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Activation of the cold-receptor TRPM8 by low levels of menthol in tobacco products

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HIGHLIGHTS

- Menthol in cigarette smoke activates the cold-receptor TRPM8 and mediates a "cooling effect" that is independent from its mint-like aroma.
- We have assessed the minimum menthol contents in cigarettes required for TRPM8 activation.
- A measurable activation of TRPM8 is expected when the content of menthol exceeds 50 μg per cigarette.

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ABSTRACT

Activation of the cold-receptor TRPM8 by menthol or other tobacco additives can suppress natural defense reactions such as coughing that usually would become effective as involuntary resistance against the inhalation of fumes. In Europe menthol is only regulated as flavor, but can be used as additive as long as no characteristic mint-like aroma will become noticeable in the end-product tobacco. The question needs to be addressed of whether such comparatively minor contents would be sufficient to trigger a measurable activation of TRPM8.

In this study, we have analyzed both the contents of menthol and other natural TRPM8 agonists in tobacco products and developed a bioassay to determine the minimum concentrations of selected agonists to activate the TRPM8 receptor in cultured cells.

The data confirm menthol as strongest natural agonist investigated. Based on these experiments and previously published data, we have estimated both the minimum menthol concentrations in cigarette smoke and in tobacco that are expected to trigger measurable physiological effects. According to our assessments, TRPM8 activation is likely to occur when cigarettes contain more than 50 micrograms of menthol. Importantly, menthol contents in cigarettes far below the typical levels that require declaration as "mentholated" would be sufficient to activate sensory receptors.

1. Introduction

Cigarette mentholation was invented in the 1920s, but the procedure did not gain a higher market share until filtered menthol cigarettes were introduced in the 1950s (Reid, 1993). Even then, marketing strategies combined associations of "cooling effects" with low tar contents (Reid, 1993). Nowadays menthol smokers account for about 30% of cigarette smokers in the USA (TPSAC, 2011). In the United States, mentholated cigarettes are preferred by adolescents (12–17 years) and used by a majority of smokers (56.7%) in this age group, followed by young adults (18–24 years) with a proportion of 45.0%. Notably, a considerable decrease in the ratio of menthol smokers (30.5–34.7%) occurs in the age group of 26 years or older (Giovino et al., 2015). This indicates that beginners start with mentholated products and switch later to alternative products. However, other factors that might affect the preference for menthol cigarettes, as for example as gender, ethnic or cultural background or changing perception of fashionable tobacco products are not yet fully understood. In Europe the proportion of menthol smokers is comparatively low and estimated to about 5%, based on cigarette sales figures (EACH, 2013).

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In light of the summarized data presented in the TPSAC report, mentholated cigarettes seem to attract adolescents and young adults and are thus believed to contribute to smoke initiation (TPSAC, 2011; Rising and Wasson-Blader, 2011). In addition to its contribution as flavoring compound in the aromatization of cigarette smoke, menthol has also physiological properties that might promote the inhalation of tobacco smoke. Sensory effects of menthol result in an increased smoothness and reduced harshness of the smoke; impact effects describe the perception of strength of a cigarette; cooling effects mask the burning/scratching properties of tobacco smoke (Ahijevych and Garrett, 2004, 2010); finally menthol also acts as anaesthetic (Wayne and Connolly, 2004). Importantly, internal documents of the tobacco industry revealed that these effects had been well known (Yerger and McCandless, 2011), and sensory properties were adopted to attract young smokers (Kreslake et al., 2008a, 2008b). Other additives such as menthol esters can be used as substitute for menthol (Bharate and Bharate, 2012) as well. Known physiological effects of menthol in the respiratory tract are bronchodilation, decreased rates of inhalation and prolonged breath holding (Henningfield et al., 2003). Menthol does also suppress tussive irritations in response to fumes (Millqvist et al., 2013) and symptoms of respiratory diseases, such as chronic cough or thick mucus production (Garten and Falkner, 2004). The physiological effects of menthol are predominantly related to the activation of the transient receptor potential melastatin 8 (TRPM8) receptor, which is also named cold-menthol receptor (McKemy et al., 2002). TRPM8 is a member of the transient receptor potential (TRP) cation channels family. TRPM8 is specifically expressed in subpopulations of neurons instrumental in sensing pain and temperature. Sensation of coldness involves various receptors that distinguish innocuous or pleasant stimuli, such as relief from previous heat or irritation, from high-threshold response levels in relation to frost as potentially harmful condition. The menthol receptor TRPM8 is predominantly involved in the transduction of innocuous "cold" stimuli, possibly paralleled by the suppression of heat responses in peripheral neurons (McKemy, 2013). The overall sensation at moderate temperature levels is pleasant and can be mimicked by chemical agonists, regardless of taste or aromatic properties.

Although physiological properties of menthol that can promote the inhalation of harsh tobacco smoke are well defined, there is ongoing debate on its relevance for smoking related health risks (Heck, 2010). Current studies do not support the conclusion that menthol in general would contribute to addictiveness (Fagan et al., 2010) or increases in lung cancer incidence because of much higher exposure levels against nicotine and prototypic carcinogens present in the smoke (Brooks et al., 2003; Carpenter et al., 1999). However, with regard to addictiveness evidence is somehow contradictory (Sidney et al., 1995). Even though menthol might not lead to additional health risks for experienced smokers (Heck, 2009), physiological properties are adequate and indeed sufficient to promote the inhalation of irritating fumes in unexperienced individuals (Rising and Wasson-Blader, 2011), similar as shown in animals (Willis et al., 2011). In support to this assumption, menthol cigarettes had been classified as starter products (Hersey et al., 2006; Kahnert et al., 2012) that are likely to facilitate product's addictiveness in adolescents (TPSAC, 2011). Menthol might increase addictiveness indirectly by two mechanisms. Firstly, impairment of physiological resistance against inhalation of irritating fumes could enable some individuals to start smoking, who otherwise would rather refrain. Secondly, menthol might alter individual smoking habits, leading to a deeper inhalation and thus uptake of higher amounts of nicotine. Again, this might primarily affect beginners who are not yet adapted to a routine inhalation of smoke. In addition, menthol was proposed to delay the metabolism of nicotine (Benowitz et al., 2004).

According to new European regulation, menthol will be restricted as a potential characterizing flavor. This raises the important question whether physiologically active levels of menthol or of other TRPM8 agonists need to be expected in conventional smoking tobacco products that are—explicitly—not sold as "menthol cigarettes" and that do not release the typical mint-like flavor. Menthol was also included in the EU priority list of tobacco additives and is subject of tighter reporting obligations (EU, 2016), partly because of its "cooling" effects". In this manuscript we have assessed whether menthol levels far below the typical contents of declared menthol cigarettes are sufficient to activate TRPM8 *via* inhalation. Our risk assessment is based on the provided experimental data and on previously published studies. We propose that TRPM8 activation can be expected when menthol levels exceed 50 μg per cigarette. These comparatively low amounts are probably adequate to facilitate inhalation, especially during the initiation phase of unexperienced smokers who are not yet adapted to the irritating properties of tobacco smoke. We also provide first data on menthol contents in cigarettes of the German market. In the products analyzed, the proposed limit for physiological effects was only exceeded in cigarettes declared as mentholated.

2. Materials and methods

2.1. Chemicals

All chemicals, analytical standards and solvents used were of analytical or LC–MS grade. Ethanol (EtOH) (99.9%), poly-L-lysine and sodium chloride (NaCl) were purchased from Merck KGaA (Darmstadt, Germany). ATP (100 mM) was bought from Sigma–Aldrich (Taufkirchen, Germany). Fluo-4 AM (1 mM), HBSS (10x) (Hank's balanced salt solution), lipofectamine 2000 and Opti-MEM were obtained from Thermo Fisher Scientific (Waltham, MA, USA).

2.2. Standard substances

All analytical standards were used as racemates (Fig. 1). 7-Hydroxycitronellal (95%), carvone (98%), eucalyptol (99%), geraniol (98%), isopulegol (98%), menthol (99%), menthone (99%) and acetophenone-β,β,β-d_3 were purchased from Sigma–Aldrich (Taufkirchen, Germany). Linalool was obtained from Merck KGaA (Darmstadt, Germany) and menthol-d_4 from TRC (Ontario, Canada).

2.3. Samples and their preparation prior to quantitative analysis

Cigarettes of four major manufacturers were obtained from retailers. From each manufacturer a typical American blend product was used as standard brand. Further, additive-free (*i.e.* non-flavored) and declared menthol variations of these standard brands were used in this study. In addition, modified cigarettes, which are sold under the same brand name but contained 0.6 mg nicotine or less, have been analyzed. The latter products are listed here as "light cigarettes", although this designation was not necessarily used by the manufacturer.

Cocoa and liquorice were also obtained from local retailers. Tobacco plants (*Nicotiana tabacum*) were grown from seeds as reference samples. Dried leafs were further processed without curing, as described for cigarette tobacco. An aliquot of each cigarette (100 mg of tobacco) was exactly weighted in 20 mL headspace vials, followed by addition of 5 mL purified water (saturated with NaCl at 60 °C). To this sample, 5 μL of an internal standard mix was added. In the case of mentholated cigarettes a standard mix containing 200 μg/mL of each menthol-d_4 and acetophenone-βββ-d_3, dissolved in EtOH, was used, while for all