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Reduced Glucocorticoid Sensitivity of Monocyte Interleukin-6 Production in Male Industrial Employees who are Vitally Exhausted

PETRA H. WIRTZ, PHD, ROLAND VON KÄNEL, MD, PIA SCHNORPFEIL, PHD, ULRIKE EHLERT, PHD, KARL FREY, PHD, AND JOACHIM E. FISCHER, MD, MSC

Objective: Proinflammatory changes are thought to link vital exhaustion with adverse cardiovascular outcomes. Monocytes play a central role in the pathogenesis of atherosclerotic lesions and are a major source of circulating cytokines. We hypothesized that vital exhaustion may alter the regulation of monocyte activity, as measured by lipopolysaccharide (LPS)-stimulated and glucocorticoid inhibited release of the proinflammatory cytokine interleukin-6 (IL-6). **Methods:** In 166 middle-aged apparently healthy men, vital exhaustion was measured by the Shortened Maastricht Exhaustion Questionnaire. Subjects in the highest quartile (highly exhausted, $N = 38$) were compared with those in the second and third quartiles (moderately exhausted $N = 89$) vs. those in the lowest quartile (nonexhausted, $N = 39$) in terms of plasma C-reactive protein (CRP) and tumor necrosis factor-α (TNF-α) levels, and as to IL-6 release after LPS stimulation in vitro. Inhibition of IL-6 release was determined by coincubation with increasing concentrations of dexamethasone. Monocyte glucocorticoid sensitivity was defined as the dexamethasone concentration inhibiting IL-6 release by 50%. **Results:** Highly exhausted individuals had higher CRP levels than nonexhausted subjects ($p = .008$). LPS-stimulated IL-6 release was not significantly different between groups. However, in highly exhausted participants, dexamethasone was less able to inhibit IL-6 release ($p = .010$), and the glucocorticoid sensitivity was lower ($p = .003$) than in nonexhausted subjects. **Conclusions:** In highly exhausted individuals, glucocorticoids exert less suppressive action on monocyte IL-6 release than in nonexhausted subjects. This finding points to altered regulation of monocyte cytokine production as one possible pathway linking exhaustion with atherosclerosis. **Key words:** Vital exhaustion, coronary artery disease, glucocorticoid sensitivity, monocytes, cytokines, interleukin-6.

ANOVA = analysis of variance; **CRP** = C-reactive protein; **GR** = glucocorticoid receptor; **IL-6** = interleukin-6; **LPS** = lipopolysaccharide; **TNF-α** = tumor necrosis factor-α.

INTRODUCTION

Vital exhaustion is characterized by a combination of unusual fatigue, loss of energy, and irritability (1, 2). Longitudinal studies have established a short- and long-term association between vital exhaustion and coronary artery disease (1, 3, 4). Data from patients undergoing angioplasty suggest that inflammatory responses are one biological pathway linking vital exhaustion with cardiovascular disease (3). Exhausted individuals show higher plasma levels of IL-1β and TNF-α than nonexhausted patients (2, 3).

Monocytes play a key role in the pathogenesis of atherosclerosis and are an important source of circulating cytokines (5, 6). The progression from early to more advanced stages of atherosclerosis hinges on the degree of inflammation within a lesion (5, 6). High levels of inflammatory activity are associated with increased plasma levels of cytokines such as IL-6, which induces hepatic production of CRP (7, 8). In prospective studies, plasma IL-6 and CRP levels have been identified as independent risk factors for cardiovascular events (9–11). Monocyte activity is regulated by a variety of stimuli. One of the most potent triggers of proinflammatory activity is LPS (12, 13).

Activated monocytes are effectively downregulated by glucocorticoids (14, 15). Endogenous glucocorticoid release is governed by the hypothalamic-pituitary-adrenal (HPA) axis. Adequate responses of the HPA axis are important to shutoff inflammatory responses (14, 16). Nicolson and van Diest (17) showed reduced release of endogenous cortisol in individuals with vital exhaustion, during baseline circadian secretion and as a trend in response to stress. Such hyporeactivity of the HPA axis may shift an individual's balance between stimulation and downregulation of inflammation toward a proinflammatory state (14, 18). Data regarding the reaction of target tissues to altered patterns of cortisol secretion are scarce. Given the observed association between vital exhaustion and coronary heart disease, circulating monocytes are the primary target cells of interest.

To investigate the effect of various conditions on monocyte activity, researchers have widely used an in vitro whole blood assay, assessing the proinflammatory cytokine production after LPS stimulation and its suppression across a range of glucocorticoid concentrations (19–21). Changes in monocyte reactivity have been found in sepsis and other inflammatory diseases as well as after physical and psychological stress (19, 21, 22). Altered glucocorticoid sensitivity of monocyte immune responses have been shown in constructs that overlap with exhaustion, such as chronic stress, depression, chronic fatigue syndrome, or fibromyalgia (23–26).

We hypothesized that altered reactivity of monocytes to external stimuli and of suppression of monocyte inflammatory response by glucocorticoids could constitute one biological mechanism linking vital exhaustion to the pathogenesis of atherosclerosis. Therefore, we investigated the in vitro monocyte reactivity in highly exhausted, moderately exhausted, and nonexhausted otherwise apparently healthy middle-aged men. To obtain a single in vitro measure to assess monocyte downregulation or glucocorticoid sensitivity, we determined the amount of dexamethasone required to suppress the LPS-stimulated IL-6 release by 50%. The rationale for choosing IL-6 for this investigation was a previous study by Ridker et al (11),

From Department of Behavioral Sciences, Swiss Federal Institute of Technology (P.H.W., R.v.K., P.S., K.F., J.E.F.), Zurich; Department of Clinical Psychology II, University of Zurich (U. E.), Zurich, Switzerland

Address reprint requests to: Joachim E. Fischer, MD, MSc, Department of Behavioral Sciences, Swiss Federal Institute of Technology, Turnerstrasse 1, CH-8092 Zurich, Switzerland. Email: fischer@ifv.gess.ethz.ch

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suggesting that IL-6 is an intermediate endpoint measure for hard cardiovascular endpoints (ie, future myocardial infarction), which has not been shown for any other proinflammatory cytokine.

METHODS

Setting and Participants

A representative sample of 647 men and women from a total of 1760 employees of an airplane manufacturing plant in Southern Germany was invited. Eventually 325 (280 male, 45 female) subjects volunteered to participate. Except for a slightly higher age, there were no baseline demographic differences between volunteers and nonparticipants (data not shown). In this study, we considered only men to avoid hormonal confounding of inflammation activity by intake of oral contraceptives or by the female cycle. All subjects with psychiatric, endocrine, cardiovascular, or other chronic diseases were excluded from this study. We also excluded subjects who were on any hormone therapy, β-blockers, psychoactive drugs, or steroids. This procedure left a study sample of 166 apparently healthy white men, whom we categorized into quartiles based on the self-reported severity of vital exhaustion. In the following, we present the comparison between three groups of individuals as per their exhaustion scores: 1) those in the highest quartile ("highly exhausted," $N = 38$); 2) those in the second and third quartiles ("moderately exhausted," $N = 89$); and 3) those in the lowest quartile ("nonexhausted," $N = 39$) of exhaustion scores. Table 1 provides health factors of the participants.

Experimental Protocol

At the study entry, participants completed a set of questionnaires, including assessment of vital exhaustion. To minimize biases arising from seasonal factors, questionnaire data from all 166 subjects were obtained within 5 days. Self-reported medical history and health behavior (smoking, alcohol intake, and physical activity) were assessed by a 96-item questionnaire derived from the Nurses Health Study (27) and from the Monitoring Trends in Cardiovascular Disease (MONICA) study (28).

Fasting blood samples were collected within the ensuing 3 weeks between 7 AM and 8:45 AM, before the participants began a morning shift, and after a normal working day. To avoid confounding by the circadian cortisol release, we collected blood approximately 2 hours after awakening in all individuals.

Blood was processed by standard techniques with the use of cooled (4°C) citrate tubes for the TNF-α assay. Urine collection for overnight cortisol secretion started at 9 PM the night before blood sampling, and it ended with the inclusion of the first void after awakening (29).

Assessment of Vital Exhaustion

Vital exhaustion was assessed with the Shortened Maastricht Exhaustion Questionnaire (9 items) (30), which was derived from the original Maastricht Questionnaire (21 items). The latter has been widely used to assess exhaustion in patients with myocardial infarction as well as in healthy subjects (1). Scores obtained from the short version correlate well with those from the original 21-item questionnaire ($r = 0.94$, $p < 0.001$, $N = 452$) (30). For the purpose of this study, the nine items were translated into German. Possible answers to each item were "no" (score = 0), "don't know" (score = 1), and "yes" (score = 2), resulting in a maximum score of 18. Rasch models (data not shown) suggested three distinct groups of individuals (highly exhausted, moderately exhausted, and nonexhausted).

Glucocorticoid Sensitivity and Assay

Monocytes are the main cytokine producing cells in LPS-stimulated whole blood (12). To assess glucocorticoid sensitivity of stimulated cytokine production, dexamethasone has been widely used (19–21). Because of its link with cardiovascular end points (31), we chose to study IL-6 plasma concentrations. The whole blood cell culture is an in vitro method to analyze cytokine secretion in a controlled environment as well as a way to study the biological effects of drugs on cytokine release (32, 33). The whole blood assay avoids possible biases during stimulation of monocytes arising from preanalytical steps by mononuclear cell separation and it also preserves the "natural environment" (including hormones) of cytokine-producing cells (33, 34).

The basic principle to assess glucocorticoid sensitivity is to use a stimulant for cytokine release coincubated with particular concentrations of a glucocorticoid. After a defined incubation period, the cytokine content of the supernatant is determined. If a stimulant like LPS is used that predominantly activates monocytes, and if a cytokine like IL-6 is assessed whose main source in whole blood are monocytes (19, 35), then one may be confident that the assay mainly reflects monocyte regulation.

The assay provides three end points: 1) the cytokine release in response to LPS (without glucocorticoid co-stimulation); 2) the characteristics of the inhibition curve plotting the cytokine concentrations against increasing glucocorticoid concentrations; and 3) the estimated glucocorticoid concentration that would exactly inhibit 50% of the LPS-stimulated cytokine release determined from the first point. The latter measure is independent of the absolute cytokine release and has been referred to as the IC_{50} or a single-measure index to describe the glucocorticoid sensitivity. To estimate IC_{50} values from the inhibition curve, we used a logistic function.

In detail, the following method was used: venous blood was collected in heparinized tubes, diluted 1:10 with saline, and subsequently incubated with LPS (*Escherichia coli*, 055:B5, catalog no. L2880, Sigma-Aldrich, Steinheim, Germany). Dexamethasone (catalog no. D8893, Sigma-Aldrich) was added at five different concentrations to a 24-well plate (no. 3047 Becton Dickinson, San Diego, CA). LPS and dexamethasone were then dissolved in a sterile saline solution (NaCl 0.9%, Fresenius Kabi, Stans, Switzerland). Diluted whole blood (400 μl) was added to 50 μl of LPS and to 50 μl of various

TABLE 1. Health Factors of Non-exhausted, Moderately Exhausted, and Highly Exhausted Men

	Nonexhausted VE Scores 0–2 (*Quartile 1*, $N = 39$)	Moderately Exhausted VE Scores 3–10 (*Quartiles 2* and *3*, $N = 89$)	Highly Exhausted VE Scores 11–18 (*Quartile 4*, $N = 38$)	*p* Value
Age, years	38.8 ± 1.6	39.5 ± 0.9	42.5 ± 1.4	.13
Body mass index, kg/m^2	26.48 ± 0.5	25.8 ± 0.3	26.53 ± 0.5	.30
Waist/hip ratio	0.92 ± 0.0	0.93 ± 0.0	0.94 ± 0.0	.39
LDL/HDL ratio	2.94 ± 0.16	2.77 ± 0.10	3.28 ± 0.16	.03
Systolic blood pressure, mm Hg	131 ± 1.9	129 ± 1.4	132 ± 2.3	.40
Diastolic blood pressure, mm Hg	80.3 ± 1.4	82.5 ± 1.1	81.1 ± 1.4	.48
Cigarettes/day	5.7 ± 1.6	5.6 ± 1.0	5.6 ± 1.8	.99
Hemoglobin A1c, %	5.12 ± 0.06	5.19 ± 0.05	5.23 ± 0.07	.71
Urinary cortisol, μg/l	47.5 ± 4.4	46.0 ± 4.8	44.4 ± 3.8	.94
Monocytes, $\times 10^5$/ml	5.92 ± 0.3	6.00 ± 0.16	5.88 ± 0.3	.92

Values are means ± SE. HDL = high-density cholesterol. LDL = low-density cholesterol. VE = vital exhaustion. N = no. of participants.

concentrations of dexamethasone. Final concentrations on the plate were 15 ng/ml LPS and 0, 10^{-10}, 10^{-9}, 10^{-8}, and 10^{-7} mol/l dexamethasone. After a 6-hour incubation time at 37°C in 5% CO_2, the plates were centrifuged for 10 minutes at 2,000 *g* at 4°C (26, 27). The supernatant was collected and stored at −80°C until assayed.

Biochemical Analyses

In vitro levels of IL-6 were determined using commercial enzyme-linked immunosorbent assay (ELISA) kits (BD Pharmingen, San Diego, CA). Highly sensitive assays were chosen to measure in vivo plasma levels of TNF-α (ELISA; Quantikine HS, R&D Systems Europe, Abington, United Kingdom) and CRP (detection limit 0.1 mg/l; Immunolite, DPC Biermann). Low- and high-density lipoprotein, hemoglobin A1c, and urinary cortisol were determined by a commercial laboratory. Monocyte counts were determined from EDTA samples within 3 hours of blood sampling with the use of a cell counter (model SE-9000, Sysmex, Norderstedt, Germany).

Statistical Analyses

All calculations were performed using SPSS version 10.0 and Curve Expert version 1.3 software packages. Data are presented as means ± SE. Results were considered statistically significant at the $p \leq .05$ level; all tests were two tailed. To approximate a normal distribution, values for IC_{50} were log transformed. In case of missing data, cases were excluded listwise. Across the three subject groups, univariate ANOVAs were calculated for health factors (Table 1) as well as for absolute numbers of monocytes, CRP, LPS-stimulated cytokine production, and IC_{50} values. Repeated-measures ANOVA were applied to dose-response curves of dexamethasone inhibition of LPS-induced cytokine production with the noninhibited LPS-stimulated value as a covariate. We applied Huynh-Feldt corrections for repeated measures. Because monocytes are the main source of cytokine production on stimulation with LPS, whole blood cytokine production was corrected for the monocyte count (21).

RESULTS

Study Population and Baseline Characteristics

Table 1 shows the baseline characteristics of the highly exhausted, moderately exhausted, and nonexhausted individuals. We observed a positive association between levels of exhaustion and CRP ($F_{2,156} = 3.726$, $p = .02$), whereas there was no such difference between groups for TNF-α ($F_{2,162} = 0.81$, $p = .45$), likely due to the high variance of plasma TNF-α levels (Figure 1).

Stimulation of Cytokine Production

After controlling for the absolute monocyte count, there was no difference in LPS-stimulated IL-6 release among highly exhausted subjects, moderately exhausted subjects, and nonexhausted individuals (Figure 2).

Inhibition of Stimulated Cytokine Production

Figure 2 shows the inhibition curve with the IL-6 release plotted against increasing concentrations of dexamethasone. Curves were different between groups ($F_{3.7,252.9} = 4.14$, $p = .004$; LPS-stimulated release as a covariate), showing reduced capacity of dexamethasone to inhibit IL-6 release in highly exhausted men as compared with nonexhausted subjects ($p = .010$).

Glucocorticoid Sensitivity of Cytokine Release

The IC_{50} of IL-6 was different among the three groups ($F_{2,142} = 5.3$, $p = .006$; Figure 3). Highly exhausted subjects

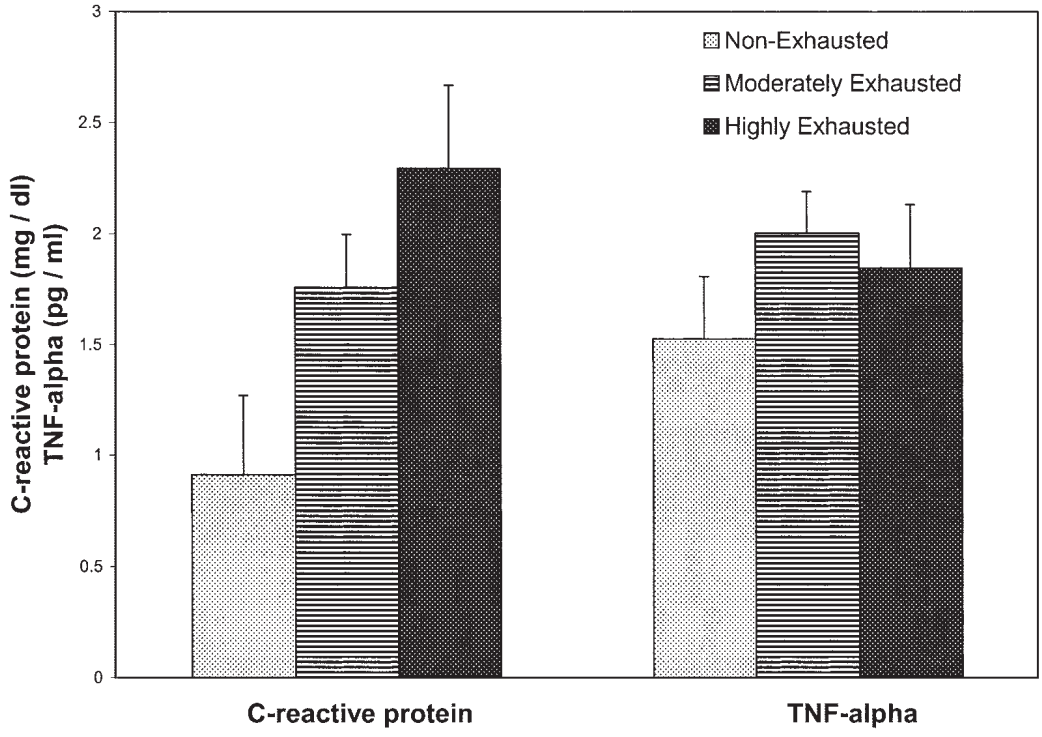

Fig 1. Plasma levels of C-reactive protein (CRP) and of unstimulated tumor necrosis factor-alpha (TNF-α) in the three groups (mean ± SEM). Highly exhausted individuals had higher levels of CRP ($p = .008$) than nonexhausted subjects, whereas there was no significant difference between groups in terms of TNF-α levels ($p = .446$).

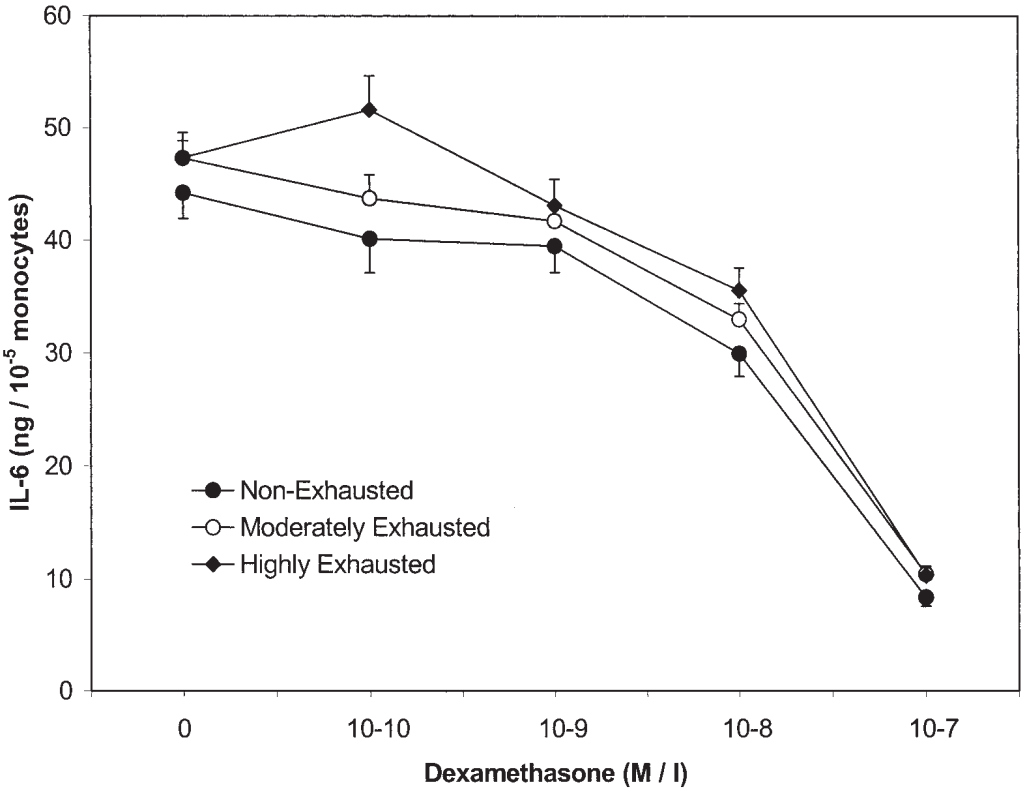

Fig. 2. LPS stimulated release of interleukin-6 (IL-6) and inhibition of IL-6 release by dexamethasone in the three groups (mean ± SEM). While there was no significant difference of IL-6 stimulation by LPS between groups, IL-6 release was less suppressed by dexamethasone in the highly exhausted subjects than in the nonexhausted subjects ($p = .010$).

had higher IC_{50} of IL-6 than nonexhausted subjects ($p = .003$). In other words, more dexamethasone was required to suppress IL-6 release in response to the same LPS stimulus in highly exhausted subjects as compared with nonexhausted individuals. Thus highly exhausted men had lower monocyte glucocorticoid sensitivity than nonexhausted men.

DISCUSSION

In this study, we compared the monocyte glucocorticoid sensitivity among highly, moderately, and nonexhausted apparently healthy men. We used an in vitro assay to study monocyte IL-6 release in response to a standardized dose of LPS, and the extent to which monocyte IL-6 release was inhibited by increasing concentrations of dexamethasone. IL-6 exerts a multitude of effects on lymphocytes and monocytes, the main effector cells of the early stages of atherosclerosis. In contrast to other proinflammatory cytokines, elevated plasma levels of IL-6 have been prospectively associated with increased risk for future myocardial infarction in apparently healthy men (11). The main finding of our study was that highly exhausted subjects required larger quantities of the glucocorticoid to inhibit LPS-stimulated IL-6 release than nonexhausted men, whereas the IL-6 release without dexamethasone coincubation was similar in the two groups.

What are the potential clinical implications of these findings and how do they relate to the observed positive dose-response relationship between exhaustion scores and plasma levels of CRP? The latter finding corroborates previous observations on increased levels of inflammatory markers in vital exhaustion (2, 3). The finding of a similar IL-6 response per monocyte in nonexhausted and highly exhausted men indicates that vital exhaustion does not affect the ability of monocytes to respond to an LPS signal, eg, from bacteria present within an atherosclerotic lesion. However, if proinflammatory activity is not appropriately shut off, but continues to smolder as a chronic inflammatory process, atherosclerosis may advance more rapidly. Consistent with this reasoning, elevated plasma levels of IL-6 and of CRP are associated with increased coronary risk (9–11).

We showed reduced glucocorticoid sensitivity in highly exhausted men, indicating that the same amount of glucocorticoid was less effective in shutting off LPS-stimulated production of IL-6. Because the major sources of cytokines after LPS stimulation are monocytes, this finding implies that the monocytes of highly exhausted men are more likely to continue producing IL-6 after having encountered a stimulus (eg, within an atherosclerotic lesion) than those of nonexhausted individuals.

We offer two possible biological mechanisms that might underlie this impaired ability to downregulate LPS-stimulated IL-6 release in monocytes of exhausted individuals. First, cellular GR expression may be downregulated in exhausted subjects by elevated endogenous glucocorticoid production. According to Appels (3), vital exhaustion may reflect a break-

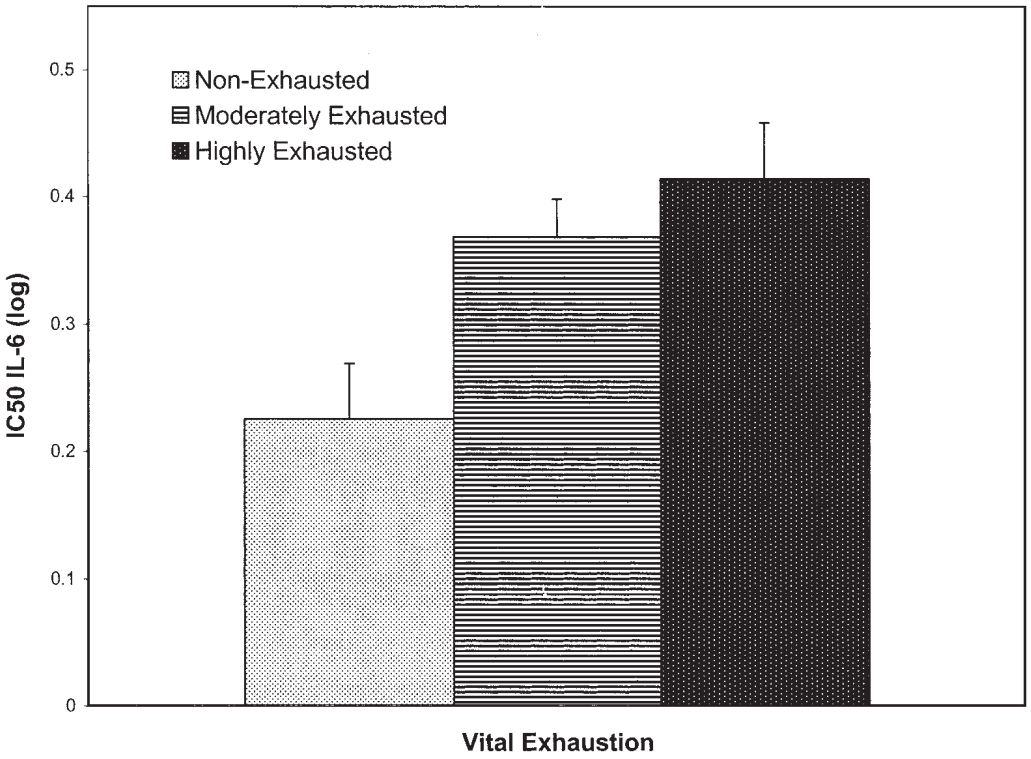

Fig. 3. Glucocorticoid sensitivity of LPS-stimulated release of interleukin-6 (IL-6) in the three groups. The IC_{50} is inversely related to glucocorticoid sensitivity; ie, higher IC_{50} indicates lower glucocorticoid sensitivity and vice versa. Highly exhausted subjects required relatively more dexamethasone to suppress LPS-stimulated IL-6 release than nonexhausted individuals ($p = .003$). In other words, monocytes of highly and of moderately exhausted subjects were less sensitive to dexamethasone suppression than monocytes of nonexhausted men.

down of the body's adaptation to prolonged mental stress. Chronic mental stress is accompanied by increased endogenous glucocorticoid secretion (36, 37). An environment with increased glucocorticoid levels effectively downregulates the level of GR expression (38) by mechanisms reviewed in detail elsewhere (39). Thus the first possible biological explanation of our findings is a reduced GR expression arising from elevated endogenous glucocorticoid release during periods of sustained stress leading to vital exhaustion.

The second possible explanation refers to the effect of cytokines on monocyte dexamethasone sensitivity. In a carefully conducted experiment, Franchimont and coworkers (40) showed that the presence of TNF-α decreases the sensitivity of LPS-stimulated monocyte-IL-6 production to dexamethasone. Our highly exhausted subjects had significantly higher plasma levels of CRP, indicating increased inflammatory activity. We observed a tendency toward higher plasma-TNF-α-levels in highly exhausted subjects, although this observation failed to reach statistical significance. We speculate that circulating monocytes of our exhausted individuals might have been primed by relatively higher TNF-α in vivo before being subjected to the in vitro condition. The study by Franchimont and coworkers (40) showed that 48 hours after exposure to TNF-α, the number of GR receptors on monocytes decreased by 60%. On a cellular level, the development of a TNF-α-mediated glucocorticoid resistance is probably related to the activity of nuclear factor-κB (NF-κB) (40, 41). NF-κB is highly expressed in inflamed tissues (42) and is involved in the transcription of many proinflammatory cytokines (including IL-6) (14, 43). Mediated via a TNF-responsive NF-κB DNA binding site, GRβ mRNA increases to a greater extent than does GRα mRNA. The increase in GRβ protein expression correlates with the development of glucocorticoid resistance and a reduction in the number of functional GR receptors on the cell surface (41).

These two biological explanations may help integrate our findings on elevated unstimulated levels of inflammatory markers, on one hand, and on reduced glucocorticoid sensitivity, on the other hand, with Appels' two-stage model in terms of the observed association between vital exhaustion and acute coronary syndromes (3). The cascade of events proposed by Appels starts with prolonged overexertion and stress. Overexertion and stress induce elevated endogenous glucocorticoid secretion and stress downregulates monocyte GR expression (38). Reduced GR expression leads to reduced sensitivity of monocyte cytokine secretion. As a consequence, monocyte activity at sites of inflammation (eg, within atherosclerotic lesions) would be less effectively downregulated. This may increase plasma levels of proinflammatory cytokines, in particular TNF-α and IL-6. Circulating monocytes exposed to and primed by elevated levels of TNF-α will also reduce their glucocorticoid sensitivity. The altered glucocorticoid sensitivity thus may be maintained–even if the cortisol secretion is no longer increased. In extremely exhausted indi-

viduals, both decrease in basal cortisol levels (23, 44) and blunted reactivity of the HPA axis to acute mental stress (23, 45) have been observed. The blunted glucocorticoid sensitivity probably contributes to maintaining a chronic proinflammatory state with persistent exhaustion and malaise (ie, "sickness behavior").

Several limitations of the present study require consideration. First, the data are of cross-sectional nature and therefore do not prove the sequence of events as outlined above. For example, we could not investigate whether there was a prospective association between changes in monocyte reactivity with alterations in levels of exhaustion. We also lack data on the concentration of GR receptors on harvested monocytes. Our investigation focused on apparently healthy men. The findings may, therefore, not be generalized to clinical populations and individuals with overt atherosclerotic disease. Furthermore, it can only be speculated whether the observed differences between exhausted and nonexhausted subjects are of clinical importance.

In summary, our data show that otherwise healthy highly exhausted men have a reduced responsiveness of monocytes to glucocorticoids. This reduced glucocorticoid sensitivity implies relatively sustained cytokine production once monocytes have encountered stressful stimuli (eg, LPS). Aside from the also elevated plasma levels of CRP, such a mechanism may be one possible biological pathway linking vital exhaustion with progression of atherosclerosis and coronary artery disease.

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