Research Paper

Menthol evokes Ca\(^{2+}\) signals and induces oxidative stress independently of the presence of TRPM8 (menthol) receptor in cancer cells

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A B S T R A C T

Menthol is a naturally occurring monoterpene alcohol possessing remarkable biological properties including antipruritic, analgesic, antiseptic, anti-inflammatory and cooling effects. Here, we examined the menthol-evoked Ca\(^{2+}\) signals in breast and prostate cancer cell lines. The effect of menthol (50–500 µM) was predicted to be mediated by the transient receptor potential ion channel melastatin subtype 8 (TRPM8). However, the intensity of menthol-evoked Ca\(^{2+}\) signals did not correlate with the expression levels of TRPM8 in breast and prostate cancer cells indicating a TRPM8-independent signaling pathway. Menthol-evoked Ca\(^{2+}\) signals were analyzed in detail in Du 145 prostate cancer cells, as well as in CRISPR/Cas9 TRPM8-knockout Du 145 cells. Menthol (500 µM) induced Ca\(^{2+}\) oscillations in both cell lines, thus independent of TRPM8, which were however dependent on the production of inositol trisphosphate. Results based on pharmacological tools point to an involvement of the purinergic pathway in menthol-evoked Ca\(^{2+}\) responses. Finally, menthol (50–500 µM) decreased cell viability and induced oxidative stress independently of the presence of TRPM8 channels, despite that temperature-evoked TRPM8-mediated inward currents were significantly decreased in TRPM8-knockout Du 145 cells compared to wild type Du 145 cells.

1. Introduction

Menthol is a naturally occurring organic compound produced synthetically or obtained from Mentha arvensis, (cornmint), Mentha x piperita (peppermint), but can also be isolated from other mint oils. Menthol is one of the most widely used natural products consumed as a spice and as a supplement in cosmetics. Menthol has been used for centuries in traditional medicines [1]. Numerous biological properties have been ascribed to menthol such as antipruritic, analgesic, antiseptic, anti-inflammatory and cooling effects [1–3]. Menthol is an agonist for the transient receptor potential cation channel melastatin 8 (TRPM8) receptor, a member of the transient receptor potential (TRP) cation channel super family. The TRP superfamily channels embrace more than 20 agonist-controlled Ca\(^{2+}\)/Na\(^{+}\) channels. They are found in many organs and fulfill various functions [4]. TRPM8 is often considered as a Ca\(^{2+}\) channel, yet TRPM8 channels have low selectivity for Ca\(^{2+}\) over Na\(^{+}\) ions compared to other TRP channel family members [5]. The ability of menthol to evoke a cold sensation is mediated by the cold-sensitive TRPM8 receptors. TRPM8 was initially identified and cloned by screening a prostate-specific subtracted cDNA library showing that TRPM8 was expressed at higher levels in prostate cancer tissue than in normal prostate tissue [6] and was furthermore observed in various other tumors [7]. Overexpression of TRPM8 was reported to be associated with poor prognosis in bladder carcinomas [8] and pancreatic adenocarcinomas [9]. Nevertheless, the precise role of TRPM8 channel in tumor progression remains still unclear. Immuno-fluorescence experiments revealed expression of TRPM8 protein in the ER (TRPM8\(_{ER}\)) and the plasma membrane (TRPM8\(_{PM}\)) in androgen-responsive LNCaP prostate cancer cells [10]. TRPM8 channels are also expressed in sensory neurons and found to play an important role in cold sensation [11].

Calcium ions (Ca\(^{2+}\)), acting as signaling molecules, are widely recognized to play a fundamental role in the regulation of various biological processes, e.g. metabolism, proliferation, secretion, and...
fertilization among others [12]. Many cellular activities carried out in the mitochondrial Ca$^{2+}$-dependent manner. Therefore, each cell possesses sophisticated mechanisms for the precise regulation of cytoplasmic ([Ca$^{2+}$]$_{cyt}$), endoplasmic reticulum luminal ([Ca$^{2+}$]$_{ER}$) and mitochondrial matrix ([Ca$^{2+}$]$_{mit}$) Ca$^{2+}$ concentrations. Although cancer cells may accumulate a vast number of mutations and are characterized by having aberrant chromosomes (size and numbers), the Ca$^{2+}$-regulating toolkit remains active and is able to produce highly organized Ca$^{2+}$ signals including intracellular Ca$^{2+}$ oscillations and moreover intercellular Ca$^{2+}$ waves between adjacent cancer cells. Since Ca$^{2+}$ regulates the cell cycle at several stages, Ca$^{2+}$ signaling is importantly involved in cell-fate determination (quiescent state, proliferation or cell death). Mitogenic compounds such as platelet-derived growth factor, vasopressin, prostaglandin, bombesin or EGF evoke Ca$^{2+}$ transients and also induce inositol trisphosphate (InsP$_3$) production [13,14]. Menthol also induces an increase in [Ca$^{2+}$]$_{cyt}$ in breast and prostate cell lines, but the published studies presented only the average of evoked [Ca$^{2+}$]$_{cyt}$ signals in the entire cell population [15,16]. This method blurs the spatio-temporal characteristic of individual intracellular Ca$^{2+}$ signals, which is essential to understand how TRP channel-mediated stimuli influence the cell behavior at the single cell level. At a single cell level intracellular Ca$^{2+}$ oscillations were reported in prostate and breast cancer cells [17,18].

The activation of TRP channels was found to cause a Ca$^{2+}$ accumulation in mitochondria that leads to excessive production of reactive oxygen species (ROS) [19,20]. Epidermal TRPM8 controls the mitochondrial Ca$^{2+}$ concentration and superoxide synthesis in keratinocytes in a cold-dependent manner [21]. ROS and mitochondria also play an important role in apoptosis induction. Cytochrome c release from mitochondria triggers caspase activation and finally apoptosis [22]. Ca$^{2+}$ entry and the extent of apoptosis in breast cancer cells is reduced by antioxidant treatments via inhibition of TRP cation channels [23,24], although the precise role of TRPM8 in these processes has not been clarified yet, neither in prostate nor in breast cancer cells. Oil extracts from leaves of Mentha piperita show anti-inflammatory and antioxidant activities [25] and induce significant cytotoxicity in human lung carcinoma, leukemia and gastric cancer cell lines [25]. However, in culture medium with reduced serum concentrations, Du 145 prostate cancer cells show a modest, yet significant increase in proliferation induced by menthol [26].

In this study, spatiotemporal recordings of cytoplasmic Ca$^{2+}$ concentrations in various cell lines were collected and analyzed. Since the intensity of the menthol-evoked Ca$^{2+}$ signals was inversely related to the expression levels of TRPM8 in prostate cancer cell lines, we consider it highly unlikely that TRPM8 mediates the effect. To clarify the issue we generated a TRPM8 knockout prostate cancer cell line using the CRISPR/Cas9 method.

2. Materials and methods

2.1. Reagents

L-menthol was purchased from Sigma-Aldrich and dissolved in ethanol (100%) at 1 M concentration. The compound was further diluted with buffer solution used for Ca$^{2+}$ imaging experiments that contained (in mM): NaCl 138, Na$_2$PO$_4$ 8, CaCl$_2$ 2, MgCl$_2$ 0.5, KCl 2.7, KH$_2$PO$_4$ 1.6; pH 7.4. In the low Ca$^{2+}$ solution, CaCl$_2$ was replaced with KH$_2$PO$_4$. 1.6; pH 7.4. The protocol of the Western blot analysis is described in detail elsewhere [29]. Briefly, protein extracts (50 µg) were loaded on 10% SDS polyacrylamide gels. After protein transfer the PVDF membranes were cut at the size of approximately 40 kDa. Antibodies used and their dilutions were: anti-TRPM8 (1:200; rabbit polyclonal, Alomone Labs #ACC-049) and anti-GAPDH (1:10,000, rabbit polyclonal, Sigma Aldrich, #SAB2100894) and anti-rabbit-HRP (1:10,000; goat secondary, Sigma-Aldrich, #AS5420). The specificity of the TRPM8 antibody was verified both by the authors [29] and by other researchers [30].

2.2. Plasmids and cell lines

The plasmid encoding the full-length human TRPM8 tagged with Myc-DDK was purchased from Origene (#RC220615). In order to get an untagged TRPM8 cDNA driven by the metallothionein gene promoter, Xhol and MluI restriction endonuclease sites were incorporated into the TRPM8 PCR fragment by amplification using specifically designed primer pairs. After cutting the PCR fragment with these enzymes, the size-separated cDNA insert was ligated into the pMTH plasmid containing compatible Xhol and MluI sites [27]. In order to generate a GFP-tagged version of TRPM8, TRPM8 cDNA was amplified with gene-specific forward (5'-GGT CAG TCG AGA CTA CTC TTC G-3') and reverse (5'-TTT GAT TTT ATT AGC AGT CTC TTT CAG AAG AC-3') primers. The amplified fragment was cloned into the Xhol and MluI sites of pEGFP-C3 plasmid (Clontech). The plasmid encoding InsP$_3$-5-phosphatase (pIRES-InsP3-5P-GFP) was a kind gift from Christophe Erneux, IRIBHM, Bruxelles [28]. The DNA sequence encoding InsP$_3$-5-phosphatase (pIRES-InsP3-5P-GFP) was a kind gift from Dario Vignali, Addgene #52115) to generate the pIRES-5P-IRES-BlueFP construct.

Human prostate (PC-3, LNCaP, Du 145) and breast (MCF7, BT-474, MDA-MB-231) cancer cell lines, as well as human embryonic kidney cells (HEK-293) were purchased from ATCC (Manassas, VA, USA). Cells were maintained in DMEM containing 10% fetal calf serum and 1% antibiotics (penicillin and streptomycin) at 37 °C in a humidified atmosphere at 5% CO$_2$.

2.3. Detection of TRPM8 transcripts

RNA isolation from cell lines was performed with TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Total RNA (600 ng) was reverse-transcribed with the RevertAid™ H Minus First Strand cDNA Synthesis Kit (Fermentas). RT-reaction products (5% of the total RT-reaction volume) were used as templates for the PCR: 95 °C denaturing for 30 s, 60 °C annealing for 30 s, and 72 °C extension time for 40 s, using Taq DNA polymerase (Fermentas). The TRPM8 transcripts of prostate and breast carcinoma cell lines were detected with specific primer pairs: 5'-CGA TTT TGT GGT CCG CCT CTT CT-3' (exon 8) and 5'-ACC GCC AGC TCC AGA CAG TT-3' (exon 10) producing a 579 bp fragment (40 cycles). GAPDH was used as a positive control (20 cycles) with the following primer pairs: 5'-GGT GTG CTC CTG CTT CAA CA-3' (exon 7) and 5'-GGT GGT GTA GCC AAA TCT GTT GT-3' (exon 8) producing a 127 bp fragment. The RT-PCR profiling of TRPM8-specific mRNA species, either from human prostate or breast carcinoma cell lines were carried out with three independent total RNA samples. PCR products were size separated on agarose gels and visualized with SYBRGreen staining.
2.5. \( \text{Ca}^{2+} \) imaging

Cells grown on collagen-coated glass bottom 35 mm dishes (MatTek Corp., Ashland, MA) were loaded with the cell permeable acetoxymethyl (AM)-ester form of the indicator dye. Fluoro-4-AM (1 \( \mu \)M; Life Technologies, Grand Island, NY) was used for monitoring changes in \([\text{Ca}^{2+}]_{\text{cyt}}\) diluted in cell culture media for 20 min at room temperature. After loading, cells were washed with buffer solution (DPBS) used for \( \text{Ca}^{2+} \)-imaging experiments that contained (in mM): NaCl 138, Na\(_2\)PO\(_4\) 8, CaCl\(_2\) 2, MgCl\(_2\) 0.5, KCl 2.7, KH\(_2\)PO\(_4\) 1.6; pH 7.4. We used an inverted confocal microscope DMi6000 integrated to a Leica TCS-SP5 workstation to examine changes in \([\text{Ca}^{2+}]_{\text{cyt}}\). The following excitation wavelengths were used to illuminate the \( \text{Ca}^{2+} \) indicators: 488 nm for Fluo-4 and 561 nm for CAR-GECO1. At the confocal microscope, fluorescence emission was recorded at 510–554 nm (Fluo-4) and 584–683 nm (CAR-GECO1). Recordings were performed at 37 °C using Temcontrol 37-2 digital, and a Heating Stage, all from PeCon GmbH (Erbach, Germany). The drugs were added to the abovementioned solutions by pipette and remained in the solution until the end of the experiments. Fluorescence images for \([\text{Ca}^{2+}]_{\text{cyt}}\) were collected. Circular-shaped regions of interest (ROI) were placed inside the cytoplasmic area of cells. The fluorescence values were calculated after background subtraction (fluorescence intensity of regions without cells). Bleaching correction was carried out, when the baseline was not stable. Each experimental procedure was repeated at least two times with similar results. Only one series of experiments is presented for each figure, but all parallel experiments were used for statistical evaluation. The LAS-AF (Leica, Wetzlar, Germany) and Prism3 (GraphPad Software, Inc., San Diego, CA) software were used for data analysis.

2.6. Statistical evaluation of the magnitude of menthol-evoked \( \text{Ca}^{2+} \) signals

The relative fluorescent unit (rfu) values were calculated for each cell; fluorescence intensities at each time point \((F(t))\) were divided by the averaged baseline fluorescence value measured during the non-treatment period \((F(0))\):

\[
\text{rfu}(t) = \frac{F(t)}{F(0)}
\]

In order to gain insight into evoked \( \text{Ca}^{2+} \) responses of the entire cell population observed under the microscope, the traces of more than 20 randomly selected cells were averaged:

\[
A(t) = \frac{1}{n} \sum_{i=1}^{n} \text{rfu}(t)
\]

where \( n \) is the number of the selected cells. The integral of the \( \text{Ca}^{2+} \) signal was calculated as

\[
\int_{t_0}^{t_1} (A(t) - 1)^*dt
\]

where \( t_0 \) is the time of the onset of \([\text{Ca}^{2+}]_{\text{cyt}}\) increment and \( t_1 \) is the endpoint of the recording period (the time when the signal usually returns to its baseline value). This integral was approximated using the trapezoidal rule. The unit for the \( \text{Ca}^{2+} \) integrals is rfu*sec. The values of integrals from at least three independent experiments were collected and analyzed further by one-way ANOVA. If the ANOVA test indicated a statistically significant difference between the groups \((p < 0.05)\), the data were further analyzed by Tukey’s multiple comparison post hoc test.

2.7. Generation of the Du 145\( ^{5\text{M8KO}} \) cell line

Du 145 cells were transduced with lentivirus produced as described before [31,32] with lentivCRISPR - EGFP sgRNA 1 (Addgene #51760) and selected with 2 \( \mu \)g/ml of Puromycin for 5 days. The sgRNA plasmids were obtained from Applied Biological Materials Inc. (Richmond, Canada, #K2536601). Cells were transduced with the lentivirus expressing sgRNA for TRPM8 and selected with 500 \( \mu \)g/ml of Geneticin for 1 week. Clones were selected, genomic DNA extracted and the region of interest (TRPM8 exon1 and 2) amplified by PCR (using Kapa High Fidelity Polymerase) using the following primers: TRPM8 FW 5'-ATG TTG CCT AGC CTG GTG TT-3'; TRPM8 RV: 5'-AAG GTG GAT GTG ACG TGG AT-3'. The correct fragment size was confirmed by agarose gel electrophoresis and sent for sequencing using the primer: TRPM8_FW 5'-ATG TTG CCT AGC CTG GTG TT-3'. One TRPM8 KO cell line clone was selected for further examination showing a frame shift due to a 1-nt insertion in the TRPM8 DNA sequence (Suppl. Fig. S1). Amplification of a TRPM8-containing DNA fragment of 96 bp containing a MwoI restriction site (GGcaagtgtgGC) allowed to distinguishing WT from mutated (GCaaggtgtgGC; 97-bp fragment) TRPM8 alleles. Obviously the mutated sequence is resistant to cleavage by MwoI (Suppl. Fig. S2).

2.8. Cell viability (MTT) assay

To assess menthol’s toxic effects on cell viability, we evaluated the mitochondrial activity of living cells by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) quantitative colorimetric assay. After treatment with menthol, the cells were washed and incubated with fresh medium containing MTT (0.5 mg/ml) at 37 °C for 90 min. Then, the supernatant was discarded and DMSO was added to dissolve the formazan crystals. The absorbance in each well was measured at 550 nm using a microplate reader (Infinite Pro200; Tecan Austria GmbH, Groedig, Austria) [33]. We performed a total of 6 experiments (n = 6) for the cell viability assays. The data were presented as percentage relative to the control.

2.9. Assay for apoptosis markers

Apoptosis was evaluated using the APOPercentage Apoptosis Assay (Biocolor, Belfast, Northern Ireland) according to the manufacturer’s instructions. In a viable cell, maintaining the asymmetric composition of membrane lipids is an energy-dependent process involving the activity of flipase enzymes. The loss of asymmetry serves as an early indicator of apoptosis. When the membrane of an apoptotic cell loses its asymmetry, the APOPercentage dye is actively transported into cells, staining apoptotic cells red, thus allowing detection of apoptosis by spectrophotometry. Absorbance was measured at 550 nm (Infinite Pro200). The data were presented as fold increase normalized to control. The determinations of caspase-3 and caspase-9 activities were based on a method previously reported [23] with minor modifications. Cleavage of the caspase-3 substrate (AC-DEVD-AMC) and caspase-9 substrate (AC-LEHD-AMC) was measured in a microplate reader (Infinite pro200) with an excitation wavelength of 360 nm and emission at 460 nm. The data were calculated as fluorescence units/mg protein and presented as fold increase normalized to control.

2.10. Intracellular ROS measurement

DHR 123 is a non-fluorescent, non-charged dye that easily penetrates cell membranes. Once inside the cell, DHR 123 gets fluorescent upon oxidation to yield rhodamine 123 (Rh 123), the fluorescence being proportional to ROS generation. The fluorescence intensity of Rh 123 was measured in a microplate reader (Infinite Pro200). Excitation was set at 488 nm and emission at 543 nm [33]. We performed a total of 6 experiments (n = 6) for the intracellular ROS assays. The data were presented as fold increase normalized to control.

2.11. Electrophysiology

Whole-cell voltage-clamp recordings were performed in Du 145\( ^{WT} \) (wild type) and Du 145\( ^{5\text{M8KO}} \) knockout cells at 15–27 °C (EPC10 patch-
clamp set, HEKA, Lamprecht, Germany). Resistances of the recording electrodes were adjusted to about 3–7 MΩ by a puller (PC-10 Narishige International Limited, London, UK). We used standard extracellular bath and pipette solutions as described in a previous study [34]. The holding potential for the patch-clamp analyses was set at −60 mV. The voltage-clamp technique was used for the analyses; the current-voltage (I-V) relationships were obtained from voltage ramps from −90 to +60 mV applied over 200 ms. All experiments were performed at room temperature (22 ± 1 °C). Cold exposure of Du 145WT and Du145ΔNKO cells was achieved in a slice mini bath chamber with a controller type TC05 (No: 200-100 500 0150, Luigs and Neumann, Ratingen, Germany) as described in a previous study [34]. After gating the channels with cold exposure in Du 145WT cells, TRPM8 channels were extracellularly blocked by administration of ACA (0.025 mM) via patch chamber. For the analysis, the maximal current amplitude (pA) in a given Du 145 cell was divided by the cell capacitance (pF), a measure of the cell surface. The results in the patch-clamp experiments are the current density relationships (pA/pF).

2.12. Mitochondrial membrane potential determination

Cells were incubated with 1 μM JC-1 for 15 min at 37 °C as previously described [35]. JC-1 is a lipophilic, cationic dye that selectively enters into mitochondria and reversibly changes color from red to green as the mitochondrial potential decreases. The green signal was measured at an excitation wavelength of 485 nm and an emission wavelength of 535 nm, the red signal at an excitation wavelength of 540 nm and an emission wavelength of 590 nm. Fluorescence values were measured using the microplate reader Infinite Pro200 and the green/red fluorescence intensity ratio was calculated. The data were presented as fold increase normalized to control.

3. Results

3.1. Expression patterns of the thermo-sensitive TRP channel TRPM8 in cancer cell lines

Three prostate (PC-3, LNCaP and Du 145) and three breast (MCF7, BT-474 and MDA-MB-231) carcinoma cell lines were tested for the presence of TRPM8 channel transcripts (Fig. 1A). The TRPM8 signal was highly variable: a very strong signal was detected in LNCaP cells, intermediate signals in the remaining ones, with the exception of MCF7, where almost no signal was detected. At the protein level, Western blot analysis showed that all cell lines expressed TRPM8 (Fig. 1B). The TRPM8 antibody revealed 130 kDa bands specific for TRPM8 protein in all cell lines including MCF7 cells, despite the very faint PCR signal for a TRPM8 transcript in these cells.

3.2. Analyses of the menthol-induced changes in [Ca2+]cyt in carcinoma cell lines

Menthol (500 μM) evoked Ca2+ oscillations in [Ca2+]cyt and intercellular Ca2+ waves in all cell lines, except in LNCaP cells (Fig. 2A-F). Very few (< 1%) of the LNCaP cells showed menthol-evoked single Ca2+ transients that never led to oscillations (Fig. 2B). In order to test whether LNCaP cells were able to produce Ca2+ oscillations at all, we used the previously used serum re-administration protocol. Serum contains a plethora of growth factors and hormones potentially activating many G protein coupled receptors. Transient serum withdrawal followed by serum re-administration is a well-known mechanism of activating the inositol phospholipid pathway [31,36]. Serum re-administration 24 h after serum deprivation induced long-lasting Ca2+ oscillations also in LNCaP cells (Fig. 2G). Comparison of the integrals of the Ca2+ response revealed that Du 145 cells produced the strongest and LNCaP cells the weakest Ca2+ response (PC-3: 194 ± 28, LNCaP: 1.6 ± 1.1, Du 145: 257 ± 41, MCF7: 217 ± 61, BT 474: 68 ± 13, MDA-MB-231: 148 ± 29, all in rfu*sec) (Fig. 2H). The relative fluorescence unit (rfu)*second (sec) values represent the magnitude of the integrals of the evoked Ca2+ signals. At a lower menthol concentration (50 µM) the integrals of Ca2+ responses were smaller (MCF7: 217 ± 61 vs 41 ± 13 rfu*sec, p < 0.05, Student t-test; PC-3 194 ± 28 vs. 31 ± 23 all in rfu*sec, p < 0.05, Student t-test), yet this menthol concentration was better suited to investigate intercellular Ca2+ waves (Fig. 2I). The waves started from few "initiator" cells and then spread more or less radially in all directions often joining with waves having started from distant "initiator" cells. The Ca2+ wave velocity was 9.5 ± 2.5 μm/s for...
Fig. 2. Menthol-evoked changes in the cytoplasmic free Ca^{2+} concentrations. A)-G) Single-cell (colored traces) and average fluorescence (grey traces) recordings from time-lapse videos show changes in [Ca^{2+}]_{cyt} after menthol administration. Bars represent standard deviations (SD). Experiments were repeated at least two times with similar results. PC-3 (A), Du 145 (C), MCF7 (D) BT-474 (E) and MDA-MB-231 (F) cells treated with menthol (500 µM) showed Ca^{2+} waves and Ca^{2+} oscillations. Very few (< 1%) LNCaP cells (B) responded to menthol with a small single Ca^{2+} transient. G) Ca^{2+} signals induced in LNCaP cells by serum re-administration. H) Statistical comparison of the integrals of menthol-evoked Ca^{2+} responses. The letters denotes the following a- significant difference from LNCaP cells b-significant difference from BT-474 cells, c-significant differences from MDA-MB-231 cells. p < 0.05, One-way ANOVA + Tukey’s post-hoc test. I) Time-lapse image series of Ca^{2+} waves. The acquisition rate was set to 3 s. Blue and red colors depict lower and higher fluorescence intensities, respectively. A Ca^{2+} wave in PC-3 (upper row) and MCF7 cells (lower row) was evoked by administration of 50 µM menthol. Scale bar represents 150 µm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).
3.3. Characterization of menthol-induced changes in [Ca^{2+}]_{cyt} in Du 145 cell lines

Since Du 145 cells showed the most pronounced response to menthol among the examined cancer cell lines, we selected this one for further analysis. ATMB hydrochloride, a specific TRPM8 antagonist [37], administered before menthol (500 µM), had no significant effect on menthol-evoked Ca^{2+} responses (257 ± 41 vs 243 ± 38 rfu*sec, p > 0.05, Student t-test) (Fig. 3A). Similarly CapZ (257 ± 41 vs 163 ± 68 rfu*sec, p > 0.05, Student t-test) (Fig. 3B) and BCTC (257 ± 41 vs 178 ± 48 rfu*sec, p > 0.05, Student t-test) (Fig. 3C), proven TRPV1 inhibitors and putative TRPM8 blockers, administered before menthol had no obvious effect on menthol-evoked Ca^{2+}
responses. These findings suggest that neither TRPM8 nor TRPV1 were involved in the menthol-evoked responses.

Removal of extracellular Ca\(^{2+}\) ions by 10 mM EGTA strongly reduced menthol-evoked responses (257 ± 41 vs 40.3 ± 7.8 rfu*sec, p < 0.05, Student t-test) and decreased the percentage of the responding cells from 91 ± 5% to 30 ± 11% (Fig. 3D). Cells pre-treated with 50 µM U-73122, a specific phospholipase C inhibitor didn’t show oscillations (257 ± 41 vs 2.5 ± 3 rfu*sec, p < 0.05, Student t-test) (Fig. 3E). Overexpression of InsP\(_3\)-5-phosphatase, an enzyme hydrolyzing InsP\(_3\) and concomitant expression of BFP using the plnPs3-5P-ires-BFP construct inhibited menthol-induced oscillations in Du 145 cells. BFP was used as a transfection control in order to identify cells overexpressing InsP\(_3\)-5-phosphatase. Blue and yellow traces represent average fluorescence recordings from cells with or without InsP\(_3\)-5P from the same culture dish, respectively (Fig. 3F). These findings indicate that InsP\(_3\) production and Ca\(^{2+}\) transport across the plasma membrane play an essential role in the menthol-evoked responses.

In order to clarify the role of TRPM8 channels in the menthol-evoked Ca\(^{2+}\) response, a TRPM8-knockout cell line was generated using the CRISPR/Cas9 technology. A TRPM8 knockout cell clone (representative for several other tested clones) responded to menthol (500 µM) in a similar manner as the parental Du 145 cells (Fig. 3G-H). No statistical differences were observed between the integrals of evoked Ca\(^{2+}\) responses (257 ± 41 vs 235 ± 47 rfu*sec, p > 0.05, Student t-test) (Fig. 3I). Moreover, icilin (50 µM), another TRPM8-activator, did not produce significant Ca\(^{2+}\) signals in Du 145 cells (257 ± 41 vs 9.2 ± 3.9 rfu*sec, p < 0.05, Student t-test) (Fig. 3J). All together, these findings demonstrate that menthol evokes Ca\(^{2+}\) signals independently of TRPM8.

Since in many cases, intracellular Ca\(^{2+}\) signals may result in the formation of Ca\(^{2+}\) waves in cell ensembles and moreover Ca\(^{2+}\) waves are a consequence of released and extracellularly propagating ATP molecules, we examined whether a blockage of this process may inhibit the menthol-evoked responses. ATP molecules acting on purinergic receptors evoke Ca\(^{2+}\) responses in many cell lines, also in Du 145 cells [17]. Suramin, a blocker of purinergic receptors strongly reduced menthol-evoked Ca\(^{2+}\) responses (257 ± 41 vs 66 ± 38 rfu*sec, p < 0.05, Student t-test) (Fig. 3K). Similarly, the presence of apyrase in the extracellular milieu, an enzyme that catalyzes the hydrolysis of ATP to yield AMP and inorganic phosphate, strongly reduced the menthol-evoked responses (257 ± 41 vs 10.9 ± 6.5 rfu*sec, p < 0.05, Student t-test) (Fig. 3L).

### 3.4. The effect of ectopically expressed TRPM8 on Ca\(^{2+}\) signals

Previously, it was reported that HEK-293 cells also show menthol-evoked Ca\(^{2+}\) signals independent of TRPM8 receptor [38] a finding that we confirmed in HEK-293 cells subjected to 500 µM menthol. The characteristics of Ca\(^{2+}\) responses were similar to responses observed in PC-3 and MCF7 cells after treatment with 50 µM menthol, also including the generation of Ca\(^{2+}\) waves spreading through an area of cells (Fig. 4A). In non-transfected HEK-293 cells the addition of menthol (50 µM) did not evoke a signal, while Ca\(^{2+}\) signals were evident in similarly treated HEK-293TRPM8 cells (Fig. 4C, yellow and green curves, respectively). Previous reports had indicated that TRPM8 is present both in the plasma membrane (TRPM8\(_{pm}\)) and in ER membranes (TRPM8\(_{ER}\)) [10]. In order to distinguish the role of TRPM8\(_{ER}\) and TRPM8\(_{pm}\) in the menthol-evoked Ca\(^{2+}\) signals, HEK-293TRPM8 cells were stimulated with menthol in a Ca\(^{2+}\)-free extracellular environment. In the absence of extracellular Ca\(^{2+}\) ions the menthol-evoked Ca\(^{2+}\) responses were strongly reduced, i.e. nearly eliminated (Fig. 4D). This indicated that TRPM8\(_{ER}\) channels have a small (if any) contribution to the menthol-evoked Ca\(^{2+}\) signals when compared to the contribution of TRPM8\(_{pm}\). We confirmed the ER localization of ectopically expressed TRPM8 in HEK 293 cells. For this we used GFP-tagged TRPM8 (green) and ER-located mCherry (red). The yellow color on the merged image indicates partial overlap (Fig. 4E).

### 3.5. Assessing the effect of menthol treatment on the viability and apoptosis of Du 145 and Du 145\(^{M8KO}\) cells

Next, we investigated how menthol influences the cell viability of Du 145 cells also in relation to TRPM8 expression. Serial dilutions of menthol were applied to Du 145\(^{WT}\) and Du 145\(^{M8KO}\) cells. Only at the highest menthol concentration (500 µM) viability was significantly decreased (p < 0.05) in both cell lines indicating that the effect was unlikely mediated by TRPM8. At lower menthol concentrations, cell viability was unaffected in both cell lines. (Fig. 5A). The effects of menthol on the induction of apoptosis were determined in two independent ways: by determination of the loss of the plasma membrane asymmetry and by measuring the increased caspase activities; caspase-3 and caspase-9 (Fig. 5B, C and D, respectively). Data analyses of the two complementary methods resulted in a rather congruent picture. A loss of membrane asymmetry (higher APOP values) and increased activities of caspase-3 and caspase-9 were observed in all menthol-treated groups; the higher the concentration of menthol applied, the higher values in all three assays were observed. In the apoptosis assay Du 145\(^{WT}\) cells showed a small but significant increase in their sensitivity to menthol-induced apoptosis compared to Du 145\(^{M8KO}\) cells, at all 3 menthol concentrations tested. No significant differences between Du 145\(^{WT}\) and Du 145\(^{M8KO}\) cells were observed in both caspase activation assays. The results are indicative of a mainly TRPM8-independent involvement of menthol in the activation of the apoptotic pathway, since activation of caspase-3 (intrinsc + extrinsic) and caspase-9 (intrinsic) pathways were in all cases of similar magnitude.

### 3.6. Effect of menthol treatment on reactive-oxygen species (ROS) production and mitochondrial membrane depolarization

Most Ca\(^{2+}\) ions entering the cytoplasm from internal Ca\(^{2+}\) stores or from the external side are taken up into mitochondria [36,39]. Subsequently, mitochondrial Ca\(^{2+}\) accumulation leads to mitochondrial depolarization, i.e. the mitochondrial membrane potential (ΔΨ\(_{m}\)) diminishes resulting in an increase in intracellular ROS production and oxidative stress [40]. Mitochondrial membrane depolarization (estimated by the JC-1 assay and reported as a ratio) and ROS production (estimated by the DHR assay) were affected by menthol treatment in a dose-dependent manner in all treatment groups (Fig. 6A and B). Again in most cases, small, but statistically significant differences were found between Du 145\(^{WT}\) and Du 145\(^{M8KO}\) cells. These results point towards an effect of menthol on mitochondrial activity and oxidative stress, partially mediated by TRPM8.

### 3.7. Effects of cold on TRPM8 channel currents in Du 145 cells with and without functional TRPM8

To corroborate the importance of cold temperature for the activation of TRPM8 channels, functional channel experiments were carried out using the patch-clamp technique using equipment allowing for controlled bath temperature. When the temperature was decreased from 27 °C to 15 °C, no temperature-dependent currents were seen in Du 145\(^{M8KO}\) cells (Fig. 7A). TRPM8 channels in Du 145\(^{WT}\) cells were clearly activated by cold exposure starting at around 19 °C (Fig. 7B). Currents induced by cold developed gradually during exposure to the cold and reached amplitudes of larger than −1.95 nA at 15 °C. These currents were reversibly blocked by ACA, a TRPM8 blocker. Replacement of external Na\(^{+}\) with NMDG\(^{+}\) also blocked the inward currents (Fig. 7B). Current density was significantly (p ≤ 0.001) higher in Du 145\(^{WT}\) cells than in Du 145\(^{M8KO}\) cells (Fig. 7C). The current density was markedly lower in ACA-treated Du 145\(^{WT}\) cells (p ≤ 0.001).
4. Discussion

In line with previous findings on elevated TRPM8 levels in tumor tissue, we demonstrated that also breast cancer- and prostate cancer-derived cell lines expressed transcripts for TRPM8. TRPM8 protein was also expressed in all 6 investigated cell lines. All but one (LNCaP) of the investigated cell lines responded to menthol stimulation at either 50 or 500 µM with elevations of [Ca2+]cyt. The type and duration of Ca2+ signals varied considerably between cell lines. Of utmost relevance is the question, whether the observed Ca2+ signals were specifically and genuinely mediated by TRPM8 channels.

Menthol is a non-specific TRPM8 agonist, yet has a significant preference for TRPM8; another cold receptor TRPA1 is stimulated by menthol only at millimolar concentrations in a heterologous expression system [41]. Moreover, the response to menthol (500 µM) is different in TRPM8-positive and TRPA1-positive neurons [42]. The same concentration (500 µM) has also been used in PC-3 cells to demonstrate the relevance of TRPM8 for menthol-induced Ca2+ responses [26]. In our experiments we did not find a positive correlation between total levels of TRPM8 expression and Ca2+ responses to menthol. This might be due in part to differences in the amount of functional TRPM8 channels in the plasma membrane and in ER compartments in the various cell lines.

Based on our experiments, only TRPM8 channels in the plasma membrane significantly contribute to Ca2+ responses. The involvement of TRPM8ER channels is likely minimal for the menthol-evoked Ca2+ responses We had previously reported similar results when comparing the contribution of TRPV1ER and TRPV1PM on capsaicin-evoked Ca2+ responses [17]. The poor responsiveness of LNCaP cells to menthol was rather surprising, given that LNCaP cell’s TRPM8 protein expression levels were the highest of all investigated cell lines (Fig. 1A-B). Moreover, others have demonstrated Ca2+ responses in LNCaP cells after menthol administration [15]. Unlike in our study using Fluo-4 to measure [Ca2+]cyt, in the previous ones Fura-2 was used. Since Fura-2 and Fluo-4 have rather similar dissociation constants (Kd) for Ca2+ binding (200 nM vs. 345 nM, respectively), we consider it highly unlikely that the choice of Ca2+ indicator dye is the reason for the different results. The different experimental settings – trypsinized floating cells measured in a cuvette for the Fura-2 measurements [15] vs. analysis of attached single cells in our measurements – are the more likely reasons for the differences in the Ca2+ responses of LNCaP cells. We can however not entirely exclude that TRPM8 in LNCaP cells contain a subtle mutation (e.g. point mutation) rendering the protein non-functional. To definitively exclude off-target effects of menthol in the tested cells as reported before [38,43], we generated a TRPM8-knockout cell line.
Fig. 5. Effects of menthol treatment on cell viability of Du 145WT and Du 145M8KO cells. A-D.) Cells were treated with menthol at different concentrations for 24 h. At this time point (24 h), MTT (A) and apoptosis assays were performed. Apoptosis was evidenced by loss of lipid asymmetry (B), caspase-3 (C) and caspase-9 activities (D). The columns represent mean ± standard deviation (SD) and n = 6 (2 independent experiments in triplicates). The letters on the columns denote the following: a - significant difference from control group, One-way ANOVA + post hoc Dunett test, b - significant difference between Du 145WT and Du 145M8KO cells at a given menthol concentration. One-way ANOVA + post hoc Sidak test.

Fig. 6. Effects of menthol treatment on mitochondrial membrane depolarization and ROS production in Du 145WT and Du 145M8KO cells (mean ± SD, n = 6, 2 independent experiments in triplicates). A-B) Cells were treated with menthol at different concentrations for 24 h. At this time point (24 h), cells were subjected to the JC-1 (A) and DHR 123 (B) assays indicating the levels of mitochondrial membrane potential and ROS production, respectively. The letters on the columns denote the following: a - significant difference from control group, p < 0.05, One-way ANOVA + post hoc Dunett test; b - significant difference between Du 145WT and Du 145M8KO cells at a given concentration of menthol; p < 0.05, one-way ANOVA + post-hoc Sidak test.
and moreover elevated concentration of extracellular ATP at the site of tissue injury or tumor mass were measured [47]. Extracellularly released ATP was found to play role in the spreading of Ca\(^{2+}\) waves between astrocytes [48] and other cell types [49]. Since the purinergic receptor blocker suramin and the presence of the ATP hydrolyzing enzyme apyrase in the extracellular milieu strongly diminished the menthol-evoked Ca\(^{2+}\) signals, we deduce that menthol induced an ATP release in tumor cells, however the identification of the precise mechanism remains elusive.

In biological systems many signaling pathways are tuned to sense stimulations in a certain physiological range; an over-stimulation often induces apoptotic processes. For instance, steroid receptor co-activators (SRC-1, SRC-2 and SRC-3) are involved in growth pathways required for tumor cell growth/proliferation, but potent SRC small molecule “stimulators” efficiently kill breast cancer cells by inducing aberrant cellular stress [50]. Prostate cancer rely on signals mediated via androgen receptors [51], but testosterone therapy for hypo-gonadal men with a history of prostate cancer lowers the recurrence of prostate cancer indicating that “overstimulation” of this signaling pathway also has beneficial anticancer effects [52]. In analogy, excess Ca\(^{2+}\) entry through plasma membrane Ca\(^{2+}\) channels induces apoptosis in many cell types [53]. A connection between excessive Ca\(^{2+}\) entry, mitochondrial Ca\(^{2+}\) accumulation and cytotoxic ROS production has also been documented in breast and prostate cancers. This, in turn, induces apoptotic processes [33,54], even if ROS production in a normal physiological range is important for growth of these cells [55]. Although menthol has antioxidant activity [56], in the current study, mitochondrial membrane depolarization, intracellular ROS production, apoptosis levels, and caspase-3 and -9 activities in Du 145 cells were increased in menthol-exposed prostate cancer cells. We hypothesize that this might be the result of an overstimulation of the purinergic pathway, but further studies are required to elucidate this question.

In conclusion, our study provided evidence that apoptotic pathways and mitochondrial ROS production through increased intracellular Ca\(^{2+}\) release were increased in Du 145 prostate cancer cells by 50–500 μM doses of menthol independently of the presence of TRPM8 channels. Moreover our data indicate that menthol affects purinergic signaling pathways.

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Conflict of interest statement

The authors declare that they have no conflicts of interest with the contents of this article.

Authors’ contributions

LP formulated the hypothesis, performed Ca\(^{2+}\) imaging and he was responsible for writing the report. MN and BC were responsible for the cell viability, apoptosis, caspase, ROS and JC-1 and electrophysiological analyses. WB, KJ, OZ performed PCR, cloning and Western blot, WB established the TRPM8 knockout cell line with the CRISPR/Cas9 method, TH performed the validation of the Du 145\(^{M8-KO}\) cells. KJ, CV, WB, TH, MN and BS contributed to writing and data analysis.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the
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