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Endocannabinoids in TNF-α and Ethanol Actions

Valeria Rettori[a] Javier Fernandez-Solari[a, b] Juan P. Prestifilippo[a]
Claudia Mohn[a] Andrea De Laurentiis[a] Stefan R. Bornstein[c]
Monika Ehrhart-Bornstein[c] Juan C. Elverdín[b] Samuel M. McCann[a]

[a]Centro de Estudios Farmacológicos y Botánicos, Consejo Nacional de Investigaciones Científicas y Técnicas (CEFYBO-CONICET) and [b]Laboratorio de Glándulas Salivales, Catedra de Fisiología, Facultad de Odontología, Universidad de Buenos Aires (UBA), Buenos Aires, Argentina; [c]Department of Medicine Carl Gustav Carus, University Hospital, University of Dresden, Dresden, Germany

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Hypothalamus · Submandibular gland · CB1 receptor · AM251

Abstract
During marijuana and alcohol consumption as well as during inflammation the reproductive axis is inhibited, mainly through the inhibition of luteinizing hormone-releasing hormone release. In male rats, this inhibitory effect is mediated, at least in part, by the activation of hypothalamic cannabinoid type 1 receptors (CB1). During inflammation, this activation of the endocannabinoid system seems to be mediated by an increase in TNF-α production followed by anandamide augmentations, while the effect of intragastric administration of ethanol (3 g/kg) seems to be due to an increase in anandamide synthase activity. On the other hand, a number of different actions mediated by the endocannabinoid system in various organs and tissues have been described. Both cannabinoid receptors, CB1 and CB2, are localized in the submandibular gland where they mediate the inhibitory effect of intrasubmandibular injections of the CB1 receptor agonist anandamide (6×10^{-5} M) on salivary secretion. Lipopolysaccharide (5 mg/kg/3 h) injected intraperitoneally and ethanol (3 g/kg/1 h) injected intragastrically inhibited the salivary secretion induced by the sialogogue metacholine; this inhibitory effect was blocked by CB1 and/or CB2 receptor antagonists. Similar to the hypothalamus, these effects seem to be mediated by increased anandamide synthase activity. In summary, similar mechanisms mediate the inhibitory actions of endocannabinoids and cannabinoids in both hypothalamus and submandibular gland during drug consumption and inflammation.

Samuel M. McCann passed away on March 16, 2007 in Buenos Aires, Argentina. The authors dedicate this manuscript to his memory.

Role of the Endocannabinoid System in the Hypothalamic-Pituitary Reproductive Axis and Salivary Secretion

It is well established that the major active ingredient of marijuana, Δ^9-tetrahydrocannabinol (THC), is capable of suppressing the reproductive function [1]. Previous studies indicate that the inhibitory effect of THC on the reproductive axis is exerted mainly at hypothalamic level by inhibiting luteinizing hormone-releasing hormone (LHRH) release with the consequent decrease in luteinizing hormone secretion by the pituitary, thereby inhibit-

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Valeria Rettori
CEFYBO-CONICET, Paraguay 2155
1121 Buenos Aires (Argentina)
Tel. +54 11 4508 3680, ext. 112, Fax +54 11 4508 3680, ext. 106
E-Mail vrettori@yahoo.com

ing gonadal function [2]. In vitro studies performed by our group in 1990 showed that THC (10^{-8} M) inhibited the norepinephrine-stimulated (NE; 5×10^{-5} M) as well as the dopamine-stimulated (5×10^{-5} M) LHRH release in medial basal hypothalamic explants (MBH) [3]. Around that time, 2 subtypes of G protein-coupled cannabinoid (CB) receptors were identified: the CB1 central receptor subtype, which is mainly expressed in the brain [4], and the CB2 peripheral receptor subtype, which appears to be particularly abundant in the immune system [5]. Both CB1 and CB2 receptors are coupled to $G_{i/o}$ proteins and respond to their ligands by inhibiting adenylyl cyclase (AC) activity [6]. A few years later, 2 endogenous cannabinoids were discovered and purified, arachidonylethanolamide (anandamide, AEA) and arachidonoyl glycerol (2-AG). Both endocannabinoids derive from arachidonic acid and bind with high affinity to CB receptors [7]. Additionally, selective antagonists have been developed for CB receptors, such as AM251 and SR141716A for CB1, and AM630 and SR144528 for CB2.

The CB1 receptors are localized in different areas of the rat brain [8]. In particular, we showed the presence of CB1 receptors in the preoptic hypothalamic area and in the periventricular MBH of male rats, regions that contain the neuronal somas and terminals involved in the synthesis and release of LHRH, respectively [9]. Using double immunohistochemistry techniques, no colocalization of CB1 receptors with LHRH-immunoreactive neurons was observed. However, CB1 receptor-immunoreactive neurons were shown adjacent to the third ventricle, an area that contains axons from LHRH neurons. Having demonstrated the presence of CB1 receptors in the MBH, we studied the effect of AEA on LHRH release. Firstly, we showed that AEA (10^{-9} M) decreased by 70% the N-methyl D-aspartic acid (NMDA)-stimulated LHRH release from MBH incubated in vitro [10]. Secondly, we demonstrated that the same concentration of AEA significantly decreased the forskolin (FRSK)-induced cyclic AMP (cAMP) content and LHRH release. These inhibitory effects of AEA were prevented by the selective CB1 receptor antagonist, AM251 (10^{-5} M), confirming the participation of the endocannabinoid system as a modulator of LHRH release in male rats. AEA (10^{-9} M) also significantly increased the release of γ-aminobutyric acid (GABA) from the MBH, but had no effect on β-endorphin release. Moreover, bicuculline (10^{-4} M), a GABAergic antagonist, was capable of totally blocking the inhibitory effect of AEA on NMDA-stimulated LHRH release. However, naltrexone (10^{-6} M), an opioid receptor antagonist, did not modify the inhibitory effect of AEA. These data confirmed the GABA-mediated inhibition of LHRH release induced by endocannabinoids. Moreover, CB1 receptors were observed on hypothalamic GABAergic neurons [M. Herkenham, pers. commun.]. An involvement of the opioid system could not be shown.

The submandibular gland (SMG) is one of the major salivary glands, together with the sublingual and parotid glands. In vitro studies on rat SMG slices showed that ^{3}H-THC was transported into and accumulated by salivary tissue [11]. Also, it has been demonstrated that THC decreased electrically stimulated salivary flow in dogs by a mechanism involving a decrease in acetylcholine release, which results in reduction of blood flow to the SMG [12].

We have shown immunohistochemically that both CB1 and CB2 receptors are present in acinar and ductal components of the SMG, and evidence of their role in diminishing saliva secretion was obtained [13]. Thus, cannabinoid receptors could be associated with the 2 fundamental mechanisms of salivary secretion: acinar release of protein and fluid, and ductal modification of the primary secretion.

Two major signal transduction pathways are implicated in salivary gland cells. One involves the generation of cAMP and the other involves the breakdown of plasma membrane polyphosphoinositides [14]. cAMP induction stimulates some salivary functions, such as salivary flow rate and secretion of proteins. CB receptors inhibit AC activity with a consequent decrease in cAMP upon binding of cannabinoids [6]. We demonstrated that AEA markedly reduced FRSK-increased cAMP levels in SMG of male rats incubated in vitro, and this effect was blocked partially by either CB1 or CB2 receptor antagonists, and completely when both antagonists were used together, indicating the involvement of both receptors in SMG physiology.

Salivation is controlled by the autonomic nervous system not only by direct activation of salivary gland receptors, but also by activation of central mechanisms. In fact, it has been reported that atropine, a muscarinic antagonist, injected intracerebroventricularly, reduced the salivation induced by pilocarpine, a muscarinic agonist, injected intraperitoneally, suggesting that pilocarpine passes the blood-brain barrier and acts on central muscarinic receptors, activating autonomic efferents to induce salivation [15]. We have recently demonstrated that the sialogogue metacholine (MC) injected through the femoral vein not only acted on its receptors in the SMG, but also on its receptors in the brain, stimulating efferent responses to the periphery that are modulated by the hypothalamic endocannabinoid system.

We also demonstrated that AEA injected intraglandularly inhibited NE- and MC-induced salivary secretion in vivo and that AM251 and AM630 prevented this inhibition, suggesting that both CB1 and CB2 are implicated in the modulation of saliva secretion [13]. CB2 mainly located at the periphery of the acini could regulate the release of saliva from these cells to the salivary ducts. The function of CB1 in the SMG is more difficult to explain, since these receptors were not immunodetected in the vicinity of the acini. However, the immunodetection of CB1 in the ductal system suggests a paracrine effect from the duct cells to the acinar cells to reduce the volume of saliva.

Additionally, since CB1 and CB2 receptor antagonists increased the salivary secretion induced by lower doses of sialogogues, these findings indicate that there is an endogenous endocannabinoid tone that could regulate salivary secretion.

These evidences suggest that the endocannabinoid system involves similar mechanisms in the brain and in peripheral organs such as the SMG. The FRSK-induced AC activity is a useful experimental procedure to study CB receptor biologic activity in the brain as well as in the periphery. At least in our experimental paradigm, CB receptors activation seems to promote inhibitory actions at brain level, such as LHRH release diminution, and peripherally, such as decrease in salivary secretion.

Role of the Endocannabinoid System in the Blockade of the Reproductive Axis and the Inhibition of Salivary Secretion during Infection

Lipopolysaccharide (LPS), an integral part of the outer membrane of Gram-negative bacteria, is a major pathogenic factor in septic shock. Macrophages are the primary target of LPS, where the toxin interacts with the CD14 protein/Toll-like receptor-4 complex to activate multiple signaling pathways. LPS induces the expression and release of cytokines such as TNF-α, interleukin (IL)-1, IL-6 and IL-8, which have been implicated in the pathophysiology of septic shock [16]. Also, LPS induces the production of different lipid mediators in macrophages, such as prostaglandins, leukotrienes and AEA [17].

Cannabinoid agonists are known to decrease neurotoxicity and AEA is able to promote anti-inflammatory responses in astrocytes via CB1 receptors [18]. In addition to modulating cellular responsiveness to various cytokines, AEA was also reported to alter its production under different conditions. Studies in human peripheral blood mononuclear cells examining a wide variety of cytokines demonstrated that AEA increased or decreased cytokine release depending upon drug concentration [19]. Also, it was reported that both synthetic and endogenous cannabinoids inhibit the LPS-induced release of TNF-α from microglial cells [20] and that LPS increased AEA levels in mouse peritoneal macrophages by inducing AEA synthesis [17].

Regarding reproductive function, it is known that during endotoxemia induced by LPS, the hypothalamic-gonadotropin axis is inhibited. Also, LPS seems to activate similar mechanisms in the inhibitory pathway of LHRH as those exerted by endocannabinoids, principally by increasing GABAergic activity [21].

It was reported that LPS leads to the suppression of LHRH pulse generator activity through a mechanism involving TNF-α. This change was faithfully reflected in the luteinizing hormone secretory pattern [22]. Furthermore, we demonstrated that AEA (50 ng/5 μl) injected intracerebroventricularly reduced plasma luteinizing hormone levels in the rat [23], similarly to the reduction of gonadotropin levels observed in sheep during endotoxemia induced by LPS [24].

In this study, we demonstrated a connection between the neuroimmune and neuroendocannabinoid systems by showing the increase in AEA synthase activity in MBH removed from rats injected with LPS (5 mg/kg/3 h, intraperitoneally) compared to rats receiving vehicle. Additionally, we demonstrated that TNF-α (100 ng/rat) injected intracerebroventricularly increased the AEA synthase activity measured ex vivo 3 h after the injections. Therefore, it is possible that LPS, through an increase in TNF-α production, enhances AEA synthase activity, thereby activating the endocannabinoid system to inhibit the release of LHRH. To confirm this hypothesis we measured the effect of TNF-α on LHRH release from MBH incubated in vitro. TNF-α significantly reduced FRSK-stimulated cAMP content and LHRH release and these effects were blocked by the CB1 antagonist AM251. These results suggest that the endocannabinoid system participates in neuroendocrine responses and immune responses mediated by TNF-α.

On the other hand, it is well known that salivary secretion is altered in different pathological states concomitantly with other physiologic parameters. We previously demonstrated that LPS injected intraperitoneally inhibits salivary secretion by increasing prostaglandin production [25]. Also, we recently demonstrated that LPS injected intraperitoneally increased AEA synthase activity in the SMG 3 h after the toxin injection. Therefore, we hypoth-

esized that the inhibition of salivary secretion observed during inflammation could be mediated by the activation of the endocannabinoid system in the SMG. In fact, both AM251 and AM630 (6×10^{-4} M), CB1 and CB2 receptor antagonists, respectively, injected intraglandularly, blocked, at least partially, the inhibitory effect of LPS on MC-induced salivary secretion. The biological activity of CB1 and CB2 receptors in the SMG was confirmed by the experimental procedure of FRSK-induced AC activity, showing that AM251 was more efficient than AM630 in blocking the inhibitory effect of TNF-α on FRSK-stimulated cAMP content in SMG incubated in vitro.

In summary, these results demonstrate that during endotoxemia induced by LPS the reproductive axis and salivary secretion are attenuated, at least in part, due to the activation of the endocannabinoid system that acts as an immunoprotector system.

Role of the Endocannabinoid System in the Ethanol-Induced Blockade of the Reproductive Axis and the Inhibition of Salivary Secretion

It is well known that ethanol (EtOH), similar to THC, can suppress reproductive function [26]. Much evidence exists that EtOH exerts its pharmacological effects in the central nervous system by modulating the function of intracellular signal transduction pathways by acting on several receptors. Since the actions of EtOH and THC on the hypothalamic-gonadotrophic axis are similar, we hypothesized that the effects of EtOH might be mediated by the endocannabinoid system.

On the basis of in vitro experiments with MBH, we demonstrated that EtOH (100 mM) inhibited the NMDA-stimulated release of LHRH by increasing the release of GABA [27] as well as AEA. Also, EtOH (100 mM) inhibited the FRSK-stimulated cAMP increase and LHRH release, inhibitory effects that were at least partially blocked by AM251, suggesting the involvement of CB1 receptors in the alcohol-induced blockade of reproductive function [10]. The incomplete inhibition could be due to the presence of a second inhibitory pathway such as the opioid system, since it has been shown that EtOH increases the release of β-endorphin which also can inhibit LHRH release [9].

In these in vitro experiments, AEA synthase activity did not change after exposing MBH to EtOH (100 mM) for 30 min. However, AEA synthase activity increased in MBH dissected 1 h after intragastric administration of EtOH (3 g/kg).

On the other hand, it is known that alcohol consumption decreases salivary secretion as does AEA. We hypothesized that EtOH might act through the endocannabinoid system to inhibit salivary secretion. Gastric administration of EtOH (3 g/kg) in adult male rats inhibited MC-stimulated saliva secretion, studied 1 h after injection, which was partially restored by intraglandular injection of AM251 and AM630 (6×10^{-4} M). Moreover, AEA synthase activity was increased significantly in SMG removed 1 h after intragastric administration of EtOH (3 g/kg). Also, EtOH (100 mM) significantly reduced the FRSK-increased cAMP content in SMG slices incubated in vitro for 30 min. This inhibitory effect was significantly blocked by the CB1 and CB2 receptor antagonists. Therefore, the hyposalia observed after alcohol consumption could be due to CB receptor activation in the salivary glands.

In summary, EtOH and the LPS-induced TNF-α release increase AEA synthesis in the MBH. This augmentation of AEA production activates CB1 receptors that reduce cAMP, thereby activating GABAergic neurons that respond by increasing the release of GABA. Finally, GABA acts on $GABA_A$ receptors located on LHRH neurons to inhibit LHRH release. Similarly, the inhibitory effect produced by alcohol and inflammation on salivary secretion is mediated, at least in part, by the endocannabinoid system. In these cases, AEA synthase activity is increased and the endocannabinoid acts on both CB1 and CB2 receptors decreasing cAMP production, thereby conducing to the diminution of salivary secretion.

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