Endotoxin-induced nonthyroidal illness in dogs

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Objective—To determine the effects of endotoxin administration on thyroid function test results and serum tumor necrosis factor-α (TNF-α) activity in healthy dogs.

Animals—6 healthy adult male dogs.

Procedures—Serum concentrations of thyroxine (T₄), 3,5,3'-triiodothyronine (T₃), 3,3',5'-triiodothyronine (rT₃), free T₄ (fT₄), and endogenous canine thyroid stimulating hormone (TSH), and TNF-α activity were measured before (day–1; baseline), during (days 0 to 3), and after (days 4 to 24) IV administration of endotoxin every 12 hours for 84 hours.

Results—Compared with baseline values, serum T₃ concentration decreased significantly, whereas rT₃ concentration increased significantly 8 hours after initial endotoxin administration. Serum T₄ concentration decreased significantly at 8 and 12 hours after initiating endotoxin administration. Serum T₃ concentration returned to reference range limits, then decreased significantly on days 6 to 12 and 16 to 20. Serum fT₄ concentration increased significantly at 12, 24, and 48 hours after cessation of endotoxin treatment, compared with baseline values. Serum rT₃ concentration returned to reference range, then decreased significantly days 5 and 7 after stopping endotoxin treatment. Serum TNF-α activity was significantly increased only 4 hours after initial endotoxin treatment, compared with baseline activity.

Conclusions and Clinical Relevance—Endotoxin administration modeled alterations in thyroid function associated with systemic nonthyroidal illness syndrome. A decrease in serum T₄ and T₃ concentrations and increase in serum rT₃ concentration indicate impaired secretion and metabolism of thyroid hormones. The persistent decrease in serum T₄ concentration indicates that caution should be used in interpreting serum T₄ concentrations after resolution of an illness in dogs. (Am J Vet Res 2003;64:229–234)

Nonthyroidal illness syndrome describes the alterations in thyroid function associated with systemic nonthyroidal illness. The changes that occur are caused by a complex interaction of disturbances at every level of the hypothalamic-pituitary-thyroid axis, as well as in transport, peripheral tissue distribution and metabolism of thyroid hormones. Results of thyroid function tests in dogs with nonthyroidal illness syndrome commonly mimic some of the abnormalities found in hypothryroidism. A decrease in serum total thyroxine (T₄) and, less frequently, total 3,5,3'-triiodothyronine (T₃) concentrations occur most commonly, whereas serum thyroid stimulating hormone (TSH) concentration is high in 8 to 14% of dogs with nonthyroidal illness syndrome. Serum free thyroxine (fT₄) concentrations are less than reference range values in many affected dogs as well. In dogs and humans, the degree of alteration of thyroid function test results is correlated with disease severity and mortality rate.

The pathogenesis of nonthyroidal illness syndrome is unclear, but various cytokines play roles. Administration of tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), interleukin-2 (IL-2), or interleukin-6 (IL-6) to dogs, humans, or laboratory animals results in abnormal findings on thyroid function tests identical to those found in euthyroid sick syndrome. Serum IL-6 and TNF-α concentrations are correlated with a decrease in serum T₄ concentration in ill humans. Conversely, the effects of endotoxin administration on thyroid function cannot be completely prevented by agents that neutralize cytokines released in response to the endotoxin.

The purpose of the study presented here was to evaluate alterations in thyroid function test results in dogs during illness induced by administration of a low dose of endotoxin and during recovery from endotoxin treatment. In addition, the association between serum TNF-α concentration and thyroid function test results was determined.

Materials and Methods

Animals—Six adult male random source dogs of unknown age, with a mean body weight of 22.6 kg (range 18.2 to 28.4 kg), were determined to be healthy on the basis of physical examination findings, CBC determination, and results of serum biochemical analysis and heartworm antigen testing. Dogs had serum T₄ responses (> 30 nmol/L) to IV administration of bovine TSHP (0.1 U/kg) that were within reference range.

Dogs were acclimated for 5 weeks prior to the study and held in indoor runs with a 12-hour light-to-dark cycle. Dogs were fed a maintenance dry food once per day.

Experimental protocol—Blood samples were obtained by jugular venepuncture and allowed to clot in siliconized glass tubes before centrifugation within 30 minutes of collection at 2000 × g for 15 minutes. Serum was harvested and stored frozen at -70°C until analysis. Blood samples were obtained immediately before and at 4, 8, 12, and 24 hours after IV administration of 10 mL of sterile saline (0.9% NaCl) solution at 7:00 AM (day–1). After collection of the 24-hour
sample, Escherichia coli O111:B4 endotoxin (1 µg/kg) in 10 mL of sterile saline (0.9% NaCl) solution was administered IV every 12 hours for a total of 8 doses (hours 0 to 84). Catheters were flushed immediately after saline solution or endotoxin administration with 5 units of heparin sulfate in 0.5 mL of saline solution. Blood samples were then obtained at 4, 8, 12, 24, 48, and 72 hours after initiation of the endotoxin treatment. Blood samples were collected at 96 and 108 hours and at 5, 6, 7, 8, 10, 12, 14, 16, 18, 20, 22, and 24 days after endotoxin administration during the recovery period. Dogs were monitored for signs of illness, and rectal body temperature was measured at 4, 8, 12, 16, and 24 hours after each morning dose of saline solution or endotoxin and on day 3. Rectal body temperature measurements and monitoring for illness was continued every 12 hours through day 7, then once per day. Food consumption and body weight were recorded on a daily basis throughout our study. The Virginia Tech University Animal Care Committee approved this protocol, and all dogs were cared for according to NIH Guide for the Care and Use of Laboratory Animals.

Serum concentrations of T₄, T₃, fT₄, 3,3',5'-triiodothyronine (reverse T₃; rT₃) and TSH were determined at each sample collection time. Tumor necrosis factor-α was measured in serum of 4 dogs at 0, 4, 8, 12, and 24 hours after saline solution administration and 4, 8, 12, 24, 48, 72, 96, and 108 hours and 5, 6, 7, 8, 10, and 24 days after initial endotoxin administration. The mean of the 5 hormone concentrations for each dog following saline solution administration was used for comparison at all subsequent times.

Analytical methods—Serum T₄, T₃, fT₄, and rT₃ were assayed by use of a radioimmunoassay previously validated for use in dogs. Serum canine TSH was measured by use of a commercial radioimmunometric assay validated for use in dogs by the manufacturer. Free T₄ was measured by use of a previously validated equilibrium dialysis radioimmunoassay. All assays were performed on duplicate samples. Intra-assay coefficients of variation were determined by serial measurement of 10 samples of pooled canine serum containing low, medium, and high hormone concentrations. Interassay coefficients of variation were determined by measuring hormone concentrations of pooled serum containing low, medium, and high hormone concentrations on 4 different dates.

Intra-assay coefficients of variation for T₄ were 7.5, 6.3, and 7.3% in low (10.3 nmol/L), medium (24 nmol/L), and high (53 nmol/L) concentration pooled serum, respectively. Intra-assay coefficients of variation for T₃ were 5.2, 4.4, and 4.9% in low (0.62 nmol/L), medium (1.62 nmol/L), and high (2.33 nmol/L) concentration pooled serum, respectively. Intra-assay coefficients of variation for rT₃ were 7.9, 6.1, and 8.8% in low (0.37 ng/mL), medium (0.93 ng/mL), and high (1.63 ng/mL) concentration pooled serum, respectively. Intra-assay coefficients of variation for TSH were 7.6, 9.2, and 7.1% in low (10 pmol/L), medium (27 pmol/L), and high (79 pmol/L) concentration pooled serum, respectively. Intra-assay coefficients of variation for TSH were 6.2 and 5.6% in low (0.19 ng/mL) and high (1.14 ng/mL) concentration pooled serum, respectively.

IV Interassay coefficients of variation for low, medium, and high concentration pooled serum were 8.2, 6.8, and 6.9% for T₄; 6.6, 5.4, and 5.4% for T₃; 6.8, 7.2, and 7.7% for rT₃; and 6.3, 7.4, and 8.2% for TSH, respectively. Interassay coefficients of variation for low and high concentration pooled serum were 5.8 and 7.1% for TSH, respectively. Reference ranges calculated as mean ± 2 SD of each hormone were derived from 56 dogs (T₄, T₃, fT₄) or 38 dogs (TSH) with serum T₄ responses to TSH within reference range as already described. Reference ranges for serum concentrations of T₄, T₃, fT₄, and TSH were 13 to 41 nmol/L, 0.7 to 2.3 nmol/L, 9 to 41 pmol/L, and 0 to 0.43 ng/mL, respectively.

Serum TNF-α bioactivity was measured as previously described with minor modifications. Briefly, LM cells, a murine fibroblast cell line that is sensitive to TNF-α, were plated in 96-well flat-bottom plates at a concentration of 4 × 10⁴ cells in 100 µL and cultured overnight. Fifty microliters of serial 2-fold dilutions of the canine serum samples was then added in triplicate along with 20 µL of culture medium containing 8.5 µg/mL actinomycin D. Serial dilutions of human recombinant TNF-α of known bioactivity were used as a standard and positive control. Following overnight incubation, 25 µL of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide at 5 mg/mL in PBS solution was added to each well. Following a 4-hour incubation, all supernatant was removed, and 100 µL of 0.01 N HCl in isopropanol was added to each well. After a 30-minute incubation, 100 µL of ddH₂O was added to each well, and absorbance was measured at 570/630 nm in an ELISA plate reader. Units of TNF-α were determined from a standard curve derived from dilutions of the human recombinant TNF-α standard.

We realize that the murine fibroblasts may be either more or less sensitive to canine TNF-α when compared with the human recombinant standard; however, the values reported are primarily for comparison purposes between dogs and treatment groups and not necessarily to reflect an absolute quantitative measurement.

Statistical analysis—Hormone concentration data obtained during the treatment phase of the experiment and data obtained after treatment were analyzed separately as the difference from the mean of all baseline hormone concentrations during saline solution infusion. A software program was used to perform a repeated measures ANOVA for each analyte. Significant time effects were further investigated by use of Bonferroni-corrected tests for significant change from baseline values at each sample collection time. Body weight and serum TNF-α activity during treatment and recovery periods were compared with baseline measurements by use of an ANOVA. Level of significance was set at P < 0.05.

Results
Each treatment of endotoxin resulted in some combination of fever, vomiting, depression, and a decrease in food consumption in all dogs. Fever resolved within 8 hours after endotoxin administration in all but 1 dog. Vomiting was infrequent but always occurred within 4 hours after endotoxin treatment. During endotoxin treatment, a significant decrease in serum T₃ concentration was first detected after 8 hours of initial treatment and continued until treatment ended (Table 1). Serum T₄ concentration was significantly decreased at 8 and 12 hours of treatment, compared with baseline values. No significant change in serum fT₄ concentration developed during treatment. A significant increase in serum rT₃ concentration was present during treatment beginning at 8 hours through the end of the treatment period (72 hours). Serum TSH concentrations did not change significantly during endotoxin administration.

During recovery, compared with baseline values, no significant effect of time on serum concentrations of T₄ or TSH was detected (Table 1). Serum T₄ concentration during recovery decreased, compared with baseline values, from days 6 to 20, with the exception of day 14. A significant effect of time on serum fT₄ concentrations was detected. Serum fT₄ concentration was significantly higher than baseline values at 96, 108, and 120 hours after initiating endotoxin treatment. A
significant effect of time on serum \( rT_3 \) concentrations was detected. Serum \( rT_3 \) concentration was significantly higher than baseline values at 96 hours, after which time it progressively decreased to become significantly decreased, compared with baseline values, at days 8 and 10.

Serum TNF-\( \alpha \) activity was \( 3.7 \pm 7.3, 83.4 \pm 94.2, 13.3 \pm 26.6, 16.5 \pm 27.0, 15.9 \pm 27.6, 11.5 \pm 23.1, 3.1 \pm 6.1, 23.8 \pm 27.5, 24.9 \pm 36.6, 2.0 \pm 4.1, 4.9 \pm 9.7, 2.3 \pm 4.6, 1.9 \pm 3.8, 2.8 \pm 5.7, 0.0 \pm 0.0 \) U/mL before and \( 4, 8, 12, 24, 48, 72, 96, \) and 108 hours and 5, 6, 7, 8, 10, and 24 days after initial endotoxin administration, respectively. A significant increase in serum TNF-\( \alpha \) activity was found 4 hours after the initial endotoxin treatment, compared with baseline values. No significant difference in serum TNF-\( \alpha \) activity was found at subsequent sample collection times during treatment or recovery periods.

### Discussion

Results of our study in dogs revealed changes in thyroid function during the acute phase of endotoxin-induced illness and during recovery from illness. Hormonal changes associated with endotoxin administration found in our study are similar in some respects to those described for dogs with spontaneous nonthyroidal illness syndrome. However, the acute nature of illness and rapid recovery following endotoxin administration may have resulted in alterations in thyroid function different from those of naturally occurring disease. Repeated administration of endotoxin was used in our study to prolong the duration of illness to allow study of subacute as well as acute disease, in addition to the period of recovery from illness.

A decrease in serum \( T_3 \) concentration was a consistent finding in dogs of our study during endotoxin administration and is also found in dogs with a variety of nonthyroidal illnesses. It is the first change to occur in most illnesses in humans, prior to a decrease in serum \( T_4 \) concentration. However, a decrease in serum \( T_3 \) concentration appears to be much less common in dogs than in humans and typically occurs with a concurrent decrease in serum \( T_4 \) concentration.

The acute nature of illness induced by endotoxin administration may account for the substantial decrease in serum \( T_3 \) concentration. The decrease in serum \( T_3 \) concentration may be the result of a rapid decrease of deiodination of \( T_4 \), either through a decrease in 5'-deiodinase activity or inhibition of entry of \( T_4 \) into cells. A decrease in 5'-deiodination would decrease \( T_3 \) production from \( T_4 \), leading to a decrease in serum \( T_3 \) concentration. This is supported by findings in our study, in which serum \( rT_1 \) concentration was increased during the same period. Reverse \( T_3 \), the inactive metabolite of \( T_4 \), undergoes deiodination catalyzed by the same 5'-deiodinase that is responsible for conversion of \( T_4 \) to \( T_3 \). It is also possible that a decrease in production of \( T_4 \) as has been found in humans with nonthyroidal illness syndrome, could decrease substrate for deiodination and thus lower serum \( T_3 \) concentration. Because serum \( T_4 \) concentration was decreased only at 8 and 12 hours during endotoxin administration...
treatment in our study, impaired deiodination is the likely cause of low serum T₃ concentration. The finding that perturbations in serum T₃ and rT₃ concentrations resolved rapidly and coincidentally after cessation of endotoxin administration is most compatible with a common mechanism for these alterations.

Similar to findings of our study, mild to moderate illness in humans results in an increase in serum FT₄ concentration. To the author's knowledge, an increase in serum FT₄ concentration has not been previously reported for spontaneous nonthyroidal illness syndrome in dogs. More severe illness is associated with a serum FT₄ concentration within reference range or a decrease in serum FT₄ concentration in humans and dogs. The abrupt increase in serum FT₄ concentration without a concurrent increase in total T₄ concentration indicates that the free fraction of T₄ was increased as a result of a decrease in hormone binding to plasma transport proteins. Impaired protein binding of T₄ may result from decreases in plasma thyroid hormone binding protein concentrations, the presence of a circulating inhibitor of binding, or changes in the avidity of carrier proteins for T₄. The fact that serum T₃ concentration was decreased or unchanged when serum FT₄ concentration was increased in our study is consistent with a decrease in T₃ production, particularly because the rate of transport of T₄ from plasma to tissues is decreased in nonthyroidal illness in humans. If this were also the case in dogs, a normal T₄ secretion rate by the thyroid gland would result in an increase in serum FT₄ concentration.

Serum T₄ concentration was decreased significantly at most measurements from day 6 to 20 during recovery from endotoxin treatment. Possible causes include a decrease in synthesis and secretion of T₄ as a result of hypothalamic, pituitary, or thyroid dysfunction; an increase in clearance of T₄; or a prolonged impairment of binding to plasma transport proteins. Endotoxin administration will result in a rapid increase in vascular permeability with movement of plasma components out of the vascular space. The initial decrease in serum T₄ concentration observed at 8 and 12 hours after endotoxin treatment may have been caused by loss of thyroid hormones and their transport proteins into the interstitial space. The more prolonged decrease in serum T₄ concentration observed later in our study was in part caused by a decrease in protein binding, because serum FT₄ concentrations were similar to baseline values during most of the recovery period and the free fraction of T₄ was increased. An increase in metabolic clearance of T₄ has been shown to occur in people with nonthyroidal illness probably primarily as a result of a decrease in protein binding. Production of T₄ is also decreased in nonthyroidal illness. This may be caused by a direct effect of illness on thyroid gland secretion or indirectly through a decrease in TSH secretion. Serum TSH concentrations in humans with nonthyroidal illness syndrome are usually within reference range or low, and TSH secretion in response to thyroid releasing hormone administration is decreased. Although no significant changes in serum TSH concentration were found at any time during our study, a decrease in TSH secretion would be predicted to occur in response to an increase in serum FT₄ concentration during endotoxin treatment. If so, a decrease in thyroid hormone secretion would result in return of serum FT₄ concentrations within reference range as seen during recovery from endotoxin treatment in our study. Alternatively, the lack of changes in serum TSH concentration may reflect a lack of sensitivity of the assay. In humans with nonthyroidal illness syndrome, a dissociation of serum thyroid hormone from TSH concentrations appears to occur, with TSH concentrations lower than expected given the low serum T₄ and FT₄ concentrations.

After cessation of endotoxin administration, serum T₃ and rT₃ concentrations rapidly returned to concentrations similar to those found prior to endotoxin treatment. This indicates that the activity of 3'-deiodinase or plasma membrane transport returns to normal rapidly after resolution of an illness. It seems unlikely that such a rapid return of these hormone concentrations to within reference range would occur in spontaneous illness in dogs, because recovery is likely to be more prolonged than in the dogs with endotoxin-induced illness in our study.

Duration of altered thyroid function in the dogs with endotoxin-induced illness in our study was approximately 16 days. Thyroxine was the last hormone to return to concentrations in serum that were within reference range and therefore may not be as useful as other thyroid function test measurements during convalescence.

Endotoxin administration causes a variety of physiologic responses, including release of cytokines. The dose of endotoxin administered to dogs in our study resulted in a moderate illness characterized by transient fever, lethargy, and inappetence. In another study in which dogs were given a single large dose of endotoxin (5 times that used in our study), clinical signs of illness were more severe despite similar changes in serum T₄, T₃, rT₃, and FT₄ concentrations at 24 hours after treatment. The dose of endotoxin used in our study was chosen because it has previously been shown in dogs to result in similar moderate clinical signs and significant increases in TNF-α and IL-6 responses. The effects of repeated administration of endotoxin on serum TNF-α activity or on thyroid function test results has, to the authors' knowledge, not been previously evaluated. Serum TNF-α activity was significantly increased only 4 hours after the first dose of endotoxin was administered. Because of this, no attempt was made to correlate hormonal changes with serum TNF-α activity. Tumor necrosis factor-α, along with IL-1, induces a proinflammatory cascade that is responsible for the changes that occur after endotoxin administration. Administration of various cytokines, including TNF-α, IL-1, IL-6, and interferon-γ, to humans, rats, or mice results in alterations at all levels of the hypothalamic-pituitary-thyroid axis similar to those of nonthyroidal illness syndrome associated with spontaneous disease. These same cytokines can suppress thyroid cell function in cell culture preparations. However, results of recent studies indicate that factors other than cytokines may play a more important role in nonthyroidal illness syndrome.
Neutralization of TNF-α, IL-1, IL-6, and interferon-γ by administering antibodies to these cytokines or blockade of IL-1 receptors prior to endotoxin administration does not prevent alterations in thyroid hormone metabolism or the pituitary-thyroid axis.45

Failure to measure consistent increases in serum TNF-α activity in our study may indicate that this cytokine is not involved in the pathogenesis of nonthyroidal illness syndrome in dogs or that sample collection times were inadequate to detect transient increases, because 12 or more hours had elapsed at sample collection times other than those following the first dose. Alternatively, TNF-α could have been acting at a local level that was not detected in serum assays. Perhaps most likely, transient increases of serum TNF-α activity played a role in the hormonal changes observed in our study through induction of other proinflammatory cytokines and acute phase reactants, including eicosanoids, the complement cascade, C-reactive protein, activation of coagulation and fibrinolysis, and other substances that in turn alter thyroid function.9,19

Nonthyroidal illness syndrome created in dogs of our study by repeated administration of endotoxin resulted in decreases in serum T₄ concentrations that were of substantial magnitude to result in a misdiagnosis of hypothyroidism. The prolonged duration of the decrease in serum T₄ concentration is of particular importance, because T₄ is probably the most common thyroid hormone evaluated in dogs suspected to have hypothyroidism. Because alterations of thyroid function test results found in our study are not identical to those found in spontaneous nonthyroidal illness syndrome, administration of endotoxin at a more frequent dosage interval, a higher dose, or for a more prolonged period may be necessary to better pattern the clinical syndrome of nonthyroidal illness.

References


