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Endocannabinoids mediate hyposalivation induced by inflammogens in the submandibular glands and hypothalamus

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ABSTRACT

Objective: The aim of this study was to investigate the factors that could participate on salivary glands hypofunction during inflammation and the participation of endocannabinoids in hyposalivation induced by the presence of inflammogens in the submandibular gland (SMG) or in the brain.

Design: Salivary secretion was assessed in the presence of inflammogens and/or the cannabinoid receptor antagonist AM251 in the SMG or in the brain of rats. At the end of the experiments, some systemic and glandular inflammatory markers were measured and histopathological analysis was performed.

Results: The inhibitory effect observed 1 h after lipopolysaccharide (LPS, 50 μg/50 μl) injection into the SMG (*ig*) was completely prevented by the injection of AM251 (5 μg/50 μl) by the same route ($P < 0.05$). The LPS (*ig*)-induced increase in PGE2 content was not altered by AM251 (*ig*), while the glandular production of TNFα induced by the endotoxin ($P < 0.001$) was partially blocked by it. Also, LPS injection produced no significant changes in the wet weight of the SMG neither damage to lipid membranes of its cells, nor significant microscopic changes in them, after hispopathological analysis, compared to controls. Finally, TNFα (100 ng/5 μl) injected intracerebro-ventricularly (*icv*) inhibited methacholine-induced salivary secretion evaluated 30 min after ($P < 0.01$), but the previous injection of AM251 (500 ng/5 μl, *icv*) prevented completely that effect.

Conclusion: We conclude that endocannabinoids mediate the hyposialia induced by inflammogens in the SMG and in the brain. The hypofunction would be due to changes on signalling pathway produced by inflammatory compounds since anatomical changes were not observed.

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1. Introduction

Saliva is a versatile substance that serves many purposes in the oral and pharyngeal environment since plays a key role in the local and systemic defense of the oral cavity, the oropharyngeal region, and the upper gastrointestinal tract.[1,2] It is a lubricant that facilitates the swallowing of food, a mechanical device that cleans the dentition, an immunological barrier, a digestive initiator and an ionic stimulator for taste. Diminished salivary output from the major and minor glands is called ''salivary hypofunction'' and the subjective complaint of a dry mouth is termed ''xerostomy''. Hyposalivation significantly affects the individual's quality of life as well as oral health. It is likely that numerous systemic diseases (such as Sjögren syndrome) and their treatments (medications, head and neck radiation, chemotherapy) contribute significantly to salivary gland hypofunction.[3–6] Whole saliva is a mixed fluid that derives predominantly from 3 pairs of major salivary glands: the submandibular (SMG), the parotid, and the sublingual glands. Approximately 90% of total salivary volume results from the activity of these 3 pairs of glands, with the bulk of the remainder from minor salivary glands located at various oral mucosal sites.[7,8] The secretion of saliva is controlled by the autonomic nervous system. The parasympathetic nervous system exerts its function through the activation of muscarinic receptors on salivary glands via impulses in the chorda tympani nerve that releases acethylcholine.[9] The sympathetic nervous system induces salivary secretion by releasing norepinephrine that stimulates α- and β-adrenergic receptors in the acini.[10] Numerous projections exist from the lateral hypothalamus to salivary nuclei located in the brain stem.[11,12] It was reported that pilocarpine, a muscarinic agonist, injected intracerebroventricularly (*icv*) induced salivary secretion by activating central autonomic efferent fibres.[13]

Cannabis consumption has been associated with a reduction in salivary flow and may lead to oral problems like progressive dental caries, fungal infection, oral pain, and dysphagia.[14,15] The main psychoactive ingredient of *Cannabis sativa*, Δ9-tetrahydrocannabinol (THC), affects different physiological functions. Twenty five years ago, two subtypes of G-protein-coupled cannabinoid (CB) receptors were identified: the CB1 central receptor subtype, which is mainly expressed in the brain,[16,17] and the CB2 peripheral receptor subtype, which appears to be particularly abundant in the immune system.[18] A few years later, anandamide and arachidonoyl glycerol, the best-known endocannabinoids, were discovered and purified. Both endocannabinoids bind with high affinity to CB receptors.[19] Selective antagonists have been developed for cannabinoid receptors, such as AM251 and SR141716A for CB1 and AM630 and SR144528 for CB2.[20–23] We reported previously that CB1 and CB2 receptors are located in the SMG and that anandamide injected into the SMG decreases the methacholine (MC)- and norepinephrine-stimulated salivary secretion by activating both receptors.[24] In contrast to classical neurotransmitters, endocannabinoids can function as retrograde synaptic messengers. They are released from postsynaptic neurons and travel backwards across synapses, activating CB1 receptors on presynaptic axons and suppressing neurotransmitter release.[25] We also previously demonstrated that anandamide acts on hypothalamic CB1 receptors to inhibit salivary secretion by attenuating parasympathetic neurotransmission to the SMG.[26] Therefore, endocannabinoids inhibit salivary secretion by acting at least at two different levels: (a) on cannabinoid receptors in the salivary glands[24] and (b) on central CB1 receptors that respond by inhibiting parasympathetic neurotransmission to the SMG.[26]

Lipopolysaccharide (LPS), an integral part of the outer membrane of gram-negative bacteria, is the main pathogenic factor that leads to infection. We have previously demonstrated that LPS (5 mg/kg/3 h) injected intraperitoneally inhibits salivary secretion by increasing the production of prostaglandins and endocannabinoids.[27,28] Also, TNFα is known to be released after LPS administration and mediates a number of effects attributed to LPS;[29] therefore it could be involved in LPS-induced inhibition of salivary secretion.[28] In fact, we previously demonstrated that the blockage of SMG cannabinoid receptors prevents the inhibition of MC-induced salivary secretion induced by TNFα injected intraglandularly.[28] In addition, anandamide content is rapidly increased in different tissues in response to LPS intraperitoneal or intravenous injections.[24,28,30] Furthermore, anandamide is able to inhibit pro-inflammatory cytokines production, including TNFα in LPS-stimulated monocytes and rat microglial cells,[31] suggesting that endocannabinoids modulate inflammatory responses.

Based in evidences presented above, the aim of this study was to investigate the factors that could participate on salivary glands hypofunction during inflammation and the participation of endocannabinoids in hyposalivation induced by the presence of inflammogens in the SMG or in the brain.

2. Materials and methods

2.1. Animals

Adult male Wistar rats (250–300 g) from our own colony were kept in group cages in an animal room having a photoperiod of 12 h of light (07:00–19:00 h), room temperature at 22–25 °C, and free access to rat chow and tap water. The animals were divided into several experimental groups with six to eight animals each and were kept in the laboratory area. Animal procedures were performed in accordance with the recommendations from the Guide for the Care and Use of Laboratory Animals of the National Research Council, USA, 1996.

2.2. Salivary secretion studies

Salivary responses were determined in anesthetized rats (100 mg/kg of chloralose, 0.5 ml of 0.9% NaCl, *iv*). The SMG ducts were cannulated with a fine glass cannula, and salivary secretion was induced by different doses of methacholine (MC, 1, 3 and 10 mg/kg in saline) administered sequentially via the right femoral vein, as previously described.[32] No resting (unstimulated) flow of saliva was observed. The secretion induced by each dose of MC during 3 min was collected on aluminium foil and weighed. There were 5–6 rats per group and results were expressed as mg of saliva/3 min.

In the first group of experiments, the rats received intraglandular (*ig*) injections of LPS (50 μg/50 μl) or saline as vehicle (control). To evaluate the participation of the endocannabinoid system in salivary responses to local inflammation, the selective antagonist for CB1 receptor, AM251 (15 μg in 50 μl of 1% dimethylsulphoxide), or its vehicle were injected into the SMG, concomitantly with LPS. The injections were performed with a 30 G needle and the substances were injected very slowly between the capsule that covers the gland and the parenchyma. One hour later, dose–response curves to MC were performed to evaluate salivary secretion. The dose of AM251 employed was obtained from our previous reports.[28]

In a second group of experiments, in order to evaluate the participation of endocannabinoids in the hypothalamic control of salivary secretion during inflammation, a cannula was implanted into the lateral cerebral ventricle under tribromoethanol anaesthesia (3.5% in saline, 1 ml/100 g animal body weight, *ip*) using a stereotaxic instrument, 1 week prior to the day of the experiment. The coordinates relative to the interaural line (AP – 0.6 mm, L – 2 mm, DV – 3.2 mm) were taken from the stereotaxic atlas of Pellegrino et al.[33] The adequate location of the cannula in the ventricle was confirmed by injecting a solution of methylene blue *icv* at the end of the experiment and confirming its position in histological sections. TNFα (100 ng/5 μl) or its vehicle (saline), were injected *icv* and 30 min later, dose–response curves were constructed to evaluate their effects on salivary secretion. To study the effect of TNFα when CB1 receptors were blocked, AM251 (500 ng/5 μl, *icv*) was injected 15 min prior to the injection of the cytokine. AM251 was first dissolved in dimethylsulphoxide and further dilutions were made in saline, with the vehicle being saline with 1% dimethylsulphoxide.

2.3. Radioimmunoassay of PGE2

To determine PGE2 content, the SMG was homogenized in 1 ml of absolute ethanol and, after centrifugation, the supernatant was dried in a Speedvac at room temperature. The residues were then resuspended with buffer; antiserum (Sigma–Aldrich) was used as described in Mohn et al.[34] The sensitivity of the assay was 12.5 pg per tube. The crossreactivity of PGE2 and PGE1 was 100%, whereas the crossreactivity of other prostaglandins was 0.1%. The intra- and interassay coefficients of variation for PGE2 were 8.2 and 12%, respectively. The results were expressed in pg of PGE per mg of tissue, since the protocol of PGE extraction from the tissue includes homogenization in ethanol that interferes with protein determination. $^{[3H]}$PGE2 was purchased from New England Nuclear Life Science Products (Boston, MA, USA).

2.4. Determination of TNFα

For TNFα preservation after extraction, the SMG were immediately homogenized in PBS buffer containing protease inhibitory cocktail for mammalian tissue extracts (Sigma–Aldrich). The concentration of rat TNFα was determined using a sandwich ELISA according to the manufacturer's instructions (BD Pharmingen, USA).

2.5. Weight and total protein content in the SMG

At the end of the experiment, the SMG were removed, weighed and total protein was determined by Bradford assay.

2.6. Thiobarbituric acid reactive species (TBARS)

The TBARS assay is well-established for screening and monitoring lipid peroxidation. Malondialdehyde (MDA), which is one of several low-molecular-weight end products formed via the decomposition of certain primary and secondary lipid peroxidation products, participates in nucleophilic addition reaction with 2-thiobarbituric acid (TBA), generating a red, fluorescent 1:2 MDA:TBA adduct at low pH and elevated temperature that absorbs at 535 nm. The method used in the present study, was previously described by Maia et al.[35] Briefly, the SMG was homogenized in phosphate buffer pH 7.4, and was transferred to a tube containing trichloroacetic acid, HCl and TBA. The mixture was boiled for 30 min and then centrifuged at 3000 rpm for 20 min at 4 °C. The absorbance in the resulting pink-stained TBARS supernatants was determined in a spectrophotometer (Genesys 10 UV, Thermo Scientific) at 535 nm. The acid did not produce colour when tested without the addition of the sample. MDA concentration was calculated using a molar absorption coefficient ε value of $1.56 \times 10^5\ M^{-1}\ cm^{-1}$ and was expressed using as pmol per milligramme of tissue.

2.7. Histopathological studies

SMG were removed and were fixed with 10% neutral buffered formalin. Tissue samples were embedded in paraffin and cut into serial sections of 4 μm thick. SMG morphology and histopathological characteristics were examined on tissue sections after haematoxylin–eosin and periodic acid–Schiff (PAS) staining. Light microscopy was performed on an Axiolab Karl Zeiss microscope (Göttingen, Germany). All photographs were taken at 630× magnification using a Canon PowerShot G5 camera (Tokyo, Japan).

2.8. Statistics

Data are presented as the mean ± SEM. Comparison between two groups were performed y Student's *t*-test. Comparisons between more than two groups were performed by one-way analysis of variance (ANOVA) followed by the Student–Newman–Keuls multiple comparison test or by two-way ANOVA followed by the Bonferroni post-test. All analyses were performed with the Graph-Pad Instat software. Differences with *P*-values < 0.05 were considered statistically significant.

3. Results

3.1. Effect of CB1 receptor antagonist on LPS (ig)-induced inhibition of salivary secretion

The inhibitory effect of LPS (50 μg/50 μl) injected into the SMG (*ig*), after 1 h, on MC (1, 3 and 10 μg/kg)-induced salivary secretion was completely prevented by *ig* injections of the CB1

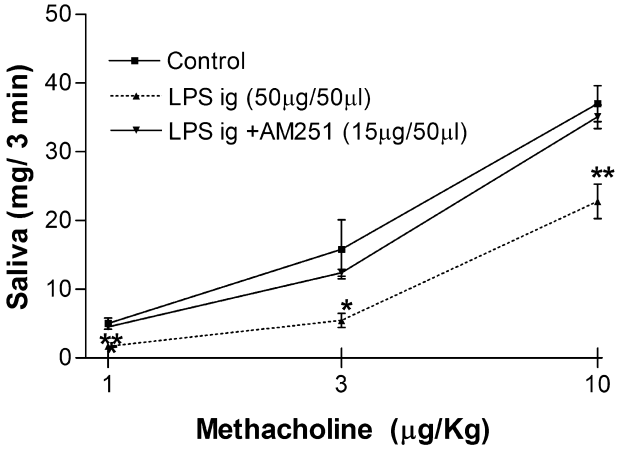

Fig. 1 – Effect of intraglandular (*ig*) injection of AM251 (15 μg/50 μl), on LPS (50 μg/50 μl/1 h, *ig*) induced inhibition on methacholine (MC)-stimulated salivary secretion. Values are mean ± SEM (6 rats per group). *P < 0.05, **P < 0.01 versus control and LPS + AM251 (two-way ANOVA followed by Bonferroni post test).

receptor antagonist, AM251 (15 μg/50 μl) ($P < 0.01$), confirming the participation of endocannabinoids in the control of salivation reduced during local inflammation (Fig. 1).

3.2. *Effects of CB1 receptor antagonist on PGE2 and TNFα increased levels induced by LPS (ig)*

TNFα released by immune cells mediates several LPS actions during infection. Therefore, we assessed the effect of LPS *ig* injection (50 μg/50 μl) on SMG's TNFα content, showing its augment, 1 h after injection, as compared to controls (injected with saline) ($P < 0.001$). Also, PGE2 content was increased in SMG 1 h after LPS injection as compared to controls ($P < 0.001$) (Figs. 2 and 3). To understand the role of endocannabinoids during SMG inflammation, we studied the effect of CB1 receptor blockage on PGE2 and TNFα glandular production, 1 h after LPS stimulation. The LPS-induced increase in PGE2 content was not altered by AM251 (50 μg/50 μl) (Fig. 2), while the glandular production of TNFα induced by LPS ($P < 0.001$) was partially blocked by it ($P < 0.05$) (Fig. 3).

3.3. *Macroscopic and histopathological analysis of SMG*

One hour after LPS injection, non-significant changes were observed in SMG wet weight as compared to control glands (Fig. 4A). However, the macroscopic observation of SMG injected with LPS shows a moderate oedema and an increased extracellular space in the area of injection. The additional treatment with AM251 did not cause appreciable changes in glandular morphology (data not shown). In addition, LPS induced a two-fold increase in the total protein glandular content as compared to control glands ($P < 0.001$), which was not modified by AM251 (Fig. 4B). AM251 treatment alone did not modify protein content as compared to controls.

Histopathological studies in SMG, evaluated 1 h after LPS injection, out of the area of injection, demonstrate non-significant microscopic changes. SMG of all groups investigated show normal structural organization of the gland without visible periductal, periacinar or caesural oedema (Fig. 5).

3.4. *Effect of the treatments on TBARS*

Since inflammation generates large amounts of reactive oxygen species (ROS) that interact with lipids, we determined the level of membrane lipid peroxidation as an index of oxidative stress in our experimental conditions.[35] LPS, at least

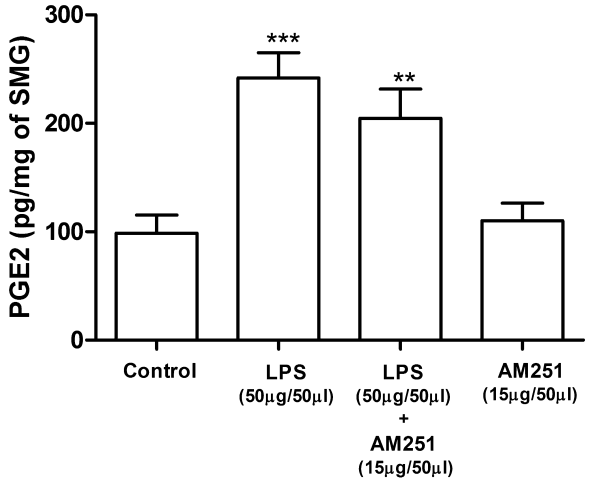

Fig. 2 – Effect of intraglandular (*ig*) injection of AM251 (15 μg/50 μl) on SMG's PGE2 content increased by LPS (50 μg/50 μl/1 h, *ig*). Values are means ± SEM (7–8 rats per group). *P < 0.05 and **P < 0.01 versus control (one-way ANOVA followed by the Student–Newman–Keuls multiple comparison test).

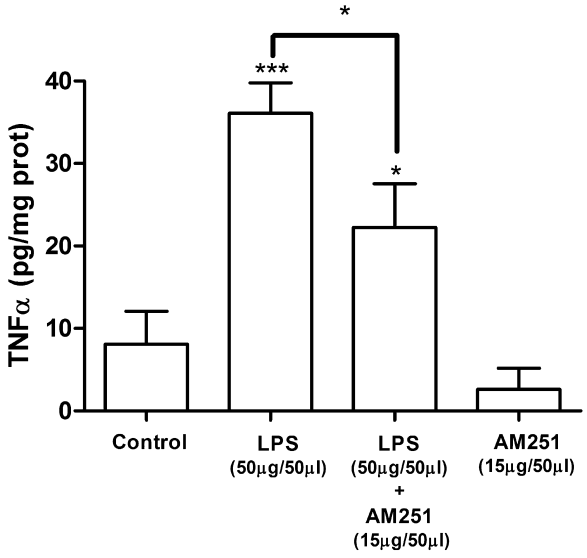

Fig. 3 – Effect of intraglandular (*ig*) injection of AM251 (15 μg/50 μl) on SMG's TNFα production increased by LPS (50 μg/50 μl/1 h, *ig*). Values are means ± SEM (7–8 rats per group). *P < 0.05 and ***P < 0.001 versus control and ¬*P < 0.05 versus LPS (one-way ANOVA followed by the Student–Newman–Keuls multiple comparison test).

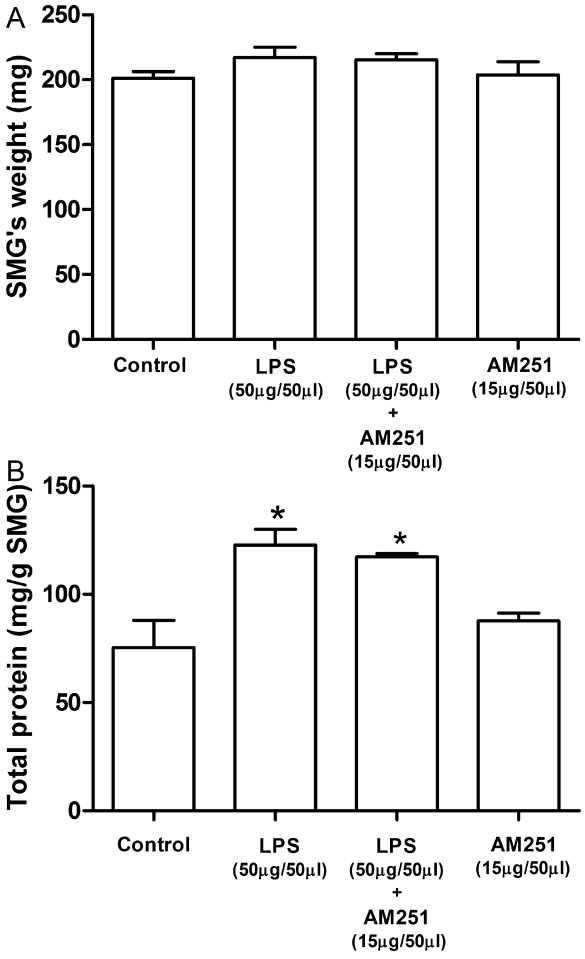

Fig. 4 – Effect of intraglandular (*ig*) injection of LPS (50 μg/50 μl/1 h) and/or AM251 (15 μg/50 μl) on (A) SMG's weight and (B) total protein content. Values are means ± SEM (6 rats per group). *P < 0.05 versus control (one-way ANOVA followed by the Student–Newman–Keuls multiple comparison test).

at the dose injected, did not show appreciable modification of TBARS at the time studied (Fig. 6).

3.5. *Effects of CB1 receptor antagonist on plasma TNFα increased by LPS (ig)*

We reported previously that LPS (5 mg/kg, intra-peritoneal) increases plasma TNFα, at least, from half an hour post injection.[28] We now demonstrated that plasma TNFα was also drastically increased 1 h after LPS (50 μg/50 μl) *ig* injection, while the concomitant injection of AM251 (15 μg/50 μl), partially but significantly blocked it ($P < 0.05$) (Fig. 7).

3.6. *Effect of TNFα injected icv on MC-stimulated salivary secretion*

Considering the increase in plasma TNFα levels after LPS (*ig*) injection, the previously reported stimulatory effect of TNFα on hypothalamic endocannabinoids production,[36] and the reported interaction between endocannabinoids and the hypothalamic centres of salivation,[26] we evaluated the interaction between TNFα and the endocannabinoid system in relation to salivary secretion. Intracerebroventricular injections of AM251 (500 ng/5 μl), 15 min before the injection of TNFα (100 ng/5 μl), blocked the inhibitory effect of the cytokine on MC-induced salivary secretion ($P < 0.01$) (Fig. 8). It is important to note that we have previously reported that AM251 injected *icv* alone did not alter MC-induced salivary secretion.[26]

4. Discussion

Bacterial LPS is the main pathogenic factor that leads to endotoxemia and is frequently used as an inflammogen in studies of cell and tissue function during inflammation.[37] It was shown that LPS infused intraductally into the rat SMG proved to be a very effective inflammogen, since there was a rapid increase in gland weight at 3 h.[38] The majority of salivary gland pathologies are inflammatory and most salivary gland inflammatory diseases have in common an associated salivary hypofunction.[39,40] The present results showed that the acute inflammatory state induced by direct administration of LPS into the SMG decreased MC-induced salivary secretion by activating the endocannabinoid system, since it was completely blocked by the administration of the selective CB1 receptor antagonist AM251 through the same route. In agreement with these results, it was described a decrease of saliva secretion in response to all types of secretagogues at early stages of inflammatory diseases.[41] Moreover, we have previously reported that endocannabinoids mediate salivary hypofunction after systemic inflammation, evaluated 3 h after of LPS injection.[28] In the present work, we evaluated the effect of LPS at shorter time (1 h) that was enough to increase TNFα glandular concentration, since the endotoxin was injected into the SMG, directly.

Immune cells are the primary target for LPS, where the endotoxin interacts with the CD14 protein/toll-like receptor-4 complex to activate multiple signalling pathways.[42,43] The downstream intracellular signalling pathway following LPS binding to TLR-4 involves the expression of inflammatory cytokines such as TNFα, interleukin-1 (IL-1), IL-6, and IL-8, iNOS and cyclooxygenase-2 (COX-2),[44] and leads to the production of different lipid mediators in macrophages, such as prostaglandins,[45] leukotrienes[46] and endocannabinoids.[30] There are several signalling pathways involved in the control of salivary secretion that can be affected by these compounds.[28,47,48] In fact, during acute inflammation of SMG, pro-inflammatory mediators such as TNFα and PGE2 are released, participating in the immune response and affecting salivary secretion.[27,28,49] We showed a significant increase of TNFα and PGE2 *ig* content after LPS *ig* injection that agrees with previous reports and supports the present model of local inflammation. Therefore, our results suggest that acute hypofunction of the SMG, is linked with early cytokine production and release by inflammatory and parenchymal cells. Additionally, the release of cytokines has been suggested to decrease the release of neurotransmitters and the response of the parenchymal cells to neurotransmitters.[50] In addition, these results are

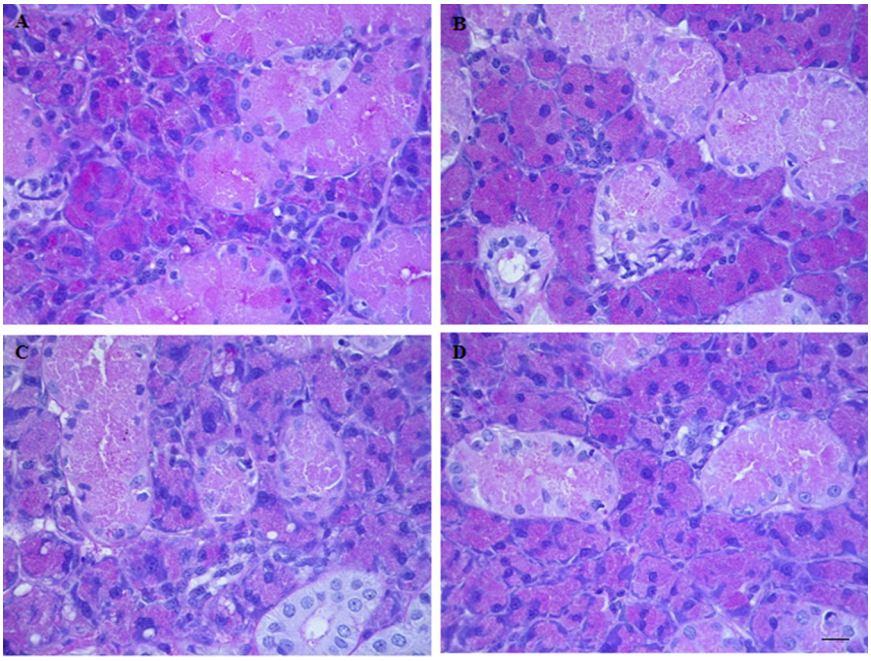

Fig. 5 – Histological appearance of SMG injected with vehicle (control) (A), LPS (B), LPS + AM251 (C), and AM251 (D), showing all few vacuoles and normal structural organization of the gland. PAS staining, 630× magnification. Scale bar = 20 μm.

supported by previous work showing that TNFα production is an important contributor to secretory dysfunction in Sjögren's syndrome by disrupting salivary epithelial cell functions necessary for saliva secretion.[51]

The reduction in salivary secretion after LPS *ig* challenge in the absence of widespread damage, suggests an influence of inflammatory mediators on parenchymal function. Also, previous report show that in inflamed SMG, there is an exudate fluid leading to increased cytokines.[52] In the same way, recent reports indicated that exocrine gland hypofunction is not necessarily correlated with the degree of secretory tissue destruction and that other non-destructive mechanisms, reducing acetylcholine release from parasympathetic nerves, may play a significant role.[40]

The increase in the concentration of total proteins in LPS-injected glands could suggest the presence of a glandular exudate that was not altered after further AM251 injections. Furthermore, LPS did not show a significant damage on lipidic membranes at least at the dose and time employed, suggesting that the mechanisms involved in the inhibition of salivary secretion in response to LPS *ig* injection are independent of

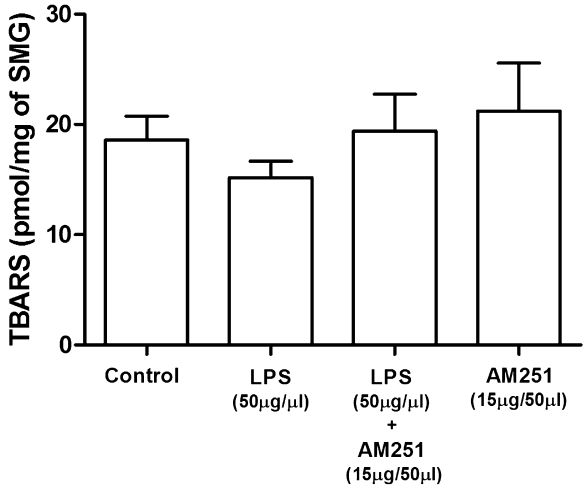

Fig. 6 – Effect of intraglandular (*ig*) injection of LPS (50 μg/50 μl/1 h) and/or AM251 (15 μg/50 μl) on TBARS in the SMG. Values are means ± SEM (6 rats per group) (one-way ANOVA followed by the Student–Newman–Keuls multiple comparison test).

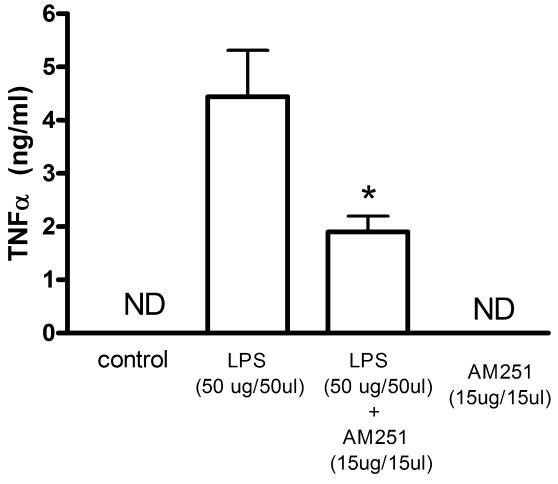

Fig. 7 – Effect of intraglandular (*ig*) injection of AM251 (15 μg/50 μl) on plasma TNFα increased by LPS (50 μg/50 μl/1 h, *ig*). Values are means ± SEM (6 rats per group). *P < 0.05 versus LPS (Student's *t*-test).

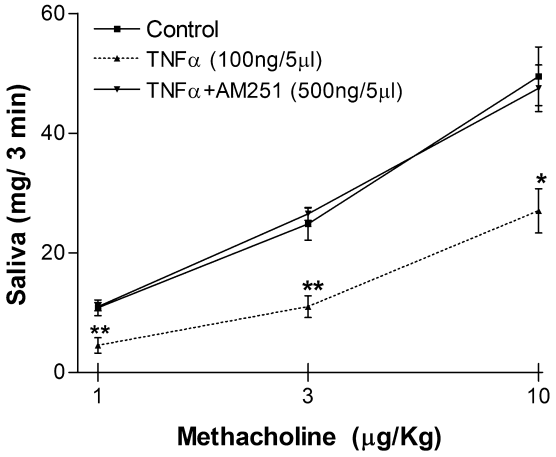

Fig. 8 – Effect of *icv* injection of AM251 (50 ng/5 μl), on TNFα (100 ng/5 μl/30 min, *icv*) induced inhibition on MC-stimulated salivary secretion. Values are mean ± SEM (6 rats per group). *P < 0.05, **P < 0.01 versus control and TNFα + AM251 (two-way ANOVA followed by Bonferroni post test).

damage-induced by lipid peroxidation. Therefore hypofunction induced by LPS in the SMG would be due to changes on signalling pathways, probably involving cytokines, prostaglandins and endocannabinoids, since anatomical changes were not observed.

Cytokine production is necessary to protect against pathogens and promote tissue repair, but excessive cytokine release can lead to systemic inflammation. In the present study, we evaluated TNFα plasma concentration and content in the SMG after LPS *ig* injection, showing that LPS not only increased TNFα concentration in SMG but also increased plasma TNFα 1 h after LPS injection. Furthermore, the *ig* injection of AM251 partially prevents the systemic augmentation of the proinflamatory citokyne, supporting the role of the endocannabinoid system as a homeostatic system activated under inflammatory conditions.

Regarding salivary glands, it was reported that IL-1/TNFα blockers partially inhibited Sjögren disease progression[53,54] and partially restore salivary gland function. Also, recent studies have shown that TNFα may act as a neuromodulator,[55] for that reason we investigated the possible role of TNFα in the central inhibition of salivary secretion.

We have previously showed the presence of CB1 receptors in the lateral hypothalamus, which is an important area for the control of salivary secretion, and that endocannabinoids inhibit salivary secretion by acting on CB1 receptors located in the brain.[26] Since LPS injected intraperitoneally as well as TNFα injected intracerebroventricularly were shown to increase anandamide in the hypothalamus,[28,36] an area that has been shown to regulate autonomic inputs to the salivary glands via endocannabinoids,[11,26] we cannot discard the participation of the hypothalamic endocannabinoid system in hyposalivation induced under systemic or brain infections. This was supported by the present results, since the inhibition of salivary secretion produced by TNFα injected *icv*, was prevented by AM251 injected by the same route, suggesting the interaction between inflammogens and the central endocannabinoid system that leads to SMG hypofunction.

We conclude that endocannabinoids mediate hyposialia induced by inflammogens in the SMG and in the brain. Therefore, our results suggest that the endocannabinoid system has a very important role in salivary function and could be a therapeutic target in salivary dysfunction caused by inflammogens.

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University of Buenos Aires and CONICET.

Competing interests

No conflicts of interest.

Ethical approval

Ethical approval by the Ethical Committee of the University of Buenos Aires, Argentina.

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