SPONTANEOUS AND LPS-STIMULATED INTERLEUKIN-6 PRODUCTION IN PERIPHERAL BLOOD MONONUCLEAR CELLS (PBMC) AND DEREGULATION OF THE HYPOTHALAMIC-PITUITARY-ADRENAL AXIS IN PATIENTS WITH MAJOR DEPRESSIVE DISORDERS

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DEREK HUMPHREYS, LIANA SCHLESINGER, MARCELO LOPEZ, A. VERONICA ARAYA

Institute of Nutrition and Food Technology (INTA), University of Chile and Institute of Nutrition and Food Technology (INTA), University of Chile

Derek HUMPHREYS, MD, PhD. University of Chile

Liana SCHLESINGER, MD. Institute of Nutrition and Food Technology (INTA), University of Chile

Marcelo LOPEZ, PhD. Institute of Nutrition and Food Technology (INTA), University of Chile

A. Veronica ARAYA, MD. Institute of Nutrition and Food Technology (INTA), University of Chile

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Running title: IL-6 and deregulation of the HPA axis in major depression

Abstract

The study of reciprocal relationships between the immune and neuro-endocrine systems has been of heuristic value in understanding the neurochemistry of major depression. There is now some evidence that major depression, disorder characterized by hypothalamic-pituitary-adrenal (HPA) axis hyperactivity, might be accompanied by a shift in immune system response with increased production of pro-inflammatory cytokines. To examine the complex reciprocal relationships between immune and HPA axis function in Major Depressive Disorder (MDD), the present study was designed to determine whether HPA axis disregulation is associated with altered cytokine production in 9 MDD patients and 11 control subjects, all drug-free. HPA axis activity, assessed through plasma cortisol, dexamethasone suppression test and ovine Corticotropine-Releasing Factor (oCRF) stimulation test, and basal and LPS-stimulated production of IL-1β, IL-6 and TNFα by peripheral blood mononuclear cells (PBMC) were determined. Basal production of IL-6 was significantly higher in depressive patients than in normal volunteers (3541.2$\pm$726.8 vs 380.4$\pm$77.5 pg/ml, $p<0.05$) but, the LPS-stimulated production was significantly lower (19867.7$\pm$3649.2 vs 33142.2$\pm$1547.2 pg/ml). There were no significant differences in IL-1β or TNFα production. No significant difference was found between depressive and controls for 24 h free urinary cortisol, diurnal cortisol rhythm and dexamethasone suppression response. The adrenocorticotropic hormone (ACTH) response to oCRF intravenous injection, evaluated as the area under the curve was decreased in patients but, a non significant statistical difference was found between both groups ($p=0.06$). These results suggest that in chronic stress conditions, a deregulation in spontaneous IL-6 production, can contribute to the HPA axis dysfunction of the depressive states.

Introduction

The state of the art in neuro-sciences establishes a clear association between Major Depressive Disorders (MDD) and hyperactivity of the Hypothalamus-Pituitary-Adrenal (HPA) axis (1-5), main homeostatic system of the organism (6,7,8). An early escape of cortisol to dexamethasone suppression (9,10), high circadian patterns for plasma cortisol and adrenocorticotropic hormone (ACTH) (3,4,11,12) and high levels of corticotropin realesing factor (CRF) in cerebro-spinal fluid have been described in depressive patients (13). CRF elevation is speculated to be of supra-hypothalamic origin, since depressive patients show a weak ACTH response to CRF injection. This unbalance has been interpreted as a central feedback dysfunction probably due to CRF-receptor downregulation (14). According to these findings, it has been postulated that CRF, a neurpopeptide of endocrine, neurochemical, immunological and behavioral effects, would be inadequately down-regulated in major depression, resulting in a persistent activation of the HPA-axis (5,6,15).

Pro-inflammatory cytokines have endocrine, electrophysiological and behavioral effects (14,16,17). They are able to activate the HPA-axis (7) at the central level, where they can act on the hypothalamus or the pituitary stimulating CRF or ACTH secretion, and they can stimulate steroidogenesis directly on the adrenal cortex, where interleukin receptors have been identified (18,19). Based on this evidence, and considering that the acute administration of cytokines may reproduce many of the physical symptoms of depression**,** it has been suggested that cytokines play an important role in the pathophysiology of depression **(16,20,21**). But, although high levels of IL-6, IL-1 and acute phase proteins have been reported in depressive disorders, a typical cytokine profile has not yet been determined and the correlation between cytokine production and HPA axis function is still subject of investigation.

Since progress in neuroscience has associated depression to a situation of physical or psychological chronic stress, in the formulation of the present study we considered an integrated view including endocrine, immune and psychiatric aspects of depression. Our aim is to determine if pro-inflammatory cytokines production is related to HPA axis dysfunction in MDD.

Patients and Methods

Patients.

A psychiatrist selected 9 patients, mainly of hispanic origin, 2 males and 7 females, aged between 19 to 46 years, with Major Depressive Disorder diagnosed according to DSM – IV criteria (22). The 21-item Hamilton Depression Rating Scale (HDRS) (23) and the Montgomery-Asberg Rating Scale for Depression (MADRS) (24) assessed severity of depression. Patients with other axis I diagnosis, with major medical illness or endocrine disorders, high suicide risk, and those who had taken anti-depressants, lithium salts, anticonvulsants or neuroleptics during the last 12 months were excluded from the study. All patients were free of drugs known to interfere with immune or endocrine function, they had never taken major psychotropic drugs (except benzodiazepines), and were not regular drinkers.

Eleven healthy controls, 3 males and 8 females, in the same range of age, were selected from volunteers with no lifetime history of axis II or I disorders and no history of axis I disorder in first-degree relatives. They were in good physical health and had not been exposed to any psychotropic medication in the last year.

For at least three weeks prior the study, all the participants did not receive non-steroidal antiinflatory drugs or glucocorticoids and they were free of any infectious disease.

Subjects with twenty-four hour free urinary cortisol (FUC) over 100 μg/24 hour were excluded from the study.

HPA Assessment

Twenty-four hour urine collections for measurement of FUC excretion were obtained during the first study day. Subjects were then demanded to come to the Endocrinology Unit of the University of Chile Clinical Hospital at 8 a.m., 3 p.m. and 11 p.m. for three separate plasma cortisol determinations. After the last baseline cortisol sampling, 1 mg. of oral dexamethasone (Oradexon®) was administered to all subjects at 11p.m and plasma cortisol levels were determined 9 hours later, according to Carroll's protocol for the dexamethasone suppression test (DST)(9).

Patients were required to return at 8 a.m. to the same hospital a week later to perform the Ovine-Corticotropine-Releasing Factor (oCRF, Bachem, Torrance, CA) stimulation test. An intravenous heparinized cannula was then inserted in each arm. After 30 minutes of supine rest period, baseline samples were obtained (-15 and 0) for basal cortisol and ACTH assessment. oCRF was administered as an intravenous bolus injection of 1μg per kilogram of body weight. Blood samples were obtained at 5, 15, 30, 45 and 60 minutes for measurements of ACTH and cortisol responses (25). An additional blood sample was drawn before the injection of oCRF for cytokine assessment. Plasma was stored at –20°C and processed within the first thirty days from sampling.

Cortisol and ACTH determinations

Cortisol and ACTH were determined by commercial chemoluminescent enzyme immunometric assays (Immulite, Diagnostic Products Corporation, Los Angeles, CA). The intraassay variation coefficient was 7%, with an interassay coefficient of 10% for both hormones.

Cytokine measurements

Induction of IL-1, TNF and IL6

Peripheral blood mononuclear cells (PBMC) were isolated by Hypaque gradient (Sigma Diagnostics, St Louis MO), washed twice in PBS, and 2.5 x 10^6 cells/ml were resuspended in RPMI 1640 culture medium supplemented with gentamicin, glutamine 0.5%, heat-inactivated AB plasma and 1 ug/ml indomethacin. Two hundred μL of cell suspension were dispensed in 96-well flat-bottom microtiter plates in the absence (spontaneous cytokine release) and presence of endotoxin: lipopolysaccharide (LPS, Sigma) per well for 24 hours at 37°C in 5% CO2/95% air. Cell viability after incubation was over 90%. Supernatants were collected and stored at –20ºC until assay for pro-inflammatory cytokines.

Cytokine Determinations

All cytokine determinations were performed using specific, commercial enzyme-linked immuno-absorbent assays (ELISA): (RD Systems, Minneapolis, MN), the assay sensibility being 3.6 pg/ml).

Statistical Analysis

Results are expressed as mean±SE**.** Parametric results were assessed by analysis of variance (ANOVA). The total and integrated ACTH and cortisol responses to oCRF were calculated by the trapezoid method and expressed as the area under the concentration-time curve (AUC) from 0-60 min. The Mann-Whitney statistic test was used to assess the significance of mean differences among both groups with 95% confidence interval. Relationships between variables were assessed by means of Spearman's rank order correlation coefficient. A p-value of less than 0,05 was considered as statistical significant.

Ethical Considerations

This study was approved by the Ethics in Human Research Committee of the Institute of Nutrition and Food Technology, University of Chile, in Santiago de Chile. Informed written consent form was obtained from each patient and control after the aims of the project had been fully explained to them.

Results

There were no significant differences in age, gender, 24-hour FUC, or plasma cortisol circadian variations (8 a.m., 3 p.m. and 11 p.m) between depressive patients and control subjects (Table 1), but, depressive patients, had higher 11 p.m. cortisol level than controls. Patients and controls were both suppressors to dexamethasone under 4 μg/dl, but we observed a suppressive response to undetectable levels of cortisol only in controls.

HPA assessment

The responsiveness of the pituitary and adrenal components of the HPA axis to administration of oCRF is shown in fig 1. The ACTH and cortisol response curve to oCRF was flatter and delayed in major depressives compared to controls. The response difference was significant at 5 minutes post stimulus for ACTH (Fig 1a) and at 45 min. for cortisol (Fig 1b) ($p < 0,05$). The AUC_{ACTH} was decreased in major depressives compared with controls; 8341±3389.9 pg/ml/min versus 18610±4104.8 pg/ml/min but, the difference was not significant (p=0.06). The $AUC_{cortisol}$ was similar in both groups, 1146.8.2±113.6 μg/dl/min in major depressives, and 1391.8±1123 μg/dl/min in controls (NS).

Cytokine measurements

PBMC spontaneous IL-6 production was significantly higher in major depressives with mean values of 3541.2±726.8 pg/ml as compared to a mean value of 380.4±77.5 pg/ml in control subjects ($p<0.001$). LPS-induced IL-6 production by PBMC was significantly lower in major depressives 19867.7±3649.2 pg/ml *versus* 33142.2±1547.2 pg/ml in healthy controls, difference that was significant ($p < 0,005$) (Fig 2).

A positive correlation between $ACTH_{AUC}$ after oCRH injection and LPS-activated IL-6 secretion was observed in major depressives, with a Spearman R of 0,75 ($p <0,005$) (Fig. 3). We found no correlation between these variables in control subjects (Fig. 3b).

No differences in LPS-stimulated IL-1β and TNFα production by PBMC between major depressives and controls could be determined. Spontaneous production was undetectable for both cytokines.

Discussion

The most consistent finding of our study was higher spontaneous IL-6 secretion by PBMC in depressive patients as compared to controls. Conversely LPS stimulated IL-6 secretion was lower in depressives. No differences in IL-1β or TNF-α production between depressives and

controls were found. An association between altered IL-6 secretion and HPA axis disorder could be established according to the criteria defined for this study.

The alteration of immune parameters in depressive disorders has been widely discussed elsewhere (5,16,20,21,26,27). Pro-inflammatory cytokine production currently represents the main subject of interest regarding this association: high levels of IL-6, IL-1 and acute phase proteins have been reported in depressive disorders. Nevertheless, no characteristic cytokine profile for depression has been determined.

The high spontaneous secretion of IL-6 in depressives found in our study agrees with the report from Maes et al (28). In another report, that included depressive inpatients under antidepressant therapy, Maes et al (16) described high IL-6 secretion in PHA-stimulated PBMC; and Seidel et al (29) have reported slightly elevated IL-1 and IL-6 levels in activated PBMC from depressive inpatients. We think, however, that skew problems should be considered in the interpretation of these studies, since inpatients represent a particular group of depressives, including chronic, treatment-resistant depressive disorders, patients with suicidal ideas, major sleep disorders and significant weight loss (30). To avoid this situation, our study design considered strict inclusion and exclusion criteria, resulting in a reduced number of patients and controls. Only healthy subjects, free of any antidepressive or hormonal therapy and within an age range normally not affected by the beginning or decline of the gonadal function were included in our study. Antidepressants are known to interfere with CRF receptor balance and are able to modify cytokine production (2,6,20). Oral contraceptives can increase pro-inflammatory cytokine production in LPS stimulated PBMC, and also with plasma cortisol determination through an increase in Cortisol Binding Globulin levels (31). Both are widely used drugs in our country. Amongst patients, only major depressives with no personality disorders were included. Moreover, two depression scales were used as a means of homogenization of the study group, considering that the severity of depression has been associated to HPA axis unbalance (20,32). Other studies on whole blood production of cytokines have also reported altered serum levels of IL-6 in depressives (33-38), but unfortunately, disaccording inclusion criteria and methodological difference with our design do not allow direct comparisons with those results. We think that serum cytokine determinations can not be

directly compared with the spontaneous production obtained from blood cells in culture. Spontaneous cytokine production could thus hypothetically explain high plasma levels of IL-6 reported in depression, but this remains a subject of speculation (39).

Based on the differences between spontaneous and stimulated cytokine production observed in our study, it could be argued that lower IL-6 secretion under conditions of stimulation with LPS are due in part to the chronic hyperactivity of the HPA axis. Considering the fact that no intracellular storage system exists for cytokines, we assume that the chronic stress underlying the depressive disorder could lead to a continuous spontaneous maximal IL-6 secretion, and a decreased LPS response.

An association between altered pro-inflammatory cytokine production and the modulation of the stress system in depressive disorders has been postulated. Our study showed a clear association between the functional HPA axis dysfunction and altered cytokine secretion, as manifested by the correlation between blunted $ACTH_{AUC}$ (40,41) and secretion of IL-6 by activated PBMC in major depressive patients but not in controls. It has been established in animal models that IL-6, a pleiotropic cytokine of immune and non-immune origin, is able to produce an increase in ACTH and corticosterone in the acute response, but in the chronic state might decrease the ACTH response, suggesting that high levels of this cytokine could modulate the activity of the HPA axis (2,42,43). IL-6 affects then CRH, ACTH and cortisol secretion (44) permanently, leading to a more global unbalance of the regulatory mechanisms evidenced as an attenuated ACTH response to oCRH injection, as seen in our patients.

These findings support the cytokine theory for depression, which establishes a pro-inflammatory cytokine disorder resulting in the HPA dysfunction that underlies the unbalance of neuro-transmission systems traditionally associated to the manifestations of depressive disorders (16,45). An alteration in the contra-regulatory mechanism of cytokines is assumed in the formulation of this theory; glucocorticoids should normally inhibit IL-6 production by PBMC, while in depressives there is a coexistence of increased spontaneous cytokine production and HPA hyperactivity (27). This theory is further supported by the fact that increased IL-6, leading to acute phase protein synthesis, is associated to a sickness behaviour syndrome that reproduces many of

the somatic and vegetative symptoms of the mood disorders. Accordingly, our results suggest that chronic increase of basal IL-6 production could affect the normal regulatory process of HPA function, thus maintaining high cytokine levels in spite of the regulatory effects of cortisol, which are at maximum functional levels, as represented by the blunted ACTH response to oCRH.

Although there were not significant differences in cortisol levels between patients and controls, depressives tend to show slightly higher plasma cortisol levels at 11 pm in depressives. The outpatient sampling conditions must be considered in the evaluation of this variable. Since healthy controls were exposed to daily stress, including that of coming to the hospital three times a day, one would expect higher cortisol levels in this group.

Considering the criteria defined by Carroll et al. for the 1 mg dexamethasone suppression test in depression, our study did not find Non-suppressors (NS) amongst depressives. Recently, different groups have proposed a cut-point lower than 4 μg/dl as suppression criteria for normal subjects, varying from 1.8 to 3 μg/dl (46-49). In agreement with this, we found that almost all the control subjects suppressed cortisol to undetectable levels and depressives showed cortisol levels over 1 μg/dl after dexamethasone. Ethnical differences in biodisponibility and pharmacokinetics of dexamethasone should also be considered. Other reports have even suggested the need to consider sub-types of depression in the interpretation of the test, since only 14% of depressive outpatients, 36% of major melancholic inpatients, and 41% of depressives with psychotic symptoms were NS in recent reports (50,51).

No difference was observed between patients and controls for the $cortisol_{AUC}$, but the difference for $ACTH_{AUC,}$ decreased in patients, indicates a tendency (p= 0,106), though not significant, considering the reduced number of cases. As it should be expected, the difference in response appears earlier for ACTH (5 min.) than for cortisol (45 min.). This could point to the direct action of IL-6 on the steroidogenic activity in the adrenal cortex.

In conditions of chronic stress such as the model of major depression proposed in this study, HPA axis regulation is altered. The blunted response of ACTH to oCRF, the increase spontaneous IL-6 production and the decrease Il-6 production after LPS challenge established in our study, is a manifestation of the chronic hyperactivity of this axis, main stress regulatory system

of the organism. Interleukin-6 determination could be considered a marker of severity of the deregulation of this system, considering that cortisol levels and dexamethasone suppression test show a great variability, are difficult to interpret or are in the case of a CRH response test too expensive to be used in ordinary practice.

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Table 1. Demographic, clinical and baseline laboratory profile of major depressive patients and control subjects

	Depressive Patients (n = 9)	Control Subjects (n = 11)
Gender (M/F)	2/7	3/8
Age	31	33
HDRS-21	35 ± 3,8	5,3 ± 1,8
MADRS	35 ± 4,6	5,3 ± 2,1
FUC μg/24 h)	54,9 ± 21,9	55,6±18,3
Plasma cortisol (μg/dl)		
8 am	20,1 ± 3,7	19,5 ± 7,7
3 pm	9,9 ± 2,3	9,6 ± 4,0
11 pm	4,8 ± 2,1	3,6 ± 1,9
Suppressors to Dexamethasone (< 4 μg/dl)	9/9	11/11

HDRS: Hamilton Depression Rating Scale; MADRS: Montgomery-Asberg Rating Scale; FUC: Free Urinary Cortisol