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PROLACTIN SECRETION IN HYPOTHYROID ENDOTOXEMIC RATS: INVOLVEMENT OF L-ARGININE AND NITRIC OXIDE SYNTHASE

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ABSTRACT—The identification of nitric oxide (NO) within the hypothalamus and pituitary gland has suggested its role as modulator of the activity on hypothalamic-pituitary axis. Hypothalamic NO synthase (NOS) is known to be regulated by thyroid hormones. We investigated the effects of previous injection of N^{ϖ}-nitro-L-arginine methyl ester (L-NAME), a NOS inhibitor, and L-arginine (L-Arg), the substrate for NO synthesis, on prolactin (PRL) secretion, through the lipopolysaccharide (LPS)-induced inflammatory response in thyroidectomized (TX) rats. TX or sham-operated (N) rats were intraperitoneally (i.p.) injected with L-NAME (10 mg kg^{-1}) or L-Arg (200 mg kg^{-1}) or the same volume of vehicle (saline solution) 30 min before endotoxemia-induction with LPS at 250 μg (100 g body weight)$^{-1}$, i.p.. In N rats, NO increased PRL release in response to endotoxemia, whereas in hypothyroid rats, NO appeared to have the opposite effect. Our data support the hypothesis that NO exerts a modulatory influence on PRL secretion after LPS-induced inflammatory response.

KEYWORDS—Lipopolysaccharide, L-NAME, L-arginine, thyroidectomy, endotoxemia, prolactin

INTRODUCTION

Nitric oxide (NO) is an important intercellular signaling molecule involved in the regulation of diverse physiological and pathophysiological mechanisms in nervous, cardiovascular, and immunological systems. It is a biological mediator with effects on smooth muscle relaxation, neurotransmission, host defense, immunity, and inflammation. High concentrations of NO produced by inducible NO synthase (iNOS) play critical roles in modulating inflammation. Lipopolysaccharide (LPS), an endotoxin of gram-negative bacteria, is considered an acknowledged trigger of the inflammatory response in the host via the macrophage-derived proinflammatory cytokines. It is well known that endotoxemia can induce an increase in iNOS expression (1).

During endotoxemia, profound changes occur in hypothalamus-pituitary-thyroid function that are known as the euthyroid sick syndrome or nonthyroidal illness. However, little is known about the mechanisms and significance of these changes. It has been postulated that proinflammatory cytokines such as interleukin (IL)-1, IL-6, and tumor necrosis factor-α (TNF-α) inhibit the hypothalamic-pituitary-thyroid axis. This response is generally believed to represent an adaptation of the organism to cope with endotoxemia, protecting it against exaggerated catabolism (2).

Prolactin (PRL) is a common mediator of the immunoneuroendocrine network and plays a significant role in regulating immune responses. The identification of NO within the hypothalamus and pituitary gland has suggested its role as modulator of the activity on hypothalamic-pituitary axis (3). The thyroid hormones, which are essential for the maintenance of secretion of neurotransmitters associated with LPS-induced inflammatory response, have also a significant impact on the immune response. Significantly, hypothalamic NOS is known to be regulated by thyroid hormones (4).

Because NO and PRL act as mediators of inflammation and low levels of thyroid hormones partially block the release of PRL in LPS-treated rats (5), we investigated the effects of previous injection of N^{ϖ}-nitro-L-arginine methyl ester (L-NAME), an NOS inhibitor, and L-arginine (L-Arg), the substrate for NO synthesis, on PRL secretion, through the LPS-induced inflammatory response in thyroidectomized (TX) rats.

MATERIALS AND METHODS

Animals

Male Wistar rats (200-260 g) were kept under standard temperatures (23°C ± 2°C), light (lights on from 6:00 a.m-6:00 p.m.), and feeding (tap water and rat chow *ad libitum*) regimens. The experiments were performed in accordance with the ethical principles of animal experimentation adopted by the Brazilian College of Animal Experimentation and in consonance with the relevant Brazilian legislation.

Hypothyroidism induction

TX was performed 15 days before the experiments on animals under 1.0 mL of 100 g per body weight (i.p.) 2.5% 2,2,2-tribromoethanol (Sigma-Aldrich, St. Louis, MO) anesthesia. Sham-operated animals were submitted to exactly the same procedures as the TX animals except that their thyroid glands were not removed. To avoid disorders of calcium metabolism induced by possible injury on parathyroid glands, TX animals were offered 1% calcium lactate in tap water.

Experimental procedures

TX or sham-operated (N = normal or euthyroid) rats, 10 animals per group, were treated with an i.p. injection of L-NAME (10 mg kg^{-1}), L-Arg (200 mg kg^{-1}), or the same volume of vehicle (saline [SL] solution) 30 min before endotoxemia induction with LPS (Sigma; *Escherichia coli* serotype 055:B5, 250 μg; 100 g of body weight^{-1}, i.p.). The day before the experiments, animals were anesthetized as before and a Silastic cannula was implanted into an external jugular vein for blood sample collection. Rats were cannulated according to the method described by Harms and Ojeda (6).

Blood samples (0.5 mL) for PRL measurements were collected into heparinized tubes before (baseline time = −30 min) and after treatment with L-NAME, L-Arg, and SL (0 min), and after 5, 15, 30, 60, 120, and 240 min of endotoxemia. Plasma

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was obtained by centrifugation for 15 min at 2500 rpm at 5°C and was immediately frozen and stored at −20°C until assayed. To avoid hypovolemia, the same volume of red blood cells from a pool of donor rats was resuspended in sterile physiological SL solution and returned to each rat through the jugular venous cannula.

At the end of the experiments, the rats were killed with an injection of thiopental sodium (50.0 mg kg^{-1}, i.v.; Abbott do Brasil, São Paulo, Brazil).

Hormone assays

Plasma PRL levels were determined using a highly sensitive double-antibody radioimmunoassay kit supplied by the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK, USA), containing ratPRL.RP3, ratPRL I_6, and anti-ratPRL S9. Intra- and interassay coefficients of variation for the assays varied between 3.0% and 12.0%, respectively.

To confirm the induction of hypothyroidism, plasma T4 levels were measured by time-resolved fluoroimmunoassay using commercial kits (EG&G do Brasil, Wallac Division, Brazil), and TSH levels were determined using a radioimmunoassay kit (NIDDK, USA) containing ratTSH-RP-3, ratTSH-I-9, and anti-rat TSH-RIA-6.

Statistical analysis

Data are expressed as mean ± SEM and were analyzed by the nonparametric Mann-Whitney test to determine the significance of differences between two groups, and by the Wilcoxon test for comparisons of endotoxemia-induced changes in each group of animals, with the level of significance set at 5%. A computer statistics package was used for statistical analyses (SPSS, version 9.0).

RESULTS

Fifteen days after the removal of thyroid glands, plasma TSH and T4 levels in hypothyroid rats were higher than 25.0 ng mL^{-1} and less than 1.3 μg dL^{-1}, respectively, whereas those of euthyroid rats were below 1.5 ng mL^{-1} and above 4.5 μg dL^{-1}.

Effect of LPS on PRL release in N and TX rats

Thyroidectomy did not alter basal levels of PRL. Plasma PRL levels in N and TX rats were 3.5 ± 0.5 ng mL^{-1} and 3.8 ± 0.6 ng mL^{-1}, respectively. As shown in Figure 1, PRL levels significantly increased after 5 min of endotoxemia in both groups (N-SL, 38.2 ± 13.7 ng mL^{-1}; TX-SL, 5.9 ± 1.6 ng mL^{-1}). Therefore, plasma PRL levels in euthyroid rats were 6-fold higher than in the hypothyroid group ($P < 0.01$). As expected, the values remained above initial levels throughout the experiment in N rats. In TX rats, plasma PRL were similar to basal levels during the experiment, except for the first 5 min.

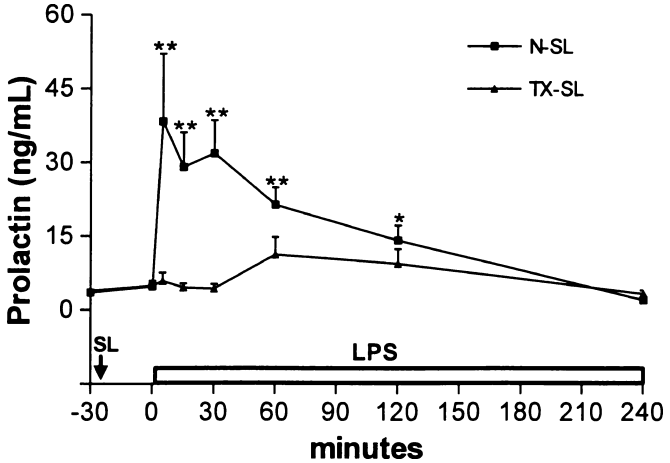

FIG. 1. **Effects of injection of LPS on the release of plasma prolactin in N and TX rats treated with SL.** The arrow represents SL injection. Endotoxemia is represented by the horizontal bar. Results are reported as means ± SEM for 10 animals in each group and were analyzed by the nonparametric Mann-Whitney *U* Test. **P* < 0.05 and ***P* < 0.01 indicate significance of differences between N-SL and TX-SL groups.

Effect of L-NAME and L-Arg on PRL release in N endotoxemic rats

Treatment with L-NAME, an NOS inhibitor, and L-Arg, the substrate for NO synthesis, did not modify basal PRL levels (N-SL, 4.7 ± 0.5 ng mL^{-1}; N-L-NAME, 4.0 ± 0.5 ng mL^{-1}; N-L-Arg 4.7 ± 0.6 ng mL^{-1}). Previous injection of L-NAME inhibited the PRL response to LPS in N rats compared with SL control (Fig. 2A; $P < 0.01$). Curiously, treatment with the NO precursor L-Arg significantly blocked PRL release during endotoxemia (Fig. 2B) when compared with the SL-injected control group.

Effect of L-NAME and L-Arg on PRL release in TX endotoxemic rats

Basal concentrations of PRL were not altered by treatment of L-NAME or L-Arg in TX animals (TX-SL, 4.9 ± 1.0 ng mL^{-1}; TX-L-NAME, 3.5 ± 0.3 ng mL^{-1}; TX-L-Arg 5.1 ± 0.7 ng mL^{-1}). Inhibition of NO production, by previous injection of L-NAME, increased PRL secretion at 60 (24.1 ± 4.9 ng mL^{-1}) and 120 min (24.5 ± 6.4 ng mL^{-1}) of endotoxemia ($P < 0.01$) in

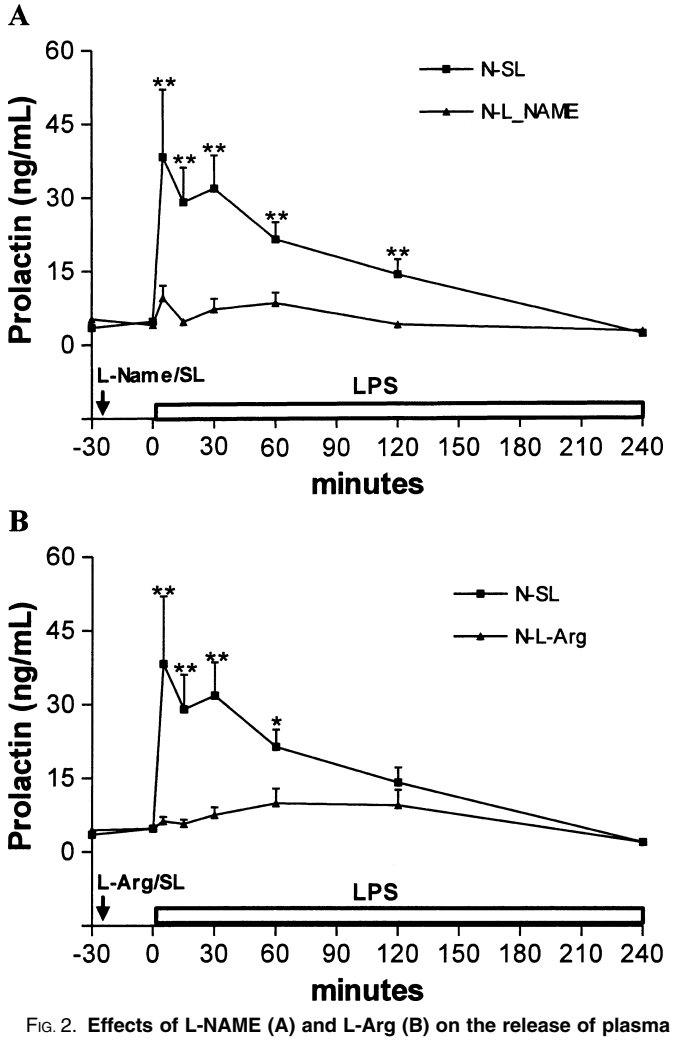

FIG. 2. **Effects of L-NAME (A) and L-Arg (B) on the release of plasma prolactin in N endotoxemic rats.** The arrows represent L-NAME, L-Arg or, SL injection. Endotoxemia is represented by the horizontal bar. Results are reported as means ± SEM for 10 animals in each group and were analyzed by the nonparametric Mann-Whitney *U* Test. **P* < 0.05 and ***P* < 0.01 indicate significance of differences between N-SL and N-L-NAME (A) and N-SL and N-L-Arg (B) groups.

TX rats compared with SL control (60 min, 11.2 ± 3.5 ng mL^{-1}; 120 min, 9.3 ± 3.0 ng mL^{-1}; Fig. 3A). Previous treatment with L-Arg also increased PRL release at 120 min (27.0 ± 8.1 ng mL^{-1}) and 240 min (22.1 ± 10.5 ng mL^{-1}) of endotoxemia ($P < 0.05$) in TX rats compared with SL-injected controls (120 min, 9.3 ± 3.0 ng mL^{-1}; 240 min, 3.2 ± 0.7 ng mL^{-1}) as shown in Figure 3B.

Effect of L-NAME and L-Arg on PRL release: a comparison of N and TX endotoxemic rats

PRL secretion in TX rats was significantly higher at 60 min (24.1 ± 4.9 ng mL^{-1}) and 120 min (24.5 ± 6.4 ng mL^{-1}) of endotoxemia, after previous injection of L-NAME when compared with N control group (60 min, 8.4 ± 2.0 ng mL^{-1}; 120 min, 3.9 ± 0.7 ng mL^{-1}; Fig. 4A). Previous treatment with L-Arg also increased ($P < 0.05$) PRL release in TX rats after LPS-induction inflammatory response at 120 min (27.0 ± 8.1 ng mL^{-1}) and 240 min (22.1 ± 10.5 ng mL^{-1}) compared with N controls (120 min, 9.5 ± 3.1 ng mL^{-1}; 240 min, 2.0 ± 0.2 ng mL^{-1}; Fig. 4B). Moreover, the PRL secretion in N SL-treated rats displayed similar levels to those of TX groups previously treated with L-NAME and L-Arg (at and after 60 min).

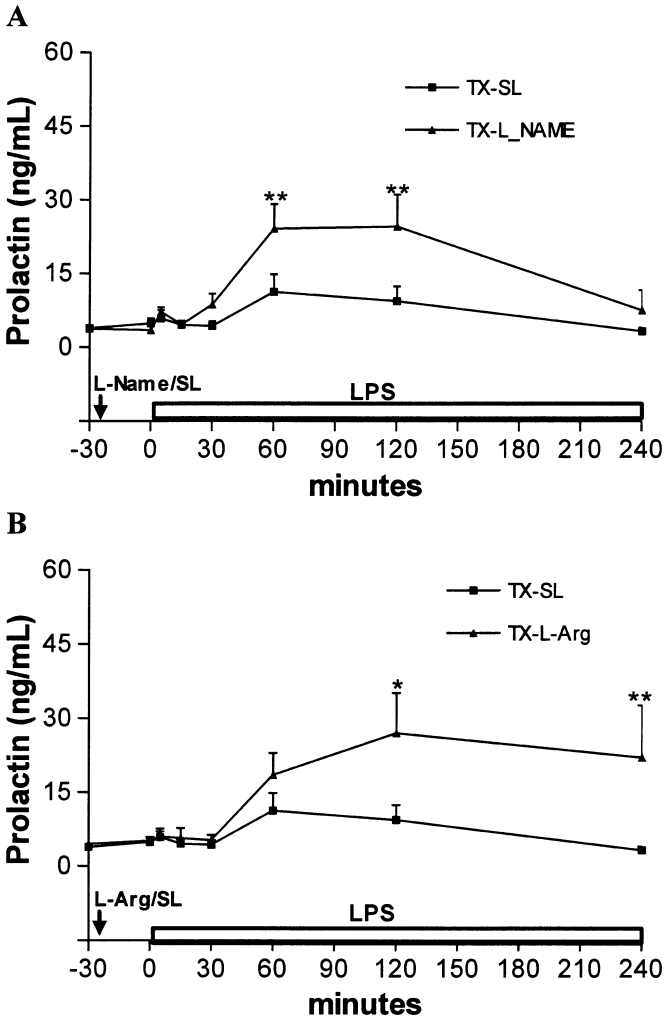

FIG. 3. **Effects of L-NAME (A) and L-Arg (B) on the release of plasma prolactin in TX endotoxemic rats.** The arrows represent L-NAME, L-Arg, or SL injection. Endotoxemia is represented by the horizontal bar. Results are reported as means ± SEM for 10 animals in each group and were analyzed by the nonparametric Mann-Whitney *U* Test. *$P < 0.05$ and **$P < 0.01$ indicate significance of differences between TX-SL and TX-L-NAME (A) and TX-SL and TX-L-Arg (B) groups.

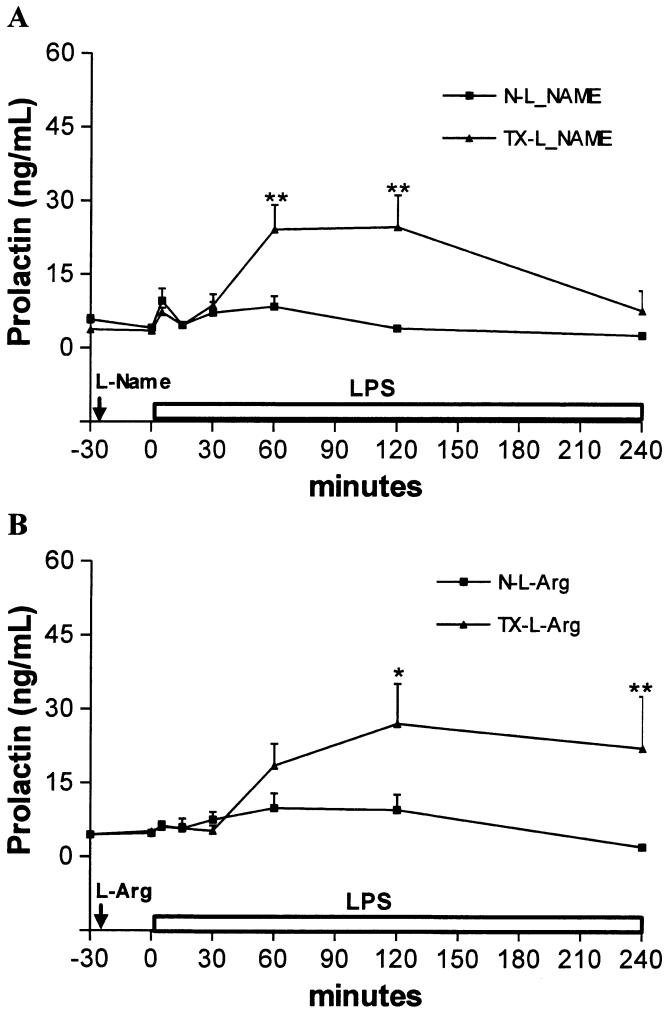

FIG. 4. **Effects of injection of LPS on the release of plasma prolactin in N and TX rats treated with L-NAME (A) and L-Arg (B).** The arrows represent L-NAME or L-Arg injection. Endotoxemia is represented by the horizontal bar. Results are reported as means ± SEM for 10 animals in each group and were analyzed by the nonparametric Mann-Whitney *U* Test. *$P < 0.05$ and **$P < 0.01$ indicate significance of differences between N-L-NAME and TX-L-NAME (A) and N-L-Arg and TX-L-Arg (B) groups.

DISCUSSION

This work presents convincing evidence that, in normal rats, NO exerts a modulatory influence on the hypothalamic pathways that lead to increased PRL secretion in response to endotoxemia, whereas in hypothyroid rats, NO appears to have an opposite effect. Although endotoxemia results in an alteration in thyroid hormone concentration (2), our experimental model demonstrated that the euthyroid status of normal endotoxemic rats did not imply hypothyroidism.

Our study demonstrated that the previous injection of L-NAME inhibited the PRL response to endotoxemia until 120 min in N rats. The neuroendocrine regulation of PRL secretion is complex and subjected to a wide variety of influences

(neurotransmitters, neuromodulators, and neuropeptides) that can act within the hypothalamus (7). It has been suggested that L-Arg may play a significant role in regulating dopamine (DA) transport in specific areas of brain (8). There are also studies showing an inhibitory action of NO on the release of norepinephrine and DA from the medial basal hypothalamus of the rat (9) and on tyrosine hydroxylase activity of the median eminence (10). PRL secretion from the pituitary gland is mainly controlled by DA released in the median eminence from tuberoinfundibular dopaminergic nerve endings, and tyrosine hydroxylase is the first and rate-limiting enzyme in DA biosynthesis (11), which further supports our data.

The results related to the previous treatment with L-Arg, the substrate for NO synthesis, which significantly blocked PRL release in N rats during 60 min of endotoxemia, may be considered controversial and are probably due to the dual personality of the free radical gas. Under normal conditions, NO produced in low concentrations acts as a signaling molecular and cytoprotective (antioxidant) factor. Alternatively, when the circumstances allow the formation of substantial amounts of NO, as in the presence of endotoxemia and/or the availability of large amounts of L-Arg, it acts as an indirect cytotoxic effector, via the formation of various reactive nitrogen species (12, 13). NO is produced by the action of endothelial NOS, neuronal NOS (both constitutives), and iNOS, which catalyzes the reaction of L-Arg with molecular oxygen to form citrulline and NO. Furthermore, NO can regulate its own production (14). Previous studies indicate that NO inhibits the activities of the constitutive isoforms of NOS (nNOS) from rat cerebellum by interacting with the heme prosthetic group (15), as well as the inducible isoform from activated rat alveolar macrophages (16). The negative feedback regulation of iNOS expression by NO in macrophages is also mediated by the inhibition of the transcription factor NF-κB activation (17), which avoids an overwhelming and uncontrolled inflammatory response.

Another possible regulation of NO production is by arginase II in the brain. *In situ* hybridization of the rat brain showed that arginase II mRNA is ubiquitous in glial cells and neurons, including nNOS-positive neurons (18). NOS and AII use arginine as a common substrate and arginase may down-regulate NO production by competing with NOS for arginine (19–22).

According to our previous work, thyroid hormones are necessary for a proper response of PRL release during endotoxemia (5). Besides DA, which exerts a physiologically relevant and inhibitory effect on PRL release, PRL-releasing factors also exist that control hormone secretion independent of, or in concert with, endogenous DA (7). Because thyroid hormones appear to act largely by influencing gene transcription, many of these PRL-releasing factors may be increased or decreased by the action of thyroid hormones.

Inhibition of NO production by L-NAME lead to increased PRL secretion at 60 and 120 min of endotoxemia induction in TX rats. Previous studies have revealed that hypothyroidism in male rats produce a highly significant reduction in NOS gene transcripts in the paraventricular and supraoptic nuclei (4). In addition, hyperthyroidism leads to a substantial increase in rat liver NOS activity, an effect that is reversed upon hormone withdrawal (23). In our work, we observed that the blockade of NO production augmented PRL secretion in hypothyroid rats. This is the first experimental study to demonstrate the participation of NO in PRL secretion through the LPS-induced inflammatory response in N and TX rats. However, the mechanisms that account for the interaction between thyroid hormones, NO and PRL release are still unknown and cannot be inferred from this work.

Treatment with L-Arg before endotoxemia also increased PRL release at 120 and 240 min of LPS-induced inflammatory response in TX rats. We suggest that NO regulates its own production even in hypothyroid animals. Although hypothyroidism in male rats produces a significant reduction in NOS production (4), excessive amount of L-Arg plus endotoxemia could bring about deleterious effects on the body proceeding from NO. Thus, the regulation of supply of arginine for NO generation in neural cells may be of pivotal importance for a delicate balance between normal and pathophysiological events in central nervous system.

Although, in the present study, surgical thyroidectomy can not assure the whole integrity of the parathyroid glands, there is no significant difference in plasma calcium levels between normal and TX rats that have received preventive calcium lactate. On the other hand, propylthiouracil (PTU) or methimazole-induced hypothyroidism methods have limitations that are not acceptable in our experimental model. PTU is a specific inhibitor of type-I deiodinase enzyme that converts T4 to the more active hormone T3 in peripheral tissues. In the pituitary and in the brain, the prevalent enzyme is type-II iodothyronine deiodinase that is insensitive to PTU. Besides, PTU-induced hypothyroidism upregulates deiodinase type II, which is thought to act primarily to maintain intracellular T3 concentration in tissues (24, 25, 26). Additionally, drugs of thionamide class as PTU and methimazole may act directly suppressing the immune response on thyrocytes. Marked changes in the proportions of activated T helper-like and T suppressor-like cells decreased prostaglandin and cytokines release from thyroid cells, and inhibition the generation of oxygen radicals in immune cells have been reported (27, 28, 29).

In summary, our results suggest a modulatory role for NO in PRL release after induction of endotoxemia in N and TX rats. Physiological amounts of NO are necessary for a proper response of PRL release during endotoxemia in N rats because inhibition of NO production or increased availability of L-Arg blocked PRL secretion after LPS injection. In hypothyroid endotoxemic rats, NO appears to have opposite and inhibitory effects on PRL secretion. These findings should be interpreted in the context of the current paradigm for NO neurotoxicity, which states that its concentration is critical in determining whether it acts as a signaling molecule or as a toxin, imposing a nitrosative stress. However, the borderline between the physiological and pathophysiological activities of NO continues to be a matter of controversy.

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