Paroxetine and other anti-depressives inhibit TDO and increase tryptophan level. In patients with anxiety disorder kynurenine level was increased while in endogenous depression it was increased (59). The precursor is tryptophan melatonin is also decreased in depressive patients. Kynurenine and tryptophan are routinely assayed by HPLC.

4.5. NO-synthase

NO is synthetised from L-arginine by NO-synthase (NOS). Until now plasma levels of nitric oxide (NO) have not been used in stress research. This is mainly due to analytical limitations because NO is rapidly degraded in the circulation to nitrate and nitrite which are both food constituents and therefor not typical for increased NO-synthesis. Interestingly though, cortisol inhibits the induction of inducible NOS (iNOS) the enzyme responsible for the bulk of NO-synthesis under pathophysiological conditions. Another relevant observation related to stress is that plasma L-arginine level is decreased for up to 6 h after cessation of stress in the rat
Decreased L-arginine plasma levels have also been reported in mood disorder (62). It is further remarkable that NK-cells in-vitro are cytotoxic only if L-arginine is available (63,64).

Stress increases neuronal expression of NOS in the adrenals and HPA-axis. Inhibitors of NOS increase corticosteron and ACTH fourfold while L-arginine is inhibitory (65). Inhibitors of NOS also stimulate release of CRF and ACTH (i.e. NO inhibits release). Immobilization stress increases the expression of nNOS mRNA in the adrenals 2.5 fold (66,67) and hippocampus (68). Animal studies further show that stress leads to excess consumption of L-arginine. One of the actions of the antidepressive drug paroxetine (serotonin re-uptake blocker) is inhibition of NO by NO-synthase (69). Finally, NO is involved in increased forearm blood flow during mental stress (70).

A major drawback of this interesting NO-pathway as stress marker is its evaluation which is sensitive to artefacts. In principle measurement of its degradation products nitrate and nitrite can be easily performed but both ions are also food constituents. Under restricted control conditions both NO-metabolites can, however, be used to monitor NO-synthase activity. The same holds for L-arginine levels.

Due to the wide interest in NO-research, various commercial assays are available to determine NO-levels. The colorimetric assay of the main metabolites nitrate and nitrite, based on the Griess reaction, is simple, not expensive, and highly reproducible. The high detection limit of this assay of 0.1-1 µM may, however, be an analytical problem to detect low NO-synthase activity.
5. IMMUNOLOGICAL MARKERS

It has been now well established that the immune system is affected by psychological stimuli. Some of these effects are mediated by circulating neurotransmitters and hormones, for which the different types of immune cells, including lymphocytes, have receptors. These changes are generally slow, and sometimes even very slow i.e. changes in the B-cells may take weeks or months to develop. Psychoneuroimmune stimuli mediated by neuronal pathways that innervate lymphoid tissues appear to be the fastest reacting system. The number of lymphocytes, in particular natural killer (NK) cells may be increased within minutes after an acute psychological stressor. These changes are so fast that it is difficult to understand what really happens (18,19). It is possible that the changes are due to cellular migration of immune cells from the blood vessel or from the extravascular space into the blood flow (18,71). Number of immune cells are routinely measured via coulter counter and FACS-analysis, while immunoglobulin levels are assayed via commercially available immuno assays.

5.1. Number of immune cell subtypes

Both meta-analytic (72) and enumerative (5,73) reviews clearly show that chronic stress decreases the number of circulating B cells, T cells, and large granular lymphocytes (NK-cells), to decreased proliferative responses of lymphocytes to several mitogens as well as to decreased natural killer cell activity (NKCA).

In response to acute stress the number of NK cells, i.e. CD57-positive cells, increase by 25 to 40 % (9,18,19). The parameters have returned to baseline values within 15 minutes after termination of the stressor. In general short lasting, relatively mild psychological stressors temporally increase the number of several cell types in peripheral blood. These stressors seem to have in common a predominant but mild effect on arousal and anxiety levels, generally accompanied by increased secretion of catecholamines. Indeed, infusion of adrenaline stimulated the redistribution of lymphocytes into the blood circulation (e.g. (74) indicating that adrenaline is one of the mediators of these effects.

Long lasting effects of changes in the immune system due to (repeated) stress have also been studied. An elegant study, performed by Kiecolt Glaser et al. (75) on persons taking care of
patients with Alzheimer’s disease, showed that this long-lasting stressful situation decreased the proliferative response to mitogens and increased the number of days with infectious disease and other health problems.

Decreased NK cell activity has been related to certain human diseases (e.g. progression of cancer, chronic viral infection and auto-immune disease). In the elderly, it has also been shown that, decreased proliferative responses to mitogens were related to increased levels of mortality and more frequent hospitalization (76,77).

5.2. Immunoglobulins

Like other immune cells, B-cell activity is down regulated by cortisol and inhibition of immunoglobulin synthesis by cortisol has been described (78,79). In general, various stressors negatively affect the serum level of IgA, IgG, IgM due to a diminished immune response during stress, which is probably mediated by increased amounts of cortisol released by the adrenals.

On the other hand, due to the diminished immune response during stress, latent viruses multiply and titers of specific antibodies directed against latent viruses such as Herpes, Epstein Barr and CMV are increased by psychological stress. Increased levels of latent viruses have been frequently and successfully applied as measure of experienced negative stress (e.g. examination stress, Alzheimer’s caregivers, etc). For review see van Amsterdam and Opperhuizen (80).

A most promising immunoglobulin as marker of stress is salivary IgA which is increased for prolonged time after stress (81). In addition to measuring antibody levels against latent viruses, one may also assay specific antibodies directed against de-novo antigens (hepatitis B, flue vaccines) and microorganisms (experimental infection studies).
6. RELATION BETWEEN PSYCHOLOGICAL AND BIOLOGICAL MARKERS

It is challenging and intriguing to relate the amount of stress as assessed by psychometric instruments with the levels of the physiological stress markers. Such relations are, however, not always observed and a number of possible causes of dyscongruence are summarized below.

a. Stressors do not lead per definition to physiological dysregulation as it largely depends on coping behavior/ability. Specific ways of dealing with stress may be differentially associated with biological functions. As shown in Figure 1 the stressor first has to be perceived, interpreted and evaluated before a psychoneuroendocrine responses develops. The subsequent emotional and behavioral response is determined by the subject’s specific coping and defense strategy and this may result in more or less activation of the HPA-axis (2). Activation of the HPA-axis is strongest where efficient coping is not possible, as with severe prolonged stress and the experience of negative affect and the "conservation-withdrawal" or "distress" reaction occurs. (82-85). On the other hand when the subject has to "fight or flight" or to put an effort to control the situation, the sympathetic adrenal medullary system (SNS) is activated that immediately responds to the threatened homeostasis via the release of catecholamines.

b. A wide range of psychological instruments is available to evaluate stressors, respectively stress. Regarding stressors, daily hassles scales usually predict the physiological stress response more adequately than major life event scales. If stress is measured, the relation to physiology depends very much on the type of stress response measured: for instance affective responses (mood-, depression-, anxiety scales), stress complaints scales, general health status, or more specific stress related disease scales (burnout- and chronic fatigue scales).

c. The time course is extremely important and may frustrate the association between the physiological and psychological markers i.e. the hormonal level may change at a time when the stressor is no longer present (86).

d. At later stages of the response, homeostatic mechanisms are active. The slower reactive hormones, such as cortisol, seem to have an important function to dampen the total stress
response. This tail of the acute reaction may, therefore, be regarded as a homeostatic device to restore the physiological dys-balance (87).

e. The physiological marker under consideration may rather be related to the disease propagated by the stressor(s) than to HPA-activation. For instance, in cancer patients the change in NK-cell activity may be initiated either by the stress the patient experiences due to the presence of this severe disease or by the tumor growth itself.
7. BEYOND THE ASSAY OF STRESS MARKERS

Correctly, cortisol represents the golden standard in stress research and the markers outlines above are all related in some way to cortisol. By assaying such cortisol-related markers in addition to cortisol, the neuroendocrinological pathways triggered by chronic stress can be characterized in more detail. One should realize that the physiological system of the body is a dynamic system with multiple feedback loops. So, cortisol release may trigger a hormonal or immunological reaction which follows a quite different time scale i.e. cortisol level may have normalized, while the immune response remains impaired for a much longer period. Thus, the measurement of additional markers provides a more complete picture which enables a better understanding of the pathophysiological effects initiated by stressors.

In addition, such assays may serve as an additional diagnostic tool to differentiate between stress related diseases like depression and chronic fatigue syndrome (CFS). Subjects experiencing chronic stress show hyper-cortisolemia and an exaggerated cortisol response to corticotropin, while CFS-patients rather show decreased plasma cortisol levels and reduced responsiveness to corticotropin (88,89).

Finally, it is an intriguing and yet unsolved question whether the body, in response to chronic stress, launches cortisol as a fine-tuned messenger or just as a blunt and crude down-regulator of immune functions. Measurement of markers of different pathways, modulated by stressful stimuli, may help to find the answer to this question.
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