

Participation of nitric oxide synthase and cyclooxygenase-2 in the salivary secretion of hypothyroid endotoxemic rats

Participação da sintase do óxido nítrico e da ciclooxigenase-2 na secreção salivar de ratos hipotireoidianos endotoxêmicos

Abstract

Purpose: In the present study the participation of nitric oxide synthase (NOS) and cyclooxygenase-2 (COX-2) on salivary secretion in endotoxemic hypothyroid rats was investigated.

Methods: Male Wistar rats with an initial weight of 180g were distributed into two groups, normal (N) or treated with propylthiouracil, 0.05 g/100 mL, administered orally for 5 weeks to induce hypothyroidism. Both groups were treated with lipopolysaccharide (LPS) (2.5 mg/kg; i.p.) to induce endotoxemia, or saline solution (SL), 90 min before salivary stimulation with pilocarpine (5 mg/kg; i.p.). Normal and PTU rats were divided into two groups each (n=07/09), receiving either L-NAME (10 mg/kg; i.p.), NOS inhibitor, or meloxicam (MLX) (0.5 mg/kg; i.p.), preferential COX-2 inhibitor, 30 min before endotoxemia challenge. Saliva was collected over a 15 min period ($\mu\text{L}/\text{min}/100\text{g}$ body wt.) from the time of the first drop of saliva.

Results: Hypothyroidism decreased salivary flow rate in both groups of rats (LPS and SL). Endotoxemia and NOS inhibition by L-NAME reduced salivary flow in N rats. Meloxicam stimulated salivary secretion in the physiological state and systemic inflammation, induced by LPS, in N and PTU rats (Mann-Whitney Test; $P < 0.05$).

Conclusion: In hypothyroid endotoxemic rats, it is COX-2 that modulates salivary secretion, not NOS.

Key words: Hypothyroidism; nitric oxide synthase; cyclooxygenase-2; saliva; endotoxemia

Resumo

Objetivo: Investigou-se a participação da sintase do óxido nítrico (NOS) e da ciclooxigenase-2 (COX-2) na secreção salivar de ratos hipotireoidianos endotoxêmicos.

Metodologia: Ratos Wistar com peso inicial de 180g foram distribuídos em dois grupos, normais (N) ou tratados com propiltiouracil (PTU) 0,05 g/100 mL, via oral, durante 5 semanas, para induzir hipotireoidismo. Ambos os grupos foram tratados com lipopolissacarídeo (LPS), 2,5 mg/kg i.p., para indução de endotoxemia ou salina (SL), 90 min antes da estimulação salivar com pilocarpina (5 mg/kg; i.p.). Os ratos N e PTU foram divididos em dois grupos cada (n = 07/09) e receberam injeções de L-NAME (10 mg/kg; i.p.), inibidor da NOS, ou meloxicam (MLX) (0,5 mg/kg; i.p.), inibidor preferencial da COX-2, 30 min antes da indução da endotoxemia. O fluxo salivar ($\mu\text{l}/\text{min}/100\text{g}$ de p.c.) foi avaliado durante um período de 15 min a partir da primeira gota de saliva.

Resultados: O hipotireoidismo diminuiu o fluxo salivar em ratos tratados ou não com LPS. A endotoxemia e a inibição da NOS, através do L-NAME reduziu o fluxo salivar em ratos N. O MLX estimulou a salivagem em situações fisiológicas e inflamatórias nos ratos N e PTU (Mann-Whitney; $P < 0,05$).

Conclusão: A COX-2, mas não a NOS, modula a secreção salivar em ratos hipotireoidianos endotoxêmicos.

Palavras-chave: Hipotireoidismo; óxido nítrico sintase; ciclooxigenase-2; saliva; endotoxemia

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Introduction

Neural control of the salivary gland secretions is primarily dependant on the muscarinic cholinergic system. Recent studies have suggested that cholinergic activation guarantees the vasodilatation necessary for their secretion through the nitergic mediation (1). Recognition of the participation of nitric oxide (NO) in cell to cell communication altered the traditional concept of neurotransmission. NO facilitates the release of excitatory neurotransmitters, possibly through the pre-synaptic cyclic GMP-dependant mechanism (2). In this context, NO modulates the salivary secretion, blood supply and neurotransmission in nerve endings of the salivary glands (3). Evaluation of the vascular capacity of NO formation in rats with thyroid dysfunction suggests that the thyroid status modulates the activity of NO synthase (NOS). Hyperthyroidism is associated with the increase in NOS activity and NO formation capacity in various tissues related to blood pressure control, in comparison with hypothyroidism (4,5). NOS activity in hypothyroidism is characterized by a pattern of heterogeneous responses. According to Quesada (5), this diversity of responses could be a result of alterations in the expression of the different isoforms of NOS.

Sepsis is a set of severe manifestations resulting from systemic inflammatory response associated with high costs and high rates of morbimortality. During sepsis, exposure to lipopolysaccharide (LPS) sets off a systemic inflammatory reaction with the participation of inflammatory mediators that require prostaglandin cascades (PGs), salivation inhibitors (6,7), with the co-participation of NO. Activation of the cascade is dependant on the cyclooxygenase enzymes (COX) and NOS. Under normal conditions, the NO produced at low concentrations, by the action of eNOS and nNOS (endothelial and neuronal), both constitutive isoforms of NOS, acts as a signaling molecule and cytoprotection factor (antioxidant). Alternatively, in situations that allow the formation of substantial volumes of NO, such as endotoxemia, the NO produced by the action of iNOS (inducible isoform) act as an indirect cytotoxic effector, through the formation of reactive nitrogen species (8,9). The systemic inflammatory response caused by severe endotoxemia frequently reduces the release of thyroid hormones, a condition known as *Low T3 Syndrome*, suggesting an overall hypofunction of the hypothalamus-hypophysis thyroid axis. Isolated thyroid dysfunction does not always show evidence of functional alterations in salivary secretion in individuals under basal conditions. In previous studies, Dixit and cols. (10) concluded that hypothyroidism and therapeutic thyroid hormone replacement in humans do not alter stimulated saliva production. However, challenges to survival, such as acute stress or sepsis in individuals with thyroid dysfunctions, rupture the homeostatic balance and show functional alterations that were previously concealed (11,12). Our recent studies showed a reduction in the volume of total saliva stimulated by the muscarinic cholinergic pathway in thyroidectomized rats. Endotoxemia aggravated the reduction in salivary flow (13).

The thyroid hormones regulate the production of body energy and contribute to homeostasis of the main metabolic

pathways (14). They modulate salivary flow through the sensitivity of salivary glands to autonomic stimulation. The salivary glands in turn, have a high rate of metabolism and blood flow; both are proportional to the rate of saliva formation (15) and indirectly dependant on thyroid function. Saliva is a complex, aqueous fluid, composed of a multiplicity of substances, mainly electrolytes and various proteins, glycoproteins and mucine, which aid digestions, lubrication, cleaning and protecting the oral cavity, the region of the oropharynx and upper digestive tract (16,17). The oral cavity is one of the ports of entry of bacteria and viruses into the body. Dental surgical procedures, such as tooth extractions translocate bacteria from the oral cavity and cause transitory bacteremia with high risk of endotoxemia. Frequently, the drugs of first choice in preventive and therapeutic treatment of pain in dental practice are non-steroid antiinflammatory drugs (NSAIDs). Particularly, the COX-2 selective inhibitors present effective antiinflammatory action and significantly lower risks of gastrointestinal toxicity, in comparison with conventional NSAIDs.

There are very few studies that investigate the interactions of inflammatory inhibitors: NO and PGs and thyroid hormones with salivary secretion in individuals submitted to endotoxemia. The aim of this study was to evaluate the participation of NOS and COX-2 in salivary flow of hypothyroid rats submitted to endotoxemia.

Methods

Ninety male Wistar rats from the Escola Bahiana de Medicina e Saúde Pública (EBMSP) animal colony, with a mean initial weight of 180 g were used. The rats were kept in a room with a temperature controlled at $22 \pm 2^\circ\text{C}$, with a regime of 12 hours of artificial light daily (06:00h to 18:00h).

For the induction of hypothyroidism the rats received tap water with propylthiouracil (PTU) 0.05 g/100 mL (Biolab, Taboão da Serra, SP, Brazil) for 5 weeks. The rats were randomly placed in groups of 4-5 per cage, separated into two groups: normal (N) and those treated with PTU. Both groups were fed on commercial rat chow – Nuvital – and the normal rats received tap water *ad libitum*. The study was approved by the Ethic Committee of Using Animals in Research of EBMSP, Protocol Number 011/2007.

On the day before the experiment, the rats were weighed, separated into individual boxes and received a liquid diet. This procedure was performed to prevent possible interference of food ingestion on salivary secretion. On the day of the experiment the two groups of rats (N and PTU) received injections of L-NAME, 10 mg/kg (12) (Sigma Chemical Co., St. Louis, MO, EUA), non-selective NOS inhibitor, or meloxicam – MLX (0.5 mg/kg; Boehringer Ingelheim, Itapeverica da Serra, SP, Brazil), preferential COX-2 inhibitor, 30 minutes before the induction of endotoxemia by LPS – 2.5 mg/kg, *Escherichia coli* serotipe 055:B5 (Sigma Chemical Co., St. Louis, MO, EUA). All the drugs were diluted in saline solution (0.9%) and injected intraperitoneally (i.p.). The control groups received a similar volume of 0.9% saline solution (SL). Ninety minutes after the injection of LPS,

the rats were anesthetized with ketamine (100 mg/kg) + xilazine (14 mg/kg), tracheostomized and stimulated to salivate with the cholinergic agonist, pilocarpine i.p. (5 mg/kg body weight). The salivary flow ($\mu\text{l}/\text{min}/100\text{ g}$ body weight) was evaluated for 15 minutes from the time of the first drop of saliva.

The rats were sacrificed after each experimental procedure by deepened anesthesia in accordance with the ethical and scientific principles of Brazilian College of Animal Experimental (COBEA) for the use of animals in research and teaching. At this time, blood was collected through the jugular vein for plasmatic dosage of the thyroid hormones, T_3 and T_4 . Dosages were performed by chemiluminescence, using commercial kits bought from Ortho-Clinical Diagnostics, Johnson & Johnson Produtos Profissionais Ltda, São Paulo, SP, Brasil.

Statistical analysis was performed by the Mann-Whitney non-parametric method for comparison of the alterations in salivary flow between the N and PTU groups of rats, whether or not they were endotoxemic, treated with L-NAME, MLX or SL. The results were expressed as means and \pm standard deviation (SD) from the mean, and differences with $P < 0.05$ were considered statistically significant.

Results

In the fifth week of treatment with PTU, the plasma concentration of T_3 and T_4 was significantly lower than that of the normal rats (Table 1). The rats treated with PTU presented reduction in body growth in comparison with their normal controls. The normal rats gained a mean of 88% weight during the 5 weeks, while the rats treated with PTU gained only 11% weight in the same period.

Table 1. Plasmatic concentration of T_3/T_4 in normal rats and propylthiouracil (PTU) treated rats.

Groups	T_3 (ng/mL)	T_4 ($\mu\text{g}/\text{dL}$)
Normal rats	0.518 ± 0.106	2.421 ± 1.179
PTU rats	$0.344 \pm 0.079^*$	$0.252 \pm 0.084^\dagger$

Note: Results are reported as means \pm standard deviation (S.D.) for 06 rats in each group and were analyzed by the non-parametric Mann-Whitney U test. * $P < 0.05$ and $^\dagger P < 0.01$ indicates significance of differences between normal (N) rats and propylthiouracil (PTU) treated rats.

The induction of hypothyroidism through treatment with PTU, markedly diminished ($P < 0.01$) salivary flow in rats whether or not they were treated with LPS. Endotoxemia also caused significant reduction ($P < 0.05$) in salivary secretion in normal rats (Fig. 1).

The prior injection of L-NAME reduced the salivary flow of hypothyroid rats when compared with their non endotoxemic N controls ($P < 0.05$), but did not modify the salivary flow in PTU rats that received LPS (Fig. 2).

A significant drop ($P < 0.01$) in the salivary flow was found in PTU rats that received previous treatment with MLX, whether or not they were submitted to endotoxemia, in comparison with the group of N rats with the same treatment (Fig. 3).

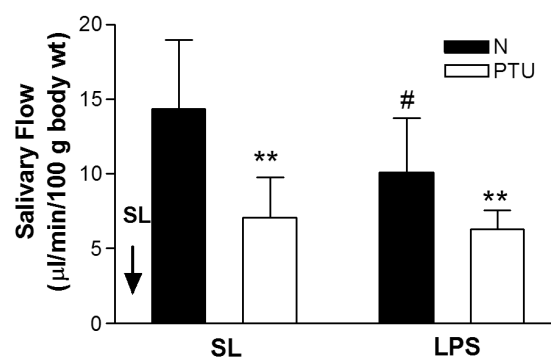


Fig. 1. Effects of propylthiouracil (PTU) and endotoxemia (LPS) on the salivary flow. Results are reported as means \pm standard deviation (S.D.) for 7-9 rats in each group and were analyzed by the non-parametric Mann-Whitney U test. ** $P < 0.01$ indicates significance of differences between normal (N) and propylthiouracil (PTU) treated rats in the same group (SL or LPS). # $P < 0.05$ indicates significance of differences between N rats after endotoxemia.

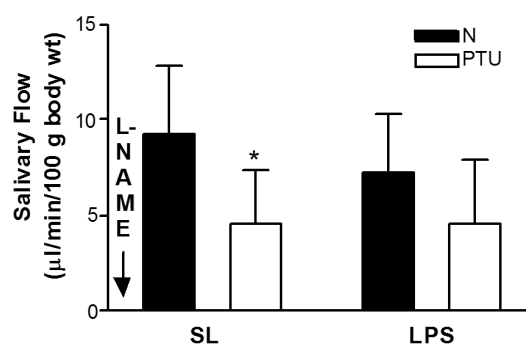


Fig. 2. Effects of previous treatment with L-NAME on the salivary flow in normal (N) and propylthiouracil (PTU) treated rats, after LPS injection. Results are reported as means \pm standard deviation (S.D.) for 7-9 rats in each group and were analyzed by the non-parametric Mann-Whitney U test. * $P < 0.05$ indicates significance of differences between N and PTU rats in the same group (SL).

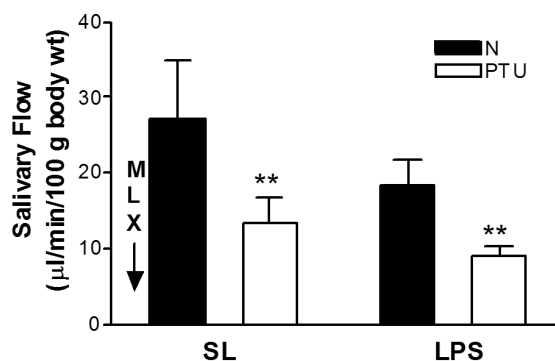


Fig. 3. Effects of previous treatment with meloxicam (MLX) on the salivary flow in normal (N) and propylthiouracil (PTU) treated rats, after LPS injection. Results are reported as means \pm standard deviation (S.D.) for 7-9 rats in each group and were analyzed by the non-parametric Mann-Whitney U test. ** $P < 0.01$ indicates significance of differences between N and PTU rats in the same group (SL or LPS).

A significant increase ($P < 0.01$) was observed in the salivary flow of hypothyroid rats previously treated with MLX and injected with saline or LPS. However, a diminished stimulatory effect of MLX was observed in the salivary secretion of hypothyroid rats after endotoxemia (Fig. 4).

Figure 5 shows that previous treatment with L-NAME reduced the release of saliva ($P < 0.05$) in the non endotoxemic N rats, but did not modify salivary secretion in rats treated with LPS. COX-2 inhibition by previous injection with MLX, markedly elevated ($P < 0.01$) the salivary flow in N rats whether or not they were submitted to endotoxemia, when compared with their respective controls. The inflammatory response promoted by LPS showed evidence of a marked decrease ($P < 0.05$) in the salivary flow of the group of N rats treated with MLX (Fig. 5).

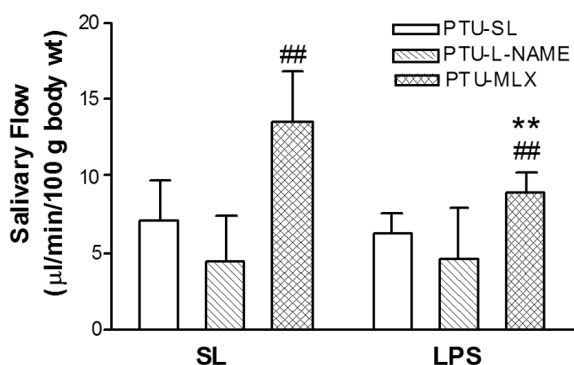


Fig. 4. Effects of previous treatment with saline (SL), L-NAME or meloxicam (MLX) on the salivary flow in propylthiouracil (PTU) treated rats, after LPS injection. Results are reported as means \pm standard deviation (S.D.) for 7-9 rats in each group and were analyzed by the non-parametric Mann-Whitney U test. ^{##} $P < 0.01$ indicates significance of differences between PTU-MLX vs. PTU-SL rats, in the same group (SL or LPS). ^{**} $P < 0.01$ indicates significance of differences between PTU rats previously treated with MLX, before and after endotoxemia.

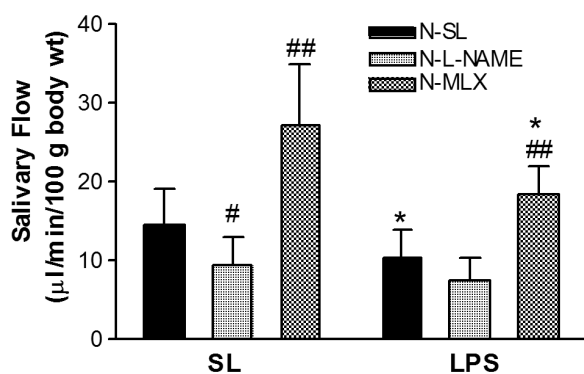


Fig. 5. Effects of previous treatment with saline (SL), L-NAME or meloxicam (MLX) on the salivary flow in normal (N) rats, after LPS injection. Results are reported as means \pm standard deviation (S.D.) for 7-9 rats in each group and were analyzed by the non-parametric Mann-Whitney U test. ^{##} $P < 0.01$ indicates significance of differences between N-MLX vs. N-SL rats, in the same group (SL or LPS). [#] $P < 0.05$ indicates significance of differences between N-L-NAME vs. N-SL rats, in the same group (SL). ^{*} $P < 0.05$ indicates significance of differences between N-SL or N-MLX rats, after endotoxemia.

Discussion

Salivary secretion is a complex phenomenon, with high energy expenditure, which depends on the active transport of solutes by the glandular tissue and significant increase in metabolic renewal under stimulation. The hypothyroidism caused by PTU, a thyroperoxidase activity inhibitor, and consequently, inhibiting thyroid hormone biosynthesis, reduced the salivary flow in both groups of rats injected with LPS or saline, in comparison with the euthyroid control rats (Fig. 1). In our previous studies (13) with thyroidectomized rats, it was demonstrated that the thyroid hormones are necessary for salivary secretion, and confirmed by the data obtained in this study with rats treated with PTU. The thyroid hormones participate in the production of body energy to meet the metabolic demands of various organic processes, for example, salivary secretion. In their target cells, they set off various biologic effects, such as the expression and activity of various enzymes of oxidative metabolism, ATPases, as well as transporters of ions and other proteins. The reduction in the salivary flow in hypothyroid rats may be explained by the lower oxygen consumption and energy production by the gland, associated with low activity and/or expression of the enzymes that guarantee the cellular ionic gradient, such as Na^+/K^+ ATPase (18).

Inadequate cell metabolism, as a result of the defects in energy production, is one of the main causes of organic dysfunction in sepsis (19). The systemic inflammatory response to LPS diminishes the availability of oxygen to the cells as a result of tissue hypoperfusion, peripheral vasodilatation, blood flow redistribution and the release of various inflammatory mediators, among them NO, pro-inflammatory cytokines and PGs. In the salivary glands of rats treated with LPS, the PG content rises in 60 minutes and remains increased for prolonged periods (6). Singh and cols. (20) demonstrated that 30 minutes after the systemic injection of LPS in low doses, there is a significant increase in the genic expression of iNOS in the brains of rats. LPS is used in a broad range of doses with various objectives, ranging from the induction of endotoxemia through to endotoxic shock, with failure of various organs. The dose of LPS chosen in this study has been used in neuroimmune endocrine studies involving thyroid function without causing lethality in the animals (11,12).

The data in Figure 1 show that endotoxemia reduces the salivary flow in euthyroid rats without modifying the salivary flow of rats treated with PTU. Possibly, the lower availability of oxygen and the production of inflammatory mediators typical of endotoxemia were incapable of potentiating the reduction in salivary secretion in the hypothyroid rats.

Constitutive production of NO is fundamental in regulating the blood flow to the salivary gland, release of neurotransmitters in the nerve endings of the gland and in the intracellular signal pathways involved in saliva production (3). Immunohistochemical studies have indicated the presence of neurons containing NOS in the exocrine glands, suggesting that

NO participates in the modulation of salivary secretion (21). NO increases the concentration of intracellular Ca^{++} in the acinar cell, which involves activation of the intracellular signal pathways and increase in salivary flow (3). Previous studies have demonstrated that inhibition of the production of NO by L-NAME reduced the salivary flow evoked by electrical stimulation of the autonomic nerves in rat salivary glands (22). A similar inhibitory effect was obtained in our present study with cholinergic stimulation. Nevertheless, the vasoconstrictor effect of L-NAME on the reduction in salivary flow cannot be excluded. As shown in Figure 5, there was a reduction in the salivary flow in N rats previously treated with L-NAME, unspecific inhibitor of NOS isoforms.

There are evidences indicating that the genic expression of constitutive NOS isoforms is also subject to the challenges of sepsis. Under this condition, the loss of modulation of nNOS and eNOS expression makes it difficult to achieve a clear classification of what is constitutive and what is inducible (23). The choice of L-NAME therefore, makes it an adequate drug in these circumstances. There was no reduction in the salivary flow of endotoxemic N rats treated with L-NAME (Figures 2 and 5). At low concentrations, NO participates as mediator of various physiological processes. During endotoxemia, the high production of NO and PGs associated with low tissue perfusion showed no evidence of the effect of L-NAME on the salivary flow of endotoxemic N rats.

In hypothyroid rats, L-NAME and saline presented similar effects on the reduction of salivary flow, suggesting the functional removal of the nitregeric pathway concomitant with hormonal deficiency. The induction of endotoxemia in hypothyroid rats previously treated with L-NAME did not alter the salivary flow. It is evident that the thyroid hormones are essential for salivary secretion and the hypothyroid status was the limiting condition in the secretion of saliva (Fig. 4).

COX-2 inhibition and consequently, synthesis of PGs by the previous injection of MLX, raised the salivary flow in the N and PTU rats whether or not they were submitted to endotoxemia (Figures 4 and 5). Previous studies (24,6) have demonstrated the activity of PGs in the reduction of salivary secretion. It is proposed that there is a biochemical signal between NO and/or NOS and the PGs in the submandibular gland of normal rats. NO or the activation of NOS is modulated by the activity of COX and the production of prostanoids, especially PGE_2 (25). Activation of the PGE_2 receptor is subject to calcium mobilization and salivary secretion requires an influx of this ion into the salivary gland. Therefore, calcium depletion, or the addition of a calcium antagonist inhibits the secretion of stimulated saliva. In the present study, MLX inhibited PG synthesis and possibly, the lack of activation of the PG receptor made intracellular Ca^{++} available for the signal pathways that stimulate the secretion of saliva. MLX completely restored the salivary secretion of hypothyroid rats in comparison with normal rats under

cholinergic stimulation. Hyperthyroidism is associated with greater phospholipase activity in the cell membrane and with an increase in the availability of precursors in the formation of PG (26). Hypothyroidism modifies both the capacity of NO formation and of the response to the NO produced (4). Considering the biochemical signal between NO and the PGs, the hypothyroidism status may be related to the low PGs production, low NOS activity and lower NO production, thus causing a reduction in salivary secretion.

The inflammatory response promoted by the LPS partially blocked the salivary flow in both the N and PTU groups of rats treated with MLX (Figures 4 and 5). The results revealed the reduction of the stimulatory effect on salivary secretion caused by the COX-2 inhibitor, MLX, possibly due to the elevated production of NO and PGs during the endotoxemia.

MLX, widely used as an antiinflammatory drug in clinical dentistry, partially recovers the defense barrier that salivary secretion represents at the port of entry into the oropharynx in normal and hypothyroid individuals.

Conclusions

1. Hypothyroidism, caused by treatment with PTU, markedly reduced salivary flow, therefore is a limiting factor for salivary secretion.
2. The endotoxemia induced by LPS diminished the salivary flow in normal rats. The effects of hypothyroidism overcame those of the endotoxemia, since the rats treated with PTU presented no alteration in salivary flow.
3. The NOS inhibition, by L-NAME, reduced the salivary flow in normal rats, suggesting that NO has stimulatory effects on the salivary gland under physiological conditions. However, during endotoxemia, NO did not modulate the salivary secretion of N rats.
4. MLX stimulated salivation in physiological and inflammatory situations in normal and hypothyroid rats, suggesting an inhibitory role of PGs in salivary secretion.
5. The endotoxemia partially reduced the stimulatory effect produced by MLX on the salivary flow of normal and hypothyroid rats.
6. The hypothyroidism status prevailed over the effects caused by L-NAME. Among the hypothyroid rats MLX restored the hyposalivation whether or not they were submitted to endotoxemia.
7. MLX completely restored the salivary secretion of hypothyroid rats in comparison with normal rats under cholinergic stimulation.

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