Epithelial expression of vanilloid and cannabinoid receptors: a potential role in burning mouth syndrome pathogenesis

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Summary. Burning mouth syndrome (BMS) is an intra-oral burning sensation for which presently no medical or dental causes have been found, and in which the oral mucosa appears normal. It remains an unknown disease for which there is still no long-term treatment. The aim of this study was to assess the epithelial alteration of transient receptor potential vanilloid channel type 1 (TRPV1) and cannabinoid receptors type 1 (CB1) and type 2 (CB2) in the human tongue. The study was performed on eight healthy controls and eight BMS patients. All patients underwent a 3-mm punch biopsy at the anterolateral aspect of the tongue close to the tip. TRPV1, CB1 and CB2 immunohistochemistry was carried out showing an altered expression of all receptors. In BMS patients there was increased TRPV1, decreased CB1 and increased CB2 expression in tongue epithelial cells also associated with a change in their distribution. It would appear that these receptors are related to BMS. These data could be useful for future characterization of BMS epithelial markers and therapy.

Key words: Burning mouth syndrome, Human tongue, Transient receptor potential vanilloid channel type 1, Cannabinoid receptor type 1, Cannabinoid receptor type 2

Introduction

The International Association for the Study of Pain defines burning mouth syndrome (BMS) as “any form of burning or stinging sensation in the mouth in association with a normal mucosa in the absence of local or systemic disease”, sometimes accompanied by taste change and xerostomia (Patton et al., 2007). BMS is a chronic condition, although there is evidence that some patients have spontaneous remission (Sardella et al., 2006). The burning has been reported to be moderate to severe in intensity and may vary over the day (Lamey and Lamb, 1988). Pain increases as the day progresses, under states of anxiety, fatigue, excessive speaking and when ingesting hot and seasoned food. Pain subsides with cold food, work and distraction (Cerchiari et al., 2006). It usually occurs in the 5th-7th decade and is seven times more common in females than males and has been reported in 10 to 40% of women (Yilmaz et al., 2007; Afrin, 2011). BMS is also known as stomatodynia, oral dysesthesia, glossodynia, glossopyrosis and stomatopyrosis. It is characterized by a continuous sensation of burning or heat in the oral cavity, mainly on the tongue, palate, lips and gingiva. The tip and the lateral borders of the tongue is the most common site of burning sensations in the oral cavity (Grushka et al., 2006; Siviero et al., 2011; Charleston, 2013).

The etiology of BMS is not well known and there may be more than one etiological factor to it (Minguez-Sanz et al., 2011). Indeed, the causes of BMS are multifactor and may include various local and systemic factors. For several years BMS was attributed mainly to
psychological causes (Shortland and Renton, 2010; Minguez-Sanz et al., 2011). To diagnose BMS correctly, it is important to exclude local and systemic pathologies such as hypoplasia, oral infection, diabetes, hormonal changes, salivary gland dysfunction, salivary component changes, autoimmune and endocrine disorders, nutritional deficiencies, allergies to metal or resins used by dentists, galvanism, dental treatment and failure, food or drugs intolerance, esophageal reflux and/or chronic pharmacological therapy (hypertension therapy, antidepressants) (Charleston, 2013). Moreover, despite the large number of epidemiological studies, the incidence and prevalence of the disease still remain uncertain. Very recent experimental studies have shown that BMS does not alter the three-dimensional architecture of the tongue (Sardella et al., 2012) or the number of fungiform papillae (Camacho-Alonso et al., 2012) but suggest that keratin 16 may be involved in the cell mechanisms underlying the syndrome (Sardella et al., 2012). One of the most widely accepted theories is the alteration of nerve function based on evidence of neuropathic mechanisms, include the loss of small fibres in oral tissues, salivary and somatosensory abnormalities, reduced corneal reflexes, peripheral nerve degeneration and gustative and olfactory nerve alterations (Lauria et al., 2005; Minguez-Sanz et al., 2011; Siviero et al., 2011; Klasser and Epstein, 2012; Tinastepe and Oral, 2013).

Recent molecular studies have uncovered six transient receptor potential (TRP) cation channels that account for thermal sensations ranging from extreme cold to extreme heat (Jordt et al., 2003; Montell, 2003). Several of these also respond to irritant chemicals. Therefore, TRPV1 (V-R-1) is activated by noxious heat and capsaicin (Caterina et al., 1997) although it is also involved in neuropathic pain transmission (Kanai et al., 2005; Costa et al., 2008). TRPV1 was initially thought to be a sensory neuron-specific molecule (Caterina et al., 1997; Tominaga et al., 1998; Michael and Priestley, 1999), although other studies have revealed that this channel protein is also expressed in other tissues and cells such as human keratinocytes (Denda et al., 2001; Inoue et al., 2002) suggesting that TRPV1 activation in these cells could help induce inflammation after thermal injury (Southall et al., 2003). Moreover, to date, few studies have described TRPV1 in the tongue. It has been found in nerve fibres of papillae (Ishida et al., 2002), in the basal layers of the healthy human tongue epithelium and in all layers of the epithelium both in precancerous and malignant human samples (Marincsák et al., 2009).

Another interesting point of the TRPV1 pathway is its involvement in the endocannabinoid system for its affinity with anandamide (Ross et al., 2001; Pertwee and Ross, 2002; Ross, 2003; Evans et al., 2004). Endocannabinoids usually function through interaction with at least two distinct receptor subtypes, CB1 (Matsuda et al., 1990) and CB2 (Munro et al., 1993), which are also involved in neuropathic pain (Rodella et al., 2005). CB1 is expressed both in the peripheral (Ahuwalia et al., 2000, 2003) and in the central nervous system, in those areas involved in nociceptive signalling processing (Tsou et al., 1998). CB2 receptors are highly expressed in the immune system and are usually found in non-neuronal cells, such as on microglia (Franklin and Stella, 2003; Zhang et al., 2003; Nunez et al., 2004; Hua et al., 2005; Tsuda et al., 2005).

Furthermore, CB2 has also been found in neurons (Brusco et al., 2008). Consistent with this finding, growing evidence has suggested that the endocannabinoid system is involved in the peripheral control of pain initiation (Calignano et al., 2000; Walker and Huang, 2002; Borsani et al., 2007), leading also to the hypothesis that CBRS present in epidermal cells might modulate cutaneous nociceptors (Nakamura and Shiono, 1999). CBRS are not restricted to the central and peripheral nervous system (Matsuda et al., 1990; Munro et al., 1993), and have been identified in the skin and more specifically in supra-basal layers of the epidermis (Casanova et al., 2003; Ibrahim et al., 2005) and in the oral cavity. Indeed, the endocannabinoid system is involved in periodontal healing (Kozono et al., 2010), regulation of periodontal inflammation (Nakajima et al., 2006) and inhibition of salivary secretion induced by lipopolysaccharide (Fernandez-Solari et al., 2010), as well as in sweet taste modulation localized in the taste buds (Yoshida et al., 2010). In the oral cavity in particular, these receptors are expressed in taste buds in both fungiform papillae and circumvallate papillae (Yoshida et al., 2010); CB2 receptors are also expressed in epithelial tissues adjacent to taste buds (Yoshida et al., 2010). Interestingly, exogenous cannabinoid agonists and antagonists are known to affect the preference for sweet compounds, e.g. administration of cannabinoid agonists increase the intake of sucrose solutions (Higgs et al., 2003) suggesting that cannabinoids can change feeding behaviour.

The aim of this study was to describe, using immune-histochemistry techniques, the localization of TRPV1, CB1 and CB2 receptors in healthy tongue epithelium and their alterations in BMS patients.

Materials and methods

Subjects

We examined 16 female patients (age range: 54-85 years with a mean of 67.5 years ± 10.7 SD), eight healthy controls and eight BMS patients referred to the Dental Clinic of the University of Brescia for unremitting sensory disturbances in the mouth for at least 6 months. Diagnosis of BMS was based on clinical criteria and quantitative sensory testing of the tongue was not performed. All patients underwent radiological and serological screening for malignancy, connective tissue, metabolic (including glucose tolerance test) and infectious disorders, vitamin deficiency, and other systemic diseases. Oral infections were ruled out by microbiological oral culture. A thorough neurological
examination was performed to exclude peripheral nervous system impairment. All patients and controls were negative for the screened pathologies. The severity of pain was assessed using the visual analogue scale (VAS). All patients complained of persisting burning pain in the anterior two-thirds of the tongue for 12±2 months. Mean VAS score was 6±2 (SD). Hard palate and/or lips were also involved in five of them. Five patients reported dysgeusia, whereas no patient reported any loss of taste or thermal sensation in the mouth. Biopsy was tolerated well and no side effects were recorded. Patients with contact sensitivity to dental materials, food allergies, tongue injuries, a regular intake of TRPV1 agonists and known causes for polyneuropathy were excluded from the study. All patients and controls gave their written informed consent to the study, which was approved by the Ethical Committee of the University and Hospital of Brescia.

Tongue biopsy

After local anesthesia with 2% lidocaine, all patients underwent a 3-mm punch biopsy at the anterolateral aspect of the tongue close to the tip (Fig. 1). Suture was applied. Biopsy was performed also in eight not age-matched healthy subjects. Specimens were immediately fixed in 2% paraformaldehyde–lysine–periodate for 24 h at 4°C, embedded in paraffin according to standard procedures and sectioned at 8 μm by a microtome.

Immunohistochemical analysis

Alternate sections were processed by immunohistochemistry or stained with hematoxylin and eosin (HE) for morphological analysis. The sections were deparaffinised, rehydrated and subjected to antigen retrieval in 0.05M sodium citrate buffer (pH 6.0) in hot water bath (98°C for 20’) (Rodella et al. 2006). The sections were processed using avidin-biotin method according to the manufacturer’s instructions (Vector, Burlingame, CA). Briefly, the sections were incubated with the following primary antibodies: rabbit anti-TRPV1 (1:1000 dilution, Abcam, ab63083, Cambridge, UK), goat N-terminus-specific anti-CB1 (1:750 dilution, Santa Cruz Biotechnology, N-15, sc-10066, Santa Cruz, CA) or goat C-terminus-specific anti-CB2 (1:50 dilution, Santa Cruz Biotechnology, C-15, sc-10073, Santa Cruz, CA). The reaction product was visualized using hydrogen peroxide and 3-3-diaminobenzidine tetrahydrochloride (Sigma, St. Louis, MO, USA) as chromogen. All sections were counterstained with hematoxylin, dehydrated and mounted.

To assess specificity of the immunostaining, primary labelling was also performed using another set of antibodies: goat C-terminus-specific anti-TRPV1 (1:50 dilution, Santa Cruz Biotechnology, D-20, sc-12502, Santa Cruz, CA); rabbit C-terminus-specific anti-CB1 (1:100 dilution, Abcam, ab23703, Cambridge, UK), goat C-terminus-specific anti-CB2 (1:1000 dilution, Santa Cruz Biotechnology, M-15, sc-10076, Santa Cruz, CA). The application of these latter primary antibodies resulted in identical staining patterns (data not shown).

The immunohistochemical control was performed by omitting the primary antibody, in presence of isotype-matched IgGs and performing pre-adsorption assay using the related peptide and gave negative results.

Quantitative analyses

The TRPV1, CB1 and CB2 immunopositivity was evaluated quantitatively. A total number of 10 sections for each sample and for each studied receptor were evaluated. For the analysis, immunopositive area was evaluated at a final X200 magnification using an optical microscope (Olympus, Hamburg, Germany). Digitally fixed images were analyzed using an image analyzer (Image Pro-Plus, Milan, Italy) by researchers unaware of the group assignment and were calculated as percentage of positive area (per-area) measuring five fields with the same area for each section.

Data were analyzed and compared by repeated-measures ANOVA, followed by Bonferroni multiple-comparison test. The level of significance was set at 5% (P<0.05).

Co-localization immunofluorescence assay

The sections, obtained using the procedure described above, were processed for double immunofluorescence. Briefly, the sections were incubated in bovine serum albumin (BSA - Sigma-Aldrich, Saint Louis, USA) blocking solution (5% BSA, 0.25% Triton X-100 in PBS 1%) and then in rabbit polyclonal primary antiserum directed against TRPV1 (1:1000 dilution, Abcam, ab63083, Cambridge, UK) with goat polyclonal primary antiserum directed against CB1 (1:750 dilution, Santa Cruz Biotechnology, N-15, sc-10066, Santa Cruz, CA) or CB2 (1:50 dilution, Santa Cruz Biotechnology, C-15, sc-10073, Santa Cruz, CA) diluted in PBS containing 3% BSA and 0.1% Triton X-100, for 24 h at +4°C. After incubation in the primary antibodies, the sections were sequentially incubated with appropriate fluorescent secondary antibodies diluted in PBS (1:200, Alexa-Fluor 488, green fluorescent dye and Alexa-Fluor 555, red fluorescent dye; Invitrogen, Carlsbad, CA, USA). The immunofluorescence control was performed by omitting the primary antibody and incubating the sections with non-immune serum. All sections were mounted using a special mounting medium (UltraCruz™ Mounting Medium, Santa Cruz Biotechnology, Santa Cruz, CA) containing DAPI (4’,6-diamidino-2-phenylindole; 1.5 μg/ml) for nuclear staining. The co-localization was evaluated on digital images acquired with laser scanning confocal microscopy (LSM 510, Zeiss, Germany) at a final magnification of 630X. The microscope is placed on a vibration isolation table in an air-conditioned room kept at constant temperature. We set the pinhole at one airy unit, for best compromise between depth
discrimination and efficacy. Moreover, we used the following as laser unit: argon to detect CB1 and CB2 immunofluorescences (wavelength 488, maximum power at 30.0 mW), HeNe 543 to detect TRPV1 immunofluorescence (wavelength 555, maximum power at 1.2 mW), and Diode 405-30 to detect DAPI (wavelength 405, maximum power at 30.0 mW).

Results

Morphological evaluation

The hematoxylin and eosin staining showed normal morphology of the filiform and fungiform papillae with a normal epithelium (Fig. 2).

Immunostaining evaluation

TRPV1 immunohistochemistry

In healthy controls, TRPV1 immunostaining was observed in the epithelial cells of the tongue and distributed heterogeneously throughout the full thickness of the epithelium, mainly in the base layer (Fig. 3a). Immunopositivity was low (9.4±0.91 SEM) in the cytoplasm (Fig. 4a).

In BMS patients, TRPV1 immunopositivity was distributed largely throughout the full thickness of the epithelium (Fig. 3b), even if a more intense staining was observed in the deeper layers. In addition, a high TRPV1 immunostaining was observed both in the cytoplasm and in the plasma membrane. Finally, an increase of the TRPV1 immunopositive area (31.85±1.64 SEM) was also found (Fig. 4a).

CB1 immunohistochemistry

In healthy controls, CB1 immunostaining was observed in the epithelial cells of the tongue and distributed heterogeneously throughout the full thickness of the epithelium (Fig. 3c), even if a more intense staining was observed in the deeper layers. The immunopositivity was moderate/high (43.23±7.73 SEM) in the cytoplasm and in the plasma membrane (Fig. 4b).

In BMS patients, CB1 immunopositivity was distributed heterogeneously throughout the full thickness of the epithelium (Fig. 3d). The cytoplasm was faintly stained and the plasma membrane virtually unstained. Finally, a decrease of the CB1 immunopositive area (24.41±5.60 SEM) was found (Fig. 4b).

CB2 immunohistochemistry

In healthy controls, CB2 immunostaining was observed in epithelial cells of the tongue and distributed heterogeneously throughout the full thickness of the epithelium, mainly on the surface and in intermediate layers (Fig. 3e). The immunopositivity was low (12.56±1.90 SEM) in the cytoplasm (Fig. 4c) with a
punctuate staining.

In BMS patients, the punctuate CB2 immunopositivity was high in the cytoplasm and mainly found localized in the inter-papillary areas and in surface layer (Fig. 3f). Finally, an increase of CB2 immuno-positive area (23.76±3.84 SEM) was found (Fig. 4c).

Co-localization immunofluorescence evaluation

The double staining for TRPV1 and CB1 or CB2 allowed us to describe their intracellular co-localization. In healthy controls, we found TRPV1 and CB1 co-localized in the same area of the cytoplasm (Fig. 5a). In BMS patients, in addition to the pattern found in healthy controls, we found increased TRPV1 close to the plasma membrane and in the plasma membrane (Fig. 5b).

In healthy controls, TRPV1 and CB2 were not co-localized even if they were both present in the cytoplasm (Fig. 5c). In BMS patients, both receptors were found clearly co-localized in the cytoplasm (Fig. 5d).

Discussion

In this study, our attention was focused principally on localization and expression of three receptors, TRPV1, CB1 and CB2 on the tongue epithelium of
Fig. 3. Microphotographs of TRPV1 (a, b), CB1 (c, d) and CB2 (e, f) immunostaining in tongue epithelium of healthy controls (a, c, e) and BMS patients (b, d, f). In healthy controls, the immunopositivity of all receptors was found in cytoplasm, only CB1 was also present in plasma membrane (c, arrow). In BMS patients, the immunopositivity of all receptors was found in cytoplasm and TRPV1 was also found in plasma membrane (b, arrow); CB2 was observed mainly in the inter-papillary areas (f inset, arrows). Bars: 20 µm; inset, 100 µm.
healthy subjects and BMS patients. TRPV1 is known to be responsible for burning-hot sensations (Cortright and Szallasi, 2004) and is well characterized in the terminals of sensory nerves involved in pain (Caterina et al., 1997; Tominaga et al., 1998). Our data highlighted the presence of TRPV1 in the epithelium of the tongue, in particular TRPV1 immunopositivity increased in BMS patients with its translocation to the plasma membrane. Our results also agree with Marincsák et al. (2009) who found TRPV1 immunopositivity in the base layer of the tongue epithelium of healthy human subjects. Moreover, several studies have indicated increased TRPV1 expression during pain states. Indeed, patients with neuropathic pain showed an up-regulation of TRPV1 in the oral mucosa (Avelino and Cruz, 2000; Yangou et al., 2001; Chan et al., 2003; Tympanidis et al., 2004; Biggs et al., 2007; Yilmaz et al., 2007). We observed an increase of TRPV1 immunostaining in BMS patients which could be correlated with the principal symptoms of the disease, in particular with its burning character (Chan et al., 2003; Yilmaz et al., 2007).

Cannabinoid receptors were also considered in this study. The activation of cannabinoid receptors has antinociceptive effects, as cannabinoid administration reduces hyperalgesia and inflammation (Richardson et al., 1998). Interestingly, both CB1 and CB2 were markedly upregulated under pathological conditions by the human gingival fibroblasts and anandamide significantly reduced the production of pro-inflammatory mediators (Nakajima et al., 2006). Moreover, in other pathologies, more CB2 expression was observed, such as in acute or chronic inflammation of the bladder (Merriam et al., 2008), or in endometrial cancer (Guida et al., 2010). Furthermore, CB1 receptor antagonists could induce pro-allergic effects in skin dermatitis (Leonti et al., 2010). In our study, we found CB1 and CB2 in the tongue epithelium of healthy controls which were altered in affected patients.

One consideration concerns the localization of CB1 and CB2 in BMS patients. CB1 decreased mainly in the plasma membrane, while CB2 only increased in the cytoplasm. We can therefore hypothesize that the increase of CB2 did not have any functional effects and may be an epiphenomenon, as the plasma membrane is the functional site of the receptor activity. On the other hand, one recent research underlined a contradictory and intriguing role of endocannabinoids in the orofacial region (cheek) as itch and pain enhancing (Spradley et al., 2012). Increasing peripheral endocannabinoid levels could be a promising therapeutic approach to treat itches arising from the lower body, but such treatment does not relieve or even exacerbate, itches and pain arising from trigeminal-innervated skin of the face or scalp (Spradley et al., 2012). Here, CB2 may surprisingly have mediated an antipruritic and antinociceptive action in the orofacial region, while CB1 does not seem to offer any relief (Spradley et al., 2012). These results also suggest that the tongue, innervated also by trigeminal nerve (lingual nerve), may suffer from this particular condition, so the decrease of CB1 and the increase of CB2 might be a physiological response to the alteration of TRPV1.

![Fig. 4. Quantitative evaluation of TRPV1 (a), CB1 (b) and CB2 (c) immunostaining in tongue epithelium of healthy controls (CTR) and BMS patients (BMS). In BMS patients, TRPV1 and CB2 increase, while CB1 decreases. Values are mean ± SEM and represent the percentage of immunopositive area; *P<0.05 vs. CTR.](image-url)
Characterization of BMS epithelial markers

Fig. 5. Double-label confocal images of tongue epithelium stained for TRPV1 (red) and CB1 (a, b) or CB2 (c, d) (green); the nuclei were stained in blue (DAPI). In healthy controls (a, c), TRPV1 and CB1 co-localized in the same area of the cytoplasm (a), while TRPV1 and CB2 were not co-localized (c). In BMS patients (b, d), TRPV1 and CB1 (b) or CB2 (d) co-localized in the same area of the cytoplasm with an increase of TRPV1 close to the plasma membrane and in plasma membrane. Bar: 8 μm.
Certain results correlated the TRPV1 and endocannabinoid pathways, as anandamide may interact with TRPV1 and cannabinoid receptors modulating their activity (Ross et al., 2001; Pertwee and Ross, 2002; Ross, 2003; Evans et al., 2004). Other studies also describe how CB1 receptor activation inhibits the flow of capsaicin-induced Ca2+ in in vitro models of diabetic neuropathy (Zhang et al., 2009). Recent data have demonstrated that epidermal keratinocytes have a dynamic, stratified neurochemical organization that may play a direct role in sensory transduction and modulation of sensory ending function (Lumpkin and Caterina, 2007; Rice and Albrecht, 2008; Hou et al., 2011). Similarly in the tongue, changes in TRPV1/CB receptor expression can cause abnormal activation of these receptors on BMS epithelial cells which releases substances from the epithelium that alter nerve fibre activity, e.g. ATP (adenosine triphosphate), growth factors, CGRP (Calcitonin Gene Related Peptide) etc. Taken together our data show more TRPV1 receptors in the tongue epithelium during BMS, supporting its “burning” character and suggesting a possible correlation with the alteration of the endocannabinoid system. The data could also be useful to identify specific markers for this pathology and finalize a specific therapy.  

References


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