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# Thyroxine Administration Prevents Streptococcal Cell Wall-Induced Inflammatory Responses*

PETER A. RITTENHOUSE AND EVA REDEI

*Neuroendocrine Research Laboratory, Departments of Psychiatry and Pharmacology, University of Pennsylvania, Philadelphia, Pennsylvania 19104*

**ABSTRACT**

Administration of streptococcal cell wall (SCW) preparation induces an inflammatory response in susceptible animals that is a model frequently used for rheumatoid arthritis. The degree of inflammation produced by SCW is greatly enhanced by low endogenous levels of glucocorticoids due to diminished hypothalamic-pituitary-adrenal activity. Because decreased glucocorticoid production is known to occur in the hypothyroid state, we tested whether hypothyroidism would increase, and conversely, whether hyperthyroidism would decrease, the inflammatory responses to SCW. Adult female Sprague Dawley rats were fed a regular diet (control), L-$T_4$ ($T_4$; hyperthyroid), or 6-propyl-thiouracil (hypothyroid) in drinking water for 7 weeks. Hypothyroidism resulted in elevated plasma levels of TSH and hypothalamic preproTRH messenger RNA (mRNA) while reducing anterior pituitary POMC mRNA and plasma ACTH and corticosterone levels. In contrast, hyperthyroid rats produced opposite results: decreased measures of central thyroid function but increased pituitary-adrenal function. Three days after administration of SCW, macrophage inflammatory protein-1$\alpha$ and interleukin-1$\beta$ mRNA expression increased dramatically in controls and even further in hypothyroid animals, as measured by Northern blot analysis. In contrast, $T_4$-treated rats showed significant inhibition of these inflammatory markers. Thus, the hyperthyroid state combined with increased endogenous glucocorticoid levels is protective against inflammatory challenges. The inverse relationship between preproTRH expression and pituitary-adrenal function suggests the possibility of a direct inhibitory link connecting the hypothalamic-pituitary-adrenal and thyroid axes, and suggests alternative sites of therapeutic intervention for rheumatoid arthritis and other inflammatory associated disorders. (*Endocrinology* **138:** 1434–1439, 1997)

STREPTOCOCCAL CELL WALL (SCW)-induced arthritis in a susceptile strain of rats is a widely used model for rheumatoid arthritis (RA) (1–3), an autoimmune disease characterized by chronic degeneration of the joints resulting from inflammation of the synovial membranes. As the most potent antiinflammatory agents, glucocorticoids are routinely prescribed as therapy for RA (4, 5), and are the treatment of choice for many autoimmune diseases (6–8) because of their ability to limit the damaging effects of inflammation (9). The cellular mechanisms by which glucocorticoids may control inflammation have recently been described (10, 11). In addition, glucocorticoids inhibit the synthesis of inflammatory cytokines (8) and limit proliferation of lymphocytes, macrophages, and other infiltrating cells at the point of inflammation (12). Thus, impaired glucocorticoid secretion due to a hypoactive hypothalamic-pituitary-adrenal (HPA) axis may exacerbate or even permit the development of an autoimmune condition. Evidence documenting such an impaired HPA axis has been shown in several autoimmune models including spontaneous thyroiditis (the obese strain chicken) (8), multiple sclerosis (experimental allergic encephalomyelitis) (13), and SCW-induced arthritis in the female Lewis rat (14).

Susceptibility to RA increases if glucocorticoid levels are diminished (15), and arthritis-induced inflammation can dramatically increase after pharmacological or surgical adrenalectomy (15). Decreased endogenous glucocorticoid production also occurs in hypothyroidism (16–18). Conversely, elevated thyroid hormone levels usually increase activity of the HPA axis, resulting in elevated glucocorticoid levels (17). Thus, hypothyroidism and the subsequent lowering of glucocorticoids may result in increased inflammatory responses to SCW administration. Inversely, hyperthyroidism may be protective by increasing endogenous glucocorticoids that diminish inflammatory responses.

In the present study, we induced hypo- and hyperthyroid states by long-term treatment with 6-propyl-thiouracil (PTU) and $T_4$, respectively. After establishing the appropriate steady state condition, SCW preparation was administered, and inflammatory responses were measured. These included messenger (RNA) mRNA levels in peritoneal macrophages of the inflammatory cytokine interleukin (IL)-1$\beta$ and the chemokine macrophage inflammatory protein-1$\alpha$ (MIP-1$\alpha$), which is specifically increased in RA compared with other forms of arthritis (19).

## Materials and Methods

### *Animals and experimental protocol*

Female rats (Harlan Sprague Dawley, Indianapolis, IN; 225–250 g) were used because of their increased propensity to develop RA (20). They were housed two per cage in a climate-controlled facility with a 12 h L, 12 h D cycle (lights on 0700 h). Six groups of rats of eight rats/group were used. They were fed rat chow and water *ad libitum* (control), 0.012% $T_4$ in drinking water (hyperthyroid), or 0.05% PTU (hypothyroid) in drinking water for 7 weeks. Rats from each group were injected ip with either SCW preparation or saline 3 days before decapitation. The SCW suspension was prepared in sterile saline and injected at a concentration of 20 $\mu$g of rhamnose per kg body weight in a volume of 100 $\mu$l/100 g body weight (20). The animal protocol was approved by

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Address all correspondence and requests for reprints to: Eva Redei, Department of Psychiatry, Northwestern University Medical School, 303 East Chicago Avenue, 9–176, Chicago, Illinois 60611-3008. E-mail: e-redei@nwu.edu.
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the University of Pennsylvania Institutional Animal Care and Use Committee.

### *RNA isolation and Northern hybridization*

The anterior pituitary and hypothalamus (optic chiasm to mammillary bodies; dorsally to the anterior commisure) were rapidly dissected on dry ice and stored at −80 C until RNA isolation. Macrophages were harvested by injecting 5.0 ml sterile RPMI into the peritoneal cavity, lavaging, and collecting the cells by centrifugation (4 C, 1000 × *g*). Total RNA was isolated using TRIzol reagent (Life Technologies, Gaithersburg, MD) following the manufacturer's protocol, with quality and quantity of RNA determined by gel electrophoresis and spectrophotometry. Total RNA (20 μg/lane) was electrophoresed in a formaldehyde denaturing 1.2% agarose gel, transferred to a nylon Hybond-N+ membrane (Amersham Life Science, Arlington Heights, IL) and UV cross-linked to the filter. Filters were prehybridized for 4 h at 42 C with 50% formamide and 50% Northern prehybridization solution (5 Prime→3 Prime, Boulder, CO). Filters were hybridized for 16 h at 42 C in 50% formamide and 50% Northern Hybridization solution (5 Prime→3 Prime, Boulder, CO) with complementary DNA (cDNA) probes $^{32}$P-labeled using a random primer labeling kit (Boehringer Mannheim, Indianapolis, IN). The filters were washed twice for 15 min each in 2× SSC/0.1% SDS at 24 C, twice for 30 min each in 0.1× SSC/0.1% SDS at 52 C, and exposed to Kodak X-OMAT-5 film (Eastman Kodak, Rochester, NY). Densitometry was carried out using an Image Analyzer, Macintosh-based BRAIN 2.1 system (Drexel University, Philadelphia, PA) with gray scale calibration. All comparisons were made from RNA samples hybridized on the same filter and normalized to the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPD). Probes were stripped from filters before rehybridization by washing in boiling water for 30 sec.

### *Probes*

Filters were hybridized with cDNA probes for preproTRH, glucocorticoid receptor (GR), CRH, POMC, IL-1β, macrophage inflammatory protein-1α (MIP-1α) and GAPD. The preproTRH probe was the 1322-bp rat preproTRH cDNA insert from a plasmid kindly provided by Dr. Stephanie Lee, Tufts University, Boston, MA (21). The GR probe was the 2.4-kilobase insert from plasmid 6RGR containing a rat GR cDNA (22) and kindly provided by Dr. Keith Yamamoto, University of California, San Francisco, CA. The CRH probe was a 320-bp *Pst*I fragment subcloned from a rat CRH cDNA kindly provided by Dr. Robert Thompson, University of Michigan, Ann Arbor, MI. The POMC probe was the 923-bp insert from plasmid pMKSU16, containing a mouse POMC cDNA (23) and kindly provided by Dr. Jim Eberwine, University of Pennsylvania. The IL-1β probe was a 245-bp PCR product generated with primers published previously (24). The MIP-1α cDNA was a kind gift of Dr. Michael Prystowsky, Albert Einstein University, New York (25). The GAPD cDNA was a 983-bp PCR product generated using primers purchased from CLONTECH (Palo Alto, CA).

### *RIAs*

Trunk blood was collected into chilled polypropylene tubes containing 0.5 ml of a 0.15 M solution of EDTA, centrifuged at 1000 × *g* for 25 min at 4 C, and the plasma stored at −80 C until assayed. All the samples were analyzed together in one assay. ACTH was measured as described previously (26) in 25 μl unextracted plasma with antiserum that recognizes ACTH 1–24 and ACTH 1–39 on an equimolar basis, with $^{125}$I-ACTH as the tracer (INCSTAR, Stillwater, MN). The assay sensitivity was 0.05 pg/tube with an intraassay coefficient of variation of 6.3%. Corticosterone (CORT) was measured in unextracted plasma as described previously (26) using antiserum raised against CORT-3-carboxymethyloxime:BSA, with $^{125}$I-CORT conjugate as the tracer (ICN Biomedicals, Carson, CA). The assay sensitivity was 0.03 ng/tube and the intraassay coefficient of variation was 12.3%. TSH standards and specific antiserum were obtained from the National Hormone and Pituitary Agency (NIDDK, Baltimore, MD). Rat TSH RP-2 was used for the iodination and standards. The assay sensitivity was 1.0 pg/tube with an intraassay coefficient of variation of 10.5%. RIAs for $T_3$ and $T_4$ were performed using ImmuChem-coated tubes purchased from ICN Pharmaceuticals (Costa Mesa, CA), following the recommended protocol provided with each kit. The sensitivity limits were 10 ng/tube and 0.5 μg/tube for $T_3$ and $T_4$, with intraassay coefficients of variation of 4.1% and 5.2%, respectively.

### *Statistical analysis*

Data were analyzed by two-way ANOVA followed by a *post hoc* Newman-Keuls comparisons test. In all statistical tests, results with $P < 0.05$ were considered statistically significant. The software used was NWA Statpak (Portland, OR).

## Results

This study was designed to analyze the role of thyroid hormonal status and the subsequent changes in HPA function in the development of SCW-induced inflammatory responses. Hypo- and hyperthyroid states were induced by treatment with PTU and $T_4$, respectively.

### *HPT axis*

Treatment with PTU and $T_4$ had highly significant effects on plasma levels of $T_4$ ($F_{[2,38]} = 73.3$; $P < 0.001$) and $T_3$ ($F_{[2,38]} = 11.9$; $P < 0.001$). PTU-treated rats had decreased plasma levels of $T_4$ (13.4 ± 1.5 *vs.* 29.6 ± 2.3 ng/ml in control) and $T_3$ (261 ± 90 *vs.* 595 ± 72 pg/ml in control). $T_4$-treated rats had elevated plasma levels of $T_4$ (142 ± 18 *vs.* 29.6 ± 2.1 ng/ml in control) and $T_3$ (711 ± 72 *vs.* 595 ± 72 pg/ml in control). As shown in Fig. 1A, PTU-treated rats had plasma TSH levels that were 20-fold higher than controls ($F_{[2,37]} = 111$; $P < 0.001$). $T_4$-treated rats had significantly reduced TSH levels that were 2.5-fold below control values ($P < 0.01$). PreproTRH mRNA levels in the hypothalamus also were significantly altered by thyroid status ($F_{[2,38]} = 6.02$, $P < 0.01$): a 20% decrease in the $T_4$-treated group and a 52% increase ($P < 0.01$) in the PTU-treated group (Fig. 1B), demonstrating feedback regulation of preproTRH synthesis by thyroid hormones. No significant changes in thyroid function were observed between saline and SCW-injected rats.

### *HPA axis*

Plasma levels of CORT in saline-injected rats displayed patterns similar to plasma $T_4$ levels: PTU produced a decrease in plasma CORT, whereas $T_4$ treatment elevated CORT levels ($F_{[2,38]} = 3.45$; $P < 0.05$, Fig. 2A). Although the changes in plasma ACTH levels were not significant in saline-injected rats (Fig. 2B), they were consistent with plasma CORT levels, as were changes in POMC mRNA levels: $T_4$-treated animals increased POMC expression by 50%, whereas POMC mRNA levels were less than half of controls in the PTU rats (Fig. 3A). Thus, the hyper- and hypothyroid states produced the expected results in HPA activity before inducing the inflammation by SCW.

Hypothalamic levels of GR ($F_{[2,37]} = 3.59$; $P < 0.05$, data not shown) and CRH mRNA ($F_{[2,37]} = 3.47$; $P < 0.05$, Fig. 3B) were also significantly modulated by thyroid status, but in a manner opposite from the CORT pattern. High CORT values in the hyperthyroid saline-injected rats were associated with lower GR and CRH mRNA levels, whereas PTU treatment produced decreased CORT levels but elevated CRH mRNA, indicating negative feedback regulation.

There was a significant effect of the SCW injection on

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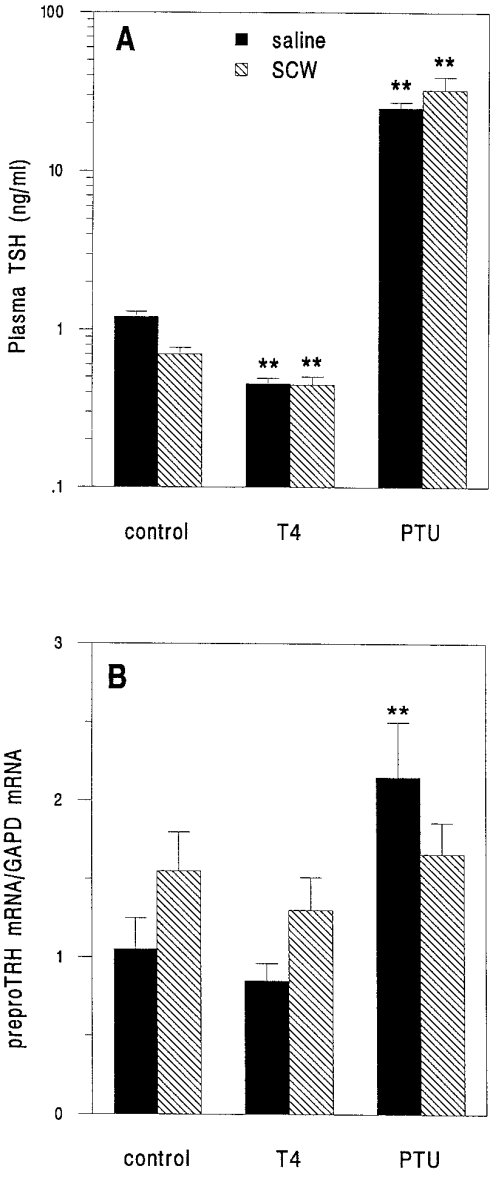


FIG. 1. A, Plasma levels of TSH; **, $P < 0.01$ compared with corresponding control groups. B, Relative preproTRH mRNA levels in hypothalamus as measured by Northern analysis. Exposure times: preproTRH, 5 days; GAPD, 15 h. **, $P < 0.01$ compared with corresponding saline-injected control group.

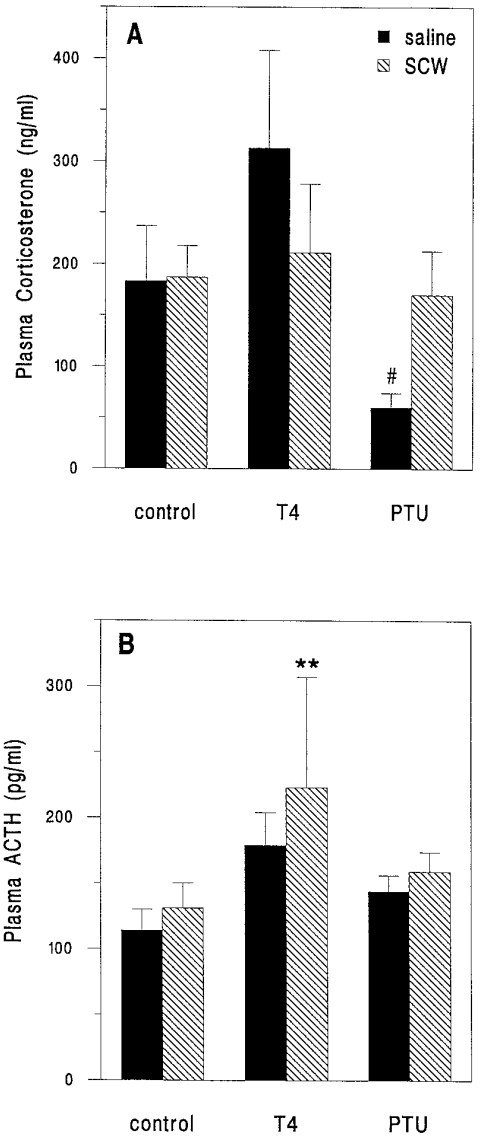


FIG. 2. A, Plasma CORT; #, $P < 0.05$ compared with saline-injected/$T_4$ group. B, plasma ACTH. **, $P < 0.01$ compared with SCW-injected/control and SCW-injected/PTU groups.

POMC expression ($F_{[1,33]} = 8.14$; $P < 0.01$, Fig. 3A) with a 3- to 4-fold increase in controls and hypothyroid rats, but no increase in hyperthyroid animals (Fig. 3A). Hypothalamic CRH mRNA levels were increased only in the control animals in response to the inflammatory challenge (interaction $F_{[2,37]} = 6.05$, $P < 0.01$; Fig. 3B).

*Inflammation*

Three days after injection, SCW produced slightly swollen hind paws and ankle joints in control and PTU-treated rats. In addition, one of eight controls and three of eight PTU-treated rats died within 48 h of SCW injection; in contrast, no $T_4$-treated rats died. In control rats SCW injection produced a dramatic induction of MIP-1$\alpha$ mRNA (Fig. 4A). SCW-injected hypothyroid rats showed a further increase by 30%. However, in SCW-injected/$T_4$-treated rats, there was a substantial 10-fold drop in MIP-1$\alpha$ expression compared with SCW-injected/control rats ($P < 0.01$). IL-1$\beta$ mRNA levels followed a pattern similar to MIP-1$\alpha$, with $T_4$ again suppressing the macrophage response to SCW (interaction $F_{[2,36]} = 8.09$; $P < 0.01$; Fig. 4B), and PTU treatment aggravating the SCW-induced macrophage IL-1$\beta$ inflammatory response ($P < 0.01$). These data demonstrate that the hypothyroid state exacerbates, whereas the hyperthyroid state nearly abolishes, these inflammatory responses to SCW administration.

## Discussion

The HPA and HPT endocrine axes have long been postulated to be linked (7, 16, 27). As such, manipulations of the thyroid axis could have profound effects on the endogenous

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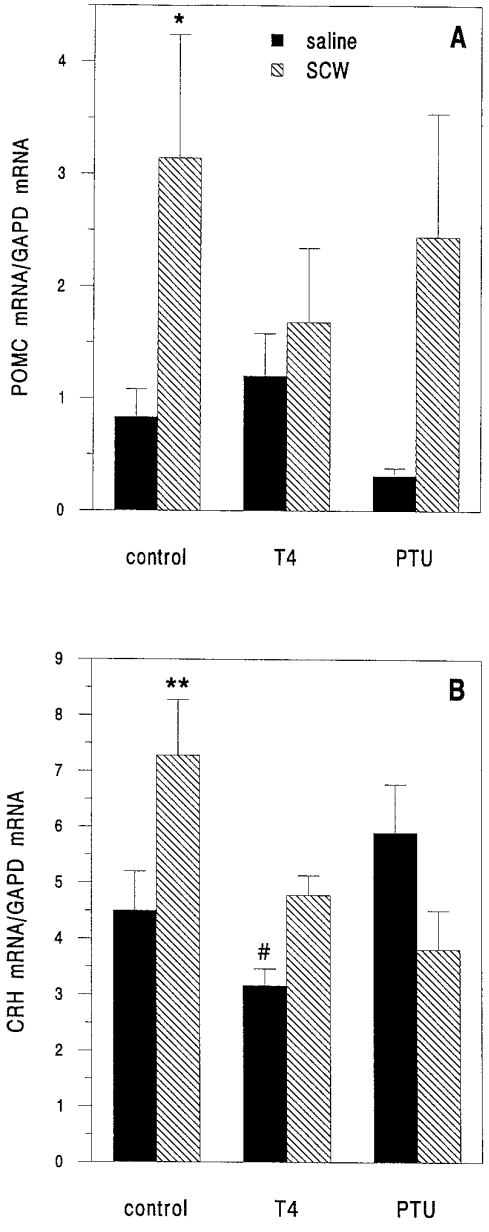


FIG. 3. A, Relative POMC mRNA levels in anterior pituitary as measured by Northern analysis. Exposure times: POMC, 2 h; GAPD, 15 h. *, $P < 0.05$ compared with saline-injected control. B, Relative CRH mRNA levels in hypothalamus. Exposure times: CRH, 4 days; GAPD, 15 h. **, $P < 0.01$ compared with saline-injected control and SCW-injected/PTU group. #, $P < 0.05$ compared with saline-injected/PTU group.

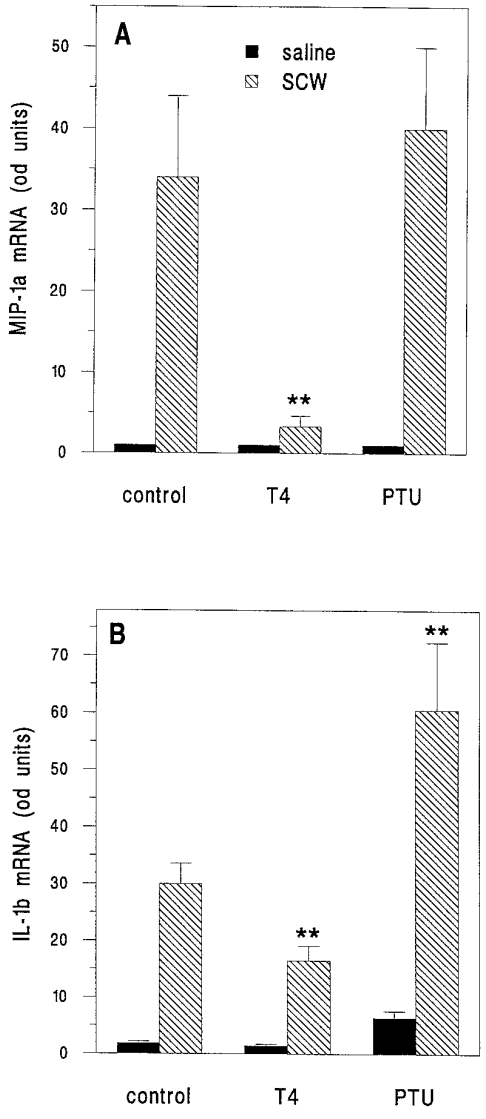


FIG. 4. Inflammatory markers are altered by thyroid condition. A, Northern analysis of mRNA in macrophages shows chemokine MIP-1$\alpha$ is reduced by $T_4$ treatment. Exposure time: 16 h. **, $P < 0.01$ compared with SCW-injected control and SCW-injected/PTU group. B, Northern analysis of mRNA in macrophages shows cytokine IL-1$\beta$ is increased by PTU treatment and decreased by $T_4$ treatment. Exposure time: 20 h. **, $P < 0.01$ compared with all groups.

glucocorticoid milieu, leading to altered susceptibility to inflammatory autoimmune illnesses. Indeed, this is the first study to show that rats made hyperthyroid with $T_4$ treatment were afforded an impressive protective advantage in reducing the macrophage cytokine/chemokine response to SCW-induced inflammation. As hypothesized, PTU-induced hypothyroidism exacerbated the inflammatory response to SCW even in a nonsusceptible rat strain. Consequently, these results have great potential for determining alternative sites of therapeutic intervention for RA and other autoimmune diseases. In support of this notion, hyperthyroidism was found to produce immunosuppression and lessen the severity of an autoimmune model for Graves' disease (28).

Intervening peptide sequences flank the five copies of TRH present within the rat preproTRH prohormone (29). Recently, our laboratory has identified that one of these peptides inhibits ACTH synthesis and secretion from anterior pituitary cells. This peptide is preproTRH 178–199 (30, 31), which has been localized to neurons within the paraventricular nucleus of the hypothalamus (32), as well as the external layer of the median eminence (33). Furthermore, this peptide has the ability to reduce stress-induced plasma ACTH and CORT levels *in vivo* (34). The *in vitro* and *in vivo* characteristics of this peptide suggest that it may be a physiological regulator of ACTH production: an endogenous cor-

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ticotropin release inhibiting factor. The discovery of a corticotropin release inhibiting factor encoded within the same precursor as TRH establishes a potential link between the HPA and HPT axes for the first time. It has been determined that diminishing the endogenous production of glucocorticoids increases the susceptibility to SCW-induced inflammation (15). Our hypothesis was that hypothyroidism would produce the same effect. Consequently, low levels of thyroid hormones could up-regulate preproTRH synthesis with an attendant increase in the secretion of preproTRH 178–199. Conversely, increased thyroid hormones could lead to elevated levels of glucocorticoids by inhibiting preproTRH mRNA and therefore preproTRH 178–199 production. Thus, hypothyroidism may contribute to the susceptibility to RA by diminishing the activity of the HPA axis and thereby reducing glucocorticoid levels at the time of the inflammatory challenge. Manipulations of the HPT axis showed that in the hypothyroid state plasma TSH was elevated, whereas $T_3/T_4$ levels were diminished, with the reverse being found in the hyperthyroid state, as previously described (35). PreproTRH mRNA followed these tendencies suggesting that, in theory, preproTRH 178–199 could be decreased or increased appropriately to regulate ACTH. Indeed, we found an inverse relationship between preproTRH and POMC gene expression similar to a previous report (16). Despite this similarity in the POMC response, Shi *et al.* (16) observed a decrease in the CRH mRNA response to hypothyroidism. This difference could be due to time course (2 weeks of PTU treatment, compared with our 7-week regimen), methodology (*in situ* hybridization compared with Northern blots), or gender differences, because they employed young male rats. In the present study, there was a significant decrease in CORT levels in saline-injected/PTU-treated rats, similar to findings by Kamilaris *et al.* (17). That this HPT-derived inhibitory influence may be an important regulatory control point of POMC synthesis is suggested by the fact that $T_4$ treatment decreased CRH but increased POMC, whereas PTU increased CRH but decreased POMC. Thus, regulation of POMC appeared to be dissociated from its primary activator CRH. Specifically, changes in hypothalamic GR and CRH mRNA levels reflected glucocorticoid negative feedback: GR and CRH expression increased when CORT was low (hypothyroid) and decreased when CORT was high (hyperthyroid). In contrast, POMC mRNA expression decreased in hypothyroid rats in the presence of low CORT and increased in hyperthyroid rats in the presence of normal to high CORT. This suggests that an additional factor other than glucocorticoid negative feedback may inhibit POMC expression.

Previous studies have demonstrated the application of SCW-induced inflammation as a model for RA (2, 3, 14, 15), with MIP-1$\alpha$ shown to be a specific marker for RA in humans (19). We found that both IL-1$\beta$ and MIP-1$\alpha$ expression were increased in response to SCW administration in control animals. Expression of IL-1$\beta$ and MIP-1$\alpha$ was increased in hypothyroid SCW-treated rats, whereas prolonged $T_4$ treatment dramatically decreased these adverse inflammatory effects of SCW. These findings suggest $T_4$ may have direct antiinflammatory effects on macrophages. Hyperthyroidism due to $T_4$ was reported to decrease cell migration and macrophage hydrogen peroxide production, whereas hypothyroidism increased macrophage phagocytic activity and glucose utilization (36). This suggests that thyroid receptors present in macrophages contribute to this response, especially considering the profound ability of $T_4$ to inhibit MIP-1$\alpha$ and IL-1$\beta$ mRNA. In a recent review, thyroid dysfunction, primarily hypothyroidism, was found in female patients with RA almost three times more often than in control women (37). Conversely, patients with subclinical hypothyroidism show improvement of symptoms after $T_4$ replacement (38). Similar beneficial effects of $T_4$ were reported in another animal model, adjuvant-induced arthritis (39).

Neuroendocrine HPA responses to an inflammatory challenge are thought to be mediated by IL-1$\beta$, because IL-1$\beta$ is produced in response to inflammatory agents (40, 41). Although IL-1$\beta$ is known to increase CRH secretion from the hypothalamus (42), we found that hypothalamic CRH expression in SCW-treated rats did not closely follow the IL-1$\beta$ pattern from macrophages in response to altered thyroid status, whereas the anterior pituitary POMC expression did. This suggests that IL-1$\beta$ may mediate a response to SCW by directly increasing POMC expression. Interestingly, POMC mRNA levels were high 3 days after SCW injection in control and hypothyroid rats, the same ones that showed very high levels of IL-1$\beta$ expression. In contrast, neither POMC nor the inflammatory markers changed in the hyperthyroid rats. We found no increases in plasma CORT or ACTH in response to SCW in either group. This phenomenon, together with the rather high CORT levels in saline-injected rats, suggests that an acute environmental stressor may have masked the subtle long-term effect of SCW. Because POMC mRNA levels are not expected to change rapidly, they are more accurate in reflecting HPA activity in response to the SCW challenge. Thus, the lower basal CORT levels found in controls and PTU-treated animals, allow higher POMC as well as inflammatory responses. These observations further suggest that hyperthyroid rats with their increased basal HPA activity could become refractory to an otherwise strong challenge, the inflammation. Thus, thyroid hormone-induced changes in basal HPA activity could determine susceptibility and responsiveness to inflammation.

The results of this study demonstrate that $T_4$ affects macrophages to suppress inflammatory responses, either through a direct or indirect mechanism. Furthermore, the evidence provided here supports the existence of a direct link connecting the HPA and HPT endocrine axes, which would have great potential for determining alternative sites of therapeutic intervention for RA. Although administration of large doses of glucocorticoids are frequently the treatment of choice for reducing the inflammation associated with RA, they nonetheless have numerous side effects. Thyroid hormones may play a fundamental role in regulating the HPA axis by adjusting the levels of preproTRH and subsequently preproTRH 178–199. If this putative corticotropin release inhibiting factor proves to be an important component in the development of RA, then regulation of preproTRH may be highly relevant to the development of other autoimmune diseases that are also exacerbated by low endogenous glucocorticoid levels.

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