ORIGINAL ARTICLE

In humans, corticotropin releasing hormone antagonizes some of the negative immunoregulatory effects of serotonin

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Abstract
Both corticotropin releasing hormone (CRH) and serotonin (5-HT) participate in the stress response and are known to modulate cytokine release by human immune cells. Extracellular 5-HT concentrations at or above the serum values have negative immunoregulatory effects by inhibiting the production of interferon-γ (IFNγ), a pro-inflammatory cytokine produced by Th-1-like lymphocytes, whereas 5-HT has no significant effects on the production of interleukin-10 (IL-10), an anti-inflammatory cytokine. In one study, CRH significantly decreases IFNγ production by cultured human peripheral blood immunocytes, whereas in other studies CRH increases the production of cytokines, such as IL-1, IL-2 and IL-6. The aims of the present study were to examine
i) the effects of CRH, 10^{-9} M, 10^{-8} M and 10^{-7} M, on the stimulated production of IFNγ, IL-10 and tumor necrosis factor-α (TNFα) by human whole blood; and
ii) whether CRH, 10^{-9} M, 10^{-8} M and 10^{-7} M, may antagonize some of the negative immunoregulatory effects of 5-HT, 1.5 μg/mL or 15 μg/mL.
We found that CRH, 10^{-9} M, 10^{-8} M and 10^{-7} M, had no significant effects either on the stimulated production of IFNγ, IL-10 or TNFα or on the IFNγ/IL-10 production ratio, which reflects the pro-inflammatory capacity of the culture. 5-HT, 1,5 μg/dL and 15 μg/dL, significantly suppressed the production of IFNγ and TNFα and the IFNγ/IL-10 production ratio. CRH, 10^{-7} M, significantly reversed the 5-HT (1.5 μg/mL and 15 μg/mL)-induced suppression of IFNγ production. CRH at all concentrations significantly blocked the 5-HT (1.5 μg/mL and 15 μg/mL)-induced suppression of TNFα production. The results suggest that CRH has no significant direct effects on the production of IFNγ, IL-10 and TNFα, but antagonizes the negative immunoregulatory effects of 5-HT on the production of IFNγ and TNFα and on the IFNγ/IL-10 production ratio.

Introduction

Corticotropin-releasing hormone (CRH), serotonin (5-HT), and the cytokine network participate in the response to psychological stress. Acute and repeated/chronic psychological stressors not only increase the production or release of CRH and 5-HT, but also enhance the synthesis or release of pro-in-

flammatory cytokines, such as interleukin-1 (IL-1), IL-6, interferon-γ (IFNγ) or tumor necrosis factor-α (TNFα) in humans or experimental animals [1–3]

CRH plays a pivotal role in integrating stress-related responses throughout the neuro-immune-endocrine axis, i.e. it acts in the CNS to integrate the autonomic, endocrine, immune and behavioral responses to stress [1]. There is some evidence that CRH may induce stimulation of some immune functions, such as lymphocyte proliferation [4]. CRH, *in vitro*, may enhance the unstimulated or stimulated production of IL-1 and IL-6, two pro-inflammatory cytokines mainly produced by monocytes, and IL-2 and the IL-2 receptor (IL-2R), produced by T cells [4–8]. In another study, however, it was found that CRH inhibits LPS-induced IL-1 and IL-6 production from human mononuclear cells and that these effects were reversed by a CRH receptor antagonist [9]. The addition of CRH to peripheral blood mononuclear cells (PBMCs) significantly decreases the production of IFNγ, a pro-inflammatory cytokine produced by Th-1-like lymphocytes [8].

Different acute and repeated/chronic stressors increase extracellular 5-HT in areas tightly linked to the control of anxiety, i.e. the prefrontal cortex, the dorsal and median raphe nuclei and the amygdala [2]. Recently, it has been shown that 5-HT has negative immunoregulatory effects through suppression of the stimulated production of IFNγ and, consequently, reduces the IFNγ/IL-10 production ratio [10], which reflects the pro-inflammatory capacity of the immunocytes producing these cytokines [11]. Some of these effects may be obtained through enhancing the production of cAMP in immunocytes: i) 5-HT stimulates the cAMP-dependent PKA pathway through different 5-HT receptors, e.g. 5-HT4, 5-HT6 and 5HT7 [12]; and ii) elevated cAMP augments the synthesis and levels of IL-10 while decreasing those of IFNγ and other pro-inflammatory cytokines, such as tumor necrosis factor-α (TNFα) [13–16]. However, to the best of our knowledge, no research has examined the effects of CRH on other cytokines, such as IL-10 and TNFα, and no research has examined whether CRH may antagonize the negative immunoregulatory effects of 5-HT.

The aims of the present study were to examine whether

i) CRH modulates the stimulated production of the pro-inflammatory cytokines, IFNγ and TNFα, and of the anti-inflammatory cytokine, IL-10; and

ii) CRH may reverse the negative immunoregulatory effects of 5-HT.

Subjects and Methods

Subjects

In the present study, 17 normal volunteers, i.e 8 women (mean age=23.3 ±1.8 years) and 9 men (mean age=28.2 ±7.4 years) participated. After an overnight fast, they had their blood sampled for the assay of the production of various cytokines by stimulated whole blood. The volunteers had a negative past and present history of axis-I psychiatric disorders, including depression, psychoses and substance abuse disorders [17]. They were non-smokers and had never been taking antidepressant or antipsychotic drugs. None was a regular drinker. The volunteers were free of chronic medical illness or drugs which modulate the immune or endocrine systems. They were free of infections, inflammatory or allergic reactions for at least two weeks. The volunteers abstained from caffeine and alcohol for at least 12 hr before blood sampling. The study design was approved by the local Ethical Committee and the volunteers gave written informed consent after the study design was fully explained.

Methods

Diluted whole blood for the assay of the stimulated production of the cytokines, IFNγ, TNFα and IL-10, was taken at 9 a.m. (±30 minutes). We examined the effects of CRH, 10^{-9} M, 10^{-8} M and 10^{-7} M (Sigma, Bornem, Belgium), and 5-HT, 1.5 μg/mL and 15 μg/mL (Sigma, Bornem, Belgium), alone and together, on the stimulated production of those cytokines. Toward this end we stimulated 1/4 diluted whole blood with PHA (1 μg/ml; Murex Diagnostics Ltd, Dartford, England) and LPS (5 μg/ml; E.coli 026:B6; lyophylized and sterilized by gamma-irradiation; Sigma, Belgium). We placed 0.75 ml RPMI-1640 medium with L-glutamine, PHA + LPS, 100 IU/mL penicillin and 100 μg/mL streptomycin (BioWhittaker, Verviers, Belgium) in 24 well cell culture plates (Falcon 353047, Becton Dickinson). CRH and 5-HT were dissolved in sterile medium, whereas medium alone served as the control. 20 μl of each of these solution was added to the wells and gently mixed with the medium. 0.25 mL of whole blood from each of the volunteers was cultured with three concentrations of CRH, 10^{-9} M, 10^{-8} M and 10^{-7} M, two concentrations of 5-HT, 1.5 μg/mL and 15 μg/mL, and the combinations between these conditions, i.e. CRH 10^{-9} M and 5-HT 1.5 μg/mL; CRH 10^{-9} M and 5-HT 15 μg/mL; CRH 10^{-8} M and 5-HT 1.5 μg/mL; CRH 10^{-8} M and 5-HT 15 μg/mL; CRH 10^{-7} M and 5-HT 1.5 μg/mL; CRH 10^{-7} M and 5-HT 15 μg/mL. The concentrations of 5-HT and CRH employed here were based on previous literature showing *in vitro* effects of these agents on the cytokine network [4, 5, 7, 8, 10]. We incubated the above solutions for 24 hours (for the assay of TNFα) and 72 hours (for the assays of IFNγ and IL-10) in a humidified atmosphere at 37°C, 5% CO2. Supernatants were taken off carefully under sterile conditions, divided into eppendorf tubes, and frozen immediately at –20°C. The three cytokines were assayed by means of ELISA methods (Eurogenetics, Tessenderlo, Belgium) based on appropriate and validated sets of monoclonal antibodies as explained previously [18, 19]. All assays were carried out on one and the same day using the same batch of antibodies applied by the same operator. The intra-assay CV values for all analytes were less than 8%.

Statistics

Analyses of variance (ANOVAs) were used to assess differences between group means. Correlations between a set of variables were assessed by means of
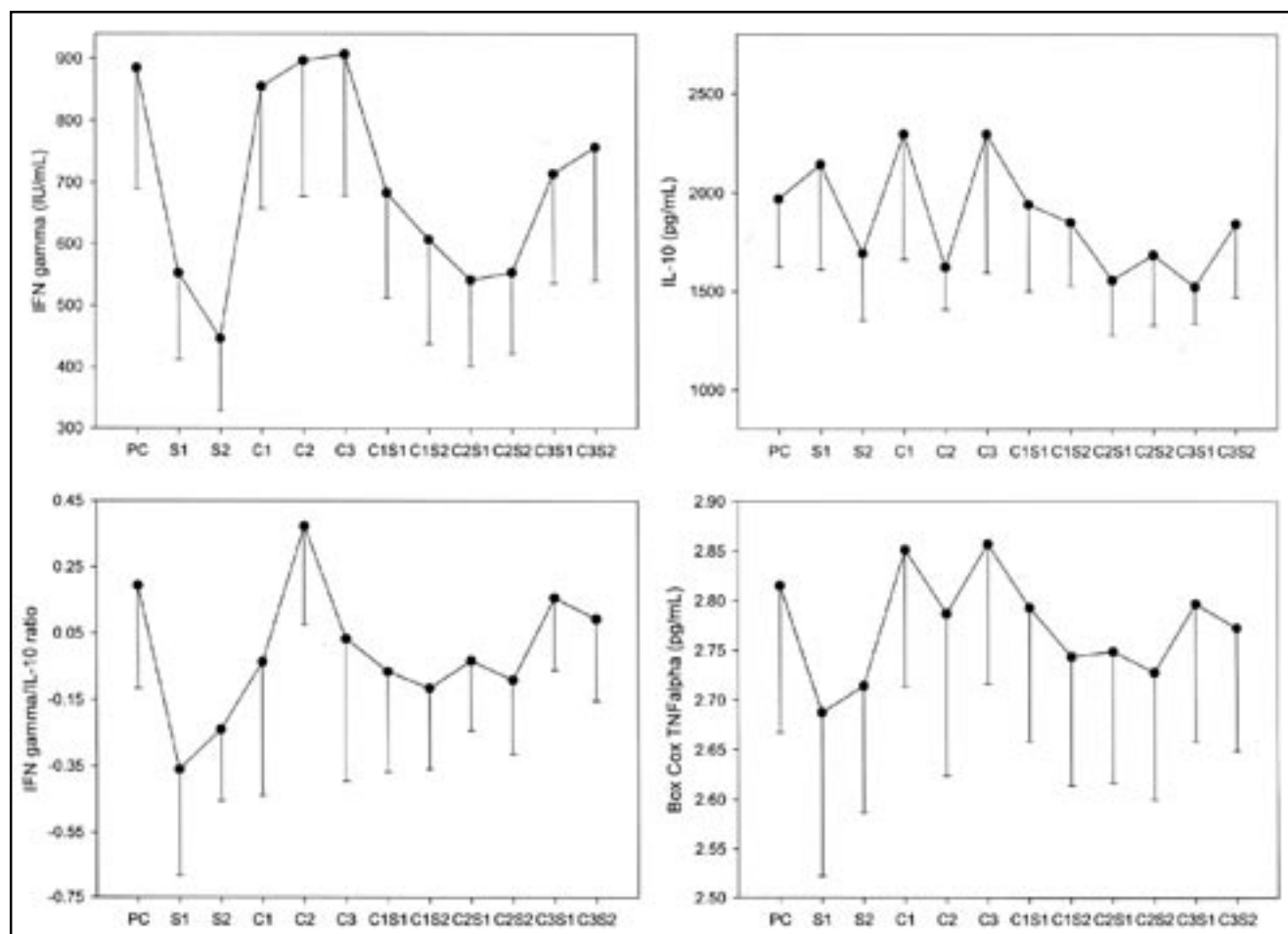
Figure 1. Effects of corticotropin releasing hormone (CRH), 10^{-9} M, 10^{-8} M and 10^{-7} M, **and serotonin (5-HT),** 1.5 μg/mL and 15 μg/mL, alone and together, **on the stimulated production of interferon-γ (IFNγ) and interleukin-10 (IL-10), the IFNγ/IL-10 ratio, and tumor necrosis factor-α (TNFα).**

PC: positive control
S1: 5-HT 1.5 μg/mL
S2: 5-HT 15 μg/mL
C1: CRH 10^{-9} M
C2: CRH 10^{-8} M
C3: CRH 10^{-7} M

C1S1: CRH 10^{-9} M + 5-HT 1.5 μg/mL
C1S2: CRH 10^{-9} M + 5-HT 15 μg/mL
C2S1: CRH 10^{-8} M + 5-HT 1.5 μg/mL
C2S2: CRH 10^{-8} M + 5-HT 15 μg/mL
C3S1: CRH 10^{-7} M + 5-HT 1.5 μg/mL
C3S2: CRH 10^{-7} M + 5-HT 15 μg/mL

Pearson's product moment correlation coefficients. We used intra-class correlations (or regression analyses pooled over the subjects) in order to assess the relationships between variables over the different conditions. We used repeated measure (RM) design ANOVAs to examine the i) within-subject variability with the control, and CRH and 5-HT, alone and together, as time conditions; and ii) between-subject variability with gender as factor. All results of the RM design ANOVAs were corrected for sphericity. Post-hoc differences among treatment means were checked with Fisher's least significant difference (LSD). The TNFα data were assessed in Box-Cox transformation. The IFNγ/IL-10 ratio was computed as: z transformed IFNγ-z transformed IL-10 [18, 19].

Results

A RM design ANOVA performed on the IFNγ values showed a significant effect of time (F=5.2, df=2/39, p=0.009). LSD showed i) a significantly lower production of IFNγ following 5-HT, 1,5 μg/mL and 15 μg/mL; ii) no significant effects of CRH 10^{-9} M, 10^{-8} M, and 10^{-7} M, on the production of IFNγ; iii) a significantly lower IFNγ production following the combination of 5-HT, 1.5 μg/mL and 15 μg/mL with CRH, 10^{-9} M and 10^{-8} M; and iv) no significant differences in IFNγ production between the positive control and the two 5-HT conditions, i.e. 1.5 μg/mL and 15 μg/mL, when combined with CRH, 10^{-7} M.

A RM design ANOVA did not show any significant differences in the production of IL-10 between the different conditions. Thus, neither 5-HT nor CRH, alone or together, had any significant effects on the production of IL-10.

A RM design ANOVA performed on the IFNγ/IL-10 ratio showed a significant effect of time (t=3.0, df=3/51, p=0.03). LSD showed: i) a significantly lower IFNγ/IL-10 production ratio following 5-HT, 1.5 μg/mL and 15 μg/mL; ii) no significant effects of CRH, 10^{-9} M, 10^{-8} M and 10^{-7} M, on the IFNγ/IL-10 production ratio; iii) no significant differences in the IFNγ/IL-10 ratio between the control condition and the combined 5-HT, 1.5 μg/dL and 15 μg/dL, and CRH, 10^{-9} M; 10^{-8} M and 10^{-7} M conditions, except for a suppressant effect of the combination of 5-HT, 15 μg/dL, with CRH 10^{-9} M.

A RM design ANOVA performed on the TNFα values showed a significant effect of time (F=2.6, df=5/78, p=0.03). LSD showed: i) a significantly lower

TNFα production following 5-HT, 1.5 μg/dL and 15 μg/dL; ii) no significant effects of CRH, 10^{-9} M, 10^{-8} M and 10^{-7} M, on the stimulated production of TNFα; iii) no significant differences in TNFα production between the positive control and the two 5-HT conditions, i.e. 1.5 μg/mL and 15 μg/mL, when combined with CRH at any of the three concentrations.

RM design ANOVA showed a significantly higher (F=7.5, df=1/15, p=0.01) production of IFNγ in men (mean=1061 $\pm$800 UI/mL) than in women (mean=294 $\pm$342 UI/mL). There was a trend toward a significantly higher (F=3.1, df=1/14, p=0.1) IL-10 production in men (mean =2426 $\pm$2193 pg/mL) versus women (mean=1258 $\pm$451 pg/mL). There were no significant differences between men and women in the IFNγ/IL-10 production ratio (F=2.6, df=1/14, p=0.13). There were no significant gender-related differences in the production of TNFα (F=2.9, df=1/15, p=0.11). There were no significant interaction patterns between time X gender for any of the cytokine data, i.e. IFNγ: F=2.7, df=3/39, p=0.06; IL-10: F=0.8, df=4/57, p=0.5; and TNFα: F=0.7, df=3/50, p=0.6. There were no significant correlations between age and the production of IFNγ (r=0.09, p=0.7), IL-10 (r=−0.09, p=0.7) and TNFα (r=−0.09, p=0.7) and the IFNγ/IL-10 production ratio (r=0.1, p=0.7).

Discussion

The first major finding of our study is that administration of CRH, 10^{-9} M, 10^{-8} M and 10^{-7} M, did not change the production rates of IFNγ, IL-10 or TNFα. Previously, it has been observed that addition of CRH to PBMCs decreases the production of IFNγ [8] in a dose dependent manner. Thus, CRH, 10^{-12} M, 10^{-9} M, and 10^{-6} M, significantly reduced the production of IFNγ by stimulated PBMCs from 8% to 21%, respectively. It should be underscored, however, that differences between studies may result from differences in the cultures employed, i.e. PBMCs [8] versus whole blood stimulated with PHA + LPS (the present study). In order to measure the production of cytokines, whole blood cultures reflect the *in vivo* immune cellular and humoral interactions and offer a more reliable and appropriate culture condition than that of PBMCs [20–21]. Other results show that CRH addition is able to increase the production of other Th-1-like cytokines or cytokine receptors, i.e. IL-2 and the IL-2R [4, 7]. To the best of our knowledge, the effects of CRH on the production rate of IL-10 or TNFα have never been examined. There are, however, a number of papers which have shown that CRH may enhance the production of other pro-inflammatory cytokines which are mainly produced by cells of the macrophage-monocyte lineage, such as IL-1 and IL-6 [4–6, 8]. The negative results of our study could also be explained by the thesis that CRH differentially modulates the cytokine system depending of the activation level of the monocytes. Thus, it has been shown that CRH is able to upregulate the IL-1 system expression in the absence of LPS, whereas in the presence of low amounts of LPS, CRH does not affect this system [5].

Previously, it has been shown that human immunocytes have specific CRH receptors. Thus, CRH may bind to human monocytes/macrophages and to T-helper but not to T suppressor or B cells [23]. Monocytes and T cells display a high affinity binding of CRH which is several times greater than that of the brain cortical cells [24]. The CRH receptor is functionally linked to a guanine nucleotide binding protein mediating stimulation of adenylate cyclase activity [1]. CRH binding is accompanied by increased intracellular levels of cAMP [23]. It is known that elevations of intracellular cAMP inhibits IFNγ mRNA expression and intracellular IFNγ concentrations and increases IL-10 levels in immunocytes [13, 14]. Thus, the negative IFNγ and IL-10 results reported here do not sustain the initial hypothesis that CRH may decrease IFNγ and /or increase IL-10 production through modulation of the cAMP dependent PKA pathway.

The results of the present study that 5-HT, 1.5 μg/mL and 15 μg/mL, significantly suppress IFNγ production and the IFNγ/IL-10 production ratio are in agreement with those of Kubera et al. [10]. The recent findings that 5-HT, 1.5 μg/mL and 15 μg/mL, may also inhibit the stimulated production of TNFα is in agreement with previous findings that 5-HT inhibits LPS-induced TNFα synthesis by human monocytes [25, 26]. As reviewed elsewhere [27], extracellular 5-HT at or above serum concentrations may suppress many aspects of cellular immunity. We have hypothesized that the negative immunoregulatory effects of 5-HT may be obtained though the stimulating effect of 5-HT on intracellular cAMP production via, for example, 5-HT6 and 5-HT7 receptors, which can be found on immunocytes [12, 28–30].

Another major finding of this study is that addition of CRH, 10^{-7} M, and CRH, at all three concentrations (except CRH 10^{-9} M when combined with the highest 5-HT concentration), are able to block the suppressive effects of 5-HT, 1.5 μg/mL and 15 μg/mL, on the stimulated production of IFNγ and the IFNγ/IL-10 ratio, respectively. Moreover, addition of CRH at all three concentrations blocks the suppressive effects of 5-HT, 1.5 μg/mL and 15 μg/mL, on the stimulated production of TNFα. Phrased differently, CRH is able to antagonize some of the negative immunoregulatory activities of 5-HT. The mechanisms which underlie these immunoregulatory effects of CRH have remained elusive. They are probably not related to the cAMP-dependent PKA pathway since CRH and 5-HT may activate this pathway. A first possibility is that CRH, through enhancing the production of other cytokines, such as IL-2 and IL-6 (see Introduction) may reverse the 5-HT-induced suppression of IFNγ production. A second possibility is that CRH may induce the leukocytic synthesis of other hormones, such as adrenocorticotropin (ACTH) and β-endorphin, which have immunoregulatory effects [31]. In any case, the findings of the present study, extent those of previous studies showing that CRH may promote several immune functions *in vitro* and *in vivo* [32].

The discovery that the negative immunoregulatory effects of 5-HT on IFNγ production is reversed in the presence of CRH is of great importance for the understanding of the neuro-endocrine-immune response to psychological stress, which encompasses enhanced release/production of CRH, 5-HT and pro-inflammatory cytokines (see Introduction). Thus, it appears that multiple reciprocal interactions between proinflammatory cytokines, CRH and 5-HT may participate in the response to psychological stress.

In conclusion, CRH *in vitro* does not exhibit immunoregulatory effects on the stimulated production of IFNγ, IL-10 and TNFα. The negative immunoregulatory effects of 5-HT on the production of IFNγ and TNFα and on the IFNγ/IL-10 production ratio are reversed in the presence of different concentrations of CRH.

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