Involvement of Thyroid Hormones in the Alterations of T-Cell Immunity and Tumor Progression Induced by Chronic Stress

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Background: Stress alters the neuroendocrine system, immunity, and cancer. Although the classic stress hormones are glucocorticoids and catecholamines, thyroid hormones have also been related to stress. We recently reported that chronic restraint stress impairs T-cell mediated immunity and enhances tumor growth in mice.

Methods: To study the participation of these hormones on the stress-induced alterations of the immune function and lymphoma growth, mice were subjected to acute or chronic stress, with or without thyroxin supplementation. Hormone levels, immune status, and cancer progression were evaluated.

Results: Differential endocrine alterations were observed in response to acute and chronic stress. Although corticosterone and noradrenaline levels were increased by acute stress, they were restored after prolonged exposure to the stressor. Instead, thyroid hormone levels were only reduced in chronically stressed animals in comparison with control subjects. Correlating, chronic but not acute stress impaired T-cell reactivity. Thyroxin replacement treatment of chronic restraint stress-exposed mice, which restored the euthyroid status, reversed the observed reduction of T-cell lymphoproliferative responses. Moreover, therapeutic thyroid replacement also reversed the alterations of lymphoma growth induced by chronic stress in syngeneic mice bearing tumors as well as Interleukin-2 production and specific cytotoxic response against tumor cells. Finally, we found that the isoforms θ and α of the protein kinase C are involved in these events.

Conclusions: These results show for the first time that thyroid hormones are important neuroendocrine regulators of tumor evolution, most probably acting through the modulation of T-cell mediated immunity affected by chronic stress.

Key Words: Catecholamines, glucocorticoids, immunity, stress, thyroid hormones, tumor progression

Stress has become a very important aspect of modern life. Importantly, stress is a key factor in the onset and outcome of several psychiatric pathologies. Nowadays it is known that acute and chronic stress have profound effects on different components of the immune system such as lymphocyte proliferation, cytokine production, and redistribution of immune cells among the different lymphoid organs (1). In addition, exposure to stressful situations is able to disrupt the normal regulation of neuroendocrine axes. Stress activates the hypothalamic-pituitary-adrenal axis (HPA) and the autonomic nervous system (ANS). Therefore, most research in this field has pointed to the classic stress hormones: glucocorticoids and catecholamines (2). These hormones have been widely proposed to be responsible for the alterations in the immune function triggered by stress.

In the past years, considerable progress toward a better understanding of the relation between stress and cancer has been made. It is suggested that the aforementioned stress hormones can alter antitumor immunity, thus promoting tumor progression (3,4). In contrast, a variety of tumor cell lines are directly regulated by several hormones. The most studied hormones are glucocorticoids, which are largely known to affect tumor growth (5,6). Also, β-adrenergic signaling pathway triggered by catecholamines can modulate cell proliferation/apoptosis in tumor cells (7–9). These findings further support the hypothesis of a stress-induced alteration of cancer prognosis mediated by neuroendocrine dysregulations.

Although hypothalamic-pituitary-thyroid axis (HPT) has not been extensively studied with regard to stress, increasing evidence indicates that thyroid hormones also participate in the response to chronic stress (10,11). Moreover, thyroid axis exerts a bidirectional modulation of the cognate immunity (i.e., hypothyroidism impairs whereas hyperthyroidism enhances T and B lymphocyte responses) (12,13). In addition, an interaction between the HPA and HPT axes under stress has been proposed (14,15). Even more, thyroid hormone receptors have been found in cancer cells of several lineages (16), and T3 and T4 are able to regulate their cell cycle, growth, and death (17). To date, there are no data linking the thyroid axis with the effects of stress on cancer, but according to the evidence as claimed in the preceding text, thyroid hormones could be participating in the alterations caused by stress in immunity and cancer.

Protein kinase C (PKC) is a family of isoforms with an important intracellular signaling role in several cell types. Its activation impacts on gene expression and cell growth and differentiation in response to diverse stimuli. Certain PKC isoforms are involved in T-cell immunity (18). Protein kinase C-θ, which is present at the immunological synapse, is a crucial regulator of T-cell activation and proliferation as well as of cytokine production (19–21). Moreover, PKC-α is also involved in T-cell proliferation acting upstream of PKC-θ (22,23). In contrast, PKC-β has been proposed to play a role in T-cell

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migration (24,25). This evidence suggests that PKC isoforms could be molecular mediators implicated in hormonal regulation of the immune function in physiological and pathological conditions.

In a recent work, we showed that chronic restraint stress selectively impairs T-cell mediated immunity, which is accompanied by an accelerated progression of a syngeneic lymphoma model (26). However, the mediators involved in these changes had not been identified yet. Thus, the aim of this work was to study the participation of the neuroendocrine axes HPA, ANS, and HPT in this experimental model. In addition, we analyzed the involvement of PKC isoforms in the alterations induced by stress.

Methods and Materials

Animals

Inbred female BALB/c (H-2^d) mice (60–100 days old) were housed in groups of four to six mice/cage and maintained on a 12/12-hour light/dark cycle under controlled temperatures between 18°C and 22°C, with food and water available ad libitum, following the Guide for the Care and Use of Laboratory Animals, U.S. National Research Council, 1996. Animals were killed between 9 AM and 11 AM together with control subjects. Samples were immediately used for cultures or quickly frozen and stored at −70°C until use.

Experimental Procedures

Acute Restraint Stress. Animals ($n = 6$) were restrained for 2 hours in well-ventilated polypropylene tubes (2.8 cm diameter × 11.5 cm length). Mice were returned to their cages, left undisturbed for 15 min, and then killed.

Chronic Restraint Stress. Animals were subjected to the same restraint explained in the preceding text for 6 hours every day. This procedure was repeated for 3 weeks, and then mice were either killed ($n = 6$) or used for tumor injection ($n = 10$), in which case procedures continued for 3 additional weeks until mice were killed.

T4 Replacement Treatment. Animals subjected or not to the chronic restraint procedure previously outlined and injected or not with tumor cells received an oral dose of 50 ng T4 dissolved in the drinking water every day ($n = 16$ each). The equal consumption of T4 in the drinking water was confirmed by measuring plasmatic thyroid hormone levels in each animal after the end of the treatment.

Control Groups. Animals from control groups were housed in normal conditions. Each experiment used the same number of control animals of the same age ($n = 22$).

Hormone Determinations

Blood was collected on ice with .1 mol/L ethylenediaminetetraacetic acid (EDTA), and plasma was separated in a refrigerated centrifuge and stored at −70°C. The T3, T4, and corticosterone levels were determined by radioimmunoassay or high-pressure liquid chromatography (10,27).

Catecholamine concentrations were determined by fluorometric assays (27,28). Briefly, spleens were homogenized in 12.5% sodium sulfite, 10% EDTA in .4 N perchloric acid. Homogenates were centrifuged, and supernatants were seeded in alumina columns. Eluates were oxidized with iodine in alkaline medium, and the fluorescence was recorded at 325/375 nm.

T-Cells Proliferation Assays

Cell suspensions from lymph nodes were prepared in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mmol/L glutamine, and 100 μg/mL streptomycin (Invitrogen, Carlsbad, California). Proliferation was determined by [^{3}H]-thymidine incorporation in cultures of 2×10^5 cells/well in microplates (26,29). Increasing concentrations of Concanavalin A (ConA; Sigma Aldrich, St. Louis, Missouri) were added to the microcultures. Twenty-four hours later, .75 μCi/mL [^{3}H]-thymidine (Amersham Biosciences, Little Chalfont, Buckinghamshire) was added. Cells were cultured at 37°C in a 5% carbon dioxide atmosphere for 72 hours. Plates were harvested, and thymidine incorporation was measured by scintillation counting after retention over GF/C glass-fiber filters (Whatman, Brentford, Middlesex, United Kingdom).

Western Blots

Suspensions of 20×10^6 lymph node cells were stimulated with 1 μg/mL of ConA for 15 min at 37°C, while basal controls were left unstimulated (30). Suspensions were centrifuged, pellets were resuspended in 10 mmol/L 2-β-mercaptoethanol, 2 mmol/L EDTA, 2 mmol/L ethyleneglycol bis-(β-aminoethyl ether)-*N,N'*-tetraacetic acid (EGTA), 1 mmol/L phenylmethyl sulfonyl fluoride (PMSF), 10 μg/mL leupeptin, and 20 mmol/L HEPES, pH 7.4 and centrifuged to obtain the soluble fractions (cytosols). The particulate fractions (membranes) were obtained treating the resulting pellet with the same buffer plus Igepal .5%. Sixty micrograms of proteins, determined by the Lowry method, of each extract were electrophoresed in polyacrylamide gels and electrotransferred to nitrocellulose membranes. The PKC isoforms expression was assessed with specific antibodies against PKC-α. (Sigma Aldrich, 1:2500), PKC-β (Sigma Aldrich, 1:1000), and PKC-θ. (Santa Cruz Biotechnology, Santa Cruz, California, 1:500), and the appropriate peroxidase-conjugated second antibody (anti-rabbit IgG, kindly given by Dr. L. Bussmann, 1:10000). Detection was performed with Amersham ECL Western Blotting Analysis System (Amersham Biosciences). Protein bands were quantified with ImageQuant software (Amersham Biosciences).

Lymphoma Model

LB cell line is an immunogenic variant of the aggressive infiltrating lymphoblastic lymphoma derived from an early T-lymphocyte precursor in BALB/c mice (31,32). The LBC cells express neither progesterone nor estrogen receptors, and hence tumor growth is not affected by medroxyprogesterone or estradiol (33). Therefore, LBC lymphoblastic leukemia constitutes a useful model for the study of human hematological malignancies, similar to the well-known murine T-cell lymphomas EL-4 and BW5147 (34). Tumor cells were cultured in supplemented medium with 50 μmol/L 2-β-mercaptoethanol. Syngeneic animals were inoculated subcutaneously with 1×10^6 LBC cells to generate solid tumors. Tumor volume was calculated as $V = \pi/6 \times \text{length} \times \text{width}^2$ (26).

Real-Time Reverse Transcriptase Polymerase Chain Reactions

Lymph nodes were homogenized in Tri-Reagent (Molecular Research Center, Cincinnati, Ohio) to isolate RNA. Complementary DNA was synthesized by retrotranscription with Omnigene kit (Qiagen, Düsseldorf, Germany) and determined in each sample with commercial master mix for real-time polymerase chain reaction (PCR) containing green fluorescent dye (Biodynamics, Buenos Aires, Argentina) in a Rotor Gene-6000 (Corbett, Life Sciences, Sydney, Australia). Oligonucleotide sequences used were: interleukin (IL)-2F 5′-CCTGAGCAGGATGGAGAATTACA-3′, IL-2R 5′-TCCAGAACATGCCGCAGAG-3′, β-actinF 3′-

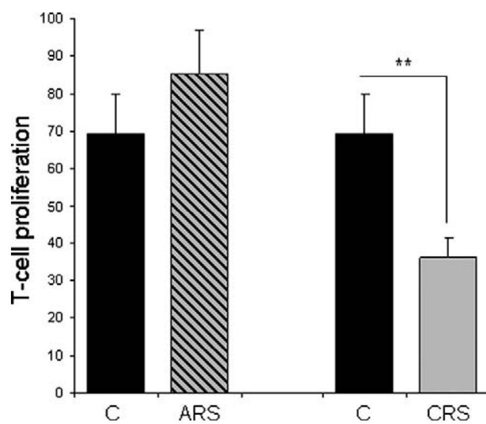

Figure 1. Effects of acute or chronic stress on T-cell proliferation. Cells from lymph nodes of mice subjected to acute (ARS) or chronic restraint stress (CRS) and of control animals (C) were stimulated with Concanavalin A at the optimal dose, and T-cell proliferation was evaluated by [^{3}H]-thymidine incorporation. Values are expressed as means ± SEM of the stimulated/basal ratio (proliferation index). Statistical significance was determined with unpaired *t* test (***p* < .01).

CAACTTGATGTATGAAGGCTTTGGT-3′, and β-actinR 5′-ACTTTTATTGGTCTCAAGTCAGTGTACAG-3′ (26,29).

Cytotoxic Activity Assays

Specific cytotoxic activity against tumor cells was evaluated according to the just another method (JAM) test (26,35). Briefly, LBC were labeled overnight with 5 μCi [^{3}H]-thymidine and co-cultured with spleen cells suspensions at different effector/target ratios for 3.5 hours. Percentages of cytotoxic activity were calculated as the relation cytotoxic activity of T lymphocytes (CTL) = 100 × (SR − ER)/SR, where SR is the spontaneous release and ER is the experimental release.

LBC Proliferation Assays

The effect of dexamethasone (Sidus, Buenos Aires, Argentina), (−)-epinephrine, T3, and T4 (Sigma Aldrich) on LBC cells proliferation was tested in 24-hour cultures of 2×10^5 cells/mL in microplates at increasing concentrations in supplemented medium (17). Thymidine incorporation was measured as described previously.

Statistical Analysis

Statistical significance ($p < .05$) was determined with unpaired two-tailed Student test and one-, two-, or three-way analysis of variance with Dunnett's and Bonferroni post-tests.

Results

Differential Effects of Acute and Chronic Stress on Corticosterone, Noradrenaline, Thyroid Hormones, and T-Cell Proliferation

To analyze the participation of HPA, HPT, and ANS in response to restraint stress, we first analyzed the effects of acute stress (a single restraint session) on the levels of the classic stress hormones glucocorticoids and catecholamines as well as on thyroid hormone levels. Acute stress induced an important increase of corticosterone and noradrenaline (Supplement 1). Instead, T3 and T4 levels remained unchanged. Chronic stress (daily restraint for 3 weeks) exerted the opposite effect: corticosterone and noradrenaline levels were restored to normal values, whereas T3 and T4 were found decreased (Supplement 1).

Given that these hormones are related to the immune function, we also studied the extent of T-cell reactivity to mitogens after exposure of the animal to acute or chronic stress. Acute stress did not significantly modify T lymphocyte proliferation induced by ConA, whereas chronic stress significantly reduced this proliferative response (Figure 1), paralleling the results observed for thyroid hormone alterations.

Chronic Stress Reduces PKC-α and PKC-θ Isoform Activation

With the aim of identifying one of the possible intracellular signalers involved in the impairment of T-cell immunity after chronic stress exposure, we evaluated the activation of PKC isoforms induced by a mitogenic stimulus in T-cells obtained from normal and stressed mice. In resting cells, PKCs are localized primarily to the cytosol and their activation leads to a rapid translocation to the membrane. As expected, immunoreactivity for PKC isoforms was reduced in the cytosol and increased in the membranes after stimulation of T-cells with ConA (Figure

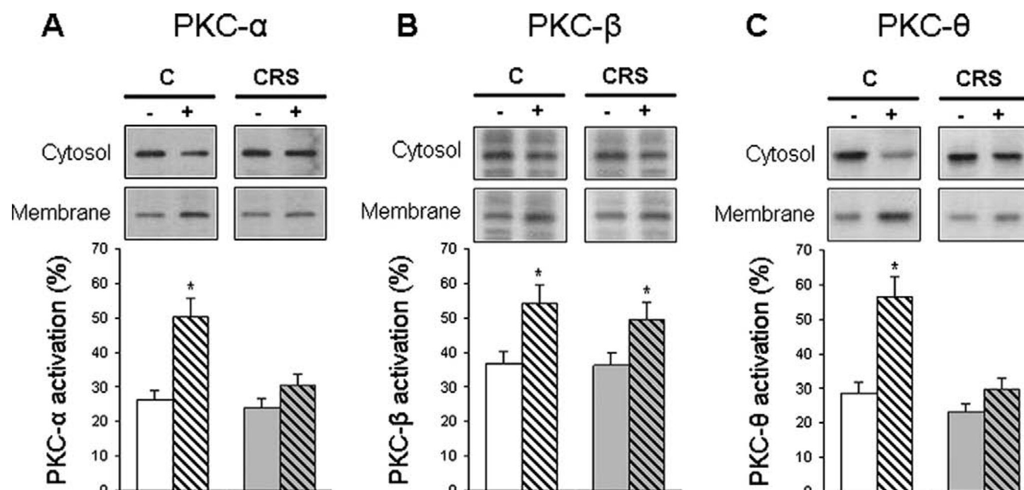

Figure 2. Protein kinase C (PKC) isoform activation after chronic stress. Lymph node cells from chronic restraint stress (CRS) and control (C) mice were stimulated with 1 μg/mL of Concanavalin A (+) or left unstimulated (−). The presence of PKC isoforms α **(A)**, β **(B)**, and θ **(C)** in cytosolic and membrane fractions was evaluated by immunoblot. The PKC activation was calculated as the value obtained after densitometric analysis for PKC in membranes related to the value obtained for total PKC. Values represent means ± SEM. Statistical significance was determined with two-way analysis of variance with Bonferroni post-test (**p* < .05).

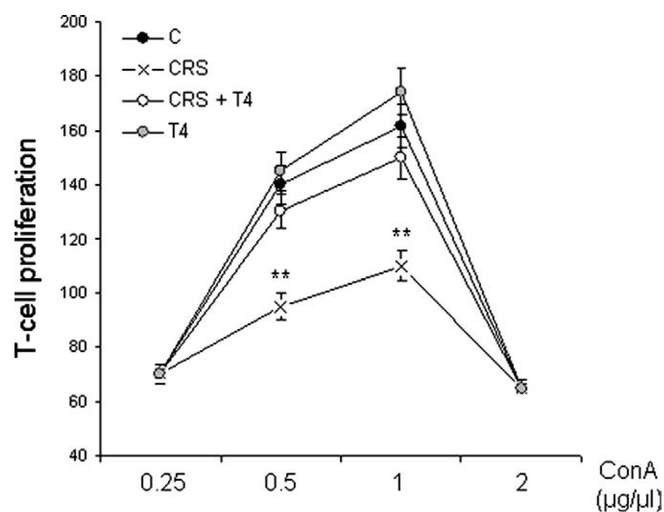

Figure 3. Effects of chronic stress and thyroxin replacement on T-cell proliferation. Chronic restraint stress (CRS) and control (C) mice were or were not treated with 50 ng/day of T4, and dose-response curves of T lymphocyte proliferation stimulated with Concanavalin A (ConA) were performed. Values are expressed as means ± SEM of the total disintegrations/min evaluated by [^{3}H]-thymidine incorporation. Statistical significance was determined with three-way analysis of variance with a 2 × 2 × 4 design followed by Bonferroni post-test (**$p < .01$).

2). In other words, T-cells stimulated displayed a translocation of PKC-α, PKC-β, and PKC-θ from the cytosol to the membrane fraction. Interestingly, stressed mice showed a reduction of PKC-α and PKC-θ translocation and hence a decrease in PKC activation (Figures 2A and 2C) (main effect CRS: $p < .01$; main effect ConA: $p < .01$; interaction CRS × ConA: $p < .05$). In contrast, PKC-β isoform activation was not altered by chronic stress when compared with the control group (Figure 2B) (main effect CRS: $p > .05$; main effect ConA: $p < .05$; interaction CRS × ConA: $p > .05$).

T4 Substitutive Treatment Reverses the Impairment of T-Cell Activation Induced by Chronic Stress

To evaluate the potential participation of thyroid hormones in the alterations of the immune function observed in this model, animals were subjected to chronic stress and concomitantly treated with a therapeutic dose of T4 (50 ng/day). This schedule was able to restore normal levels of T3 and T4 in plasma (T3: main effects CRS and T4: $p < .05$; interaction CRS × T4: $p < .05$; T4: main effects CRS and T4: $p < .01$; interaction CRS × T4: $p < .01$) (see Supplement 2). It is to be noted that this dose did not affect T3 and T4 levels in normal animals, given that it is inside the physiologic range used for hormonal replacement therapy in humans. Hence, we tested whether lymphoproliferative response reduction in stressed mice is reversed by restoring normothyroid status. Hormone replacement was able to prevent the reduction of T-cell proliferation in stressed animals, as can be seen in Figure 3, restoring normal values at physiologically relevant ConA concentrations (main effects CRS, T4, and ConA: $p < .01$; interaction CRS × T4: $p < .01$; overall interaction ConA × CRS × T4: $p < .05$). Finally, we analyzed whether T4 substitutive treatment in chronically stressed mice was able to restore normal values of PKC activation. We found that the activation blockade of PKC-α and PKC-θ observed after chronic stress was counteracted by thyroxin administration (Figure 4) (for both isoenzymes: main effects CRS, T4 and ConA: $p < .01$; interaction CRS × T4: $p < .01$; overall interaction CRS × T4 × ConA: $p < .05$).

T4 Substitutive Treatment Prevents the Enhancement of Tumor Progression Induced by Chronic Stress

Next, we evaluated whether thyroid hormone alterations in response to stress are also related to cancer prognosis. With this purpose, chronically stressed and normal syngeneic mice were injected subcutaneously with 1 × 10^6 LBC T lymphoma cells to generate a solid tumor. Animals bearing tumors that had been subjected to chronic stress also showed a reduction in T3 and T4 levels, when compared with tumor-bearing control animals, and in thyroxin-replaced mice T3 and T4 concentrations in plasma were restored to control levels (T3: main effects CRS and T4: $p < .05$; interaction CRS × T4: $p < .05$. T4: main effects CRS and T4: $p < .01$; interaction CRS × T4: $p < .05$) (Supplement 3).

As expected, tumor growth was significantly increased in chronically stressed mice as compared with control animals (Figure 5A). We next evaluated whether the increase of tumor proliferation induced by chronic stress mice can be reversed by T4 substitutive treatment. Stressed animals daily treated with T4 showed the same lymphoma progression as their normal counterparts (Figure 5A) (main effects CRS, T4, and Day: $p < .001$; interaction CRS × T4: $p < .001$; overall interaction CRS × T4 × Day: $p < .01$). Also, unstressed animals treated with T4 displayed

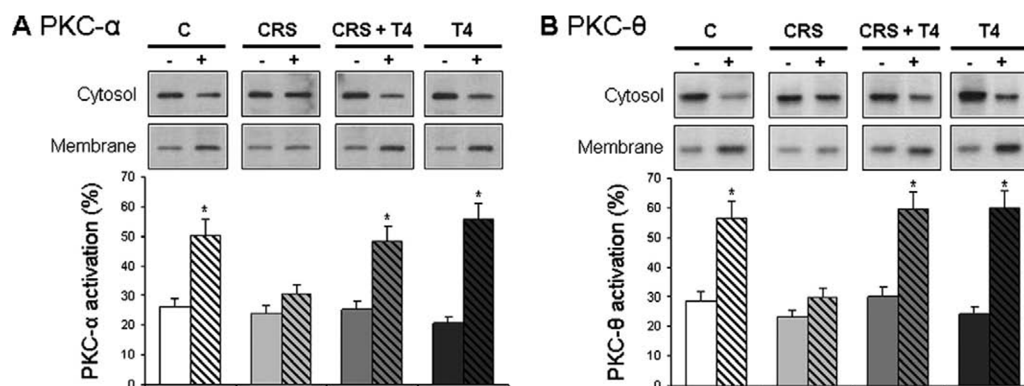

Figure 4. Protein kinase C (PKC) isoforms activation after chronic stress and T4 treatment. Lymph node cells from CRS and control (C) mice treated or not with 50 ng/day of T4 were stimulated with 1 μg/mL of ConA (+) or left unstimulated (−). The presence of PKC isoforms α **(A)** and θ **(B)** in cytosolic and membrane fractions was evaluated by immunoblot. The PKC activation was calculated as the value obtained after densitometric analysis for PKC in membranes related to the value obtained for total PKC. Values represent means ± SEM. Statistical significance was determined with three-way analysis of variance with fixed factors in a 2 × 2 × 2 design and Bonferroni post-test comparisons (*$p < .05$). Abbreviations as in Figure 3.

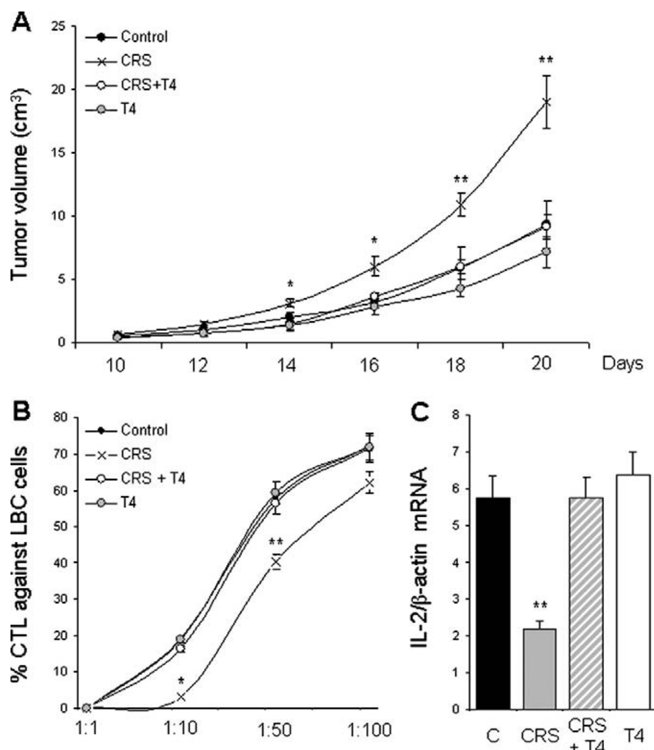

Figure 5. Effects of chronic stress and thyroxin administration on lymphoma progression. Normal animals or those subjected to chronic restraint stress (CRS) and/or T4 treatments were inoculated subcutaneously with 1×10^6 LBC cells to generate a solid tumor. **(A)** Tumor length and width were measured, and tumor volume was calculated as $V = \pi/6 \times L \times W^2$. **(B)** For the evaluation of specific cytotoxic activity against LBC cells, spleen cells were co-cultured with tumor cells labeled with [^{3}H]-thymidine, and its release was evaluated as an indicator of cytolysis. The percentage of cytotoxic activity at different effector/target ratios is shown. **(C)** Cytokine expression was evaluated by real time reverse transcriptase polymerase chain reaction with messenger RNA isolated from lymph node cells of tumor-bearing animals subjected to the different treatments. Interleukin-2 expression was normalized with β-actin as housekeeping gene. Values are expressed as means ± SEMs. Statistical significance was determined in **(A)** and **(B)** with three-way analysis of variance with of $2 \times 2 \times 6$ and $2 \times 2 \times 4$ designs, respectively, and in **(C)** with two-way analysis of variance with Bonferroni post hoc analysis. *$p < .05$, **$p < .01$. CTL, cytotoxic activity of T lymphocytes.

a tumor behavior similar to control animals (Figure 5A). This is consistent with euthyroid levels of T3 and T4 observed in plasma, which are not significantly different from those of untreated animals (Supplement 3).

Finally, we analyzed the ability of the immune system of animals subjected to the aforementioned treatments to respond to tumor inoculation. We found that T4 treatment counteracted the reduction of the specific cytotoxic response against LBC tumor cells induced by chronic restraint stress (Figure 5B) (main effects CRS, T4, and E/T ratio: $p < .001$; interaction CRS × T4: $p < .01$; overall interaction E/T × CRS × T4: $p < .05$). In accordance with these results, we also found that chronic stress decreased IL-2 production in tumor-bearing animals, and thyroxin administration reversed this reduction (Figure 5C) (main effect CRS and T4: $p < .01$; interaction CRS × T4: $p < .01$).

Direct Effects of Hormones on Tumor Cell Proliferation

Finally, we studied which hormones are able to regulate the growth of LBC lymphoma cells by direct actions. With this purpose, we evaluated the effects of dexamethasone, epinephrine, triiodothyronine, and thyroxin on tumor cell proliferation in vitro. We found that dexamethasone induced a marked inhibition of LBC cells proliferation at concentrations $\geq 10^{-6}$ mol/L (Figure 6A). Instead, epinephrine induced a dose-dependent reduction of tumor cell replication at doses as low as 10^{-7} mol/L, having a more pronounced effect at 10^{-4} mol/L (Figure 6B). No differences were found in LBC cell proliferation when non-synchronized tumor cells where cultured in the presence of T3 or T4 at increasing concentrations (Figures 6C and 6D). Therefore, LBC cells seem to be negatively regulated by glucocorticoids and catecholamines but not by a direct effect of thyroid hormones.

Discussion

Here we showed that thyroid hormones are important mediators of the impairment of T-cell mediated immunity and the enhancement of tumor growth induced by chronic exposure to a stressor. To our knowledge, this is the first study to demonstrate a relationship between stress, thyroid axis, immunity, and cancer.

Glucocorticoids and catecholamines are the classic hormones related to stress. In this experimental model, we found that a single session of restraint increases corticosterone and noradrenaline, whereas chronic exposure to the stressor normalized their levels. In contrast, thyroid hormones were decreased only after chronic restraint stress. These results were paralleled by alterations on T-cell reactivity to mitogens: chronic but not acute stress reduced T lymphocyte proliferation in response to ConA. Acute stress has been widely related to increases in the levels of glucocorticoids and catecholamines (1,2,28). However, for chronic stress controversial results have been reported. Although most works show that this increase is persistent, other works indicate that repeated exposure to the stressor leads to the exhaustion of the axes (10,14). Moreover, other authors demonstrated that whereas chronic stressors are deleterious to immune function, very brief stressors enhance some aspects of immune function, such as trafficking of cells from lymphoid organs to the peripheral blood and the skin. These effects seem to be mediated by glucocorticoid- and adrenaline-induced stress responses (36). Therefore, although this short-run response is proposed to be beneficial for the adaptation of the organism, the long-term exposure to the stressor ultimately results deleterious to the individual (2), which is in accordance with the hypothesis of the allostatic load of the organism produced by chronic stress (37). This would provide an adaptative explanation to the results presented herein regarding restoration of classic stress hormone levels after prolonged restraint stress. In contrast, thyroid hormones have been shown to be downregulated by chronic stress in other models of stress (14,15). Moreover, in other models of stress, a relationship between downregulation of the thyroid axis and depression of the immune function has been observed (10,11). Results presented herein are consistent with prior evidence that demonstrates that experimental hypothyroidism leads to a general depression of the immune system (12,13). Furthermore, it has been shown that concentrations of T3 and T4 up to 10^{-9} mol/L and 10^{-7} mol/L, respectively, are able to stimulate T-cell proliferation elicited by a mitogen in a direct manner (17). Strengthening our hypothesis of a relation between thyroid hormones and lymphoma progression, the reduction of T3 and T4 levels was also observed in chronically stressed mice bearing solid tumors. Thyroxin replacement in these animals prevented the exacerbation of tumor growth and the inhibition of cytotoxic response against lymphoma cells as well as of cytokine expres-

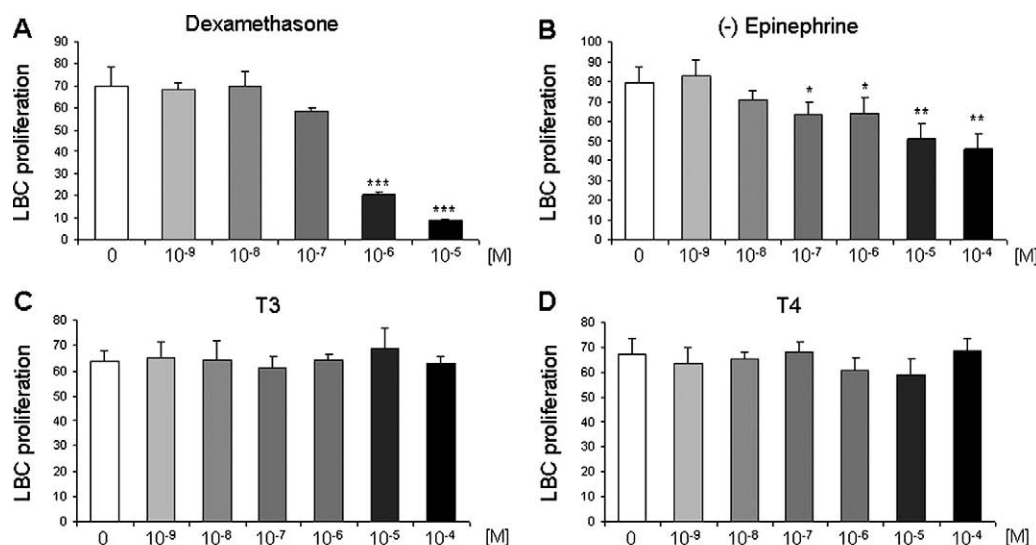

Figure 6. Direct effects of hormones on tumor cells proliferation. LBC cells were cultured in the presence of **(A)** dexamethasone, **(B)** epinephrine, **(C)** T3, or **(D)** T4, and proliferation was evaluated by [^{3}H]-thymidine incorporation in triplicates. Values are expressed as means ± SEM of the total disintegrations/min, and statistical differences were analyzed with one-way analysis of variance and compared with basal values cultured in supplemented medium alone with Dunnett's contrast. *$p < .05$, **$p < .01$, ***$p < .001$.

sion. The lack of effects observed in non-stressed animals treated with thyroxine that displayed euthyroid levels is due to a negative feedback mechanism that controls T4 secretion through thyrotropin (TSH) regulation. The reversion of the chronic stress-induced alterations of immune response and tumor progression by the administration of thyroxin supports our hypothesis pointing to the relevance of HPT dysregulation in response to the stressor.

The present results also indicate that certain PKC isoforms are involved in the events induced by chronic restraint stress. We found that PKC-α and PKC-θ translocation from the cytosol to the membrane after mitogenic stimulation is reduced in chronically stressed animals and that hormonal replacement reverses this effect. These are the most important PKC isoenzymes involved in T-cell function (18). The PKC-θ is essential for T lymphocyte activation through T-cell receptor (TCR) antigen signaling in the T-cell immunological synapses (38,39). Furthermore, PKC-θ mediates activation of the transcription factors activator protein-1 (AP-1) and nuclear factor κB (NF-κB) via TCR/CD28 co-stimulation in T-cells (40,41). In contrast, PKC-α translocated to the cell membrane serves as an active second messenger of the signal delivered by the TCR-CD3 complex for proliferation and IL-2 production (22). In addition, PKC-α (but not PKC-β) acts upstream of PKC-θ to activate the I κ*B* kinase (IκB) complex and NF-κB in T lymphocytes after TCR activation (23). Hence, the impairment of T-cell mediated immunity observed in chronically stressed mice could be attributed, at least in part, to the blockade of PKC-θ and PKC-α activation observed in these animals.

We also found that stressed animals bearing solid tumors displayed a reduction of the cytotoxic response against lymphoma cells and a decrease of IL-2 production as well. It has been shown that PKC plays an important role in $CD8^+$ cytotoxic T lymphocyte effector responses during tumor rejection (42). In addition, PKC-θ also modulates IL-2 transcription in T-lymphocytes through TCR/CD28-induced NF-κB and AP-1 activation (20,21). Knockout mice for PKC-θ exhibit reduced T-cell proliferation and IL-2 production, whereas knock-in mice for PKC-α display the opposite phenotype (21,22). We found, in accordance with this evidence, that chronic stress also reduces T-cell proliferation, IL-2 production, and cytotoxicity against tumor cells. Therefore, we postulate that PKC (isoforms θ and α) might be one of the major intracellular signalers involved in the actions of thyroid hormones in antitumor immunity after chronic stress, a process that in turn alters tumor prognosis.

Protein kinase C-β is implicated in the migration of T-cells, promoting cell polarization and enhancing cell motility (18,25). Moreover, intercellular cell-adhesion molecule 1 (ICAM-1)-mediated cross-linking of lymphocyte-function–associated antigen-1 (LFA-1) recruits PKC-β to microtubules (24). These results support a role of PKC-β on T-cell recruitment during inflammation. Interestingly, stress affects the redistribution of immune cells among the different lymphoid organs (36). However, we did not find any difference on PKC-β activation between chronic stressed and control animals, ruling out its participation in the model studied at present.

Tumor cell lines are often sensitive to hormones. Glucocorticoid hormones activate survival genes that protect cancer cells from the effects of chemotherapy both in vitro and in vivo (5,6). In addition, cathecolamines have been shown to participate in cancer cell migration, invasion, and metastasis through β-adrenergic receptors (43,44). During stress, β-adrenergic activation of the cyclic AMP (cAMP)-protein kinase A (PKA) signaling pathway enhances tumor angiogenesis (7). Moreover, differential production of matrix metalloproteinases MMP-2 and MMP-9 as well as of vascular endothelial growth factor (VEGF) regulated by catecholamines is involved in the promotion of invasive cell growth (45,46). However, in the particular case of leukemias/lymphomas, both hormones often induce apoptosis in tumor cells rather than proliferation (8,47,48). The findings that dexamethasone and epinephrine blocked LBC cell proliferation in vitro rule out the participation of classic stress hormones in the enhanced tumor growth observed in our model after chronic stress. With respect to thyroid hormones, the T-cell lymphoma BW5147 is stimulated by short-term exposure to T3 or T4, leading to increased proliferation and cell signaling activation (17). In contrast, after prolonged exposure, thyroid hormones

drive apoptosis of BW5147 cells, due to sustained activation of these pathways. Hence the alterations of thyroid hormones could directly modulate tumor growth in stressed animals. However, we did not find a direct effect of thyroid hormones on LBC lymphoma proliferation in vitro, supporting the hypothesis that these hormones affect tumor progression by modulating T-cell immunity.

In humans, exposure to stressful situations has been associated with the onset and outcome of specific neuropathologies such as anxiety, depression, and posttraumatic stress disorder (PTSD) among others. Chronic restraint stress in rodents provides a useful model for the study of said psychiatric disorders (49–51). Restrained mice display the typical behavioral and hedonic disturbances observed in other well-validated models, including learned helplessness, olfactory bulbectomy, and genetically manipulated animals (52). Epidemiologic studies revealed that thyroid hormones are reduced in East Germany refugees suffering anxiety and depression (53). Vietnam veterans and civilian PTSD patients display a downregulation of the HPT axis (54,55). These alterations are associated with an abnormal function of the immune system (56). Additionally, stress has been implicated in autoimmune thyroid diseases such as Hashimoto's and Grave's thyroiditis, probably through deregulations of the immune-endocrine function (57). Besides, these neuropsychiatric diseases have been implicated in the prognosis of human cancers including hematological malignancies (58,59). We propose that this effect could be mediated by the immunosuppressive state associated with stress-induced hypothyroidism, supported by the new experimental evidence presented in this work.

Taken together, the present results show for the first time that thyroid hormones are implicated in the impairment of T-cell–mediated immunity and the enhancement of tumor progression induced by chronic restraint stress in a murine model of lymphoma and that isoforms α and θ of PKC are molecular signalers involved in these events. These findings also indicate a potential therapeutic action of thyroxin in the adjuvant treatment of stress-related disorders such as immunosuppression and cancer.

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Supplementary material cited in this article is available online.

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