Investigation of the mechanisms of hyperthermia induced by endogenous and exogenous substances: characterization of the thermoregulatory effects of cholecystokinin and menthol

PhD Thesis

Patrik Kéringer, MD

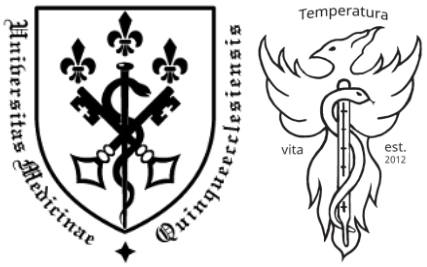
Department of Thermophysiology, Institute for Translational Medicine

Medical School, University of Pécs, Pécs, Hungary

Doctoral School Leader: Erika Pintér, MD, PhD, DSc

Program Leader: Péter Hegyi, MD, PhD, DSc

Supervisor: András Garami, MD, PhD



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1. List of abbreviations

BMI: body mass index
CCK: cholecystokinin
CI: confidence interval
COX: cyclooxygenase
DA: dorsal hypothalamic area
GABA: gamma aminobutyric acid
icv: intracerebroventricular
ip: intraperitoneal
iv: intravenous
LPS: lipopolysaccharide
MPO: medial preoptic area
NK1R: neurokinin-1 receptor
PBS: phosphate-buffered saline
PGE_2: prostaglandin E_2
rRPA: rostral raphe pallidus
T_a: ambient temperature
T_b: body temperature
TC: thermal comfort
TRP: transient receptor potential
TRPM8: transient receptor potential melastatin-8
TS: thermal sensation
TT: time trial
TTE: time-to-exhaustion
VMH: ventromedial hypothalamus
WMD: weighted mean of differences

2. Introduction

2.1. Hyperthermia

Body temperature (T_b) in warm-blooded animals is maintained at a relatively steady level called balance point. When the T_b is increased above this level, fever or hyperthermia occurs (Romanovsky 2018). An elevated T_b can be helpful, e.g., fever in fighting against microorganisms crossing the barriers of the body. However, excessive hyperthermia may be harmful causing irreversible tissue damages e.g., in case of a heat stroke. Hyperthermia can be triggered by endogenous and exogenous stimuli. Some of the endogenous hyperthermia-inducing substances are the alpha-melanocyte-stimulating hormone (Rostas et al. 2015), somatostatin (Wakabayashi, Tonegawa, and Shibasaki 1983), leptin (Rostas et al. 2016), substance P (Pakai et al. 2018), or cholecystokinin (CCK) (Szelenyi et al. 1994). Exogenous ligands can also induce a hyperthermic response: capsaicin – the pungent compound in hot peppers – increases energy expenditure in humans via activation of the transient receptor potential (TRP) vanilloid-1 ion channel (Zsiboras et al. 2018) , while menthol increases deep T_b via the activation of the TRP melastatin-8 (M8) channel in rats (Almeida et al. 2012). Different drugs can also cause hyperthermia, such as anesthetics or antidepressant agents, as well as psychostimulant drugs like amphetamine, cocaine, and LSD (Gomez 2014).

2.2. CCK as an endogenous hyperthermia-inducing substance

CCK, a gut hormone and brain neurotransmitter, evokes its effects mainly through two receptors: CCK_1, located primarily in the gastrointestinal tract, and CCK_2, expressed predominantly in the central nervous system (Noble et al. 1999). The contribution of CCK to the regulation of complex energy balance was well established by the discovery of decreased food intake induced by CCK administration in rats, monkeys, and humans (Gibbs, Young, and Smith 1973, Gibbs and Smith 1977, Kissileff et al. 1981). In the early 1980s, a role for CCK in the thermoregulation system, which is also part of energy balance (Garami and Székely 2014), was suggested (Clark and Lipton 1985), and later it was concluded that activation of the two CCK receptors differently affects body temperature (Szelenyi et al. 2004). When administered peripherally, CCK caused hypothermia, which was mediated by CCK_1 receptors (Szelenyi et al. 1994, Rezayat, Ravandeh, and Zarrindast 1999), while the administration of CCK into the central nervous system resulted in fever-like hyperthermia through the activation of CCK_2 receptors (Szelenyi et al. 1994, Sugimoto, Simons, and Romanovsky 1999). The hyperthermic response to CCK suggested a link between central CCK signaling and systemic inflammation-associated fever.

2.3. The role of cyclooxygenase (COX) enzymes in lipopolysaccharide (LPS) induced fever

In animal models, the administration of bacterial lipopolysaccharide (LPS) in a thermally neutral environment is commonly used to induce fever, which is typically polyphasic (Garami,

Steiner, and Romanovsky 2018). In rats, the febrile response entails the activation of cold-defense mechanisms, which include behavioral (warmth seeking) and autonomic thermoeffectors (skin vasoconstriction and non-shivering thermogenesis). Fever is mediated by the cyclooxygenase (COX)-2 – prostaglandin (PG) E_2 pathway, which is activated first in hepatic and pulmonary macrophages and later in brain endothelial cells (Steiner et al. 2006). In the brain [for reviews, see (Garami et al. 2018, Morrison and Nakamura 2019)], PGE_2 acts on EP3-expressing, γ-aminobutyric acid (GABA)ergic preoptic neurons in the preoptic area of the hypothalamus, which tonically inhibit cutaneous vasoconstriction through projections to the rostral raphe pallidus (rRPa) as well as non-shivering thermogenesis in brown adipose tissue through projections to the dorsal hypothalamic area (DA) (Figure 1). Hence, PGE_2 reduces the activity of GABAergic preoptic neurons, and thereby it disinhibits downstream neural substrates (in rRPa and DA) to activate the cold-defense effectors (skin vasoconstriction and thermogenesis), resulting in fever response (for reviews, see Morrison and Nakamura 2019 and Garami et al. 2018).

2.4. Modulators of the COX-PGE pathway in fever

While endogenous PGE_2 is well known to play a fundamental role in the mediation LPS fever (Saper, Romanovsky, and Scammell 2012, Roth and Blatteis 2014, Garami et al. 2018), other pyrogenic substances have been also identified, which can be associated with the COX-PGE pathway [e.g., neurokinin-1 signaling (Pakai et al. 2018, Keringer and Rumbus 2019), kallikrein-kinin system (Soares et al. 2017), and hydrogen sulfide (Kwiatkoski et al. 2013) or

act through PG-independent mechanisms, [e.g., interleukin-8 (Zampronio et al. 1994) and platelet-activating factor (Steiner and Romanovsky 2015)]. Based on similarities between the thermoregulatory effects of centrally administered CCK and PGE, it was suggested that CCK also participates in the modulation of the febrile response to LPS, but its relation to the COX-PGE pathway remained controversial (Szelenyi et al. 2004).

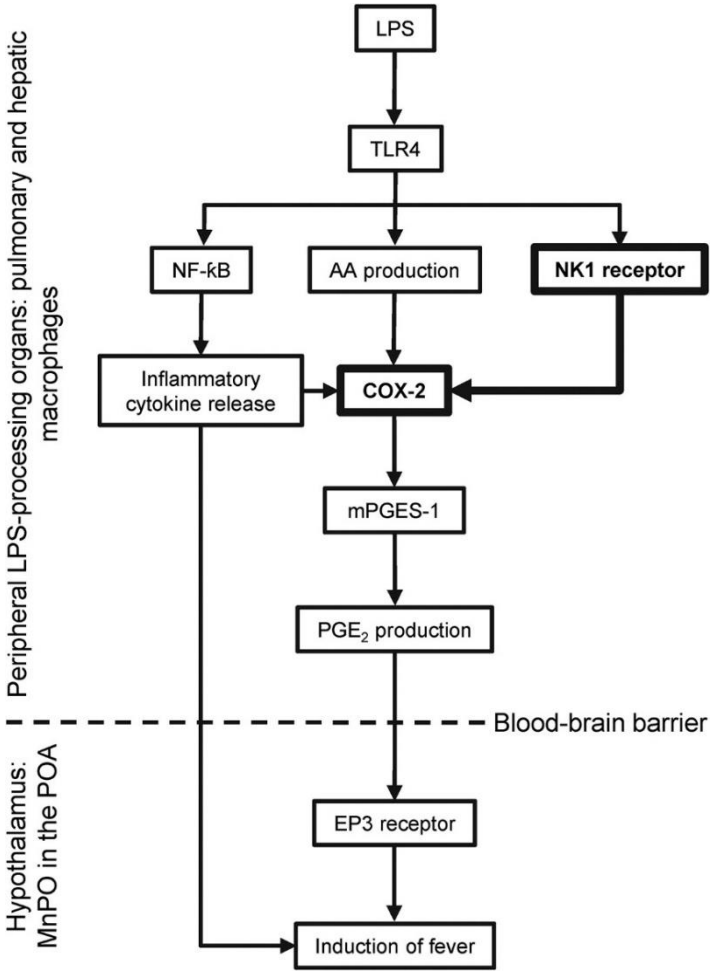

Figure 1. Simplified schematic of the mechanisms in endotoxin fever, highlighting the interaction between NK1 signaling and the cytokine-COX-2-PGE_2 axis. AA: arachidonic acid; COX-2: cyclooxygenase-2; EP3: prostaglandin EP3 receptor; LPS: lipopolysaccharide; MnPO: median preoptic nuclues; mPGES-1: microsomal PGE_2 synthase-1; NK1: neurokinin-1; PGE_2: prostaglandin E_2; POA: preoptic area; TLR4: Toll-like receptor 4 (Keringer and Rumbus 2019).

2.5. The interaction between CCK and the COX-PGE pathway

Similarly to the PGE-induced rise in deep T_b, skin vasoconstriction and enhanced thermogenesis were also observed to contribute to the hyperthermic response to intracerebroventricularly (icv) administered CCK octapeptide (CCK-8) (Szekely, Szelenyi, and Balasko 1994, Szelenyi et al. 1994, Sugimoto et al. 1999). Moreover, the first phase of LPS fever was attenuated by a pharmacological antagonist of the CCK_2 receptor (Szekely et al. 1994), while the genetic disruption of the CCK_2 receptor gene suppressed the early and late changes in T_b induced by LPS (Weiland, Voudouris, and Kent 2007), suggesting that central CCK signaling modulates the fever response. However, COX inhibition with indomethacin did not affect CCK-8-induced hyperthermia (Kandasamy and Williams 1983, Szekely et al. 1994) and PGE-induced hyperthermia was not influenced by CCK receptor blockers (Szelenyi et al. 1994), which results question the interaction between the thermal actions of CCK signaling and the COX–PGE pathway.

2.6. Menthol as an exogenous hyperthermia-inducing substance

Menthol (2-isopropyl-5-methylcyclohexanol) is a lipophilic, organic compound which can be extracted from essential oils of aromatic plants or produced synthetically (Eccles 1994, Kamatou et al. 2013). The most common naturally occurring form of menthol is the L-isomer, which is used in various products, e.g., candies, beverages, cigarettes, and toothpastes, mainly because of its cooling, analgesic, and anti-inflammatory effects (Patel, Ishiuji, and Yosipovitch 2007, Kamatou et al. 2013). From animal experiments it is known that the TRPM8 channel,

formerly called as menthol receptor, is a universal cold sensor in the thermoregulation system. The pharmacological modulation of TRPM8 changes the activity of the cold-activated neural pathway (Almeida et al. 2012), which raises the possibility that activation of TRPM8 with ligand agonists like menthol can have similar effects to physical cooling before or during physical exercise. Indeed, in human studies menthol administration resulted in increased thermogenesis (Bongers, Hopman, and Eijsvogels 2017), decreased sweating (Kounalakis et al. 2010), and more pronounced skin vasoconstriction (Lee et al. 2012, Gillis et al. 2015), consequently in elevated deep T_b (Gillis, House, and Tipton 2010); that is the same pattern of thermoregulatory effector recruitment which can be observed as part of the cold-defence responses (Romanovsky 2018).

2.7. The effect of menthol during physical exercise

It has long been assumed that menthol might improve different aspects of physical performance such as endurance, speed, strength, and joint range of motion, consequently it is often used by athletes in the form of sprays, creams, tapes, beverages, etc. (Stevens and Best 2017, Best et al. 2018). Warming-up before an exercise is often used to optimize muscle temperature and, thereby, maximal muscle power production, however, at high ambient temperatures (T_a), it increases the thermal and circulatory strain (Racinais, Cocking, and Periard 2017). Endurance exercise capacity at a high T_a is impaired by heat stress prior to exercise (Otani et al. 2017), and hyperthermia induces fatigue during short intense activities and prolonged exercise in the heat (Nybo 2008). On the contrary, physical cooling of the body before and during exercise in the

warmth improves exercise endurance and reduces cardiovascular strain (Hasegawa et al. 2006). A recent meta-analysis of 45 studies also concluded that physical cooling improves aerobic and anaerobic exercise performance in hot conditions (Douzi et al. 2019).

2.8. The presumable risk of menthol use during physical exercise

Importantly, the menthol-induced decrease in heat loss and elevation in deep T_b can increase the risk for heat exhaustion and adverse cardiovascular events in the warmth (Gillis et al. 2010, Kounalakis et al. 2010), therefore, the safety of menthol application in physical exercise, especially at high T_a, remains questionable. In contrast with the aforementioned studies showing an increased risk for the onset of heat-related illnesses in association with menthol application, several human studies showed beneficial effects of menthol on physiological, psychological, and performance parameters during physical exercise (Mundel and Jones 2010, Sonmez et al. 2010, Schlader et al. 2011, Riera et al. 2014, Stevens et al. 2016, Flood, Waldron, and Jeffries 2017, Stevens et al. 2017, Jeffries, Goldsmith, and Waldron 2018, Rinaldi et al. 2018), while a decent number of studies found no effect (Barwood et al. 2012, Barwood, Corbett, and White 2014, Barwood et al. 2015, Shepherd and Peart 2017). The observed discrepancies among the studies may originate from differences in study designs, application methods (route of administration, dosage, location of the administration, and the surface area), and experimental conditions (e.g., T_a).

Menthol-containing products can be administered externally (e.g., in spray or gel form) or internally (e.g., mouth rinse, beverage consumption). External application has been shown to

be more beneficial than the internal in sports physiology and on endurance performance (Kounalakis et al. 2010, Schlader et al. 2011, Flood et al. 2017, Barwood, Kupusarevic, and Goodall 2019), whereas other authors found that internally applied menthol is more effective (Mundel and Jones 2010, Riera et al. 2014, Stevens et al. 2016, Stevens et al. 2017, Jeffries et al. 2018), and yet others showed no effect of menthol independently from the application method (Sonmez et al. 2010, Barwood et al. 2012, Barwood et al. 2014, Barwood et al. 2015, Shepherd and Peart 2017).

3. Aims

The current study investigated the development and action mechanisms of hyperthermia induced by an endogenous or an exogenous substance (CCK and menthol, respectively). In particular, the goals of our study were the followings:

- to investigate in an animal model whether the hyperthermic and satiety responses to central administration of CCK (as an endogenous substance) depend on the COX pathway. To this end, we studied whether COX inhibitors affect the T_b responses and neuronal activation patterns in thermoregulation-related brain structures in rats treated centrally with CCK (Keringer et al. 2022);

- to study in a meta-analysis how menthol administration (as an exogenous hyperthermia-inducing substance) affects the changes in perceptual and physiological parameters of thermoregulation, and in indicators (*viz.*, power output and performance time) of the overall endurance performance during physical exercise in healthy humans (Keringer et al. 2020).

4. Materials and methods

4.1. CCK as an endogenous hyperthermic substance

4.1.1. Animals

Our experiments were performed in 220 adult male Wistar rats (Keringer et al. 2022). The rats were housed in standard plastic cages kept in a room with an ambient temperature maintained at 21-23°C and humidity at 30-40%. The room was on a 12/12-hour light/dark cycle (lights on at 5:00 a.m.). Standard rodent chow and tap water were available *ad libitum*. At the time of the experiments, the rats weighed 300-400 g. The rats were extensively handled and habituated to staying inside wire-mesh cylindrical confiners, as in earlier studies (Romanovsky, Ivanov, and Shimansky 2002, Garami et al. 2018). The cylindrical confiner prevented the animal from turning around but allowed for some back-and-forth movements; it was used throughout the thermometry experiments and for substance administration at the beginning of the feeding experiments (see below). All procedures were conducted under protocols approved by the Institutional Animal Use and Care Committee of the University of Pecs and followed the directives of the National Ethical Council for Animal Research and those of the European Communities Council (86/609/EEC).

4.1.2. Surgeries

Each rat was implanted with an icv cannula and with either an intraperitoneal (ip) or an intravenous (iv) catheter in the same anesthesia as described below. Rats were anesthetized with

ip administration of a ketamine-xylazine cocktail (78 and 13 mg/kg, respectively) and received antibiotic protection intramuscularly (gentamycin, 6.7 mg/kg). During ip and iv catheter implantation, the rats were heated with a temperature-controlled heating pad (model TMP-5a; Supertech Instruments UK Ltd., London, UK) placed under a surgery board. The experiments were performed 4-7 days after the surgery. Implantation of the icv cannula was performed as described earlier (Banki et al. 2014). In brief, each rat was fixed to a stereotaxic apparatus, the scalp was incised; the periosteum was removed; the skull was cleaned; two supporting microscrews (Fine Science Tools, Heidelberg, Germany) were driven into the skull, and a small hole was drilled in the skull 1 mm posterior from bregma and 1.5 mm lateral from midline. A 22-gauge steel guide cannula was attached to a plastic tube fitted into a stereotaxic manipulator (David Kopf Instruments, Tujunga, CA, USA). The tip of the cannula was placed within the right lateral ventricle (3.8 mm from dura) (Paxinos and Watson 2007). The cannula was secured to the supporting microscrews with zinc phosphate cement (Adhesor, SpofaDental, Jicin, Czech Republic) and released from the manipulator. The guide cannula was closed by a dummy cannula and covered by an adhesive tape.

For ip catheter implantation, a small midline incision was made on the abdominal wall, and then a polyethylene (PE)-50 catheter filled with pyrogen-free saline was inserted into the peritoneal cavity. The internal end of the catheter was fixed to the left side of the abdominal wall with a suture; the free end of the catheter was tunneled under the skin to the nape where it was exteriorized and heat-sealed. The surgical wound was sutured in layers. The catheter was flushed with 0.25 ml of saline on the day after the surgery and every other day thereafter. For iv catheterization, a small longitudinal incision was made on the ventral surface of the neck,

left to the trachea. The left jugular vein was exposed, cleared from its surrounding connective tissue, and ligated. A silicone catheter with 0.5 mm inner and 0.9 mm outer diameter filled with heparinized saline (10 U/ml) was passed into the superior vena cava through the jugular vein and secured in place with ligatures. The free end of the catheter was knotted, tunneled under the skin to the nape, and exteriorized. The wound on the ventral surface of the neck was sutured. The iv catheters were flushed with heparinized saline (10 U/ml) on the day after the surgery and then every other day.

4.1.3. Thermocouple thermometry

In the thermocouple thermometry setup, the rat was placed in a cylindrical confiner and equipped with a copper-constantan thermocouple (Omega Engineering, Stamford, CT, USA) to measure colonic temperature (T_c). The colonic thermocouple was inserted 10 cm deep beyond the anal sphincter and was fixed to the base of the tail with a loop of adhesive tape. The thermocouple was plugged into a data logger device (Cole-Palmer, Vernon Hills, IL, USA) connected to a computer. Rats in their confiners were then placed into a temperature-controlled incubator (model BJPX-Newark; Biobase, Jinan, China) set to a T_a of ~30°C, which is at the lower end of the thermoneutral zone for rats in this setup, and also neutral for adult rats in similar setups (Romanovsky et al. 2002). A needle injector was fitted into the icv guide cannula and connected to a PE-50 extension, which was passed through a port of the incubator and connected to a 10-µl syringe (model 701N, Hamilton, Reno, NV, USA). The ip or iv catheter was also connected to a PE-50 extension filled with the drug of interest or saline.

4.1.4. CCK-induced anorexia test

The anorexigenic response to CCK was tested by measuring the changes in the body mass of the rats after a 24-hour food deprivation. On the morning of the experiment, the rat was placed in a restrainer and infused ip with metamizol or saline. Thirty minutes later the rat was injected icv with CCK or saline and was kept in the restrainer for another 30 minutes after the injection. Then, the rat was weighed and returned to its home cage, where standard rodent chow was available *ad libitum*. Three hours later the rat was weighed again and the difference in body mass between 0 and 3 hours was expressed as percentage.

4.1.5. Tissue harvesting

Each rat was implanted with an icv cannula and an ip catheter and extensively adapted to the experimental setup. On the day of the experiment, each rat was placed in a confiner and transferred to an incubator chamber (Biobase), which was set to a T_a of 30°C. PE-50 extensions were connected to the icv injector and to the ip catheter as in the thermometry experiments. Rats were left to acclimate for ~2 hours; infused ip with metamizol or saline and 30 minutes later administered with CCK or saline icv. Two hours after the icv injection, the rats were anesthetized with a ketamine-xylazine cocktail injected through the extension of the ip catheter. Each rat was perfused through the left ventricle with 0.1 M phosphate-buffered saline (PBS), followed by perfusion with 4% paraformaldehyde in 0.2 M Millonig's phosphate buffer, then the entire brain was removed and post-fixed in the same fixative for 12 hours.

4.1.6. Immunohistochemistry

The c-Fos staining was performed as in earlier studies (Banki et al. 2014, Kovacs et al. 2018). Coronal sections (30 μm) were prepared on vibratome (Lancer, Ted Pella Inc., Redding, CA, USA) and stored in anti-freeze solution at −20 °C. The sections were washed 6 × 10 min in PBS, incubated in 0.5% Triton X-100 (Sigma Chemical, Zwijndrecht, The Netherlands) and, subsequently, in 2% normal goat serum (Jackson Immunoresearch Europe Ltd., Ely, UK) in PBS for 30 min. Then, sections were incubated overnight at room temperature in a rabbit polyclonal c-Fos antiserum (sc-52; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) diluted to 1:500 in PBS. Sections were treated with biotinylated goat anti-rabbit IgG (Vectastain Elite ABC Kit, Vector Laboratories, Burlingame, CA, USA) diluted to 1:200 in PBS and with 2% normal goat serum for 2 hours. Sections were rinsed in PBS and treated with avidin-biotin complex (Vectastain Elite ABC Kit) in PBS for 1 hour. After 3 × 10 min PBS rinses, the immunolabeling was visualized in Tris buffer (pH = 7.6) for 10 min. Finally, preparations were treated with 0.05% diaminobenzidine in the Tris buffer with 0.03% H_2O_2 (Sigma Chemical); the latter reaction was controlled under a stereomicroscope and stopped with PBS. Sections were mounted on gelatin slides, treated with xylene (Merck, Leicester, UK), air-dried, coverslipped with DePex mounting medium (Fluka, Heidelberg, Germany). The specificity and sensitivity of the primary and secondary antisera were carefully tested earlier in the rat (Kovacs et al. 2018). For this study, this was also confirmed: the omission of the primary or secondary serum and their replacement with nonimmune sera prevented the immunolabeling (images not shown).

The regions of interest were photographed with a Spot RT color digital camera using the Spot advanced imaging software (Nikon, Tokyo, Japan). In each brain, the cell counts positive for c-Fos were determined in five serial sections, each interspaced by 60 μm in the medial preoptic area (MPO), DA, rRPa, and ventromedial hypothalamus (VMH) according to the atlas by Paxinos and Watson (Paxinos and Watson 2007). Cell counting was carried out on non-edited digital images using ImageJ software (version 1.37, NIH, Bethesda, MD, USA). Quantitation was performed in a double-blind setup by a colleague who is an expert of rodent neuroanatomy but was blinded to the identity of preparations.

4.1.7. Substance administration

Sulfated CCK-8 was purchased from Bachem (Bubendorf, Switzerland). A stock solution of CCK-8 (1 μg/μl) in pyrogen-free saline was aliquoted and stored at –20°C. On the day of the experiment, an aliquot was diluted with saline to a final concentration of 0.1 μg/μl, which was injected icv (3.4 μl/kg/min for 5 min) to deliver CCK-8 at a total dose of ~1.7 μg/kg. Control rats were infused with saline. The selective CCK_2 receptor antagonist YM022 was purchased from Tocris (Bristol, UK). Aliquots of an ethanolic stock solution of YM022 (6 μg/μl) were stored at –20°C. On the day of experiment, the stock solution was diluted with saline to a give a working solution of YM022 at 0.6 μg/μl in 10% ethanol. By infusing this working solution of YM022 into the lateral ventricle (3.3 μl/kg/min for 5 min), a total dose of ~10 μg/kg was delivered icv. Control rats were infused with the vehicle (10% ethanol in saline).

On the day of the experiment, metamizol (Sanofi, Budapest, Hungary), a nonselective COX inhibitor, as well as, two selective COX-2 inhibitors, *viz.*, meloxicam (Boehringer Ingelheim International GmbH, Ingelheim, Germany) and etoricoxib (Merck & Co., Kenilworth, NJ, USA), were dissolved in saline at concentrations of 120, 10, and 10 mg/ml, respectively. Metamizol (120 mg/kg), meloxicam (10 mg/kg), and etoricoxib (10 mg/kg) were infused through the pre-implanted ip catheter (0.08 ml/kg/min for 12.5 min).

LPS from *Escherichia coli* 0111:B4 was purchased from Sigma-Aldrich (St. Louis, MO, USA). A stock suspension of LPS (5 mg/ml) in pyrogen-free saline was stored at −20°C. On the day of the experiment, the stock was diluted with saline to a final concentration of 10 μg/ml. The diluted LPS suspension or saline was infused (0.33 ml/kg/min for 3 min) through the extension of the iv catheter to deliver LPS at a final dose of 10 μg/kg.

4.1.8. Data processing and analysis

Changes in T_b were compared by two-way ANOVA, while changes in body mass and the numbers of the c-Fos positive cells were compared with one-way ANOVA, as appropriate. ANOVA was followed by the Student-Newman-Keuls post hoc test. Sigmaplot 11.0 (Systat Software, San Jose, CA, USA) was used for statistical analysis. The effects were considered significant when $P < 0.05$. All data are reported as mean ± standard error (SE).

4.2. Menthol as an exogenous hyperthermic substance

Our meta-analysis (Keringer et al. 2020) was conducted in accordance with the guidelines of the Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) protocols (Moher et al. 2009). The analysis was based on the Participants, Intervention, Comparison, and Outcome model: in physically active, healthy participants, we investigated the effects of menthol application compared to controls (i.e., no menthol or placebo treatment) on physiological and perceptual parameters and on indicators of endurance performance during physical exercise. The protocol for this meta-analysis was registered on PROSPERO (registration number: CRD42019125034).

4.2.1. Search strategy

A search of the PubMed, EMBASE, and Cochrane Controlled Trials Registry databases was performed until May 2020 using the following search key: "(menthol OR mint OR peppermint OR mentha OR spearmint) AND (temperature OR "heart rate" OR "oxygen uptake" OR lactate OR "sweat rate" OR "physical performance" OR exhaustion)". We restricted our search to randomized controlled human trials published in English without time period limitations. A manual search of the reference lists of identified full-text articles was also performed in Google Scholar for eligible studies. The search was conducted separately by two authors, who also assessed study eligibility and extracted data from the selected studies independently. Disagreements were resolved, if needed, by a third party.

4.2.2. Study selection and data extraction

After screening on the titles and abstracts of the identified publications, the full texts of eligible articles were obtained. We included studies which reported at least one of the following values: thermal sensation (TS), thermal comfort (TC), T_b, sweat production, heart rate, performance time, and power output in menthol-treated and control healthy subjects before and during physical exercise. For all parameters, the maximal change from baseline after menthol treatment (and the corresponding value at the same time point in the control group) was extracted to assess the acute effect of menthol. In each study we calculated the difference between the menthol-treated and control groups, which was then included in the analyses. This approach allowed for taking into considerations differences in experimental protocols between studies. From all included articles, we extracted the group size, the reported mean values and standard deviations (SD) of the parameters of interest, and the level of statistical significance (P value). To analyze the effects of menthol under different conditions, we also divided the studies into subgroups, which were determined on the basis of known influencing factors (Wegmann et al. 2012, Campos et al. 2018), and data availability. The main influencing factors were grouped in three categories: characteristics of the subject (body mass index [BMI] and heat acclimation), study protocol (trial type and menthol administration method), and environmental circumstances (airflow and T_a).

4.2.3. Statistical analysis

In each study, we calculated the maximum change in the outcome parameter from baseline after menthol application and the change from baseline until the same time point in the control group. Then, we calculated the weighted mean difference (WMD) with 95% confidence interval (CI) in the change of the parameter between the menthol-treated and the control groups. The statistical analysis was performed according to the standard methods of meta-analysis by using a random effects model. The effects were considered significant when $P < 0.05$. Using both P value and CI allowed us to detect physiologically relevant differences between the groups even in the case of overlapping CIs (du Prel et al. 2009).

To study perceptual responses, data on TS and TC were collected. By definition, TS identifies the relative intensity of the temperature being sensed, and, as such, provides the body with information about the thermal environment, while TC means subjective indifference with the thermal environment, so that thermal pleasure is perceived when a stimulus aims to restore TC (for a comprehensive review, see Flouris & Schlader (2015)). In the analyzed studies, TS scales were used with ranges of 7-point (Schlader et al. 2011), 9-point (Jeffries et al. 2018), and 20-point (Barwood et al. 2014, Barwood et al. 2015, Barwood et al. 2019), while in case of TC, the authors used 4-point (Schlader et al. 2011), 7-point (Flood et al. 2017, Jeffries et al. 2018, Rinaldi et al. 2018), and 20-point ranges (Barwood et al. 2014, Barwood et al. 2015, Barwood et al. 2019). Since TS and TC were determined by different visual analogue scales in the studies, in order to make the reported TS and TC values comparable for our meta-analysis, while also minimizing the need for conversion of the originally reported data in the studies, if required, the reported values were extrapolated into a unified scale, ranging from 0 to 20. The scales were

bilateral, i.e., 0 corresponded to neutral, in several studies. For example, TS was assessed with a 9-point scale ranging from very cold (− 4) to neutral (0) to very hot (4). Thus, after extrapolation of the endpoints of the original scales to 0 and 20, in case of TS the middle of the unified scale (10) represented the neutral situation and an increase in TS between 0 (very cold) and 20 (very hot) indicated a weaker cold or stronger warmth sensation. To assess TC, three types of scales were used in the analyzed studies: 20-cm visual analogue scale (0: very uncomfortable, 20: very comfortable) (Barwood et al. 2014, Barwood et al. 2015, Barwood et al. 2019), 7-point scale (− 3: much too cool, 0: comfortable, 3: much too warm) (Flood et al. 2017, Jeffries et al. 2018, Rinaldi et al. 2018), and 4-point scale (1: comfortable, 4: very uncomfortable) (Schlader et al. 2011). In the studies using 7-point scale, we did not find any value smaller than 0, thus we considered the scale from 0 (comfortable) to 3 (uncomfortable) and these endpoints were extrapolated to 20 and 0, respectively. In the 4-point scale, the 1 (comfortable) and 4 (uncomfortable) endpoints were extrapolated to 20 and 0, respectively. As result, the unified 20-point scale, ranging from very uncomfortable to very comfortable, resembled the one used in the pioneer study by Gagge, Stolwijk, and Saltin (1969). In this scale, a higher TC value between 0 (very uncomfortable) and 20 (very comfortable) corresponded to more pleasant comfort feeling.

With regards to performance time, it must be noted that depending on the exercise protocol its decrease and increase can both indicate an improved endurance performance. In time-to-exhaustion (TTE) protocols, a longer performance time indicates improved endurance as the subjects are able to perform the exercise for a longer time period, whereas in time-trial (TT) protocols the subjects aim at finishing a predefined exercise task as fast as they can, thus longer

performance time indicates reduced endurance in these tasks. Therefore, in our analyses, we always separated the TTE and TT protocols in different groups when the investigated outcome was performance time, similarly as in previous studies (Goulet 2013, Campos et al. 2018).

Inter-study heterogeneity was tested with the Q homogeneity test and with the I^2 statistical test, where I^2 is the proportion of total variation attributable to between-study variability (an I^2 value of more than 50% was considered as an indication of considerable heterogeneity). To evaluate the quality of the included trials, two independent reviewers assessed the risk of bias according to the Cochrane Handbook (Higgins et al. 2011). The methodology described for random sequence generation, allocation concealment, blinding of participants and personnel, blinding of outcome assessment, completeness of outcome data, and selective outcome reporting was assessed, similarly as in our recent study (Olah et al. 2018).

All analyses were performed using the Comprehensive Meta-Analysis software (version 3.3; Biostat, Inc., Engelwood, NJ).

5. Results

5.1. CCK as an endogenous hyperthermic substance

5.1.1. Dependence of the hyperthermic and anorexic effects of centrally administered CCK on COX enzymes in rats

First, we characterized the thermoregulatory effect of CCK administered icv in rats (Keringer et al. 2022). As expected based on previous studies (Szekely et al. 1994, Szelenyi et al. 1994, Ghosh, Geller, and Adler 1997, Ghosh et al. 1998, Sugimoto et al. 1999), in response to CCK the rats developed a marked elevation in T_b, whereas administration of saline did not cause any effects (Figure 2). The hyperthermic response to CCK developed promptly (in less than 10 min) and T_b reached the highest mean increase of 0.4 ± 0.1°C at 20 min ($P = 0.007$), then it gradually decreased, but remained elevated compared to saline treatment throughout the experiment.

In order to study the involvement of the COX enzymes in the development of CCK-induced hyperthermia, the rats were treated with the nonselective COX inhibitor metamizol (120 mg/kg; ip) 30 minutes preceding the icv administration of CCK. The effect of the pretreatment was significant on the T_b response in CCK-treated rats [ANOVA, $F_{(1,304)} = 62.994$, $P < 0.001$]. In the metamizol-pretreated rats the hyperthermic response to CCK was abolished as compared to ip saline pretreatment, reaching the level of significance at 10-40 and 110-160 min between the pretreatment groups ($P < 0.05$) (Figure 2).

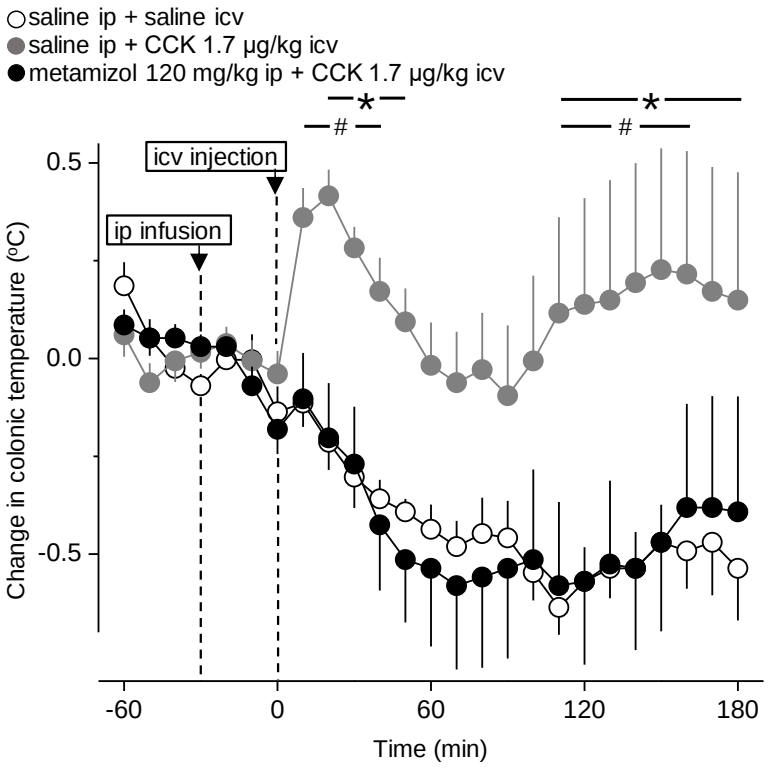

Figure 2. Deep (colonic) T_b responses of rats to icv administration of CCK (1.7 µg/kg) or saline after pretreatment at -30 min with an ip infusion of metamizol (120 mg/kg) or saline. For each group, n = 9. *P < 0.05, icv CCK vs. saline difference in ip saline-pretreated rats; $^{\#}P < 0.05$, ip metamizol vs. saline difference in icv CCK-treated rats as determined by two-way ANOVA followed with Student-Newman-Keuls test. Data are presented as mean ± SE (Keringer et al. 2022).

We also wanted to know whether the inhibition of COX enzymes attenuates the anorexic effect of CCK. For that reason, in another set of experiments, 24-hours fasted rats were treated with metamizol or saline before the icv administration of CCK or saline. As expected, in saline-pretreated rats, the injection of CCK significantly reduced the gain of body mass during 3-hour refeeding as compared to icv saline injection (1.6 ± 0.3 vs. 2.6 ± 0.3%, $P < 0.05$) (Figure 3).

Importantly, however, we did not detect any significant difference in CCK-induced anorexia between the metamizol- and saline-pretreated rats [ANOVA, $F_{(1,21)} = 0.532$, $P = 0.474$].

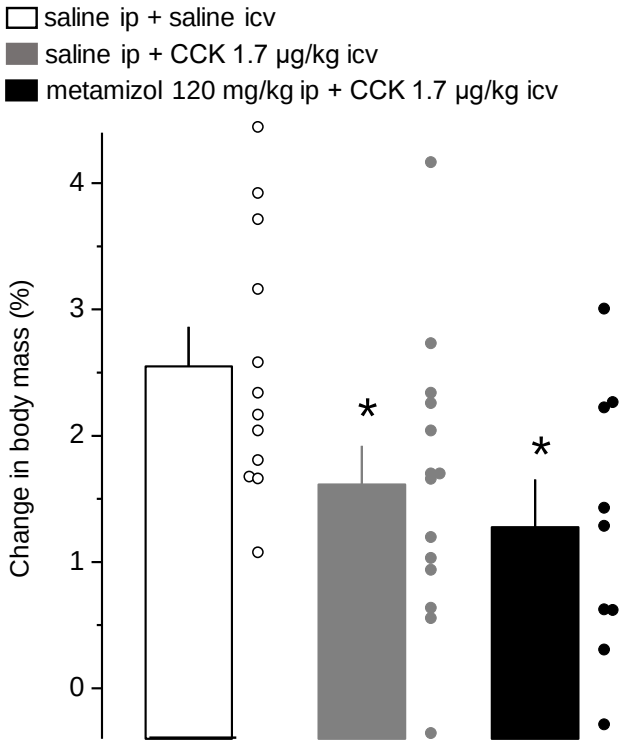

Figure 3. Changes in body mass of rats in response to ip and icv administration of saline (n = 12) and to icv administration of CCK (1.7 µg/kg) after pretreatment at -30 min with an ip infusion of metamizol (120 mg/kg; n = 9) or saline (n = 14). *P < 0.05, icv CCK vs. saline difference as determined by one-way ANOVA followed with Student-Newman-Keuls test. Bars represent group means (± SE), individual data are shown as circles (Keringer et al. 2022).

5.1.2. CCK-induced changes in c-Fos expression in thermoregulation- and feeding-related brain nuclei and their dependence on COX activation

Knowing that CCK-induced hyperthermia entails autonomic thermoeffector responses, i.e., activation of brown fat thermogenesis and cutaneous vasoconstriction (Szekely et al. 1994, Szelenyi et al. 1994), we hypothesized that blocking CCK-induced hyperthermia with a COX-inhibitor changes the activation of hypothalamic efferent neurons controlling these responses. To test this hypothesis, we measured expression of the inducible transcription factor c-Fos, a marker of neuronal activation (Sagar, Sharp, and Curran 1988) in the MPO, DA, and rRPa (Figure 4), which nuclei contribute to the autonomic thermoregulatory responses to cooling (Nakamura and Morrison 2008, Wanner et al. 2017) and PGE_2 (Saper et al. 2012). We found a significant decrease in the number of c-Fos positive cells in the MPO in response to CCK as compared to icv administration of saline (8.3 ± 0.9 vs. 27.3 ± 1.1, $P < 0.001$), whereas CCK increased c-Fos immunoreactivity expression in the DA (69.1 ± 1.9 vs. 31.9 ± 3.2, $P < 0.001$) and the rRPa (11.3 ± 1.6 vs. 5.1 ± 0.9, $P < 0.01$) compared to saline. Pretreatment of the rats with metamizol ip completely reversed the CCK-induced changes in the number of c-Fos positive cells in the MPO (36.1 ± 5.9, $P < 0.001$), DA (28.7 ± 3.9, $P < 0.001$), and rRPa (5.4 ± 1.07, $P < 0.01$) compared to ip saline pretreatment.

We wanted to confirm that CCK-induced anorexia involves changes in the neuronal activation of the VMH, which harbors neurons involved in the regulation of food intake (Wen et al. 2019), and to study whether the observed changes can be influenced by the inhibition of COX (Figure 5). CCK induced an elevation in c-Fos positive cell number in the VMH (91.2 ± 6.7 vs. 35.7 ± 13.0, $P < 0.001$) compared to icv saline administration. In contrast with our results in thermoregulatory nuclei, the ip pretreatment with metamizol had no effect on the CCK-induced neuronal activation in the VMH (98.7 ± 13.3, $P = 0.505$) compared to saline pretreatment.

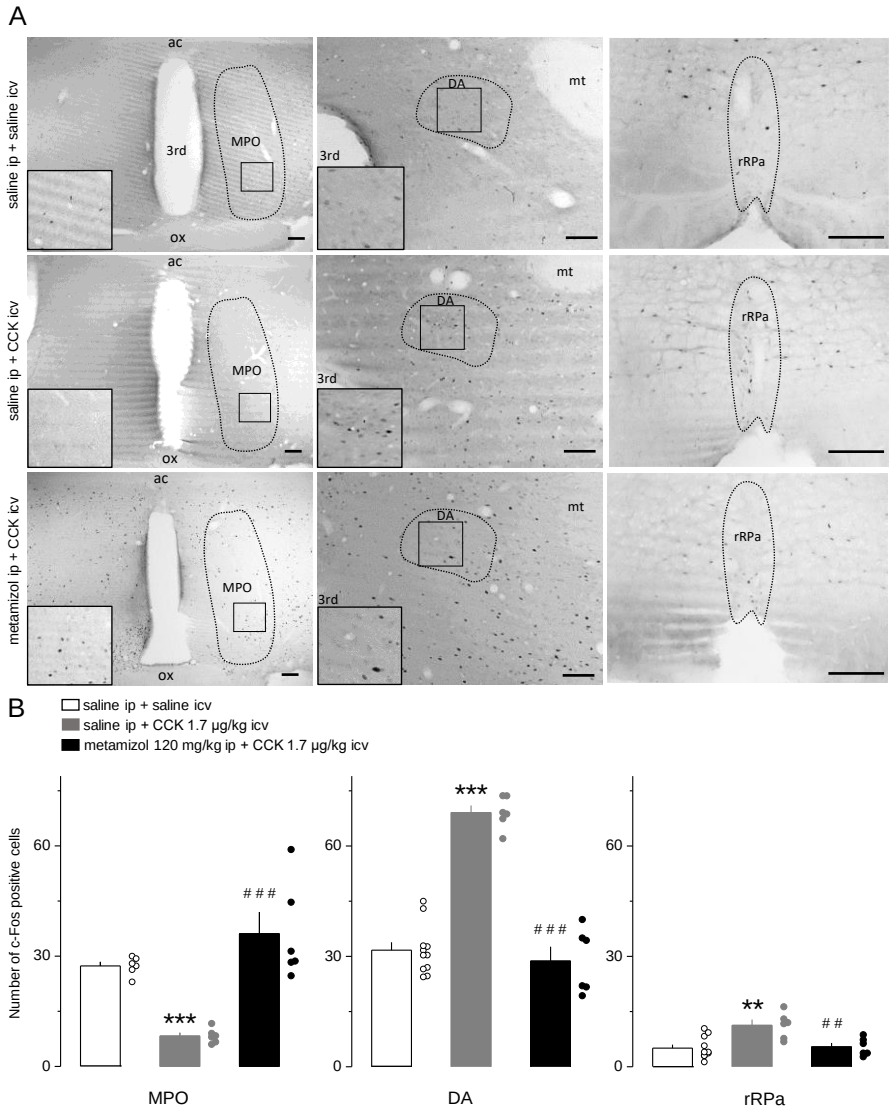

Figure 4. The expression of c-Fos in thermoregulatory nuclei of rats in response to icv administration of CCK (1.7 µg/kg) or saline after pretreatment at -30 min with an ip infusion of metamizol (120 mg/kg) or saline. A: representative photomicrographs of coronal sections from MPO, DA, and rRPa at -0.48, -2.76, and -10.68 mm to Bregma, respectively. The anterior commissure (ac), third ventricle (3rd), optic chiasm (ox), and mamillothalamic tract (mt) are shown as landmarks. Scale bar = 100 µm. B: quantitative analyses of c-Fos immunoreactive cells in the MPO, DA, and rRPa. For each group in MPO, n = 6. For treatment groups in DA and rRPa, n = 11 for saline + saline and n = 6 for saline + CCK and for metamizol + CCK. **P < 0.01 and ***P < 0.001, icv CCK vs. saline difference in ip saline-pretreated rats; $^{\#\#}$P < 0.01 and $^{\#\#\#}$P < 0.001, ip metamizol vs. saline difference in icv CCK-treated rats as determined by one-way ANOVA followed with Student-Newman-Keuls test. Bars represent group means (± SE), individual data are shown as circles (Keringer et al. 2022).

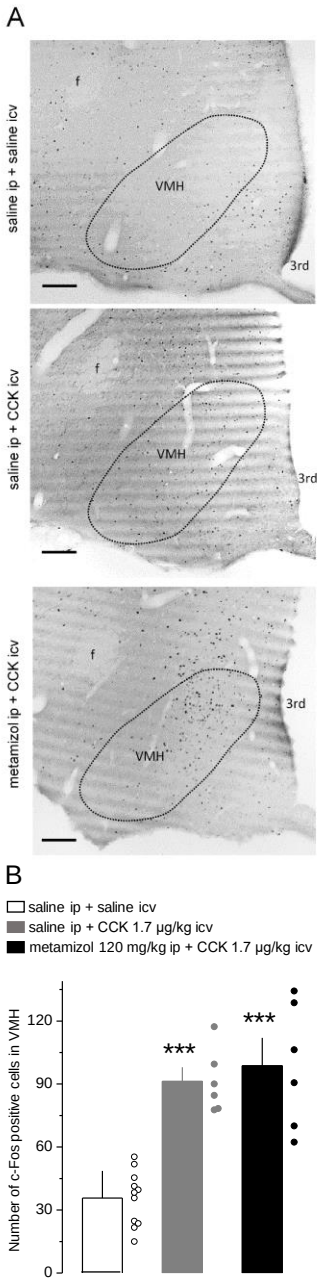

Figure 5. The expression of c-Fos in the VMH of rats in response to ip and icv administration of saline (n = 11) and to icv administration of CCK (1.7 µg/kg) after pretreatment at -30 min with an ip infusion of metamizol (120 mg/kg; n = 6) or saline (n = 6). A: representative photomicrographs of coronal sections of VMH at -2.40 mm posterior to Bregma. The third ventricle (3rd) and fornix (f) are shown as landmarks. Scale bar = 100 µm. B: quantitative analyses of c-Fos immunoreactive cells in the VMH. ***P < 0.001, icv CCK vs. saline difference as determined by one-way ANOVA followed with Student-Newman-Keuls test. Bars represent group means (± SE), individual data are shown as circles (Keringer et al. 2022).

5.1.3. Effects of selective COX-2 inhibitors on CCK-induced hyperthermia

We showed that metamizol blunts the effects of CCK, however metamizol inhibits both isoforms of COX. In systemic inflammation-associated thermal changes the two COX isoforms play different roles: COX-2 is essential in the development of fever, whereas COX-1, and not COX-2, is the isoform that mediates the hypothermic response (Steiner et al. 2009). Since CCK induced a rise in T_b, we hypothesized that COX-2 is responsible for the mediation of its thermal effect. To test our hypothesis, we studied the effects of two different preferential COX-2 inhibitors, meloxicam and etoricoxib, on CCK-induced hyperthermia (Figure 6). As expected, the hyperthermic effect of icv administered CCK was significant compared to saline [ANOVA, $F_{(1,285)} = 30.386$, $P < 0.001$] in ip saline-pretreated rats. However, when the rats were pretreated with meloxicam or etoricoxib ip, the icv injection of CCK did not cause any change in T_b of the rats. As compared to ip saline pretreatment, the effect was significant for both etoricoxib [ANOVA, $F_{(1,247)} = 105.804$, $P < 0.001$] and meloxicam [ANOVA, $F_{(1,266)} = 82.613$, $P < 0.001$]. The CCK-induced hyperthermia was attenuated by meloxicam at 20-30, 50, 80, and 100-180 min ($P < 0.05$), and by etoricoxib at 20-50, 70-80, and 100-180 min ($P < 0.05$) during the experiments (Figure 6).

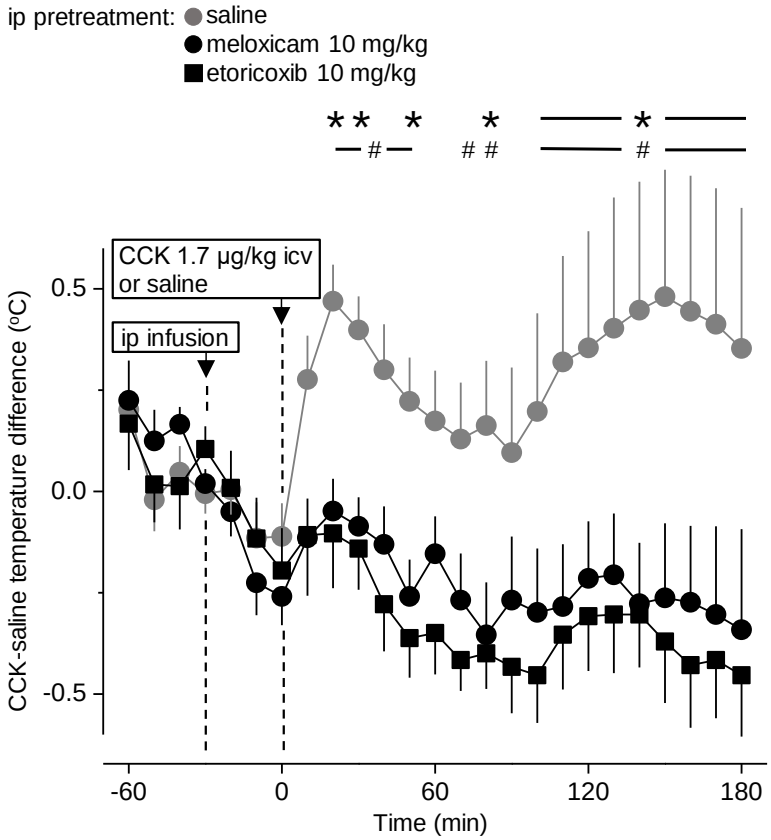

Figure 6. The difference in deep (colonic) T_b between rats treated icv with CCK (1.7 µg/kg) and saline after pretreatment at -30 min with an ip infusion of meloxicam (10 mg/kg; n = 7 and 8, respectively), etoricoxib (10 mg/kg; n = 6 and 8, respectively) or saline (n = 9 and 8). *P < 0.05, ip meloxicam vs. saline difference in icv CCK-treated rats; $^{\#}$P < 0.05, ip etoricoxib vs. saline difference in icv CCK-treated rats as determined by two-way ANOVA followed with Student-Newman-Keuls test. Data are presented as mean ± SE (Keringer et al. 2022).

5.1.4. Effect of the CCK_2 antagonist YM022 on LPS-induced fever

After we showed that the hyperthermic response to CCK is mediated by COX-2, we wanted to know whether CCK signaling in the central nervous system contributes to fever induced by bacterial endotoxin, which response is known to be mediated by COX-2 (Garami et al. 2018). Previous studies showed that CCK-induced hyperthermia is triggered mainly via CCK_2 receptors in the brain (Szelenyi et al. 1994, Weiland et al. 2007), thus in our experiments we focused on the role of the CCK_2 receptor in LPS-induced fever. As expected, the iv infusion of low-dose LPS in a thermoneutral environment caused a polyphasic febrile response in rats administered icv with the vehicle of YM022 before LPS; the three phases were peaking at 50-60, 100-120, and 300-330 min (Figure 7). When the rats were infused icv with YM022 before the LPS infusion, the first two phases of the fever response to LPS did not differ from what was observed in vehicle-pretreated rats, however the third febrile phase was markedly attenuated, reaching the level of significance ($P < 0.05$) at 280 and 300-360 min (Figure 7).

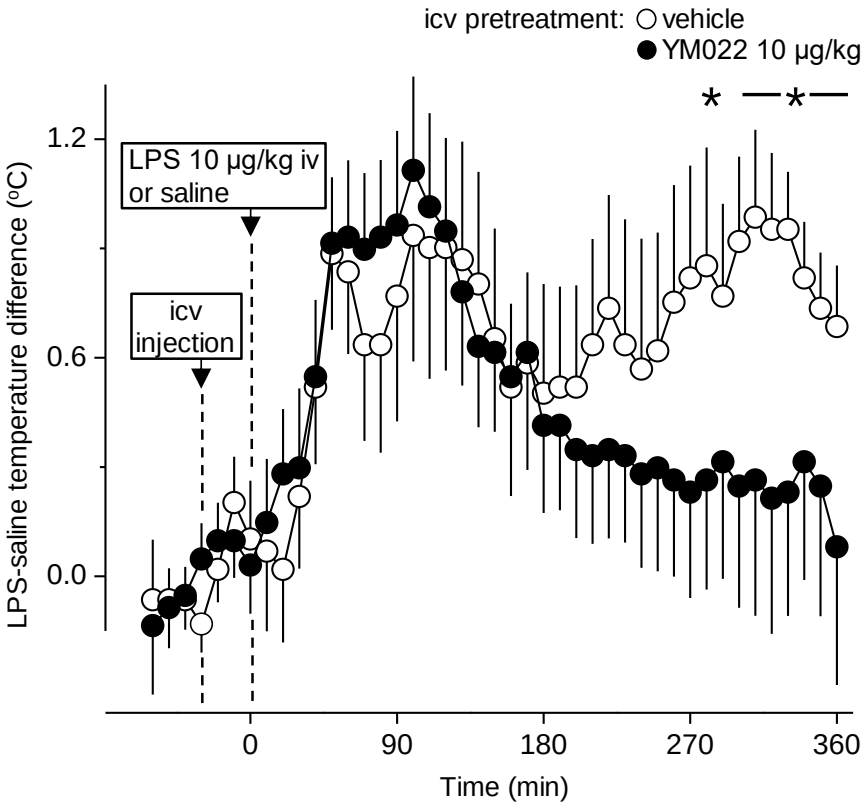

Figure 7. The difference in deep (colonic) T_b between rats treated iv with LPS (10 µg/kg) and saline after pretreatment at -30 min with an icv injection of YM022 (10 µg/kg) or its vehicle. For each group, n = 6. *P < 0.05, icv YM022 vs. vehicle difference in ip LPS-treated rats as determined by two-way ANOVA followed with Student-Newman-Keuls test. Data are presented as mean ± SE (Keringer et al. 2022).

5.2. Menthol as an exogenous hyperthermic substance

5.2.1. Study selection and characteristics

After characterizing the mechanisms of the hyperthermic response to CCK in rats, in the remaining of the work, we wanted to know whether the application of a hyperthermic substance (i.e., menthol) in humans influences different physiological and mental parameters of physical activity. For that we conducted a meta-analysis (Keringer et al. 2020). The flowchart of study selection is presented in Figure 8.

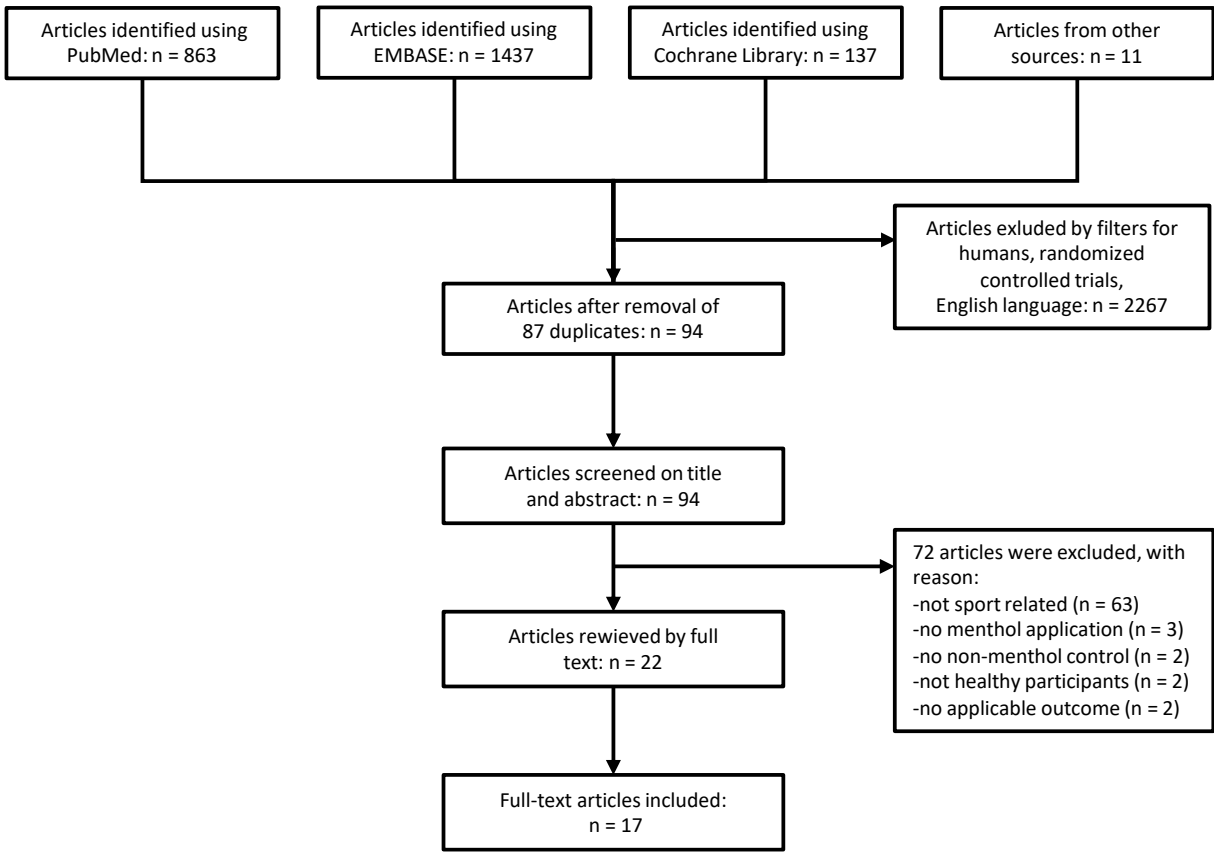

Figure 8. Flowchart of study selection and inclusion (Keringer et al. 2020).

Until May 2020, a total of 2,448 records were retrieved from the PubMed (n = 863), EMBASE (n = 1,437), and Cochrane (n = 137) databases and 11 records from other sources (e.g., Google Scholar). After removing duplicates and enabling filters for human studies, randomized controlled trials, and English language, 94 articles remained. By screening on title and abstract, further 72 records were excluded from the analysis because (1) the required outcome parameters were not reported, (2) menthol-treated or control group was absent, (3) not only healthy participants were recruited, and (4) no original data was reported. The full texts of 22 articles were reviewed in detail, from which 17 papers provided eligible data for qualitative and quantitative analyses (Gillis et al. 2010, Kounalakis et al. 2010, Mundel and Jones 2010, Sonmez et al. 2010, Schlader et al. 2011, Barwood et al. 2012, Barwood et al. 2014, Riera et al. 2014, Barwood et al. 2015, Stevens et al. 2016, Flood et al. 2017, Shepherd and Peart 2017, Stevens et al. 2017, Jeffries et al. 2018, Rinaldi et al. 2018, Barwood et al. 2019, Saldaris, Landers, and Lay 2020). All included studies had randomized, crossover design and included data from a total of 177 athletes. The participant characteristics in the studies are presented in Table 1.

Table 1. Characteristics of participants in the studies included in the meta-analysis (Keringer et al. 2020).

Study	Number of participants	Mean age (years)	Mean BMI (kg/m^2)	Trained?	Acclimated?
Barwood (2012)	11	30	24.0	Yes	No
Barwood (2014)	6	21	24.4	Yes	NR
Barwood (2015)	8	21	25.4	Yes	NR
Barwood (2019)	8	22	NR	Yes	NR
Flood (2017)	8	26	24.2	NR	No
Gillis (2010)	12	22	23.6	Yes	NR
Jeffries (2018)	10	33	23.7	NR	No
Kounalakis (2010)	8	28	22.2	Yes	NR
	8	21	23.2	Yes	NR
Mundel (2010)	9	25	25.0	NR	No
Riera (2014)	12	42	22.8	Yes	Yes
Rinaldi (2018)	8	24	NR	Yes	Yes
Saldaris (2020)	12	25	23.4	Yes	No
Schlader (2011)	12	23	25.6	Yes	NR
Shepherd (2017)	7	25	24.3	Yes	NR
Sonmez (2010)	16	22	NR	Yes	NR
Stevens (2016)	11	29	23.4	Yes	Yes
Stevens (2017)	11	30	23.4	Yes	Yes

BMI, body mass index; NR, not reported

The majority of the studies was conducted in males, except for an article which included participants of both sexes (Shepherd and Peart 2017) and possibly another one which did not report the sex ratio in the sample (Sonmez et al. 2010). In 12 trials, the participants were refrained from strenuous exercise, alcohol and caffeine intake before the experiments (Kounalakis et al. 2010, Mundel and Jones 2010, Schlader et al. 2011, Barwood et al. 2012, Barwood et al. 2014, Barwood et al. 2015, Stevens et al. 2016, Flood et al. 2017, Stevens et al. 2017, Jeffries et al. 2018, Barwood et al. 2019, Saldaris et al. 2020), two studies included unspecified training limitations (Riera et al. 2014, Rinaldi et al. 2018), and three articles did not report any limitations (Gillis et al. 2010, Sonmez et al. 2010, Shepherd and Peart 2017).

The mean exercise duration was 30 min in TT tests, ranging from 1 min (Sonmez et al. 2010) to 71 min (Barwood et al. 2012), while in TTE protocols, it ranged between 1 min (Saldaris et al. 2020) and 61 min (Mundel and Jones 2010), with an average of 27 min. In two studies the subjects exercised for a fixed time duration of 45 min (Gillis et al. 2010) or 2 × 20 min (Rinaldi et al. 2018), while the exercise duration was calculated from distance and speed in one of the studies (Sonmez et al. 2010). In all studies we considered the beginning of the exercise as the baseline, which was before the menthol treatment, except for two studies, in which menthol administration was before the start of the trial (Schlader et al. 2011, Flood et al. 2017). It should be noted that the TTE test was preceded by 45-min exercise without interruption in the study by Barwood et al. (Barwood et al. 2019), during which the subjects were repeatedly treated with menthol, thus we considered the beginning of the 45-min preliminary fatiguing task as the baseline for TS, TC, and deep T_b. The TTE test was also preceded by physical exertion and repeated menthol administration in the study by Saldaris et al. (2020), but in that study the 3 × 30 min trials were interrupted by breaks and resting between the trials, as well as, before the TTE test. Therefore, we considered the beginning of the TTE test as the baseline in that study.

The used doses of menthol varied due to differences in administration method (i.e., internal or external), concentration (0.01–8%), volume (25–500 ml), and surface of the treated body area (4–91%). The most commonly used concentrations and volumes were, respectively, 0.01% and 25 ml in internal, while 0.2% and 100 ml in external administration routes. The details of the used administration routes, doses, and experimental procedures are summarized in Table 2.

Table 2. Menthol application methods, exercise protocols, and environmental conditions in the studies eligible for quantitative analysis in our meta-analysis (Keringer et al. 2020).

First author (publication year)	Menthol concentration (%); volume (ml)[a]	Administration method; body surface area (%)[a]	Warm up	Exercise protocol; approx. duration (min)	T_a (°C)[b]	Relative humidity (%)[b]
Barwood (2012)	0.05; 100	External (spray on top wear); 36	Yes	Cycling TT; 71	31.5 (0.7)	53 (5)
Barwood (2014)	0.2; 100	External (spray on top wear); 36	Yes	Running TT; 28	33.9 (0.1)	55
Barwood (2015)	0.2; 100	External (spray on top wear); 36	Yes	Cycling TT; 33	33.5 (0.5)	33 (5)
Barwood (2019)	0.2; 100	External (spray on top wear); 36	Yes	Cycling TTE; 49	34.6 (1.2)	22 (1)
Flood (2017)	0.01; 25	Internal	Yes	Cycling TTE; 23	35.0 (0.8)	48 (2)
Gillis (2010)	0.05; 100	External (spray on top wear); 36	No	Cycling FTE; 45	26.3 (0.9)	72 (3)
Jeffries (2018)	0.01; 25	Internal	Yes	Cycling TTE; 25	35.0 (0.2)	40 (1)
Kounalakis (2010)	[c]; 100	External (cream on whole body); 91	No	Cycling TTE; 27	24.1 (1)	46 (4)
Mundel (2010)	0.01; 25	Internal	No	Cycling TTE; 61	34.0 (1)	27 (4)
Riera (2014)	0.01; 190	Internal	Yes	Cycling TT; 37	30.7 (0.8)	78 (0)
Rinaldi (2018)	0.1; NR	External (whole-body immersion); 91	Yes	Cycling FTE; 2 x 20	29.1 (1.5)	62 (4)
Saldaris (2020)	0.1; 25	Internal	No	Running TTE; 1	35.3 (0.3)	59 (3)
Schlader (2011)	8; [d]	External (gel on face); 4	Yes	Cycling TTE; 21	20.3 (0.2)	48 (3)
Shepherd (2017)	0.01; 500	Internal	No	Cycling TTE; 10	NR	NR
Sonmez (2010)	NR	Internal	No	Running TT; 1	NR	NR
Stevens (2016)	0.01; 25	Internal	Yes	Running TT; 26	32.6 (0.2)	46 (6)
Stevens (2017)	0.01; 25	Internal	No	Running TT; 14	32.5 (0.1)	47 (8)

[a]according to the Wallace rules of nine (Wallace, A. B. The exposure treatment of burns. *Lancet*, **257**, 501–504 [1951]), by omitting the area of the head from whole-body and of the arms from top wear applications; [b]data are shown as mean (standard deviation); menthol was applied at [c]4.6 g and [d]0.5 g/100cm^2; FTE, fixed-time exercise; NR, not reported; T_a, ambient temperature; TT, time trial; TTE, time to exhaustion.

5.2.2. Perceptual responses

First, we studied how menthol application influences perceptual responses, *viz.*, TS and TC during exercise. As it could be expected based on to the cold-mimicking effect of menthol-containing products (Eccles 1994), the TS score decreased in the menthol-treated groups as

compared to controls in seven studies (Schlader et al. 2011, Barwood et al. 2014, Barwood et al. 2015, Stevens et al. 2016, Jeffries et al. 2018, Barwood et al. 2019, Saldaris et al. 2020), while two studies reported a slight increase in TS (Schlader et al. 2011, Flood et al. 2017). Accordingly, the overall WMD between the menthol-treated and control groups was − 1.65 (95% CI, − 2.96 to − 0.33; P = 0.014) (Figure 9). The TC score decreased during physical exercise compared to baseline in all groups, but the magnitude of the decrease was smaller in the menthol-treated group than in controls by a WMD of 1.42 (95% CI, − 0.13 to 2.96; P = 0.073) (Figure 10), which indicates that the perceived temperature was more comfortable (i.e., not so hot) after menthol administration compared to controls.

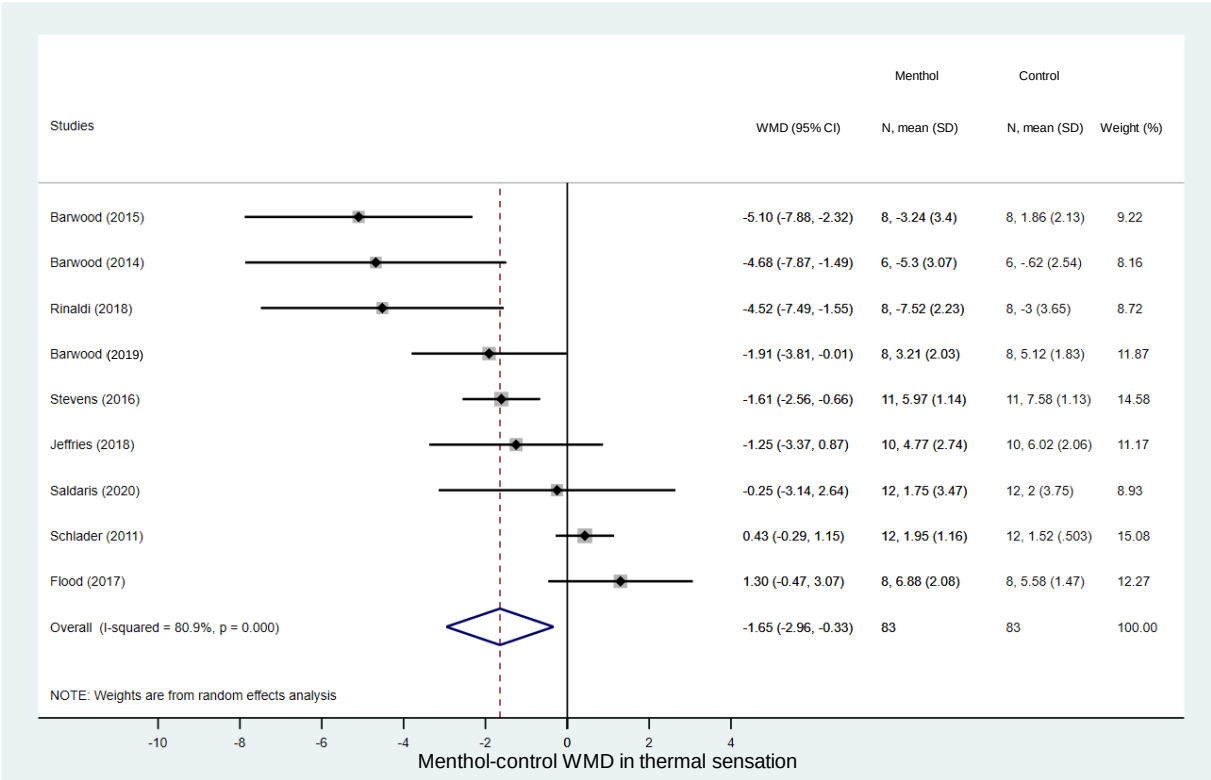

Figure 9. Forest plot of the weighted mean differences (WMDs) showing the effect of menthol on thermal sensation during exercise. The black circles represent the WMD for each study, while the left and right horizontal arms of the circles indicate the corresponding 95% confidence intervals (CI) for the WMD. The size of the grey box is proportional to the sample size; bigger box represents larger sample size, thus bigger relative weight of the study. The diamond represents the average WMD calculated from the WMDs of the individual studies. The left and right vertices of the diamond represent the 95% CI of the average WMD. The vertical dashed line is determined by the low and top vertices of the bottom diamond and indicates the value of the average WMD of all studies in the forest plot. A WMD lesser than 0 indicates that the thermal sensation value (intensity of cold sensation) is higher in menthol-treated group, whereas a WMD higher than 0 indicates that thermal sensation is higher in control group (Keringer et al. 2020).

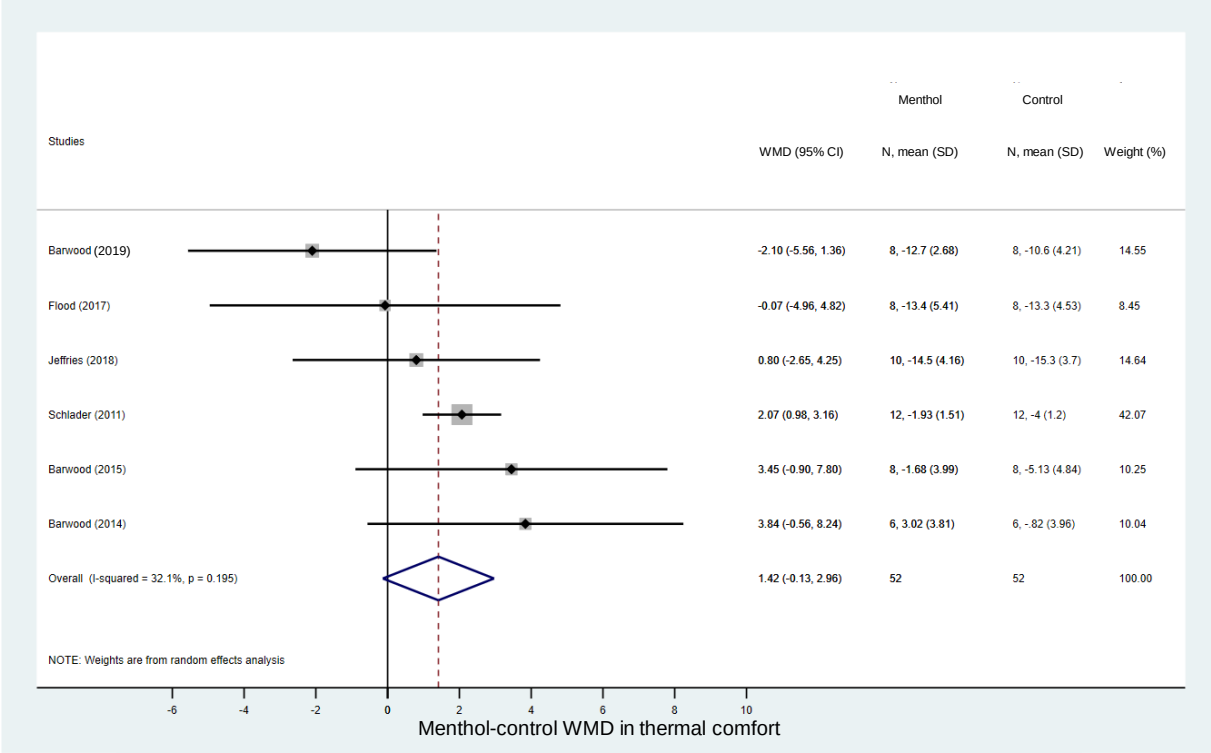

Figure 10. Forest plot of the weighted mean of diferences (WMDs) for thermal comfort showing the effect of menthol during exercise (Keringer et al. 2020).

5.2.3. Thermophysiological responses

We could extract sufficient data for the analysis of three thermoregulatory parameters: sweat production (an indicator of the activity of autonomic heat-dissipating mechanisms), heart rate (a nonspecific indicator of metabolic rate), and deep T_b (i.e., the tightly controlled parameter in thermoregulation). We found that the volume of sweat production did not differ significantly between the menthol-treated and control groups during exercise (WMD = − 24.10 ml; 95% CI, − 139.59 to 91.39 ml) (Figure 11). Similar to sweat production menthol also did not have a meaningful effect on the exercise-induced increase in deep T_b compared to the control group

(WMD = 0.02°C; 95% CI, − 0.11 to 0.15°C) (Figure 12). Furthermore, there was no significant difference in exercise-induced elevation of heart rate between the treatment groups (WMD = 2.67 bpm; 95% CI − 0.74 to 6.09 bpm) (Figure 13).

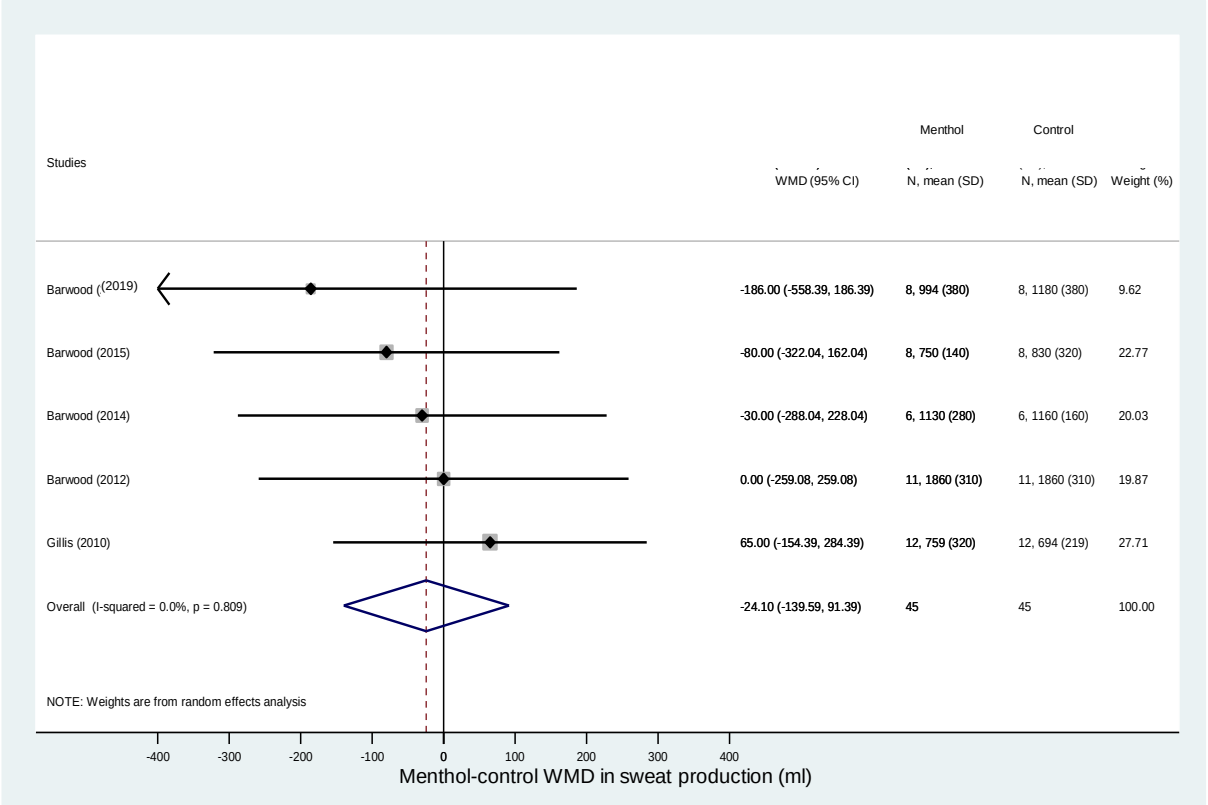

Figure 11. Forest plot of the weighted mean of diferences (WMDs) for sweat production showing the effect of menthol during exercise (Keringer et al. 2020).

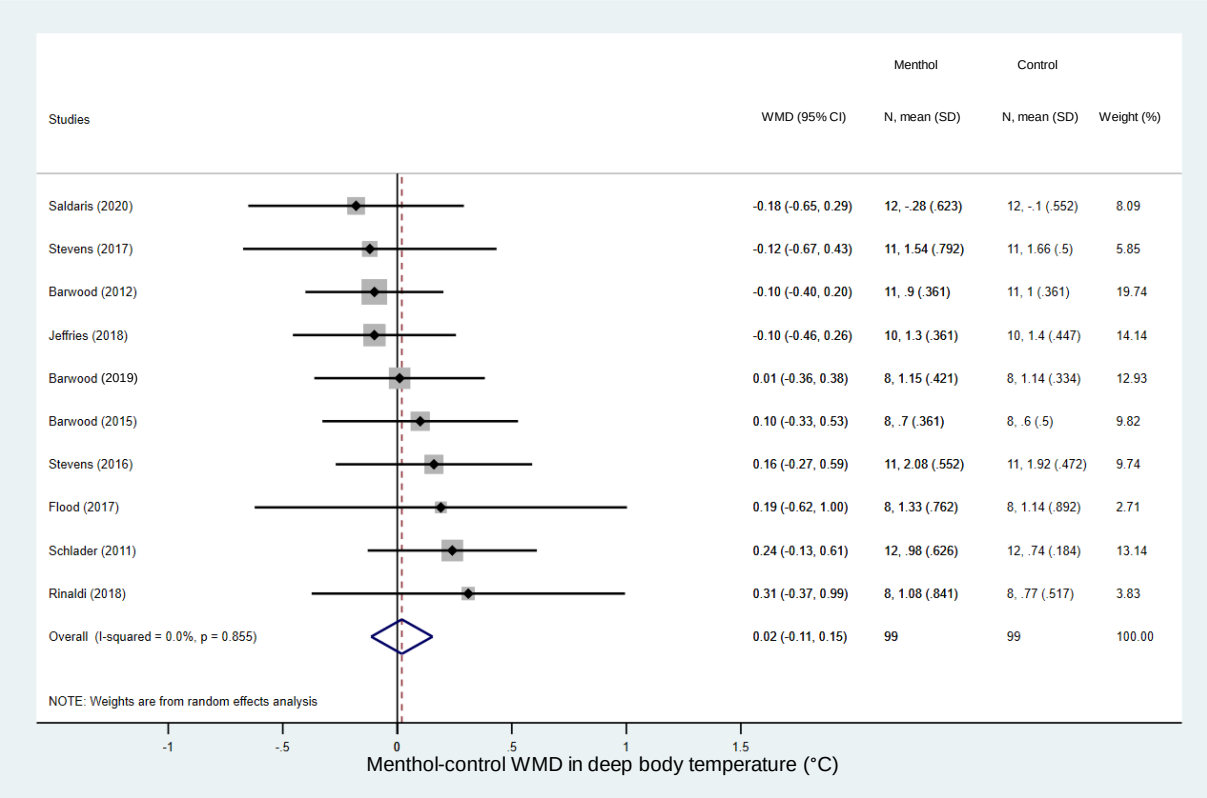

Figure 12. Forest plot of the weighted mean of diferences (WMDs) for deep T_b showing the effect of menthol during exercise (Keringer et al. 2020).

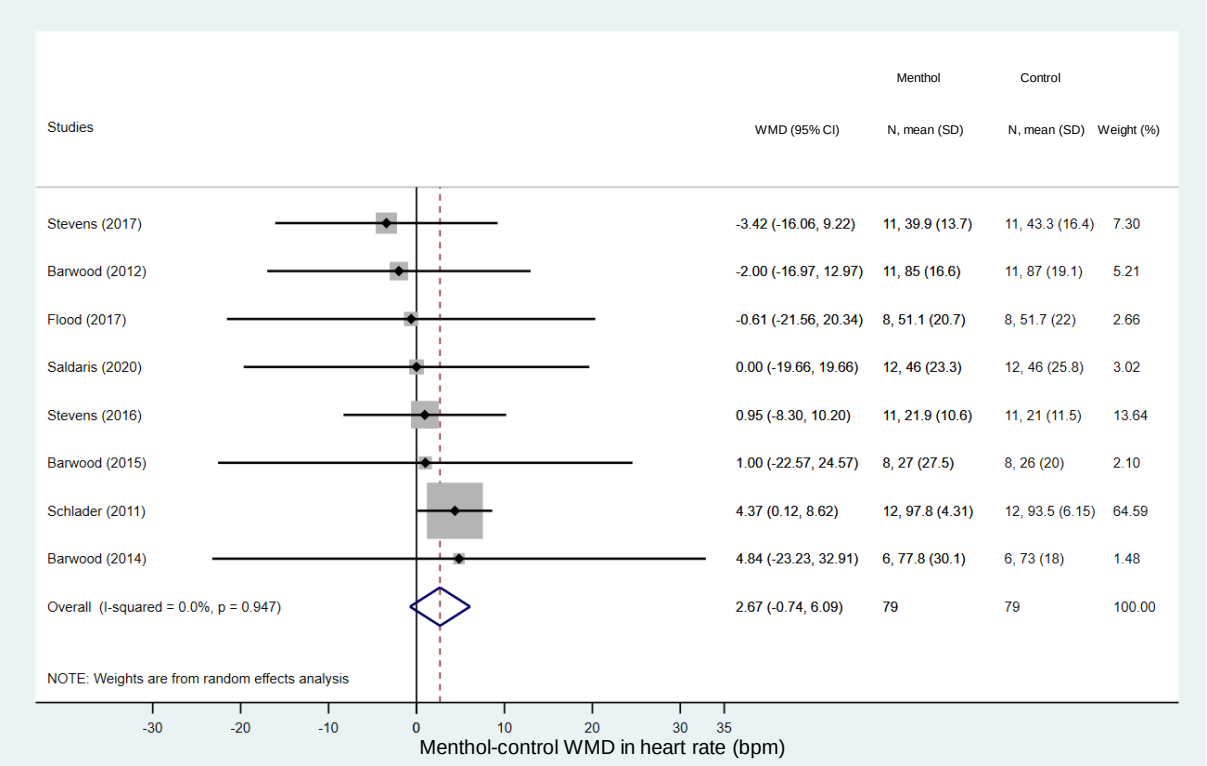

Figure 13. Forest plot of the weighted mean of diferences (WMDs) for heart rate showing the effect of menthol during exercise (Keringer et al. 2020).

5.2.4. Performance time

Overall, the performance time did not differ statistically between menthol-treated and control groups in TT protocols (WMD = − 0.52 min; 95% CI, − 1.37 to 0.34 min) (Figure 14a) and TTE tests (WMD = 1.04 min; 95% CI, − 0.47 to 2.55 min) (Figure 14b).

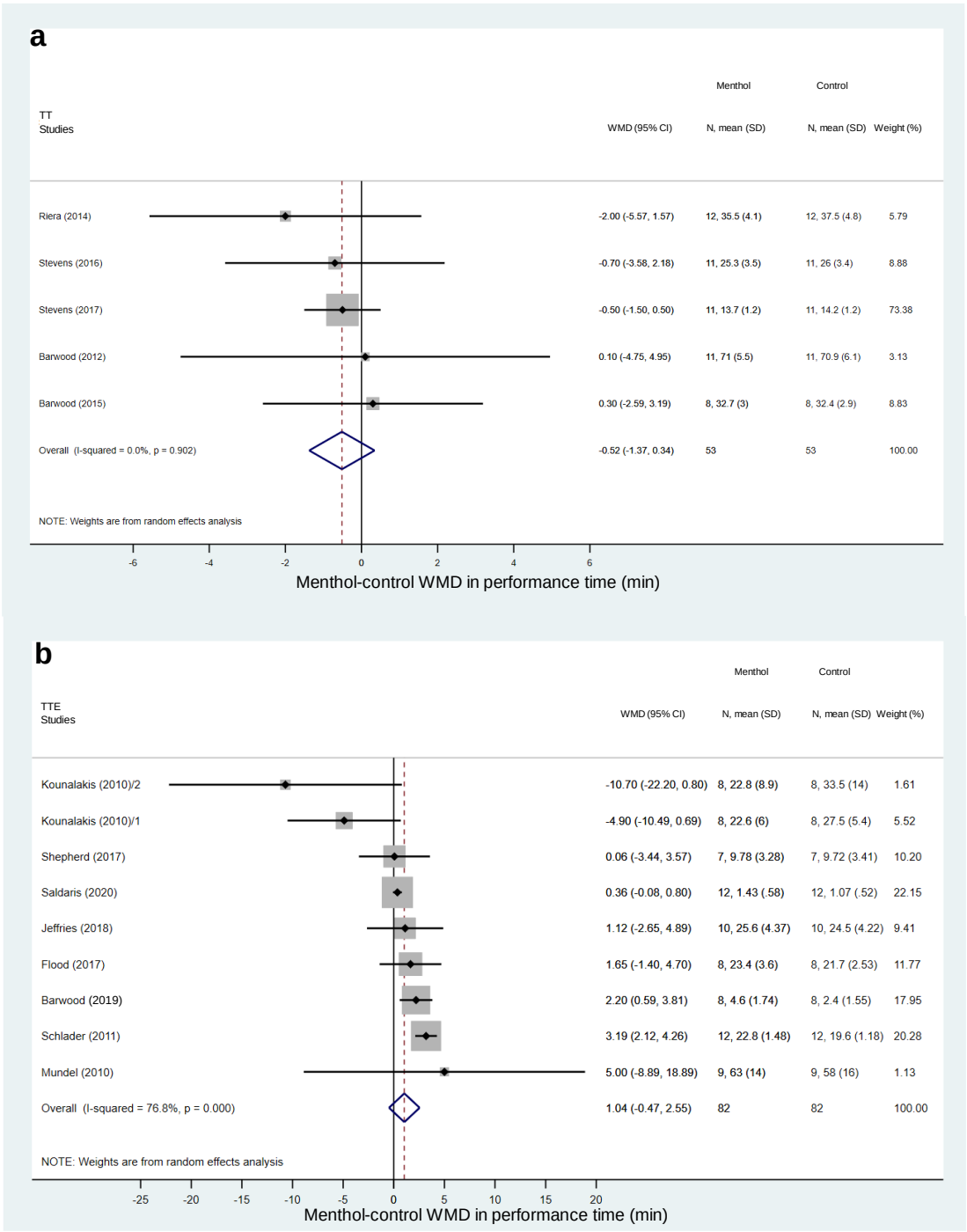

Figure 14. Forest plot of the weighted mean of differences (WMDs) for performance time in (**a**) time-trial (TT) and (**b**) time-to-exhaustion (TTE) tests showing the effect of menthol during exercise (Keringer et al. 2020).

In the TT protocols, no meaningful difference was observed in the effect of menthol between subgroups of higher (above 23.5) BMI and lower (21.4–23.5) BMI (Figure 15a). However, in the TTE tests, among athletes with higher BMI, performance time increased significantly in the menthol-treated group compared to controls (WMD = 2.57 min; 95% CI 1.76 to 3.39 min), whereas menthol tended to decrease performance time in the lower BMI group (WMD = − 3.20 min; 95% CI − 8.81 to 2.42 min) (Figure 15b). The WMD between the treatment groups was markedly bigger in the higher than in the lower BMI subgroup ($p < 0.001$). We also analyzed whether acclimation of the subjects to exercising in warmth influences the effects of menthol. In TT protocols, we did not find meaningful difference in menthol's effect on performance time between non-acclimated and acclimated participants. In TTE protocols, all of the studies were performed in non-acclimated participants and the effect of menthol was also not significant in the group [for details, see Keringer et al. (2020); also enclosed in the appendix]. When we compared the effect of external and internal menthol application on endurance performance, we found that external application of menthol markedly increased performance time compared to internal application in TTE exercise protocols (WMD = 0.83 min; 95% CI − 1.95 to 3.60 min versus 0.40 min, 95% CI, − 0.03 to 0.83 min; $P < 0.001$), while in the other subgroups no significant effect was detected (Figure 16).

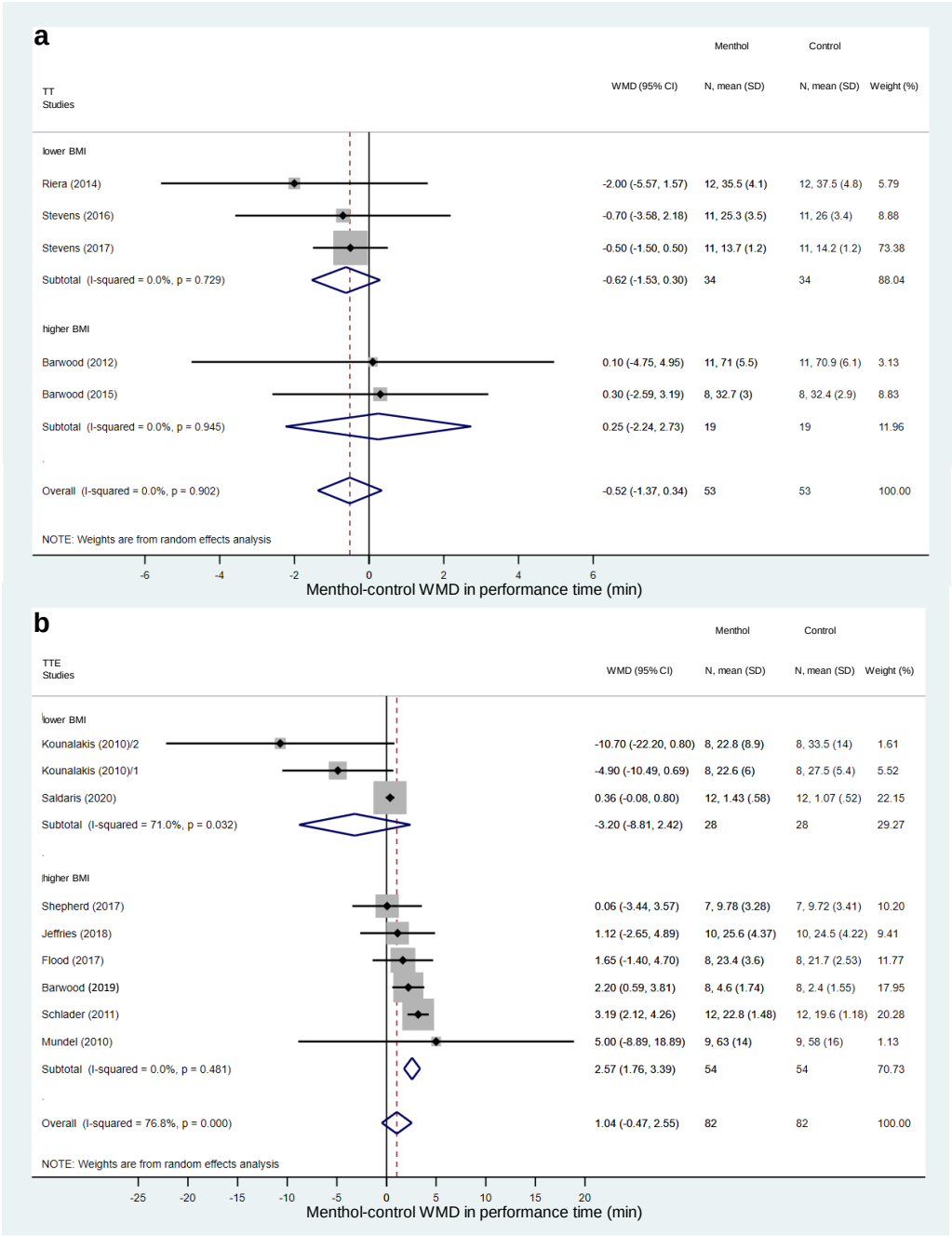

Figure 15. Forest plot of the weighted mean of differences (WMDs) for performance time showing the effect of menthol in (a) time-trial (TT) and (b) time-to-exhaustion (TTE) tests of athletes with lower (<23.5) and higher (>23.5) body mass index (BMI). The diamonds in the panels represent the average WMD calculated from the WMDs of the individual studies in each subgroup (top and middle) or in all studies (bottom) (Keringer et al. 2020).

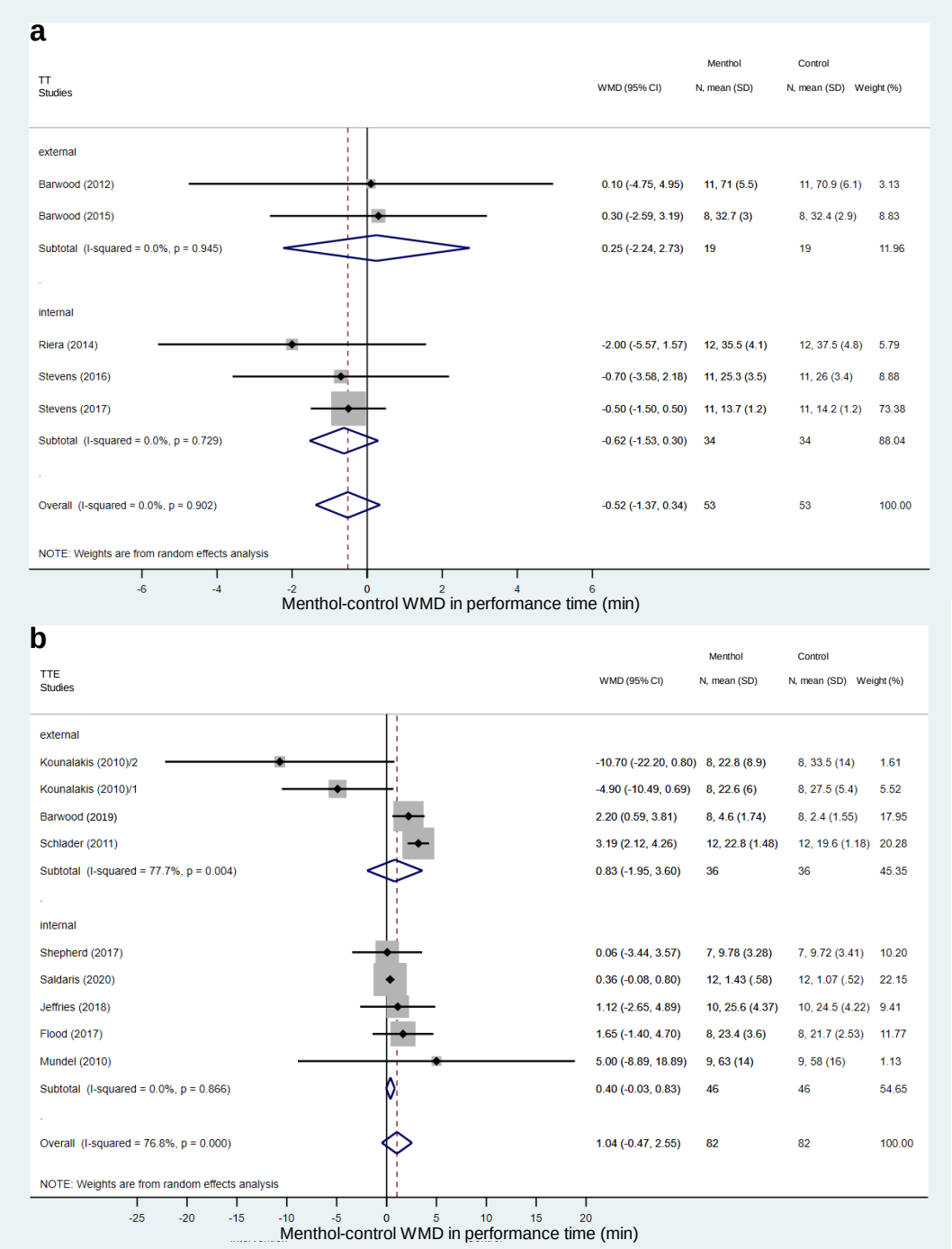

Figure 16. Forest plot of the weighted mean differences (WMDs) for performance time showing the effect of menthol in (**a**) time-trial (TT) and (**b**) time-to-exhaustion (TTE) tests in subgroups of external and internal application (Keringer et al. 2020).

The external menthol application methods were different in the studies: spray on the top wear (Barwood et al. 2012, Barwood et al. 2014, Barwood et al. 2015, Barwood et al. 2019), whole-body creaming (Kounalakis et al. 2010) or immersion (Rinaldi et al. 2018), and gel on the face (Schlader et al. 2011) (for details, see Table 2 above). The location of the administration and the surface area may also influence the effect since thermal signals from hairy skin provide more important feedback signals for the thermoregulation system than thermal signals from non-hairy skin; the latter functioning predominantly as an effector rather than a sensor (Romanovsky 2014). To examine the possibility that treatment of a certain area of the body (e.g., face) with menthol has bigger impact on endurance performance than other areas in TTE protocols, we performed a sensitivity analysis (i.e., iteratively removing one study from the analyses and recalculating WMD to investigate the impact of each individual study on the summary estimate), which showed no difference in the final pooled results. Among the environmental factors, no meaningful difference was observed between subgroups with and without airflow in TT protocols. In TTE tests, we found that menthol increased performance time when a fan (i.e., airflow) was present compared to no use of a fan [for details, see Keringer et al. (2020); also enclosed in the appendix]. However, the averaged result of the subgroup with airflow should be taken with scrutiny due to the low number (n = 2) of studies in this subgroup. Furthermore, in TTE tests at higher T_as (above 31°C), performance time was significantly increased in response to menthol compared with T_as ranging from ~20 to 30°C, while there was no significant difference between the subgroups in TT protocols [for details, see Keringer et al. (2020); also enclosed in the appendix].

6. Discussion

In our study, we investigated different aspects of hyperthermia (for summary, see Figure 17). First, we studied the mechanisms of the hyperthermic effects of CCK in a rat model and identified the involved molecular pathways and neural structures. Then, as a translational approach to show the importance of the use of hyperthermia-inducing substances in humans, we analyzed the effects of menthol application on thermophysiological parameters and on sport performance in human subjects and demonstrated its beneficial effects and safety.

In the first part of our work (Keringer et al. 2022), for the first time of our knowledge, we showed that the hyperthermic response to the icv administration of CCK involves changes in the activity of thermoregulatory nuclei, *viz.*, the MPO, DA and rRPa that belong to the efferent neuronal pathways of autonomic thermoeffectors. Inhibition of the COX pathway with selective COX-2 and non-selective COX inhibitors attenuated these thermoregulatory effects of central CCK, which were novel findings. We also showed that pharmacological blockade of CCK_2 receptors reduces the late phase of LPS-induced fever. These findings suggest an interaction between central CCK signaling and the COX pathway in CCK-induced hyperthermia and in the maintenance phase of endotoxin-induced fever. In contrast with the thermoregulatory effects, CCK-induced satiety was not influenced by COX inhibition, indicating that, unlike LPS-induced anorexia, the effects of CCK on food intake are independent from the COX pathway.

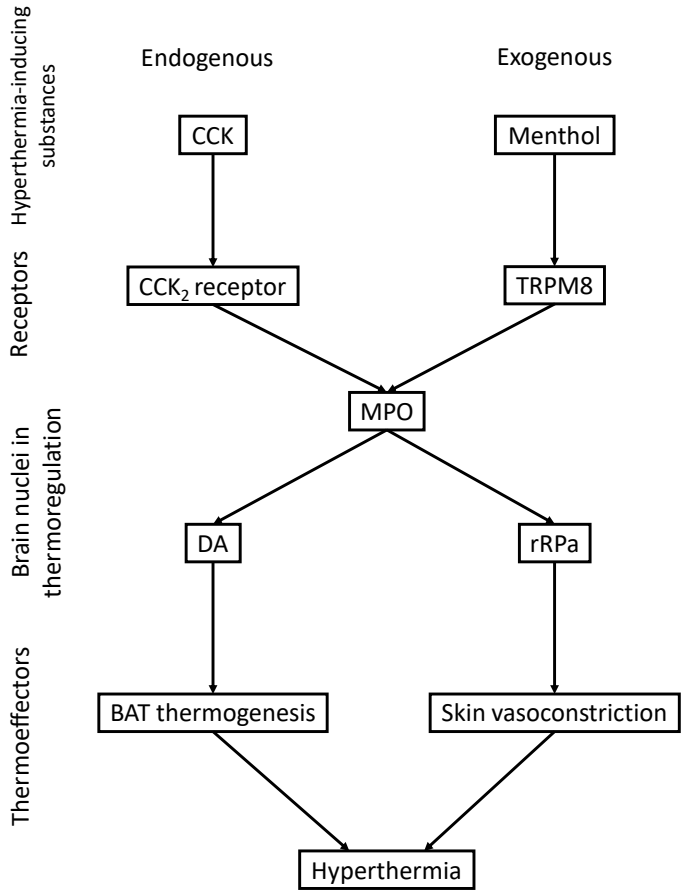

Figure 17. Summary of the neural processes in hyperthermia induced by endogenous or exogenous substances. BAT: brown adipose tissue; CCK: cholecystokinin; DA: dorsal hypothalamic area; MPO: medial preoptic area; TRPM8: transient receptor potential melastatin-8.

The hyperthermic effect of central CCK has been known for long (Szekely et al. 1994, Szelenyi et al. 1994). It was also shown that it involves the increased activity of the two main autonomic cold-defense effectors: cutaneous vasoconstriction and non-shivering thermogenesis (Szekely et al. 1994, Szelenyi et al. 1994, Sugimoto et al. 1999). The centrally induced hyperthermic

effect of CCK is mediated by the CCK_2 receptor, which is in contrast with the CCK_1 receptor-mediated hypothermia in response to peripheral CCK administration (Szelenyi et al. 1994). In a recent study, the importance of CCK neuron populations in the lateral parabrachial nucleus was shown, suggesting that these neurons are involved in the transmission of warmth afferent signals from the periphery to the central nervous system, thereby recruiting autonomic heat-defense mechanisms and preventing the elevation of deep T_b (Yang Wen et al. 2020). These findings (Yang Wen et al. 2020) are well in accordance with the body temperature-decreasing effect of peripherally administered CCK, but they do not explain the development of hyperthermia in response to centrally administered CCK. We showed that the icv administration of CCK caused changes in the neuronal activation in the MPO, rRPa, and DA, which brain structures are well-established portions within the efferent pathways of autonomic thermoeffector responses (Nakamura 2011, Wanner et al. 2017, McAllen and McKinley 2018, Morrison 2018, Romanovsky 2018). In our study (Keringer et al. 2022), centrally administered CCK decreased the c-Fos immunoreactivity in the MPO, but increased it in the DA and the rRPa. The MPO harbors GABAergic neurons, which tonically suppress brown adipose tissue thermogenesis and skin vasoconstriction (Nakamura et al. 2002, Osaka 2004) through their inhibitory projections to the DA and rRPa, from where the sympathoexcitatory drive to brown adipose tissue and skin vessels, respectively, is provided (Nakamura and Morrison 2007, Rathner, Madden, and Morrison 2008). Therefore, our findings suggest that CCK reduces the activity of GABAergic neurons in MPO, thereby disinhibits the excitatory DA and rRPa resulting in an increased sympathetic drive to the autonomic cold-defense effectors. A possible explanation for the CCK-induced changes in hypothalamic neuronal activity could be a direct

action of CCK on CCK_2 receptors expressed by these cells. In support of such a scenario, CCK_2 receptors are found in the hypothalamus of adult rats (Micevych et al. 1987, Ito et al. 1993, Mercer et al. 2000). However, in the preoptic area, the level of CCK receptors is lower than in other hypothalamic regions (Gaudreau et al. 1983), and CCK-immunoreactive neurons are restricted to the periventricular and paraventricular hypothalamic nuclei, whereas different preoptic structures have few CCK-immunoreactive cells (Tsukahara and Yamanouchi 2003). Moreover, the CCK_2 receptor was not detected in the MPO and DA (Honda et al. 1993). Therefore, an indirect action of CCK on the hypothalamic thermoregulatory neurons is more plausible.

It was observed that the physiological mechanisms of PGE_1 and CCK-8 hyperthermia are similar in that they both have the same dependence of the effector pattern on the initial body temperature, and both substances increase body temperature to a level that depends on the dose but does not depend on the initial body temperature (Szelenyi, Szekely, and Romanovsky 1992, Szelenyi et al. 1994). Furthermore, both PGE_1 and CCK-8, when the hyperthermic response resolves, cause imprecise body temperature regulation (Szelenyi et al. 1992), which is characteristic of the later febrile phases: initially, deep T_b (colonic) is regulated very tightly due to constant minor adjustment in the skin vasomotor tone (tail skin temperature), but at the end of the response, large fluctuations occur in the colonic temperature caused by large waves of vasoconstriction/vasodilation (Vybiral et al. 1987). At the time of those observations, no information was available on the possibility of a CCK-mediated link to the febrile response, but later, interactions between CCK signaling and the arachidonic acid cascade were found. Activation of the CCK_2 receptor by CCK-8 leads to arachidonic acid production in different

cell cultures (Keiko, Taku, and Tetsuro 1997, Pommier et al. 2003), and a CCK_2 receptor-mediated increase in COX-2 mRNA and protein expression, followed by PGE_2 secretion, was shown in several cell lines (Guo et al. 2002, Slice, Hodikian, and Zhukova 2003, Colucci et al. 2005). Accordingly, the contribution of CCK signaling to the mediation of fever was proposed [for review, see (Szelenyi et al. 2004)]. In our study (Keringer et al. 2022), we provided thermophysiological and immunohistochemical evidence for the close interaction between CCK signaling and the COX pathway. First, we showed that that the hyperthermic effect of centrally (icv) administered CCK can be completely abolished by nonselective inhibition of COX enzymes with metamizol (also known as dypirone). Then, we demonstrated that the same inhibition also prevented the CCK-induced changes in c-Fos expression observed in the thermoregulatory nuclei (i.e., in the MPO, DA, and rRPa) of the efferent autonomic effector pathway. We also revealed that selective inhibition of COX-2 with two different drugs (*viz.*, meloxicam and etoricoxib) blunted the CCK-induced hyperthermia practically to the same extent as the nonselective COX enzyme inhibitor.

At the applied dose of 120 mg/kg, metamizol could be expected to exert maximal inhibition of both COX-1 and COX-2 enzymes, as in humans it elicits nearly complete (94-97%) COX inhibition already at 14 mg/kg (Hinz et al. 2007). It is also important to note that, at 120 mg/kg dose, metamizol blocked LPS-induced fever, but by itself had no effect on the body temperature of rats, unlike higher doses (240-360 mg/kg), which caused hypothermia (Malvar et al. 2014). In accordance, metamizol did not cause any meaningful change in the T_b of the rats compared to control (saline-treated) rats in this study (Figure 2). Our findings seem to contradict a previous report which showed that the subcutaneous pretreatment with 10 mg/kg of

indomethacin (a nonselective COX inhibitor) had no effect on the hyperthermic response to icv CCK (Szekely et al. 1994). However, when indomethacin was injected ip to mice at 10 mg/kg, the indomethacin content of the brain was very low and it did not reduce brain inflammation, even though it effectively suppressed peripheral inflammation (Gamache and Ellis 1986). Indomethacin (10 mg/kg; intramuscularly) also failed to alter arachidonic acid-induced brain edema in another study in rats (Chan et al. 1983). These results suggest that the applied dose of indomethacin in the study by Szekely et al. (1994) was not high enough to efficaciously block COX enzymes in the brain. In contrast with indomethacin, the metabolites of metamizol were present in appreciable concentrations in the cerebrospinal fluid of humans after oral administration of metamizol at 14 mg/kg (Cohen et al. 1998). Detectable levels of metamizol metabolites were also shown in the brain and spinal cord of mice fed metamizol via drinking water (Rogosch et al. 2012), and in the hypothalamus and cerebrospinal fluid of rats administered ip with metamizol at 120 mg/kg (Aguiar et al. 2013). Similarly to metamizol, the ability to penetrate the blood-brain barrier was also shown for meloxicam (Jolliet et al. 1997, Tegeder et al. 2000) and etoricoxib (Renner et al. 2010). Both of these drugs inhibit COX-2 more potently than COX-1: the 50% inhibitory concentrations for COX-2 compared to COX-1 are 2 times lower for meloxicam and 106 times lower for etoricoxib (Riendeau et al. 2001). Our finding that these two different, selective COX-2 inhibitors caused a practically identical suppression of CCK-induced hyperthermia as metamizol exclude the possibility that the observed effects of metamizol were independent from the COX pathway. Moreover, they indicate that the activity of COX-2 is required in the hyperthermic response.

An endproduct of the COX-2 pathway is PGE_2, which is known as the key mediator of systemic inflammation-associated fever (Ivanov and Romanovsky 2004, Saper et al. 2012, Roth and Blatteis 2014, Garami et al. 2018). According to the classical concepts of febrigenesis, PGE_2 binds to EP3-expressing GABAergic neurons in the preoptic area (including the MPO and the median preoptic nucleus) and suppresses their activity, thereby disinhibiting downstream targets such as the DA and rRPa, which leads to autonomic heat conservation and heat production (Nakamura et al. 2002, Saper et al. 2012, Garami et al. 2018, Morrison and Nakamura 2019). It should be mentioned that a recent study challenged the classical concept by showing that EP3-expressing glutamatergic neurons in the median preoptic nucleus mediate the febrile response (Machado et al. 2020). PGE_2 is thought to inhibit the activity of preoptic neurons (Nakamura et al. 2002, Nakamura 2011, Tan and Knight 2018), and the aforementioned PGE_2-induced changes in the activity of neurons within the efferent thermoeffector pathways are similar to our immunohistochemistry results observed after the central CCK administration (Figure 4). Furthermore, the c-Fos expression changes observed in our study were completely blocked by inhibition of COX, thereby supporting the contribution of the COX-2-PGE_2 pathway to CCK-induced hyperthermia.

Last, we showed that the icv administration of the selective CCK_2 receptor antagonist YM022 attenuated endotoxin-induced fever, which is in harmony with previous results obtained with a different CCK_2 antagonist (Szekely et al. 1994) and with mice genetically lacking the CCK_2 receptor (Weiland, Voudouris, and Kent 2007). These findings provide further evidence for the interaction between CCK signaling and the COX-2 pathway. Earlier it was also found that the inhibition of CCK_2 receptors did not attenuate icv PGE-induced hyperthermia (Szelenyi et al.

1994), which indicates that CCK most likely modulates the production of PGE and not its effect on the receptor. In the present study, the CCK_2 receptor blocker suppressed the late (maintenance) phase of LPS-induced fever. Since the later phases of fever are mediated by PGE_2 produced mainly in the preoptic hypothalamus (Garami et al. 2018), our results suggest that a CCK_2 receptor-mediated effect on cells in this region contributes to the development of fever.

Upon inflammatory stimulation, PGE_2 can be produced by different cell types in the brain, including endothelial cells (Cao et al. 1996, Matsumura et al. 1998), perivascular macrophages (Elmquist et al. 1997, Schiltz and Sawchenko 2002) and microglia (Elmquist et al. 1997, Zhang et al. 2009), astrocytes (Pistritto et al. 1999, Molina-Holgado et al. 2000), and hypothalamic neurons (Lacroix and Rivest 1998). Amongst these cell types, the CCK_2 receptor is abundantly expressed in astrocytes (Hösli and Hösli 1994, Müller, Heinemann, and Berlin 1997, Crosby et al. 2018), and its stimulation with CCK-8 leads to the release of arachidonic acid due to diacylglycerol lipase and phospholipase A_2 activation (Noble and Roques 1999, Pommier et al. 2003), which biosynthetic pathways are also involved in the COX-2-mediated synthesis of PGE_2 during the febrile response to systemic inflammation (Garami et al. 2018). Based on these findings, it can be assumed that the COX-2-PGE_2 pathway functions as a downstream mediator of CCK_2 receptor activation in astrocytes, which cells may be the link between CCK signaling and the COX reaction. In line with that assumption, COX-2 was suggested as a downstream player of the CCK_2 receptor activation in epithelial, fibroblast, and adenocarcinoma cell lines (Zeng et al. 2020). Alternative possibilities for the interaction between the two systems cannot be excluded, for example, via a direct neuronal mediation, since a CCK_2 receptor-mediated

PGE_2 release was detected in the cerebrospinal fluid after microinjection of CCK-8 into the rostral ventromedial medulla (Marshall et al. 2012), which structure contains sympathetic premotor neurons for autonomic thermoeffectors (Morrison and Nakamura 2019).

Finally, it should be also mentioned that in the present study metamizol did not influence CCK-induced satiety and neuronal activation in the VMH. We observed reduced fasting-induced food intake after central administration of CCK, which is in accordance with earlier findings (Willis, Hansky, and Smith 1984, Shiraishi 1990). In the VMH, which is a feeding-related brain region expressing CCK_2 receptors (Noble et al. 1999), CCK caused an increase in c-Fos expression, which is in line with previous results about increased neuronal activity in the VMH after icv CCK administration in vivo (Shiraishi 1990) or direct CCK application in vitro (Kow and Pfaff 1986). Importantly, however, the inhibition of COX enzymes did not influence either of these effects. These results indicate that in contrast with the hyperthermic effect, the satiety effect of CCK is independent of the COX pathway. The different dependence of the thermal and the feeding effects on COX distinguishes the CCK-induced responses from fever and anorexia associated with endotoxin-induced systemic inflammation, in which both fever and anorexia are dependent on COX-2, even though they are triggered by distinct cell types (Nilsson et al. 2017).

In summary, we showed the dependence of central CCK-induced hyperthermia on the COX-2 pathway, and that central CCK_2 receptors are involved in the maintenance of fever. These findings advance our understanding of the interactions between CCK signaling and the COX pathways in the brain, and as a perspective may identify the CCK_2 receptor as a target in the management of fever response.

In the second part of our work, we moved from endogenous to exogenous hyperthermia-inducing substances and examined the effects of menthol application during physical exercise with a meta-analysis (Keringer et al. 2020). This was important because via its hyperthermic effect menthol may increase the risk of exertional heat stroke in athletes. We showed that the application of menthol improves TS, TC, and power output during physical exercise. Our results about thermal perception are in harmony with the findings of a previous meta-analysis (Jeffries and Waldron 2019), which also showed beneficial effects of menthol on TS in exercise performance; however, in that study the thermophysiological effects of menthol were not analyzed and influencing factors (e.g., acclimation, T_a) of menthol's effect were not investigated. In our study, we aimed at filling those gaps by studying the effects of menthol also on thermophysiological parameters, i.e., sweat production, heart rate, and deep T_b, and by identifying different phenotypes and environmental factors which can augment or attenuate menthol's effects. We showed that the use of menthol did not lead to compromised warmth-defense responses during physical exercise, since it does not affect sweat production, heart rate, and deep T_b. We also identified bodily (*viz.*, higher BMI), methodological (i.e., external menthol administration), and environmental factors, such air movement (fan use) and higher T_a, which enhance the beneficial effects of menthol on performance time.

Thermoregulatory changes, particularly in TS and TC, during physical exercise are of high importance, as they are considered among the limiting factors of endurance performance, and, as such, play a role in the development of fatigue (Marcora 2008, Schlader, Stannard, and Mündel 2011). The active muscle generates heat during physical exercise, thereby constituting an internal heat stress for the body, which is further augmented when physical activity is

performed in the heat (Szekely, Carletto, and Garami 2015). The heat load leads to worsening of TS and TC (Schlader et al. 2011), while behavioral and autonomic warmth-defense mechanisms are recruited to prevent an excessive increase in deep T_b (Szekely et al. 2015). When the defense mechanisms are compromised or exhausted, bodily homeostasis cannot be maintained, and heat-related illnesses, such as exertional heat stroke in the most severe forms, develop (Schlader et al. 2011). Efforts should be made to prevent the simultaneous presence of severe external and internal heat load to the individuals. There are, however, certain scenarios, when prevention of these conditions is not possible. The most obvious examples include the strenuous physical activity of firefighters, soldiers, and professional athletes in hot environments.

As part of the global climate change, the incidence of heat waves has increased in different countries, including, for example, the UK (Chapman, Watkins, and Stainforth 2019), France (Planton et al. 2008), the US (Habeeb, Vargo, and Stone 2015), Australia (Perkins-Kirkpatrick et al. 2016), and Japan (Kissling, Akerman, and Cotter 2019). These countries can be actual or potential hosts of upcoming worldwide, summertime sport events, e.g., Summer Olympic Games, thus pre-cautions should be implemented in order to prevent heat-related illnesses of the athletes during the games. In addition to physical methods of cooling, menthol may be also used as a pharmacological cooling intervention prior to and during exercise in hot conditions (Kissling et al. 2019). The improved TC in response to menthol can increase the thermal tolerance in athletes (Kissling et al. 2019), which can lead to better performance. It should be noted, however, that menthol may not be safely used to improve TC in athletes competing at

cold environments (e.g., at T_a below 20°C). Our analysis, to our knowledge for the first time, also showed that the application of menthol did not result in compromised warmth defenses.

Menthol has been identified earlier as a cold-mimicking substance, and its beneficial effects on sport performance have been also reported in a recent review (Jeffries and Waldron 2019). It is also known that menthol evokes its thermoregulatory effects through the TRPM8 channel, which, at least in rodents, serves as a universal cold receptor for the body (Romanovsky 2018). The activation of TRPM8 (e.g., by cold or menthol) leads to the recruitment of cold-defense responses, which aim at elevating (but at least preventing the drop in) deep T_b (Almeida et al. 2012). These thermophysiological effects of menthol, which were mostly discovered in animal experiments, imply a risk of menthol application in humans during physical exercise, since an adverse thermoregulatory effect, *viz.*, an overt increase in deep T_b, can not be ruled out. However, in humans thermal signals from the skin are less important for autonomic thermoregulation because the greater thermal inertia makes transient thermal exposures less threatening, thus decreases the importance of signals from the skin (Romanovsky 2014). Hence, activating peripheral cold receptors, such as TRPM8, with menthol in humans can have smaller effects on T_b than in rodents. It should be also noted that signals used for behavioral thermoregulation, which can be triggered through altered TS or TC, can differ from signals for autonomic thermoregulation (Flouris 2011). For example, antagonists of the TRP vanilloid-1 channel readily affect autonomic thermoeffectors in rats (Garami et al. 2018), but fail to affect the behavioral thermoeffectors in the same species, at least as concluded from one study (Steiner et al. 2007). Moreover, the mode of action for the thermal effect of TRP vanilloid-1 channel antagonists differs between rodents and humans (Garami et al. 2020). Therefore,

activation of peripheral thermosensation with menthol in humans can have smaller effects on deep T_b than in rodents.

In our study, we collected the available information about the thermoregulation homeostasis in menthol-treated athletes performing exercise, and conducted meta-analysis of the obtained data. We showed that at the used doses, menthol exerted beneficial effects on endurance performance, but it had no significant effect on any of the thermoregulation-related parameters, which included sweating production, heart rate, and deep T_b. It should be noted that sweat rate could be also an important indicator of thermoregulatory warmth defense. We found only three studies (Mundel and Jones 2010, Schlader et al. 2011, Barwood et al. 2019), which reported sweat rate, but in all of them only the averaged sweat rate was reported for the treatment groups. In two studies (Mundel and Jones 2010, Schlader et al. 2011), there was no significant difference in sweat rate between menthol-treated and control groups, whereas in the third study the average sweat rate was significantly reduced after menthol treatment (Barwood et al. 2019). However, sweat rate is not steady, but rather a dynamic parameter during exercise. It was shown that during exercise sweating rate increased abruptly for 8 min after the onset of sweating and then continued increasing at a much lower rate (Kondo et al. 2001), therefore the average sweat rate for the entire duration of the exercise should be interpreted with caution. As an alternative, we compared the exercise durations between the menthol-treated and control groups of the studies that reported sweat production and found that the difference between the treatment groups was less than 3 min in all studies (Gillis et al. 2010, Barwood et al. 2012, Barwood et al. 2014, Barwood et al. 2015, Barwood et al. 2019). We believe that such minimal difference in exercise duration between treatment groups of the same study did not have a significant

influence on sweat production. Our results suggest that with regards to thermoregulation homeostasis, menthol can be safely applied during physical exercise in humans. Nevertheless, it is also possible that the administered doses of menthol and the treated surface area were not sufficient in the most of the analyzed studies to trigger cold-defense responses, thereby leading to a change in thermophysiological parameters, including deep T_b. Furthermore, we pointed out different influencing factors, which can help to augment the performance-improving effects of menthol. Among environmental factors, we found that the use of a fan (i.e., wind effect) and higher T_a increased the efficacy of menthol on endurance performance. The beneficial effects of menthol were more pronounced in subjects with higher BMI, while acclimation to heat did not influence the effects.

Some limitations of our meta-analysis should be also mentioned. There were inter-study differences in the design of the analyzed studies regarding, for example, the sample population, the menthol administration route and dose, the exercise protocol, and the measurement of the outcome parameters. For example, the assessment of power output differed in the three analyzed studies (Barwood et al. 2012, Barwood et al. 2015, Rinaldi et al. 2018). The study with the biggest effect size showed a significant improvement in power output (Rinaldi et al. 2018), whereas power output did not differ statistically between the menthol-treated and control groups in either of the studies with smaller effect size (Bright et al. 2019, Gibson, Wrightson, and Hayes 2019). Based on the risk of bias assessment, we found that blinding was not feasible in many studies, because of the characteristic odor of menthol. Furthermore, the allocation concealment was not indicated in some articles (Gillis et al. 2010, Kounalakis et al. 2010, Mundel and Jones 2010, Schlader et al. 2011, Riera et al. 2014, Shepherd and Peart 2017,

Rinaldi et al. 2018), which could have also influenced the effectiveness of menthol application. These methodological and medical differences in study design can explain the considerably high between-study heterogeneity (indicated by an I^2 of more than 50%), as observed in our analysis. To account for the presence of heterogeneity, we used the random effects model in all forest plots of our meta-analyses. However, it is still possible that, despite all of our approaches to reduce methodological errors, the high heterogeneity of the analyzed studies might have negatively impacted our results.

As a summary of the second part of our work, our findings suggest that menthol can be safely used during physical exercise to improve thermal perception. Due to its beneficial effects on TS and TC, it can be used as an alternative to mitigate the impact of heat exposure on the individuals. External application of menthol in a warmer environment with air movement is more efficient, especially in subjects with higher BMI than 23. The validation of our results in targeted human trials is subject for future research.

7. Conclusions

In our work, we studied hyperthermia from two different aspects: 1) we investigated the effect of CCK as an endogenous hyperthermic substance and modulator of fever in an experimental model, and 2) we assessed the risk of the hyperthermic effect of menthol during physical performance in human subjects with a meta-analysis. It is well established that increased deep T_b can be a useful tool to fight infections, but in certain cases it may be harmful for the host, e.g., in severe systemic inflammation (like septic shock) or in heat stroke caused by excessive heat load. In such cases the extremely high temperature can lead to irreversible brain damage, thus it is important to know the regulatory factors, mediators, and modulators of elevated deep T_b. As conclusion of our work, we found that COX-2 mediated the hyperthermic response to CCK, which also plays an important role in the maintenance of fever, thereby it may serve as a therapeutic target in treatment of systemic inflammation (Keringer et al. 2022). Furthermore, we assessed whether the application of menthol during physical exercise increases the risk of exertional heat stroke and we found that menthol does not influence the thermophysiological parameters (including deep T_b) in humans, therefore it can be safely applied to improve thermal tolerance and sport performance (Keringer et al. 2020).

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10. Publications and presentations

Sum of all impact factors: 42.868

Sum of impact factors from publications related to the topic of PhD thesis: 8.689

All citations: 16

Independent citations: 10

Publications related to the topic of the PhD thesis:

Keringer P, Furedi N, Gaszner B, Miko A, Pakai E, Fekete K, Olah E, Kelava L, Romanovsky AA, Rumbus Z, Garami A: The hyperthermic effect of central cholecystokinin is mediated by the cyclooxygenase-2 pathway. American Journal of Physiology-Endocrinology and Metabolism. 2022; 322:E10-E23. **(IF 4.31 [2020])**

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Patrik Keringer, András Garami: Plants under pressure: Do not step on the grass! Summer School on Stress: From Hans Selye's original concept to recent advances. St. Petersburg, Russia, June 25-28, 2019.

Patrik Keringer, Eszter Pakai, Valeria Tekus, Csaba Zsiboras, Zoltan Rumbus, Emoke Olah, Nora Khidhir, Robert Matics, Laszlo Deres, Katalin Ordog, Nikolett Szentes, Krisztina Pohoczky, Agnes Kemeny, Peter Hegyi, Erika Pinter, Andras Garami: The role of neurokinin signalling receptor in the development of endotoxin fever. 16th Annual Conference of the Hungarian Neuroscience Society, Debrecen, January 17-18, 2019.

P. Keringer, Zoltan Rumbus, Alexandra Miko, Alexandra Csenkey, Petra Gaspar, Eszter Pakai, Emoke Olah, Nora Khidhir, Nora Furedi, Zoltan Horvath-Szalai, Csaba Zsiboras, Margit Solymar, Eva Polyak, Balazs Gaszner, Andras Garami: Acute effects of saccharin on the energetic homeostasis in rodents. PPTR 7th International Conference on the Physiology and Pharmacology of Temperature Regulation, Split, October 7-12, 2018

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Kéringer P.: Different ways of body composition measurement in rats. 5th Central European Congress on Obesity, November 1-3, 2015

National oral and poster presentations:

Kéringer Patrik, Erdélyi Attila, Oláh Emőke, Tékus Valéria, Solymár Margit, Pákai Eszter, Rumbus Zoltán, Kemény Ágnes, Gaszner Balázs, Pintér Erika, Garami András: A tranziens receptor potenciál ankyrin-1 szerepe hidrogén-szulfid által indukált hipotermiában. Magyar Kísérletes és Klinikai Farmakológiai Társaság, Magyar Anatómus Társaság, Magyar Mikrocirkulációs és Vaszkuláris Biológiai Társaság, Magyar Élettani Társaság Közös Vándorgyűlése – FAMÉ 2019, Budapest, 2019. június 5-8.

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The interaction between neurokinin-1 receptors and cyclooxygenase-2 in fever genesis

Patrik Keringer & Zoltan Rumbus

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FRONT MATTER: DISCOVERY

The interaction between neurokinin-1 receptors and cyclooxygenase-2 in fever genesis

Comment on: Pakai E, Tekus V, Zsiboras Cs, Rumbus Z, Olah E, Keringer P, Khidhir N, Matics R, Deres L, Ordog K, Szentes N, Pohoczky K, Kemeny A, Hegyi P, Pinter E, Garami A. The neurokinin-1 receptor contributes to the early phase of lipopolysaccharide-induced fever via stimulation of peripheral cyclooxygenase-2 protein expression in mice. Frontiers in Immunology. 2018;9:166. doi: 10.3389/fimmu.2018.00166.

Fever is a common thermoregulatory manifestation in systemic inflammation, which can be triggered by different stimuli, such as infection, head trauma, and neurological disorders. In experimental animals, fever is often induced by administration of bacterial lipopolysaccharide (LPS), and it is associated with the release of a wide range of proinflammatory mediators, including also substance P (SP). The role of SP signaling was sought for long in inflammatory processes, yet its involvement in the induction of systemic inflammation-associated fever has remained unclarified. In our recent study [1], we aimed at identifying the receptorial and molecular mechanisms of the SP-neurokinin (NK)-1 receptor system that are involved in the development of LPS fever.

We studied the fever response to LPS in mice with the *Tacr1* gene, i.e. the gene encoding the NK1 receptor, homozygously present ($Tacr1^{+/+}$) or absent ($Tacr1^{-/-}$). In the absence of the NK1 receptor, LPS-induced fever was attenuated starting from the early phase (from ~40 min). On the contrary, we found no difference in the febrigenic effect of prostaglandin (PG) E_2 administered into the brain between $Tacr1^{-/-}$ and $Tacr1^{+/+}$ mice. At 40 minutes after LPS administration, serum concentrations of pyrogenic cytokines and COX-2 mRNA expression in the lungs, liver, and brain did not differ between the genotypes, whereas COX-2 protein expression was lower in the lungs and, to a lesser extent, in the liver of $Tacr1^{-/-}$ mice than in their $Tacr1^{+/+}$ littermates [1]. These results show that the NK1 receptor is involved in fever genesis through the facilitation of COX-2 protein expression in peripheral LPS-processing organs such as the lungs and the liver (Figure 1).

When a microorganism crosses the physical barriers (e.g. in the skin, or in the gastrointestinal or respiratory tract) of the host, the innate immune response is initiated. LPS is recognized by the Toll-like receptor 4 (TLR4), which is expressed in immune cells, including monocytes and macrophages. TLR4 activation results in the translocation of NF-κB into the nucleus, which mounts the gene expression and synthesis of various inflammatory mediators, including pyrogenic cytokines, such as interleukin-1, -6, and tumor necrosis factor-α. Besides cytokine production, the arachidonic acid (AA) cascade is also activated in leukocytes, thereby yielding considerable amounts of lipid-derived mediators. Of these, PGE_2, the end-product of the COX-2-microsomal PGE_2 synthase-1 (mPGES-1) pathway, is the key mediator of fever (Figure 1) [2].

The initial phase of fever depends mainly on PGE_2 produced in peripheral organs, likely in lung and liver macrophages, while the later phases of fever are mediated by PGE_2 synthetized within the blood-brain barrier. In the central nervous system, PGE_2 interacts with EP3-expressing neurons in the median preoptic nucleus (MnPO) of the preoptic area (POA) of the hypothalamus, thereby, triggering the activation of autonomic cold defense thermoeffectors (e.g. skin vasoconstriction and nonshivering thermogenesis), and leading to the induction of fever (Figure 1) [2].

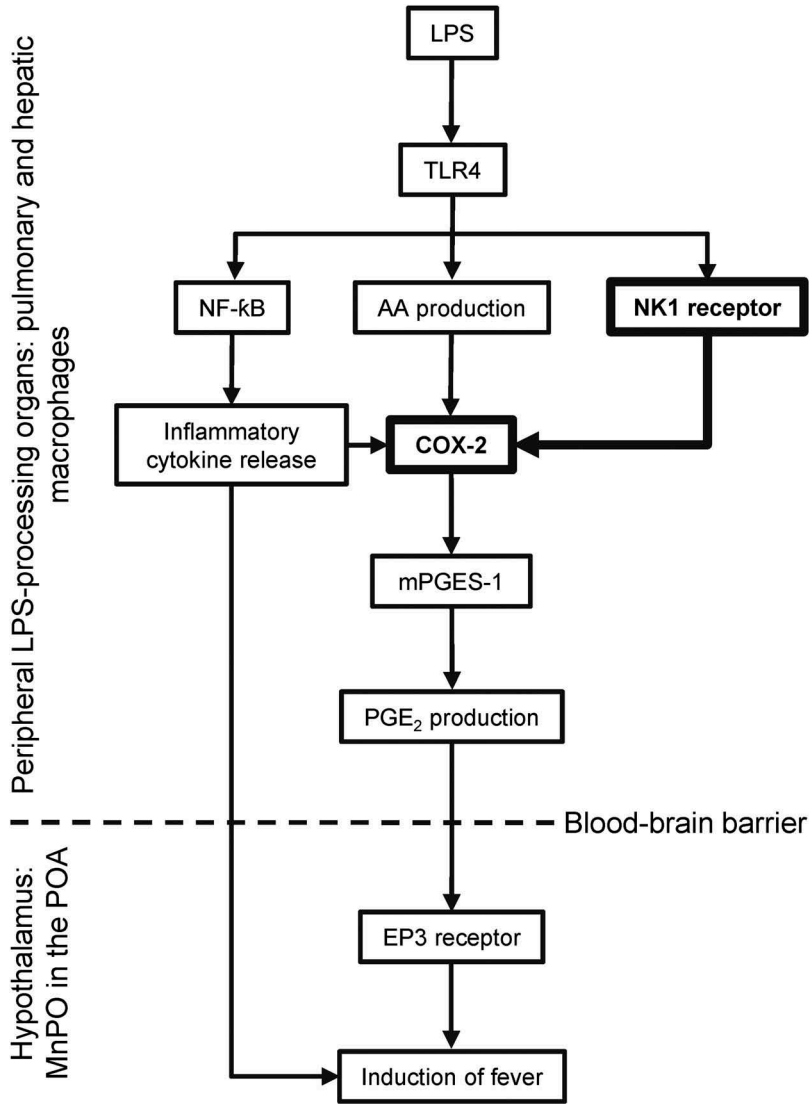

Figure 1. Simplified schematic of the mechanisms in endotoxin fever, highlighting the interaction between SP signaling and the cytokine-COX-2-PGE_2 axis based on the study in focus [1] (see text for explanation). AA: arachidonic acid; COX-2: cyclooxygenase-2; EP3: prostaglandin EP3 receptor; LPS: lipopolysaccharide; MnPO: median preoptic nuclues; mPGES-1: microsomal PGE_2 synthase-1; NK1: neurokinin-1; PGE_2: prostaglandin E_2; POA: preoptic area; TLR4: Toll-like receptor 4.

The potential roles of SP and its receptors cover a wide range in systemic inflammation and in its common clinical manifestation, sepsis. NK1 receptors are expressed in leukocytes, in different peripheral tissues (e.g. vessels, intestines, lungs, liver), and in the brain [3]. Activation of immune cells, release of inflammatory mediators, vasodilation and edema formation, regulation of apoptosis and bacterial translocation in the gut, as well as, a possible role in lung and liver injury can be all associated with SP signaling in sepsis [3]. As discussed in the highlighted study [1], NK1 receptors are expressed in macrophages and granulocytes, and the SP-NK1 receptor pathway can trigger the activation and trafficking of these cells, as well as, the upregulation of COX-2 and the production of proinflammatory mediators. It is suggested that SP-induced activation of NK1 receptors on immune cells exacerbates systemic inflammation via recruitment of leukocytes and induction of inflammatory mediator expression [1,3]. Indeed, in septic and aseptic (LPS) animal models of systemic inflammation, the levels of SP were elevated in the lungs and the liver [3], i.e. in the organs which harbor the peripheral macrophages that trigger fever [2]. The study in focus [1] suggests that the activation of NK1 receptors in these hepatic and pulmonary macrophages contributes to the augmentation of COX-2 protein expression, thereby, resulting

in increased PGE_2 production, and in the initiation of the fever response [1] (Figure 1). Whether the activity of other enzymes downstream or upstream COX-2 (e.g. phospholipase A_2, monoacylglycerol lipase, and mPGES-1) is also affected by the SP-NK1 receptor pathway remains subject for further research.

The importance of the changes in body temperature, viz., fever and hypothermia, in systemic inflammation is well established [2]. Fever is associated with decreased, whereas hypothermia with increased rate of death in sepsis [2,4], though it has to be noted that body temperature per se does not serve as the cause of the outcome, but instead, it is an indicator of the actual coping strategy of the host, and, as such, it gives information about the severity of the disease [4]. Similar to body temperature, an association between SP levels and mortality was also observed in septic patients [5]. The risk of death was lower in patients who had persistently higher levels of SP starting from the time of diagnosis of sepsis, whereas non-survivors had sustained low serum levels of SP [5]. Based on the results of the study in focus [1], the similarity between the predictive roles of SP and body temperature on the mortality rate in sepsis can be explained: SP signaling is involved in the development of fever, hence an elevated SP level also means higher PGE_2 production, and, therefore, a more pronounced fever response. Due to their causative relationship, it is evident that increased serum SP concentration and elevated body temperature both predict lower risk of death in sepsis, as previously known about each of them separately [4,5].

Due to the better understanding of the interaction between SP signaling and the cytokine-COX-2-PGE_2 axis, the findings of the highlighted study [1], in theory, also raise the possibility of the application of NK1 receptor antagonists as antipyretic drugs. Some NK1 receptor antagonists are already used in clinical practice as antiemetic drugs, however, their use in systemic inflammation to reduce fever is doubtful, as the anti-inflammatory efficacy of different NK1 antagonists could not be firmly confirmed in human studies. Moreover, even if the use of NK1 antagonists as antipyretics is confirmed in clinical trials, the question of when to use these drugs still remains, since their inappropriate application may counteract the beneficial impact of higher SP levels and elevated body temperature on the outcome of the disease in systemic inflammation.

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Patrik Keringer and Zoltan Rumbus
Institute for Translational Medicine, Medical School, University of Pecs, Pecs, Hungary
zoltan.rumbus@aok.pte.hu

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Menthol can be safely applied to improve thermal perception during physical exercise: a meta-analysis of randomized controlled trials

Patrik Keringer[1], Nelli Farkas[2,3], Noemi Gede[2], Peter Hegyi[2,4], Zoltan Rumbus[1], Zsolt Lohinai[5], Margit Solymar[1], Kasidid Ruksakiet[5,6], Gabor Varga[6] & Andras Garami[1]✉

Menthol is often used as a cold-mimicking substance to allegedly enhance performance during physical activity, however menthol-induced activation of cold-defence responses during exercise can intensify heat accumulation in the body. This meta-analysis aimed at studying the effects of menthol on thermal perception and thermophysiological homeostasis during exercise. PubMed, EMBASE, Cochrane Library, and Google Scholar databases were searched until May 2020. Menthol caused cooler thermal sensation by weighted mean difference (WMD) of − 1.65 (95% CI, − 2.96 to − 0.33) and tended to improve thermal comfort (WMD = 1.42; 95% CI, − 0.13 to 2.96) during physical exercise. However, there was no meaningful difference in sweat production (WMD = − 24.10 ml; 95% CI, − 139.59 to 91.39 ml), deep body temperature (WMD = 0.02 °C; 95% CI, − 0.11 to 0.15 °C), and heart rate (WMD = 2.67 bpm; 95% CI − 0.74 to 6.09 bpm) between the treatment groups. Menthol improved the performance time in certain subgroups, which are discussed. Our findings suggest that different factors, viz., external application, warmer environment, and higher body mass index can improve menthol's effects on endurance performance, however menthol does not compromise warmth-defence responses during exercise, thus it can be safely applied by athletes from the thermoregulation point of view.

Menthol (2-isopropyl-5-methylcyclohexanol) is a lipophilic, organic compound which can be extracted from essential oils of aromatic plants or produced synthetically[1,2]. The most common naturally occurring form of menthol is the L-isomer, which is used in various products, e.g., candies, beverages, cigarettes, and toothpastes, mainly because of its cooling, analgesic, and anti-inflammatory effects[2,3]. It has long been assumed that menthol might improve different aspects of physical performance such as endurance, speed, strength, and joint range of motion, consequently it is often used by athletes in the form of sprays, creams, tapes, beverages, etc.[4,5].

Warming-up before an exercise is often used to optimize muscle temperature and, thereby, maximal muscle power production, however, at high ambient temperatures (T_a), it increases the thermal and circulatory strain[6]. Endurance exercise capacity at a high T_a is impaired by heat stress prior to exercise[7], and hyperthermia induces fatigue during short intense activities and prolonged exercise in the heat[8]. On the contrary, physical cooling of the body before and during exercise in the warmth improves exercise endurance and reduces cardiovascular strain[9]. A recent meta-analysis of 45 studies also concluded that physical cooling improves aerobic and anaerobic exercise performance in hot conditions[10]. From animal experiments it is known that the transient receptor

[1]Department of Thermophysiology, Institute for Translational Medicine, Medical School, University of Pecs, 7624 Pecs, Hungary. [2]Institute for Translational Medicine, Szentagothai Research Centre, Medical School, University of Pecs, 7624 Pecs, Hungary. [3]Institute of Bioanalysis, Medical School, University of Pecs, 7624 Pecs, Hungary. [4]Department of Translational Medicine, First Department of Medicine, Medical School, University of Pecs, 7624 Pecs, Hungary. [5]Department of Conservative Dentistry, Faculty of Dentistry, Semmelweis University, 1088 Budapest, Hungary. [6]Department of Oral Biology, Faculty of Dentistry, Semmelweis University, 1089 Budapest, Hungary. ✉email: andras.garami@aok.pte.hu

potential (TRP) melastatin-8 (M8) channel, formerly called as menthol receptor, is a universal cold sensor in the thermoregulation system. The pharmacological modulation of TRPM8 with systemic (intravenous or intraperitoneal) administration of an antagonist changes the activity of the cold-activated neural pathway[11], which raises the possibility that activation of TRPM8 with ligand agonists like menthol can have similar effects to physical cooling before or during physical exercise. Indeed, in exercising humans menthol administration resulted in increased thermogenesis[12], decreased sweating[13,14], and more pronounced skin vasoconstriction[15,16], consequently in elevated deep body temperature (T_b)[17,18]; that is the same pattern of thermoregulatory effector recruitment which can be observed as part of the cold-defence responses[19]. Importantly, the menthol-induced decrease in heat loss and elevation in deep T_b can increase the risk for heat exhaustion and adverse cardiovascular events in the warmth[13,17], therefore, the safety of menthol application in physical exercise, especially at high T_a, remains questionable. In contrast with the aforementioned studies showing an increased risk for the onset of heat-related illnesses in association with menthol application, several human studies showed beneficial effects of menthol on physiological, psychological, and performance parameters during physical exercise[18,20–27], while a decent number of studies found no effect[28–31].

The observed discrepancies among the studies may originate from differences in study designs, application methods (route of administration, dosage, location of the administration, and the surface area), and experimental conditions (e.g., T_a). Menthol-containing products can be administered externally (e.g., in spray or gel form) or internally (e.g., mouth rinse, beverage consumption). External application has been shown to be more beneficial than the internal in sports physiology and on endurance performance[13,14,23,25], whereas other authors found that internally applied menthol is more effective[20–22,24,27], and yet others showed no effect of menthol independently from the application method[26,28–31].

In our meta-analysis, we analysed how menthol administration affects the changes in perceptual and physiological parameters of thermoregulation, and in indicators (viz., power output and performance time) of the overall endurance performance during physical exercise in healthy humans.

Methods

Our meta-analysis was conducted in accordance with the guidelines of the Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) protocols[32] (Supplementary Table S1). The analysis was based on the Participants, Intervention, Comparison, and Outcome model: in physically active, healthy participants, we investigated the effects of menthol application compared to controls (i.e., no menthol or placebo treatment) on physiological and perceptual parameters and on indicators of endurance performance during physical exercise. The protocol for this meta-analysis was registered on PROSPERO (registration number: CRD42019125034).

Search strategy. A search of the PubMed, EMBASE, and Cochrane Controlled Trials Registry databases was performed until May 2020 using the following search key: "(menthol OR mint OR peppermint OR mentha OR spearmint) AND (temperature OR "heart rate" OR "oxygen uptake" OR lactate OR "sweat rate" OR "physical performance" OR exhaustion)". We restricted our search to randomized controlled human trials published in English without time period limitations. A manual search of the reference lists of identified full-text articles was also performed in Google Scholar for eligible studies. The search was conducted separately by two authors (PK, AG), who also assessed study eligibility and extracted data from the selected studies independently. Disagreements were resolved, if needed, by a third party (ZR).

Study selection and data extraction. After screening on the titles and abstracts of the identified publications, the full texts of eligible articles were obtained. We included studies which reported at least one of the following values: thermal sensation (TS), thermal comfort (TC), T_b, sweat production, heart rate, performance time, and power output in menthol-treated and control healthy subjects before and during physical exercise. For all parameters, the maximal change from baseline after menthol treatment (and the corresponding value at the same time point in the control group) was extracted to assess the acute effect of menthol. In each study we calculated the difference between the menthol-treated and control groups, which was then included in the analyses. This approach allowed for taking into considerations differences in experimental protocols between studies. From all included articles, we extracted the group size, the reported mean values and standard deviations (SD) of the parameters of interest, and the level of statistical significance (p value). To analyse the effects of menthol under different conditions, we also divided the studies into subgroups, which were determined on the basis of known influencing factors[33,34], and data availability. The main influencing factors were grouped in three categories: characteristics of the subject (body mass index [BMI] and heat acclimation), study protocol (trial type and menthol administration method), and environmental circumstances (airflow and T_a).

Statistical analysis. In each study, we calculated the maximum change in the outcome parameter from baseline after menthol application and the change from baseline until the same time point in the control group. Then, we calculated the weighted mean difference (WMD) with 95% confidence interval (CI) in the change of the parameter between the menthol-treated and the control groups. The statistical analysis was performed according to the standard methods of meta-analysis by using a random effects model. The effects were considered significant when $p < 0.05$. Using both p value and CI allowed us to detect physiologically relevant differences between the groups even in the case of overlapping CIs[35].

To study perceptual responses, data on TS and TC were collected. By definition, TS identifies the relative intensity of the temperature being sensed, and, as such, provides the body with information about the thermal environment, while TC means subjective indifference with the thermal environment, so that thermal pleasure is perceived when a stimulus aims to restore TC (for a comprehensive review, see Flouris & Schlader[36]). In the

analysed studies, TS scales were used with ranges of 7-point[25], 9-point[22], and 20-point[14,29,30], while in case of TC, the authors used 4-point[25], 7-point[18,22,23], and 20-point ranges[14,29,30]. Since TS and TC were determined by different visual analogue scales in the studies, in order to make the reported TS and TC values comparable for our meta-analysis, while also minimizing the need for conversion of the originally reported data in the studies, if required, the reported values were extrapolated into a unified scale, ranging from 0 to 20. The scales were bilateral, i.e., 0 corresponded to neutral, in several studies. For example, TS was assessed with a 9-point scale ranging from very cold (− 4) to neutral (0) to very hot (4). Thus, after extrapolation of the endpoints of the original scales to 0 and 20, in case of TS the middle of the unified scale (10) represented the neutral situation and an increase in TS between 0 (very cold) and 20 (very hot) indicated a weaker cold or stronger warmth sensation. To assess TC, three types of scales were used in the analysed studies: 20-cm visual analogue scale (0: very uncomfortable, 20: very comfortable)[14,29,30], 7-point scale (− 3: much too cool, 0: comfortable, 3: much too warm)[18,22,23], and 4-point scale (1: comfortable, 4: very uncomfortable)[25]. In the studies using 7-point scale, we did not find any value smaller than 0, thus we considered the scale from 0 (comfortable) to 3 (uncomfortable) and these endpoints were extrapolated to 20 and 0, respectively. In the 4-point scale, the 1 (comfortable) and 4 (uncomfortable) endpoints were extrapolated to 20 and 0, respectively. As result, the unified 20-point scale, ranging from very uncomfortable to very comfortable, resembled the one used in the pioneer study by Gagge et al.[37]. In this scale, a higher TC value between 0 (very uncomfortable) and 20 (very comfortable) corresponded to more pleasant comfort feeling.

With regards to performance time, it must be noted that depending on the exercise protocol its decrease and increase can both indicate an improved endurance performance. In time-to-exhaustion (TTE) protocols, a longer performance time indicates improved endurance as the subjects are able to perform the exercise for a longer time period, whereas in time-trial (TT) protocols the subjects aim at finishing a predefined exercise task as fast as they can, thus longer performance time indicates reduced endurance in these tasks. Therefore, in our analyses, we always separated the TTE and TT protocols in different groups when the investigated outcome was performance time, similarly as in previous studies[33,38].

Inter-study heterogeneity was tested with the Q homogeneity test and with the I^2 statistical test, where I^2 is the proportion of total variation attributable to between-study variability (an I^2 value of more than 50% was considered as an indication of considerable heterogeneity). Publication bias was assessed by Egger's test and visual inspection of funnel plots (Supplementary Figs. S7-S10). To evaluate the quality of the included trials, two independent reviewers (PK and ZR) assessed the risk of bias according to the Cochrane Handbook[39]. The methodology described for random sequence generation, allocation concealment, blinding of participants and personnel, blinding of outcome assessment, completeness of outcome data, and selective outcome reporting was assessed (Supplementary Table S2), similarly as in our recent study[40].

All analyses were performed using the Comprehensive Meta-Analysis software (version 3.3; Biostat, Inc., Engelwood, NJ).

Results

Study selection and characteristics. The flowchart of study selection is presented in Fig. 1. Until May 2020, a total of 2,448 records were retrieved from the PubMed (n = 863), EMBASE (n = 1,437), and Cochrane (n = 137) databases and 11 records from other sources (e.g., Google Scholar). After removing duplicates and enabling filters for human studies, randomized controlled trials, and English language, 94 articles remained. By screening on title and abstract, further 72 records were excluded from the analysis because (1) the required outcome parameters were not reported, (2) menthol-treated or control group was absent, (3) not only healthy participants were recruited, and (4) no original data was reported. The full texts of 22 articles were reviewed in detail, from which 17 papers provided eligible data for qualitative and quantitative analyses[13,14,17,18,20–31,41]. All included studies had randomized, crossover design and included data from a total of 177 athletes. The participant characteristics in the studies are presented in Supplementary Table S3. The majority of the studies was conducted in males, except for an article which included participants of both sexes[31] and possibly another one which did not report the sex ratio in the sample[26]. In 12 trials, the participants were refrained from strenuous exercise, alcohol and caffeine intake before the experiments[13,14,20–23,25,27–30,41], two studies included unspecified training limitations[18,24], and three articles did not report any limitations[17,26,31].

The mean exercise duration was 30 min in TT tests, ranging from 1 min[26] to 71 min[28], while in TTE protocols, it ranged between 1 min[41] and 61 min[20], with an average of 27 min. In two studies the subjects exercised for a fixed time duration of 45 min[17] or 2 × 20 min[18], while the exercise duration was calculated from distance and speed in one of the studies[26]. In all studies we considered the beginning of the exercise as the baseline, which was before the menthol treatment, except for two studies, in which menthol administration was before the start of the trial[23,25]. It should be noted that the TTE test was preceded by 45-min exercise without interruption in the study by Barwood et al.[14], during which the subjects were repeatedly treated with menthol, thus we considered the beginning of the 45-min preliminary fatiguing task as the baseline for TS, TC, and deep T_b. The TTE test was also preceded by physical exertion and repeated menthol administration in the study by Saldaris et al.[41], but in that study the 3 × 30 min trials were interrupted by breaks and resting between the trials, as well as, before the TTE test. Therefore, we considered the beginning of the TTE test as the baseline in that study.

The used doses of menthol varied due to differences in administration method (i.e., internal or external), concentration (0.01–8%), volume (25–500 ml), and surface of the treated body area (4–91%). The most commonly used concentrations and volumes were, respectively, 0.01% and 25 ml in internal, while 0.2% and 100 ml in external administration routes. The details of the used administration routes, doses, and experimental procedures are summarised in Supplementary Table S4.

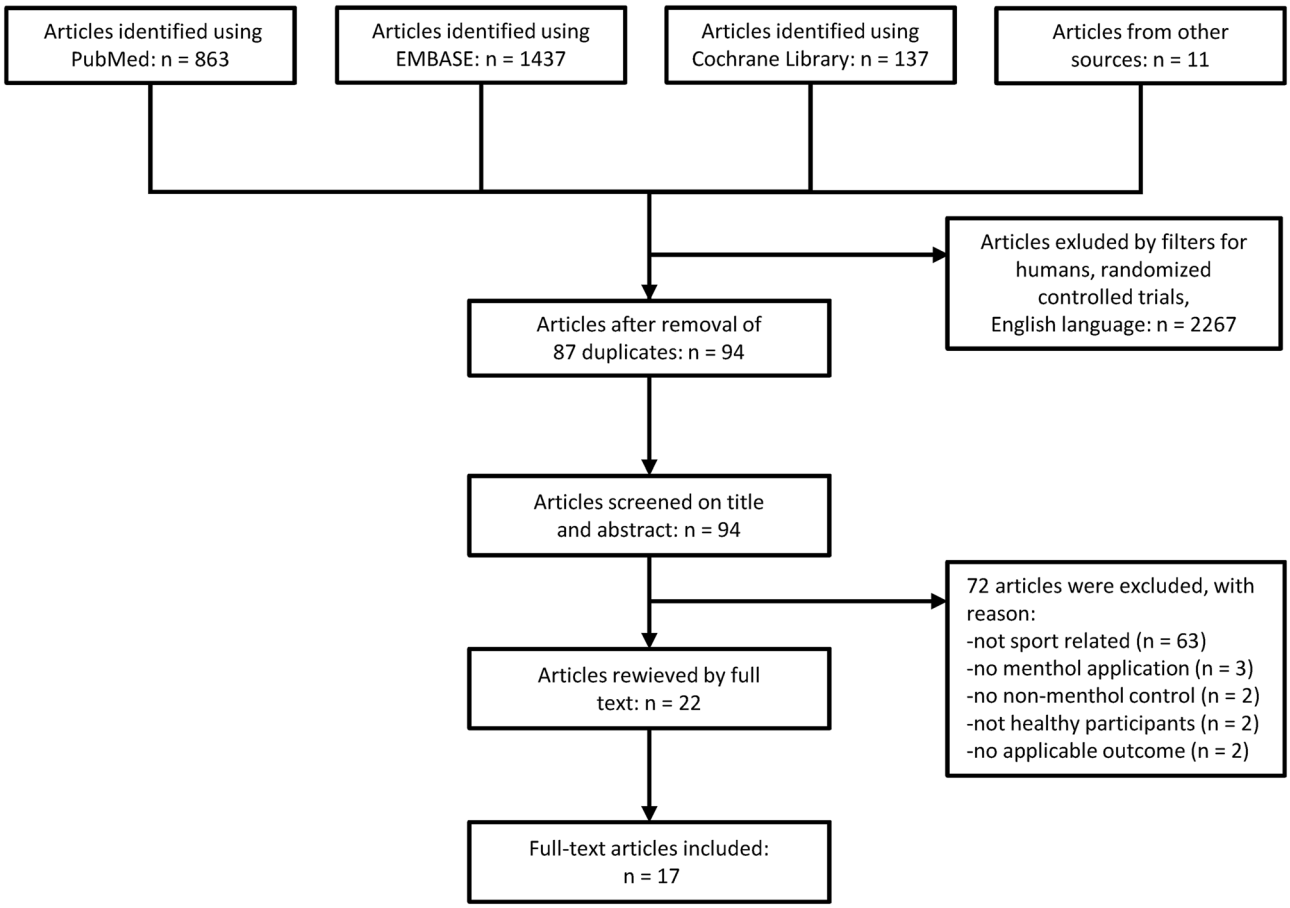


Figure 1. Flowchart of study selection and inclusion.

Perceptual responses. First, we studied how menthol application influences perceptual responses, viz., TS and TC during exercise. As it could be expected based on to the cold-mimicking effect of menthol-containing products[1], the TS score decreased in the menthol-treated groups as compared to controls in seven studies[14,21,22,25,29,30,41], while two studies reported a slight increase in TS[23,25]. Accordingly, the overall WMD between the menthol-treated and control groups was − 1.65 (95% CI, − 2.96 to − 0.33; p = 0.014) (Fig. 2). The TC score decreased during physical exercise compared to baseline in all groups, but the magnitude of the decrease tended to be smaller in the menthol-treated group than in controls by a WMD of 1.42 (95% CI, − 0.13 to 2.96; p = 0.073) (Fig. 3), which indicates that the perceived temperature was more comfortable (i.e., not so hot) after menthol administration compared to controls. In subgroup analysis, we found that the absence of airflow further decreased the TS in the menthol-treated group (WMD = − 2.86; 95% CI, − 4.51 to − 1.22), whereas the menthol-induced drop was not significant when the fan was used (WMD = − 1.12; 95% CI, − 3.10 to 0.86) (Supplementary Fig. S1). The TS-decreasing effect of menthol differed significantly (p < 0.001) between the two subgroups. We also analysed whether the menthol-induced improvements in TS and TC are associated with an increased power output (an indicator of exercise intensity) during physical exercise and found that the power output remained higher in the menthol-treated groups compared to controls in all of the individual studies[18,28,30], and accordingly, their overall average was also higher by a WMD of 31.52 W (95% CI, 22.52 to 40.53 W) (Fig. 4).

Thermophysiological responses. We could extract sufficient data for the analysis of three thermoregulatory parameters: sweat production (an indicator of the activity of autonomic heat-dissipating mechanisms), heart rate (a nonspecific indicator of metabolic rate), and deep T_b (i.e., the tightly controlled parameter in thermoregulation). We found that the volume of sweat production did not differ significantly between the menthol-treated and control groups during exercise (WMD = − 24.10 ml; 95% CI, − 139.59 to 91.39 ml) (Fig. 5). Similar to sweat production menthol also did not have a meaningful effect on the exercise-induced increase in deep T_b compared to the control group (WMD = 0.02 °C; 95% CI, − 0.11 to 0.15 °C) (Fig. 6). Furthermore, there was no significant difference in exercise-induced elevation of heart rate between the treatment groups (WMD = 2.67 bpm; 95% CI − 0.74 to 6.09 bpm) (Fig. 7).

Performance time. Overall, the performance time did not differ statistically between menthol-treated and control groups in TT protocols (WMD = − 0.52 min; 95% CI, − 1.37 to 0.34 min) (Supplementary Fig. S2a) and TTE tests (WMD = 1.04 min; 95% CI, − 0.47 to 2.55 min) (Supplementary Fig. S2b). In the TT protocols, no meaningful difference was observed in the effect of menthol between subgroups of higher (above 23.5) BMI and

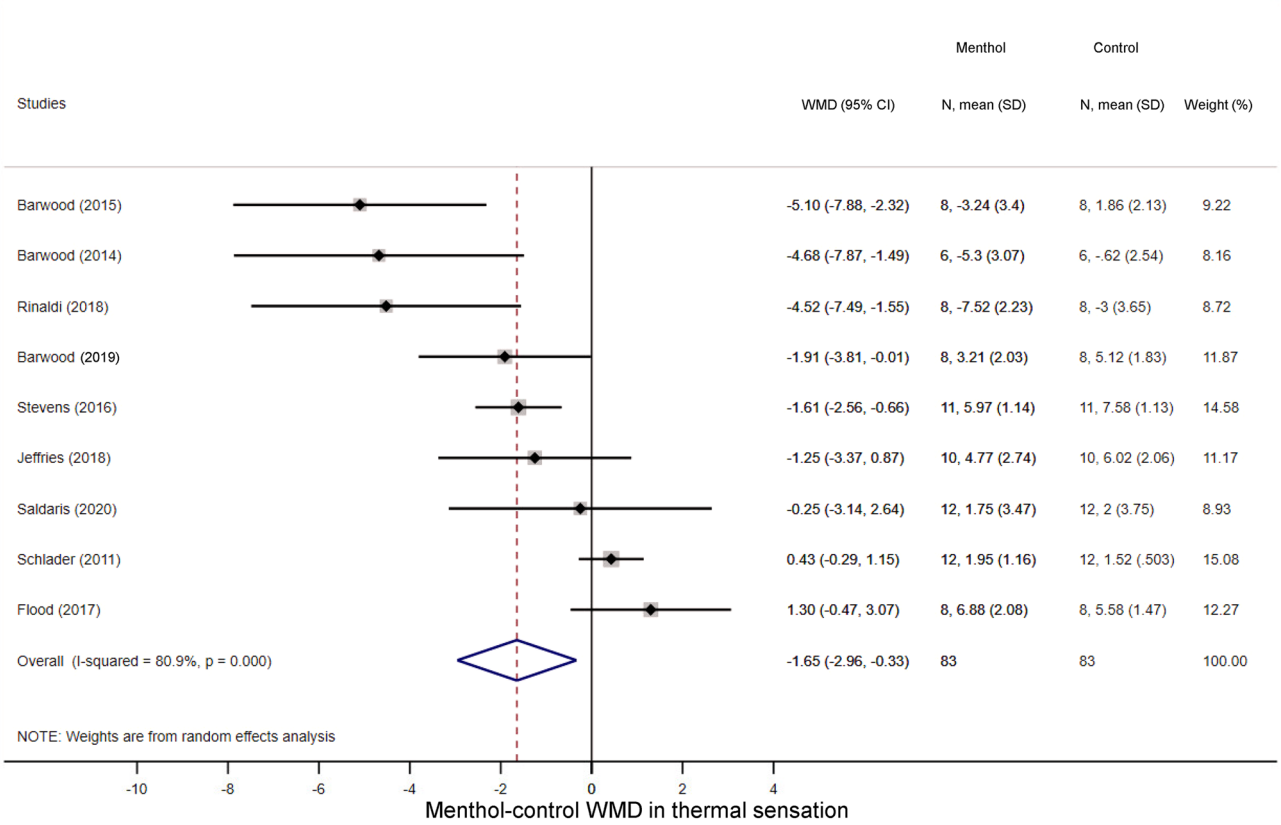


Figure 2. Forest plot of the weighted mean differences (WMDs) showing the effect of menthol on thermal sensation during exercise. Here, and in Figs. 3, 4, 5, 6, 7, and 8, black circles represent the WMD for each study, while the left and right horizontal arms of the circles indicate the corresponding 95% confidence intervals (CI) for the WMD. The size of the grey box is proportional to the sample size; bigger box represents larger sample size, thus bigger relative weight of the study. The diamond represents the average WMD calculated from the WMDs of the individual studies. The left and right vertices of the diamond represent the 95% CI of the average WMD. The vertical dashed line is determined by the low and top vertices of the bottom diamond and indicates the value of the average WMD of all studies in the forest plot. A WMD lesser than 0 indicates that the thermal sensation value (intensity of cold sensation) is higher in menthol-treated group, whereas a WMD higher than 0 indicates that thermal sensation is higher in control group.

lower (21.4–23.5) BMI (Fig. 8a). However, in the TTE tests, among athletes with higher BMI, performance time increased significantly in the menthol-treated group compared to controls (WMD = 2.57 min; 95% CI 1.76 to 3.39 min), whereas menthol tended to decrease performance time in the lower BMI group (WMD = − 3.20 min; 95% CI − 8.81 to 2.42 min) (Fig. 8b). The WMD between the treatment groups was markedly bigger in the higher than in the lower BMI subgroup ($p < 0.001$). We also analysed whether acclimation of the subjects to exercising in warmth influences the effects of menthol. In TT protocols, we did not find meaningful difference in menthol's effect on performance time between non-acclimated and acclimated participants (WMD = 0.25 min; 95% CI − 2.24 to 2.73 min versus WMD = − 0.62 min; 95% CI, − 1.53 to 0.30 min) (Supplementary Fig. S3a). In TTE protocols, all of the studies were performed in non-acclimated participants and the effect of menthol was also not significant in the group (WMD = 1.13 min; 95% CI, − 0.52 to 2.77 min) (Supplementary Fig. S3b). When we compared the effect of external and internal menthol application on endurance performance, we found that external application of menthol markedly increased performance time compared to internal application in TTE exercise protocols (WMD = 0.83 min; 95% CI − 1.95 to 3.60 min versus 0.40 min, 95% CI, − 0.03 to 0.83 min; $p < 0.001$), while in the other subgroups no significant effect was detected (Supplementary Fig. S4). The external menthol application methods were different in the studies: spray on the top wear[14,28–30], whole-body creaming[13] or immersion[18], and gel on the face[25] (Supplementary Table S4). The location of the administration and the surface area may also influence the effect since thermal signals from hairy skin provide more important feedback signals for the thermoregulation system than thermal signals from non-hairy skin; the latter functioning predominantly as an effector rather than a sensor[42]. To examine the possibility that treatment of a certain area of the body (e.g., face) with menthol has bigger impact on endurance performance than other areas in TTE protocols, we performed a sensitivity analysis (i.e., iteratively removing one study from the analyses and recalculating WMD to investigate the impact of each individual study on the summary estimate), which showed no difference in the final pooled results (Supplementary Table S5). Among the environmental factors, no meaningful difference was observed between subgroups with and without airflow in TT protocols (Supplementary Fig. S5a). In TTE tests, we found that menthol increased performance time when a fan (i.e., airflow) was present

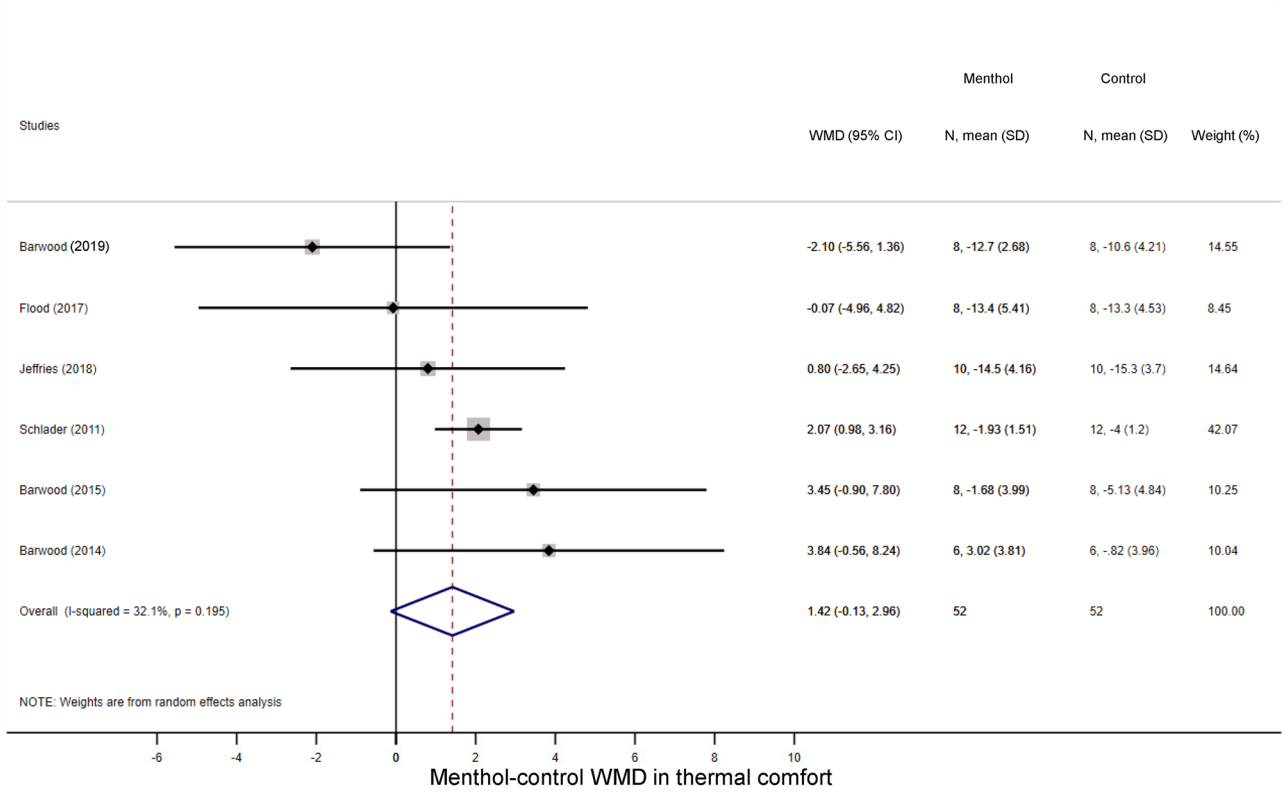


Figure 3. Forest plot of the weighted mean of differences (WMDs) for thermal comfort showing the effect of menthol during exercise.

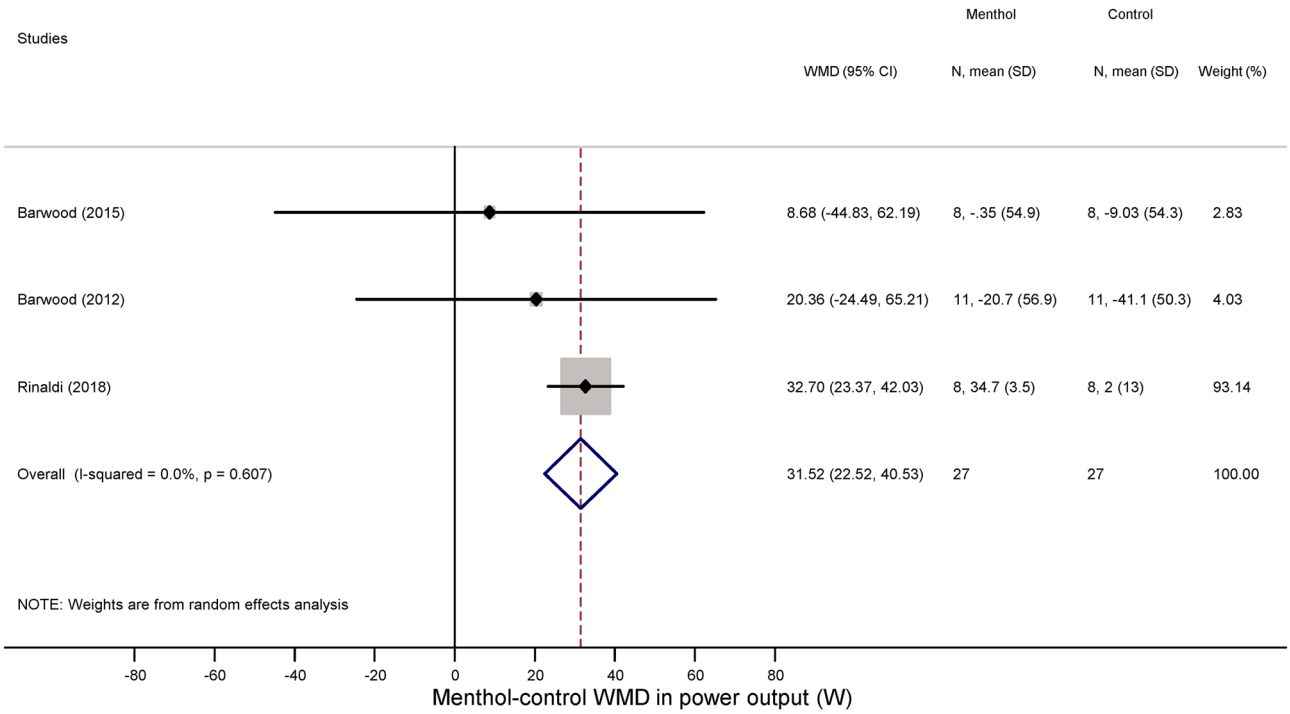


Figure 4. Forest plot of the weighted mean of differences (WMDs) for power output showing the effect of menthol during exercise.

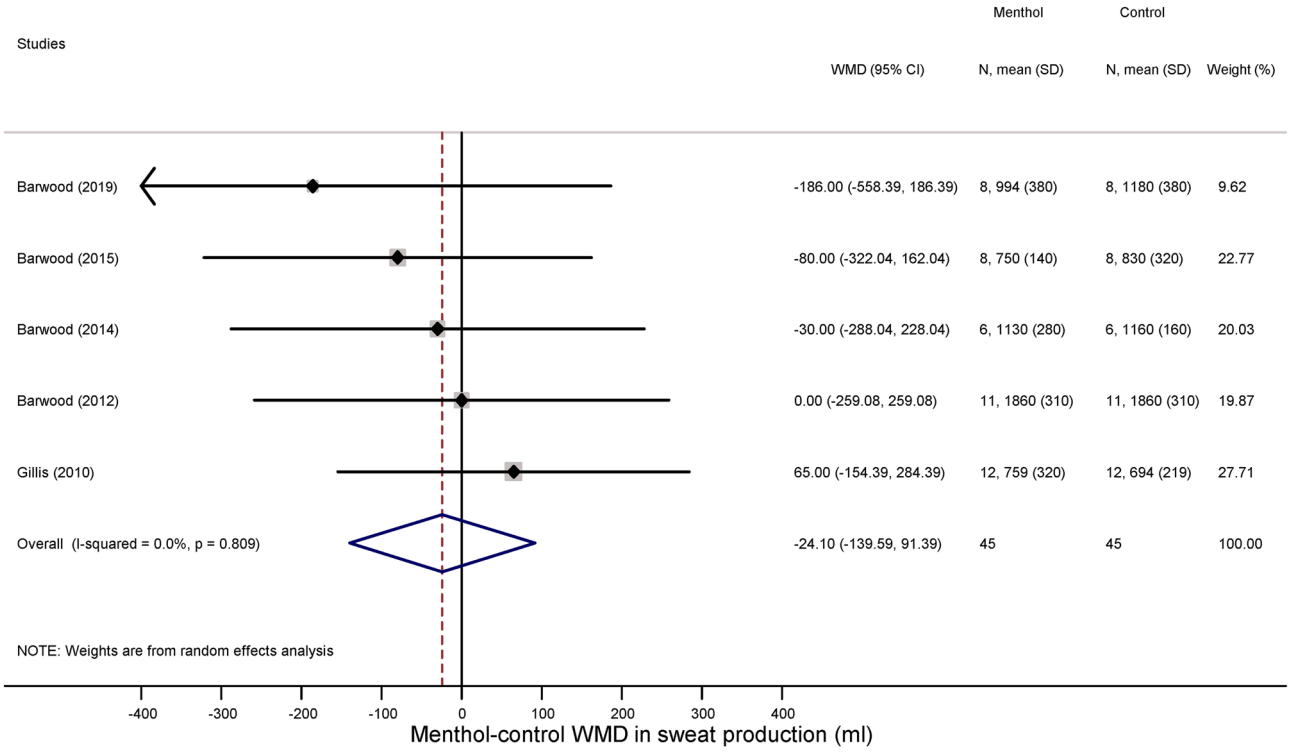


Figure 5. Forest plot of the weighted mean of differences (WMDs) for sweat production showing the effect of menthol during exercise.

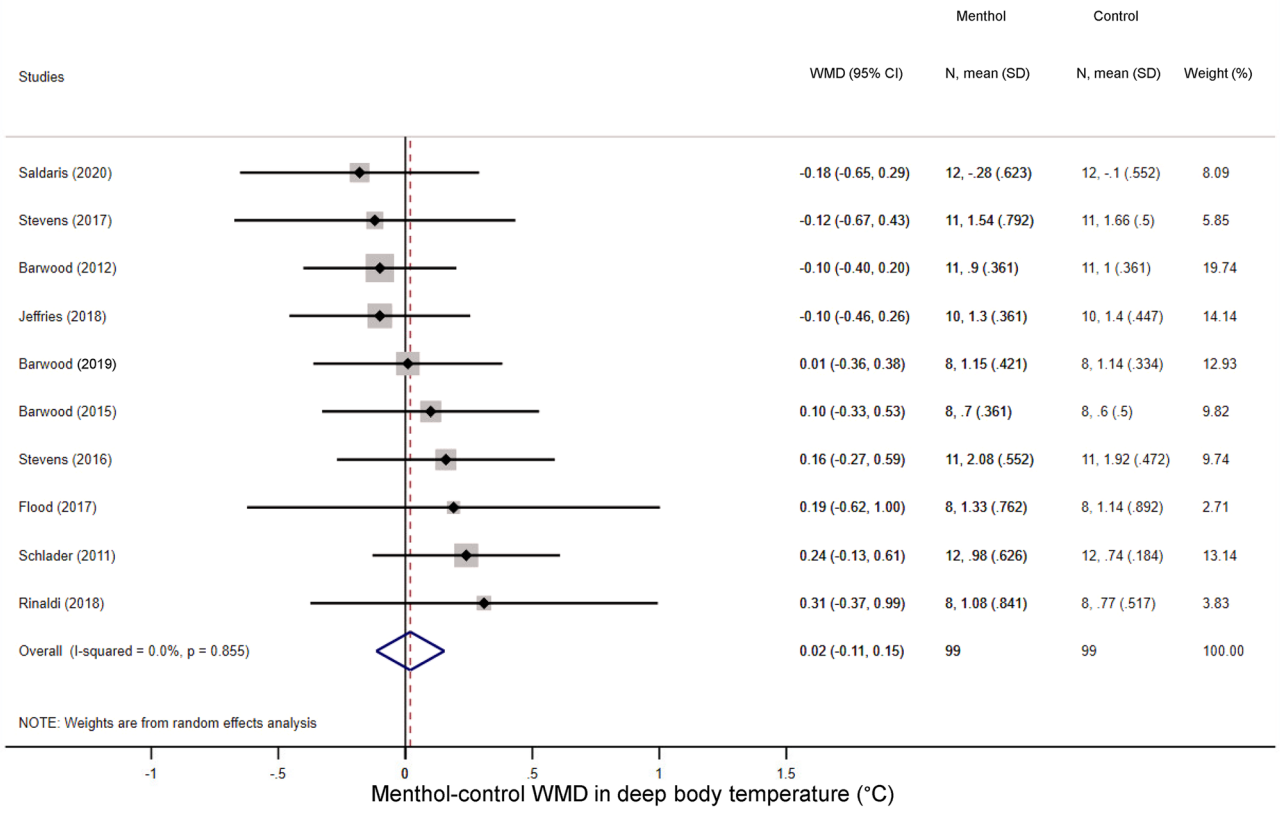


Figure 6. Forest plot of the weighted mean of differences (WMDs) for deep body temperature showing the effect of menthol during exercise.

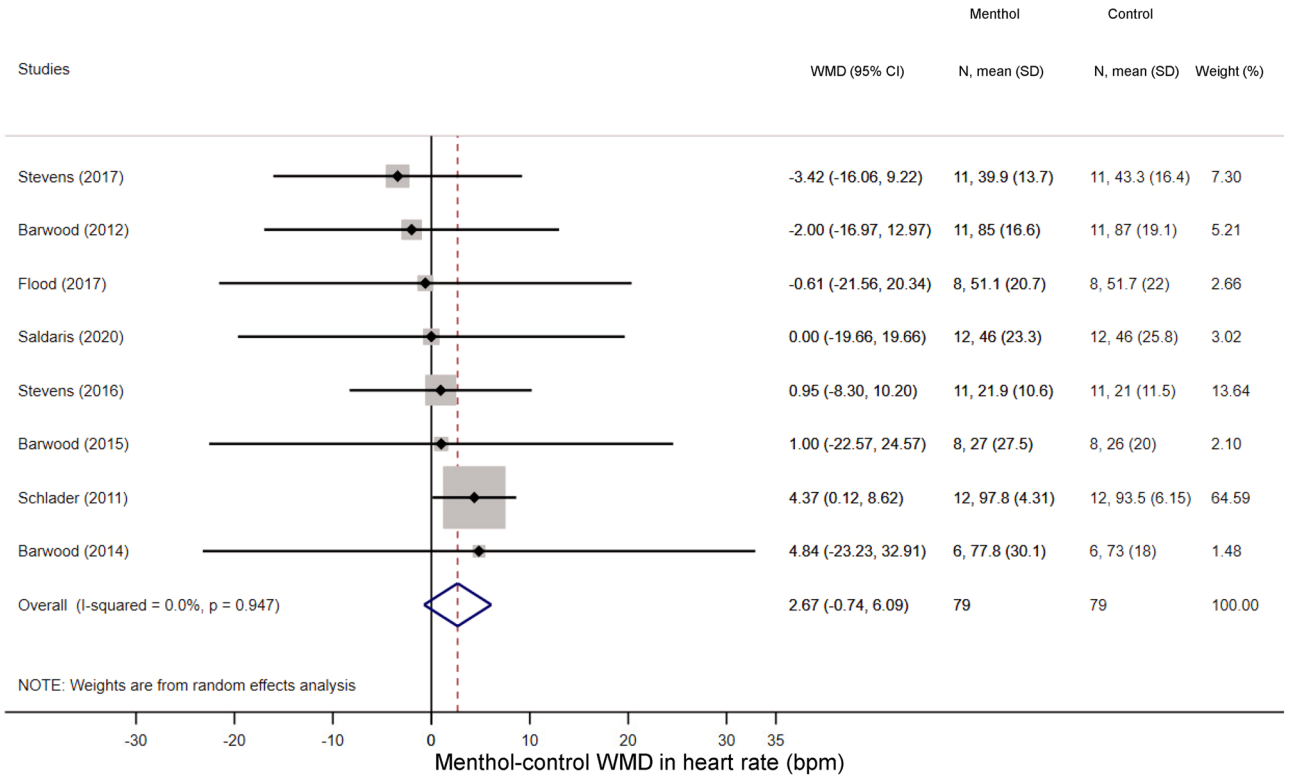


Figure 7. Forest plot of the weighted mean of differences (WMDs) for heart rate showing the effect of menthol during exercise.

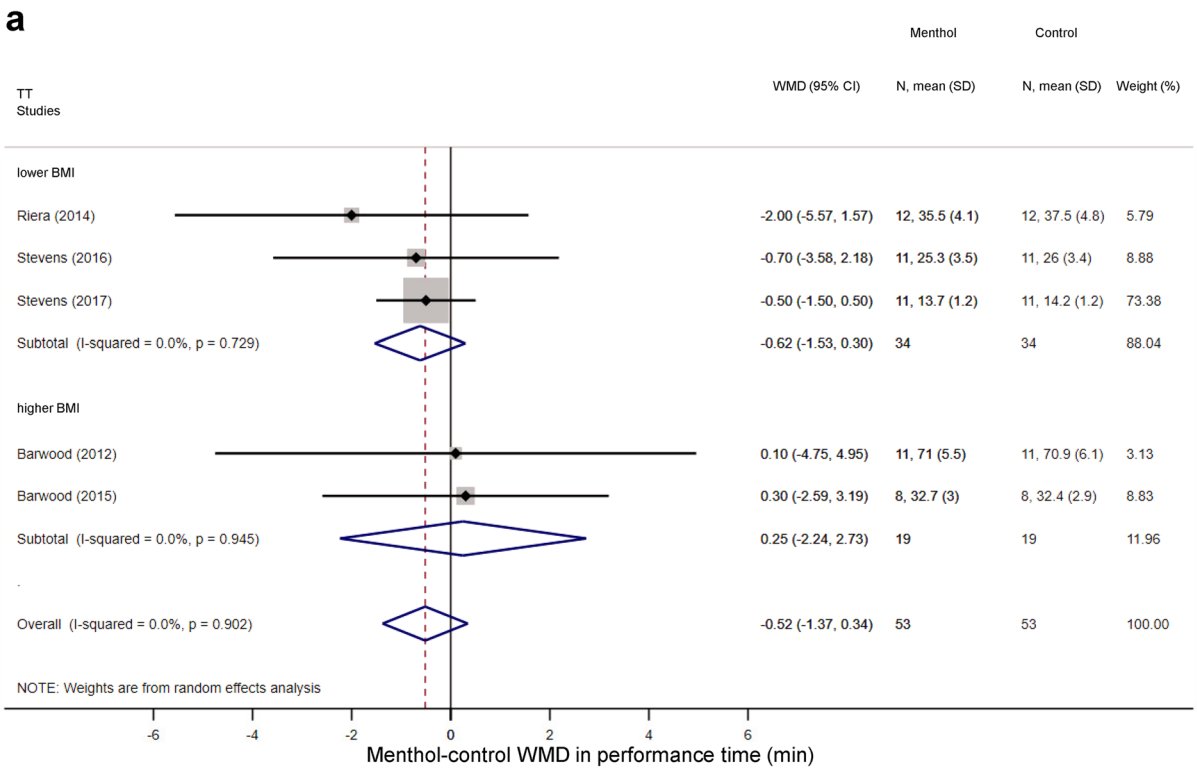
(WMD = 2.24 min; 95% CI 0.63 to 3.84 min) compared to no use of a fan (WMD = 0.64 min; 95% CI − 1.17 to 2.45 min) (Supplementary Fig. S5b). However, the averaged result of the subgroup with airflow should be taken with scrutiny due to the low number (n = 2) of studies in this subgroup. Furthermore, in TTE tests at higher T_as (above 31 °C) performance time was significantly increased in response to menthol compared with T_as ranging from ~ 20 to 30 °C (WMD = 1.02; 95% CI, − 0.01 to 2.04 min versus WMD = − 2.77 min; 95% CI, − 10.66 to 5.12 min; $p < 0.001$), while there was no significant difference between the subgroups in TT protocols (Supplementary Fig. S6).

Discussion

In the present study, we show that the application of menthol improves TS, TC, and power output during physical exercise. Our results about thermal perception are in harmony with the findings of a previous meta-analysis[43], which also showed beneficial effects of menthol on TS in exercise performance; however, in that study the thermophysiological effects of menthol were not analysed and influencing factors (e.g., acclimation, T_a) of menthol's effect were not investigated. In our study, we aimed at filling those gaps by studying the effects of menthol also on thermophysiological parameters, i.e., sweat production, heart rate, and deep T_b, and by identifying different phenotypes and environmental factors which can augment or attenuate menthol's effects. We show that the use of menthol does not lead to compromised warmth-defence responses during physical exercise, since it does not affect sweat production, heart rate, and deep T_b. We also identify bodily (viz., higher BMI), methodological (i.e., external menthol administration), and environmental factors, such air movement (fan use) and higher T_a, which enhance the beneficial effects of menthol on performance time.

Thermoregulatory changes, particularly in TS and TC, during physical exercise are of high importance, as they are considered among the limiting factors of endurance performance, and, as such, play a role in the development of fatigue[44,45]. The active muscle generates heat during physical exercise, thereby constituting an internal heat stress for the body, which is further augmented when physical activity is performed in the heat[46]. The heat load leads to worsening of TS and TC[44], while behavioural and autonomic warmth-defence mechanisms are recruited to prevent an excessive increase in deep T_b[46]. When the defence mechanisms are compromised or exhausted, bodily homeostasis cannot be maintained, and heat-related illnesses, such as exertional heat stroke in the most severe forms, develop[44]. Efforts should be made to prevent the simultaneous presence of severe external and internal heat load to the individuals. There are, however, certain scenarios, when prevention of these conditions is not possible. The most obvious examples include the strenuous physical activity of firefighters, soldiers, and professional athletes in hot environments.

As part of the global climate change, the incidence of heat waves has increased in different countries, including, for example, the UK[47], France[48], the US[49], Australia[50], and Japan[50]. These countries can be actual or potential hosts of upcoming worldwide, summertime sport events, e.g., Summer Olympic Games, thus pre-cautions should be implemented in order to prevent heat-related illnesses of the athletes during the games. In addition to



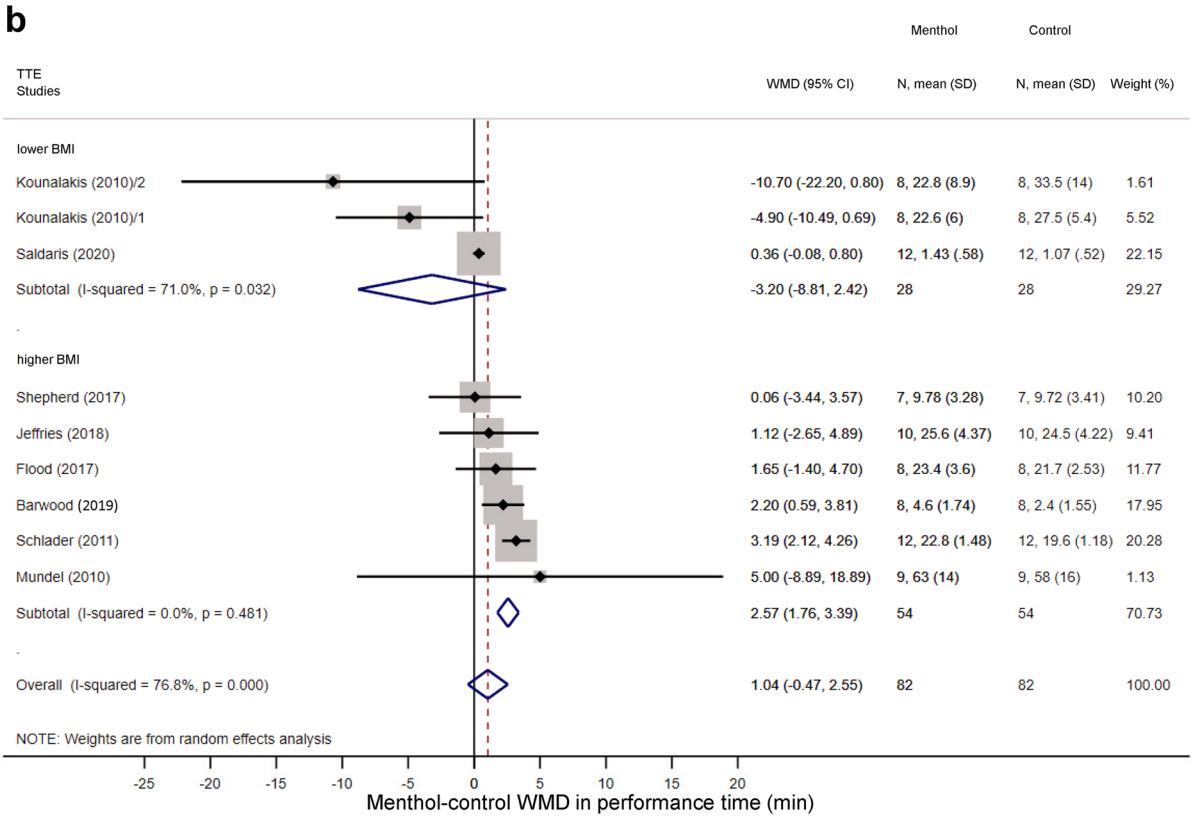


Figure 8. Forest plot of the weighted mean of differences (WMDs) for performance time showing the effect of menthol in (**a**) time-trial (TT) and (**b**) time-to-exhaustion (TTE) tests of athletes with lower (< 23.5) and higher (> 23.5) body mass index (BMI). The diamonds in the panels represent the average WMD calculated from the WMDs of the individual studies in each subgroup (top and middle) or in all studies (bottom).

physical methods of cooling, menthol may be also used as a pharmacological cooling intervention prior to and during exercise in hot conditions[50]. The improved TC in response to menthol can increase the thermal tolerance in athletes[51], which can lead to better performance. It should be noted, however, that menthol may not be safely used to improve TC in athletes competing at cold environments (e.g., at T_a below 20 °C). Our analysis, to our knowledge for the first time, also shows that the application of menthol did not result in compromised warmth defences.

Menthol has been identified earlier as a cold-mimicking substance, and its beneficial effects on sport performance have been also reported in a recent review[43]. It is also known that menthol evokes its thermoregulatory effects through the TRPM8 channel, which, at least in rodents, serves as a universal cold receptor for the body[19]. The activation of TRPM8 (e.g., by cold or menthol) leads to the recruitment of cold-defence responses, which aim at elevating (but at least preventing the drop in) deep T_b[11]. These thermophysiological effects of menthol, which were mostly discovered in animal experiments, imply a risk of menthol application in humans during physical exercise, since an adverse thermoregulatory effect, viz., an overt increase in deep T_b, can not be ruled out. However, in humans thermal signals from the skin are less important for autonomic thermoregulation because the greater thermal inertia makes transient thermal exposures less threatening, thus decreases the importance of signals from the skin[42]. Hence, activating peripheral cold receptors, such as TRPM8, with menthol in humans can have smaller effects on T_b than in rodents. It should be also noted that signals used for behavioural thermoregulation, which can be triggered through altered TS or TC, can differ from signals for autonomic thermoregulation [52]. For example, antagonists of the TRP vanilloid-1 channel readily affect autonomic thermoeffectors in rats[53], but fail to affect the behavioural thermoeffectors in the same species, at least as concluded from one study[54]. Moreover, the mode of action for the thermal effect of TRP vanilloid-1 channel antagonists differs between rodents and humans[55]. Therefore, activation of peripheral thermosensation with menthol in humans can have smaller effects on deep T_b than in rodents.

In the present study, we collected the available information about the thermoregulation homeostasis in menthol-treated athletes performing exercise, and conducted meta-analysis of the obtained data. We showed that at the used doses, menthol exerted beneficial effects on endurance performance, but it had no significant effect on any of the thermoregulation-related parameters, which included sweating production, heart rate, and deep T_b. It should be noted that sweat rate could be also an important indicator of thermoregulatory warmth defence. We found only three studies[14,20,25], which reported sweat rate, but in all of them only the averaged sweat rate was reported for the treatment groups. In two studies[20,25], there was no significant difference in sweat rate between menthol-treated and control groups, whereas in the third study the average sweat rate was significantly reduced after menthol treatment[14]. However, sweat rate is not steady, but rather a dynamic parameter during exercise. It was shown that during exercise sweating rate increased abruptly for 8 min after the onset of sweating and then continued increasing at a much lower rate[56], therefore the average sweat rate for the entire duration of the exercise should be interpreted with caution. As an alternative, we compared the exercise durations between the menthol-treated and control groups of the studies that reported sweat production and found that the difference between the treatment groups was less than 3 min in all studies[14,17,28–30]. We believe that such minimal difference in exercise duration between treatment groups of the same study did not have a significant influence on sweat production. Our results suggest that with regards to thermoregulation homeostasis, menthol can be safely applied during physical exercise in humans. Nevertheless, it is also possible that the administered doses of menthol and the treated surface area were not sufficient in the most of the analysed studies to trigger cold-defence responses, thereby leading to a change in thermophysiological parameters, including deep T_b. Furthermore, we pointed out different influencing factors, which can help to augment the performance-improving effects of menthol. Among environmental factors, we found that the use of a fan (i.e., wind effect) and higher T_a increased the efficacy of menthol on endurance performance. The beneficial effects of menthol were more pronounced in subjects with higher BMI, while acclimation to heat did not influence the effects.

Some limitations of our study should be also mentioned. There were inter-study differences in the design of the analysed studies regarding, for example, the sample population, the menthol administration route and dose, the exercise protocol, and the measurement of the outcome parameters. For example, the assessment of power output differed in the three analysed studies[18,28,30]. The study with the biggest effect size showed a significant improvement in power output[18], whereas power output did not differ statistically between the menthol-treated and control groups in either of the studies with smaller effect size[57,58]. Based on the risk of bias assessment, we found that blinding was not feasible in many studies, because of the characteristic odour of menthol. Furthermore, the allocation concealment was not indicated in some articles[13,17,18,20,24,25,31], which could have also influenced the effectiveness of menthol application. These methodological and medical differences in study design can explain the considerably high between-study heterogeneity (indicated by an I^2 of more than 50%), as observed in our analysis (Figs. 2 and 8b; Supplementary Figs. S1-S6). To account for the presence of heterogeneity, we used the random effects model in all forest plots of our meta-analyses. However, it is still possible that, despite all of our approaches to reduce methodological errors, the high heterogeneity of the analysed studies might have negatively impacted our results.

Our findings suggest that menthol can be safely used during physical exercise to improve thermal perception. Due to its beneficial effects on TS and TC, it can be used as an alternative to mitigate the impact of heat exposure on the individuals. External application of menthol in a warmer environment with air movement is more efficient, especially in subjects with higher BMI than 23. The validation of our results in targeted human trials is subject for future research.

Data availability

All data generated or analysed during this study are included in this published article.

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Author contributions

Conceptualization: P.K., A.G. Data curation: P.K., Z.R. Formal analysis: P.K., N.G., Z.R., A.G. Funding acquisition: A.G., P.H. Investigation: P.K., Z.R., A.G. Methodology: N.F., N.G., Z.L., M.S., K.R., G.V., A.G. Project administration: P.K., N.G. Resources: A.G., P.H. Software: N.F., N.G. Supervision: Z.R., A.G., P.H. Visualization: P.K., N.G. Writing—original draft: P.K., A.G. Writing—review & editing: N.F., N.G., P.H., Z.R., Z.L., M.S., K.R., G.V.

Competing interests

The authors declare no competing interests.

Additional information

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Correspondence and requests for materials should be addressed to A.G.

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Supplementary Information

Menthol can be safely applied to improve thermal perception during physical exercise: a meta-analysis of randomized controlled trials

Patrik Keringer, Nelli Farkas, Noemi Gede, Peter Hegyi, Zoltan Rumbus, Zsolt Lohinai, Margit Solymar, Kasidid Ruksakiet, Gabor Varga, Andras Garami

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Figure S3. Forest plot of the weighted mean differences (WMDs) for performance time showing the effect of menthol in (**a**) time-trial (TT) and (**b**) time-to-exhaustion (TTE) tests in subgroups of non-acclimated and acclimated participants.

Figure S4. Forest plot of the weighted mean differences (WMDs) for performance time showing the effect of menthol in (**a**) time-trial (TT) and (**b**) time-to-exhaustion (TTE) tests in subgroups of external and internal application.

Figure S5. Forest plot of the weighted mean differences (WMDs) for performance time showing the effect of menthol in (**a**) time-trial (TT) and (**b**) time-to-exhaustion (TTE) tests in subgroups without and with airflow.

Figure S6. Forest plot of the weighted mean differences (WMDs) for performance time showing the effect of menthol in (**a**) time-trial (TT) and (**b**) time-to-exhaustion (TTE) tests in subgroups of lower (< 31°C) and higher (31°C and above) ambient temperature (T_a).

Figure S7. Funnel plot of the studies that were included in the forest plot of menthol's effect on thermal sensation (n = 9, Egger's test: p = 0.073). Here, and in Figures S8-S10, the dots represent results from studies included in the forest plot.

Figure S8. Funnel plot of the studies that were included in the forest plot of menthol's effect on core temperature (n = 10, Egger's test: p = 0.341).

Figure S9. Funnel plot of the studies that were included in the forest plot of menthol's effect on heart rate (n = 8, Egger's test: p = 0.064).

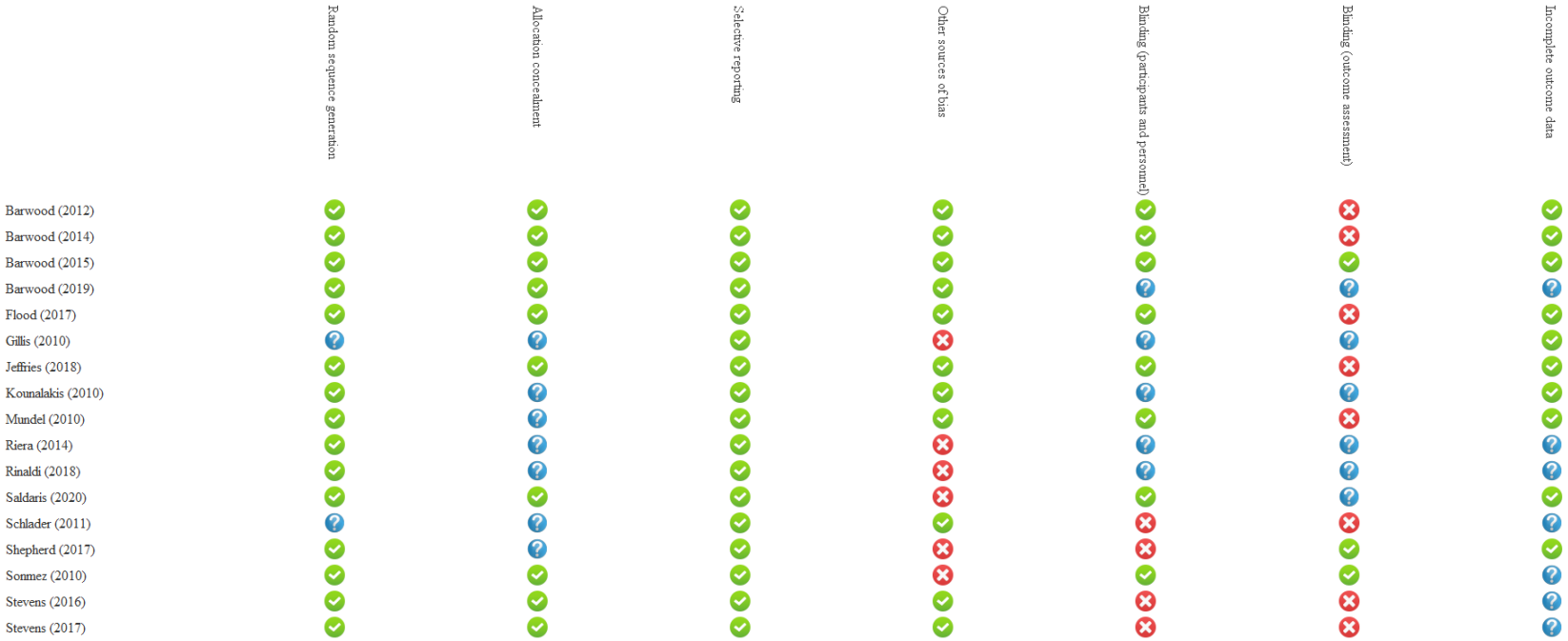
Figure S10. Funnel plot of the studies that were included in the forest plot of menthol's effect on performance time in time-to-exhaustion exercise protocols (n = 9, Egger's test: p = 0.877).

Table S1. PRISMA checklist**.**

Section/topic	#	Checklist item	Reported on page #
TITLE			
Title	1	Identify the report as a systematic review, meta-analysis, or both.	1
ABSTRACT			
Structured summary	2	Provide a structured summary including, as applicable: background; objectives; data sources; study eligibility criteria, participants, and interventions; study appraisal and synthesis methods; results; limitations; conclusions and implications of key findings; systematic review registration number.	1
INTRODUCTION			
Rationale	3	Describe the rationale for the review in the context of what is already known.	1-2
Objectives	4	Provide an explicit statement of questions being addressed with reference to participants, interventions, comparisons, outcomes, and study design (PICOS).	2
METHODS			
Protocol and registration	5	Indicate if a review protocol exists, if and where it can be accessed (e.g., Web address), and, if available, provide registration information including registration number.	2
Eligibility criteria	6	Specify study characteristics (e.g., PICOS, length of follow-up) and report characteristics (e.g., years considered, language, publication status) used as criteria for eligibility, giving rationale.	2
Information sources	7	Describe all information sources (e.g., databases with dates of coverage, contact with study authors to identify additional studies) in the search and date last searched.	2
Search	8	Present full electronic search strategy for at least one database, including any limits used, such that it could be repeated.	2
Study selection	9	State the process for selecting studies (i.e., screening, eligibility, included in systematic review, and, if applicable, included in the meta-analysis).	2
Data collection process	10	Describe method of data extraction from reports (e.g., piloted forms, independently, in duplicate) and any processes for obtaining and confirming data from investigators.	2
Data items	11	List and define all variables for which data were sought (e.g., PICOS, funding sources) and any assumptions and simplifications made.	2
Risk of bias in individual studies	12	Describe methods used for assessing risk of bias of individual studies (including specification of whether this was done at the study or outcome level), and how this information is to be used in any data synthesis.	2
Summary measures	13	State the principal summary measures (e.g., risk ratio, difference in means).	2

Synthesis of results	14	Describe the methods of handling data and combining results of studies, if done, including measures of consistency (e.g., I^2) for each meta-analysis.	2-3
Risk of bias across studies	15	Specify any assessment of risk of bias that may affect the cumulative evidence (e.g., publication bias, selective reporting within studies).	2-3
Additional analyses	16	Describe methods of additional analyses (e.g., sensitivity or subgroup analyses, meta-regression), if done, indicating which were pre-specified.	2-3
RESULTS			
Study selection	17	Give numbers of studies screened, assessed for eligibility, and included in the review, with reasons for exclusions at each stage, ideally with a flow diagram.	3; Fig. 1
Study characteristics	18	For each study, present characteristics for which data were extracted (e.g., study size, PICOS, follow-up period) and provide the citations.	3; Tables S3-S4
Risk of bias within studies	19	Present data on risk of bias of each study and, if available, any outcome level assessment (see item 12).	3; Table S2
Results of individual studies	20	For all outcomes considered (benefits or harms), present, for each study: (a) simple summary data for each intervention group (b) effect estimates and confidence intervals, ideally with a forest plot.	4-8; Figures 2-8; Figures S1-S6
Synthesis of results	21	Present results of each meta-analysis done, including confidence intervals and measures of consistency.	4-8; Figures 2-8; Figures S1-S6
Risk of bias across studies	22	Present results of any assessment of risk of bias across studies (see Item 15).	4-8; Table S2
Additional analysis	23	Give results of additional analyses, if done (e.g., sensitivity or subgroup analyses, meta-regression [see Item 16]).	4-8; Table S5
DISCUSSION			
Summary of evidence	24	Summarize the main findings including the strength of evidence for each main outcome; consider their relevance to key groups (e.g., healthcare providers, users, and policy makers).	8-10
Limitations	25	Discuss limitations at study and outcome level (e.g., risk of bias), and at review-level (e.g., incomplete retrieval of identified research, reporting bias).	10
Conclusions	26	Provide a general interpretation of the results in the context of other evidence, and implications for future research.	10
FUNDING			
Funding	27	Describe sources of funding for the systematic review and other support (e.g., supply of data); role of funders for the systematic review.	12

Table S2. Risk of bias assessment using the Cochrane Risk of Bias Tool for Randomized Controlled Trials.



According to the Cochrane Risk of Bias Tool, 7 studies are considered overall as poor quality (Gillis 2010, Riera 2014, Rinaldi 2018, Schlader 2011, Shepherd 2017, Stevens 2016, Stevens 2017), as two of the points were determined as “potentially high risk of bias”; 9 studies as moderate quality (Barwood 2012, Barwood 2014, Barwood 2019, Flood 2017, Jeffries 2018, Kounalakis 2010, Mundel 2010, Saldaris 2020, Sonmez 2010), since one criterion was determined as “potentially high risk of bias” or two criteria as “unclear risk of bias”; and one study as high quality (Barwood 2015), for every points were determined as “low risk of bias”.

, low risk; , high risk; , unclear risk of bias.

Table S3. Characteristics of participants in the studies included in the meta-analysis.

Study	Number of participants	Mean age (years)	Mean BMI (kg/m^2)	Trained?	Acclimated?
Barwood (2012)	11	30	24.0	Yes	No
Barwood (2014)	6	21	24.4	Yes	NR
Barwood (2015)	8	21	25.4	Yes	NR
Barwood (2019)	8	22	NR	Yes	NR
Flood (2017)	8	26	24.2	NR	No
Gillis (2010)	12	22	23.6	Yes	NR
Jeffries (2018)	10	33	23.7	NR	No
Kounalakis (2010)	8 8	28 21	22.2 23.2	Yes Yes	NR NR
Mundel (2010)	9	25	25.0	NR	No
Riera (2014)	12	42	22.8	Yes	Yes
Rinaldi (2018)	8	24	NR	Yes	Yes
Saldaris (2020)	12	25	23.4	Yes	No
Schlader (2011)	12	23	25.6	Yes	NR
Shepherd (2017)	7	25	24.3	Yes	NR
Sonmez (2010)	16	22	NR	Yes	NR
Stevens (2016)	11	29	23.4	Yes	Yes
Stevens (2017)	11	30	23.4	Yes	Yes

BMI, body mass index; NR, not reported

Table S4. Menthol application methods, exercise protocols, and environmental conditions in the studies eligible for quantitative analysis.

First author (publication year)	Menthol concentration (%); volume (ml)	Administration method; body surface area (%)[a]	Warm up	Exercise protocol; approx. duration (min)	T_a (°C)[b]	Relative humidity (%)[b]
Barwood (2012)	0.05; 100	External (spray on top wear); 36	Yes	Cycling TT; 71	31.5 (0.7)	53 (5)
Barwood (2014)	0.2; 100	External (spray on top wear); 36	Yes	Running TT; 28	33.9 (0.1)	55
Barwood (2015)	0.2; 100	External (spray on top wear); 36	Yes	Cycling TT; 33	33.5 (0.5)	33 (5)
Barwood (2019)	0.2; 100	External (spray on top wear); 36	Yes	Cycling TTE; 49	34.6 (1.2)	22 (1)
Flood (2017)	0.01; 25	Internal	Yes	Cycling TTE; 23	35.0 (0.8)	48 (2)
Gillis (2010)	0.05; 100	External (spray on top wear); 36	No	Cycling FTE; 45	26.3 (0.9)	72 (3)
Jeffries (2018)	0.01; 25	Internal	Yes	Cycling TTE; 25	35.0 (0.2)	40 (1)
Kounalakis (2010)	[c]; 100	External (cream on whole body); 91	No	Cycling TTE; 27	24.1 (1)	46 (4)
Mundel (2010)	0.01; 25	Internal	No	Cycling TTE; 61	34.0 (1)	27 (4)
Riera (2014)	0.01; 190	Internal	Yes	Cycling TT; 37	30.7 (0.8)	78 (0)
Rinaldi (2018)	0.1; NR	External (whole-body immersion); 91	Yes	Cycling FTE; 2 x 20	29.1 (1.5)	62 (4)
Saldaris (2020)	0.1; 25	Internal	No	Running TTE; 1	35.3 (0.3)	59 (3)
Schlader (2011)	8; [d]	External (gel on face); 4	Yes	Cycling TTE; 21	20.3 (0.2)	48 (3)
Shepherd (2017)	0.01; 500	Internal	No	Cycling TTE; 10	NR	NR
Sonmez (2010)	NR	Internal	No	Running TT; 1	NR	NR
Stevens (2016)	0.01; 25	Internal	Yes	Running TT; 26	32.6 (0.2)	46 (6)
Stevens (2017)	0.01; 25	Internal	No	Running TT; 14	32.5 (0.1)	47 (8)

[a]according to the Wallace rules of nine (Wallace, A. B. The exposure treatment of burns. *Lancet*, **257**, 501–504 [1951]), by omitting the area of the head from whole-body and of the arms from top wear applications; [b]data are shown as mean (standard deviation); menthol was applied at [c]4.6 g and [d]0.5 g/100cm^2; FTE, fixed-time exercise; NR, not reported; T_a, ambient temperature; TT, time trial; TTE, time to exhaustion

Table S5. Sensitivity analysis for performance time showing the effect of menthol in time-to-exhaustion tests in different external menthol application methods (spray on the top wear [Barwood 2019], cream over the whole body [Kounalakis 2010] and gel on the face [Schlader 2011]).

Study omitted	Estimate	95% confidence interval	
Kounalakis (2010)/2	1.730273	-0.61056	4.071108
Kounalakis (2010)/1	2.278419	0.103842	4.452996
Barwood (2019)	-2.76844	-10.6567	5.119824
Schlader (2011)	-2.9609	-10.0378	4.116044
Combined	0.825719	-1.94855	3.599985

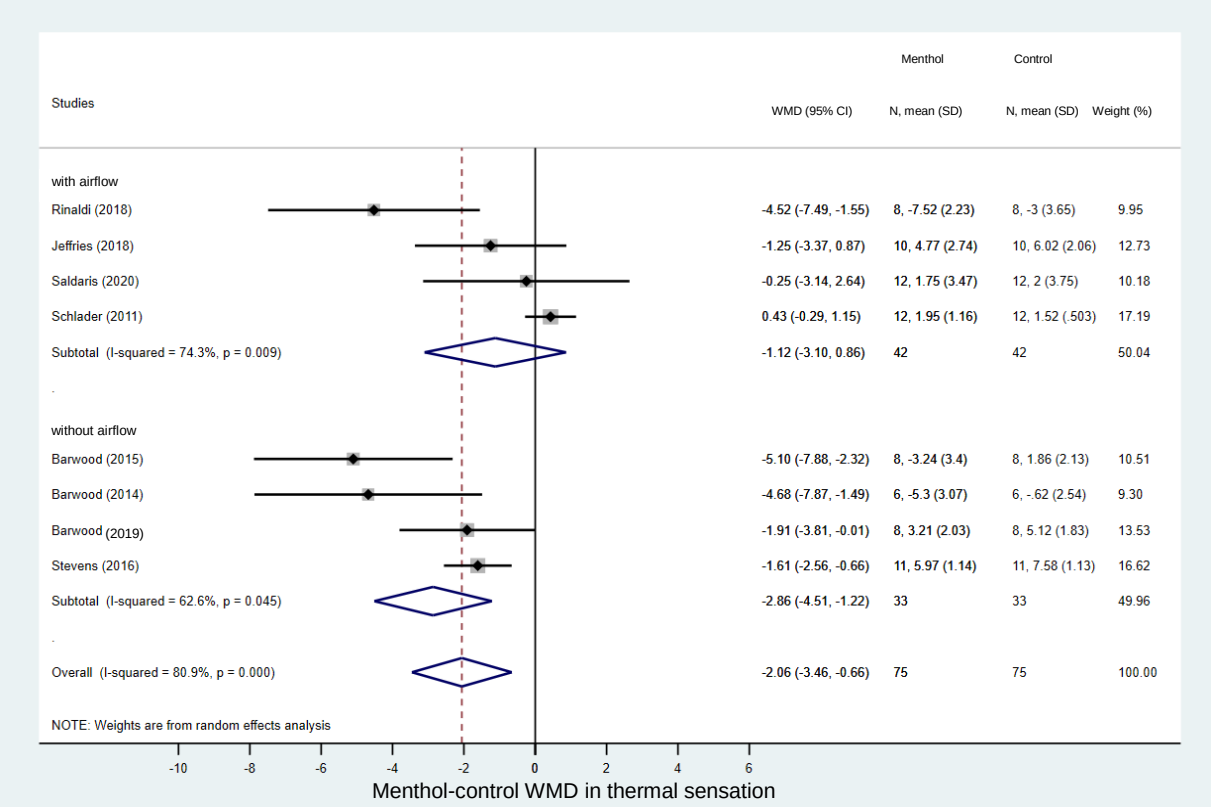


Figure S1. Forest plot of the weighted mean differences (WMDs) for thermal sensation showing the effect of menthol in subgroups with (top) and without (bottom) airflow.

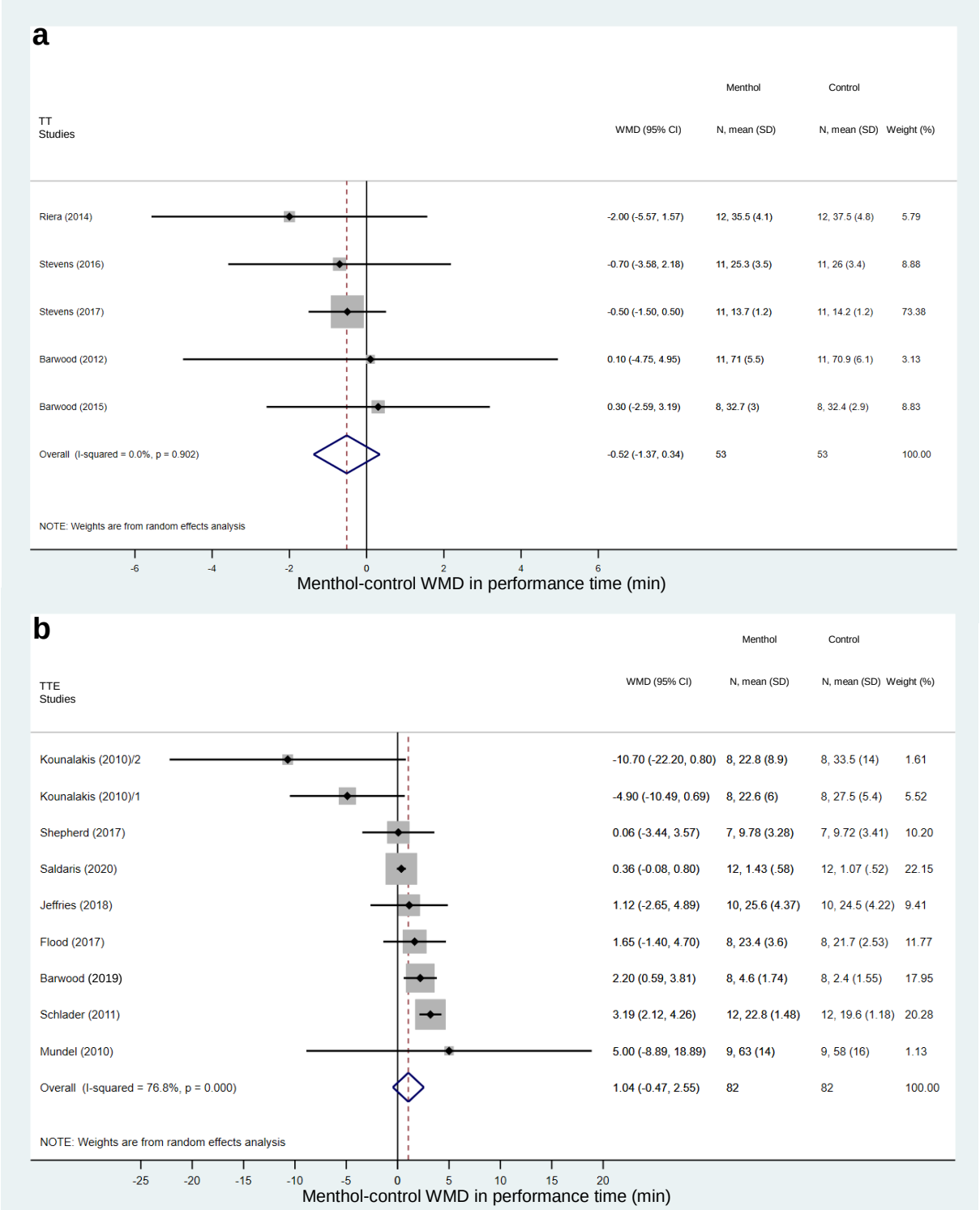


Figure S2. Forest plot of the weighted mean of differences (WMDs) for performance time in (**a**) time-trial (TT) and (**b**) time-to-exhaustion (TTE) tests showing the effect of menthol during exercise.

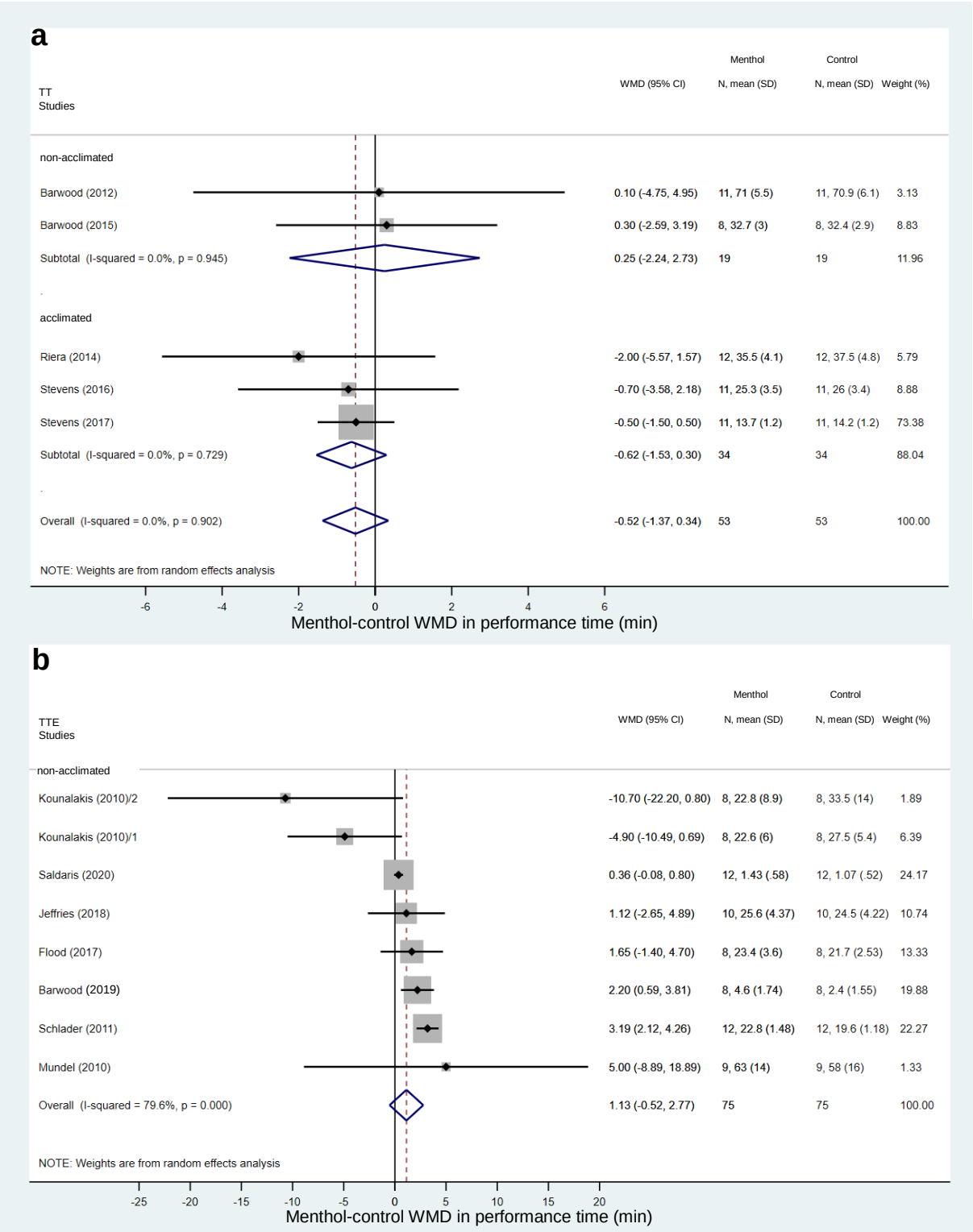


Figure S3. Forest plot of the weighted mean differences (WMDs) for performance time showing the effect of menthol in (**a**) time-trial (TT) and (**b**) time-to-exhaustion (TTE) tests in subgroups of non-acclimated and acclimated participants.

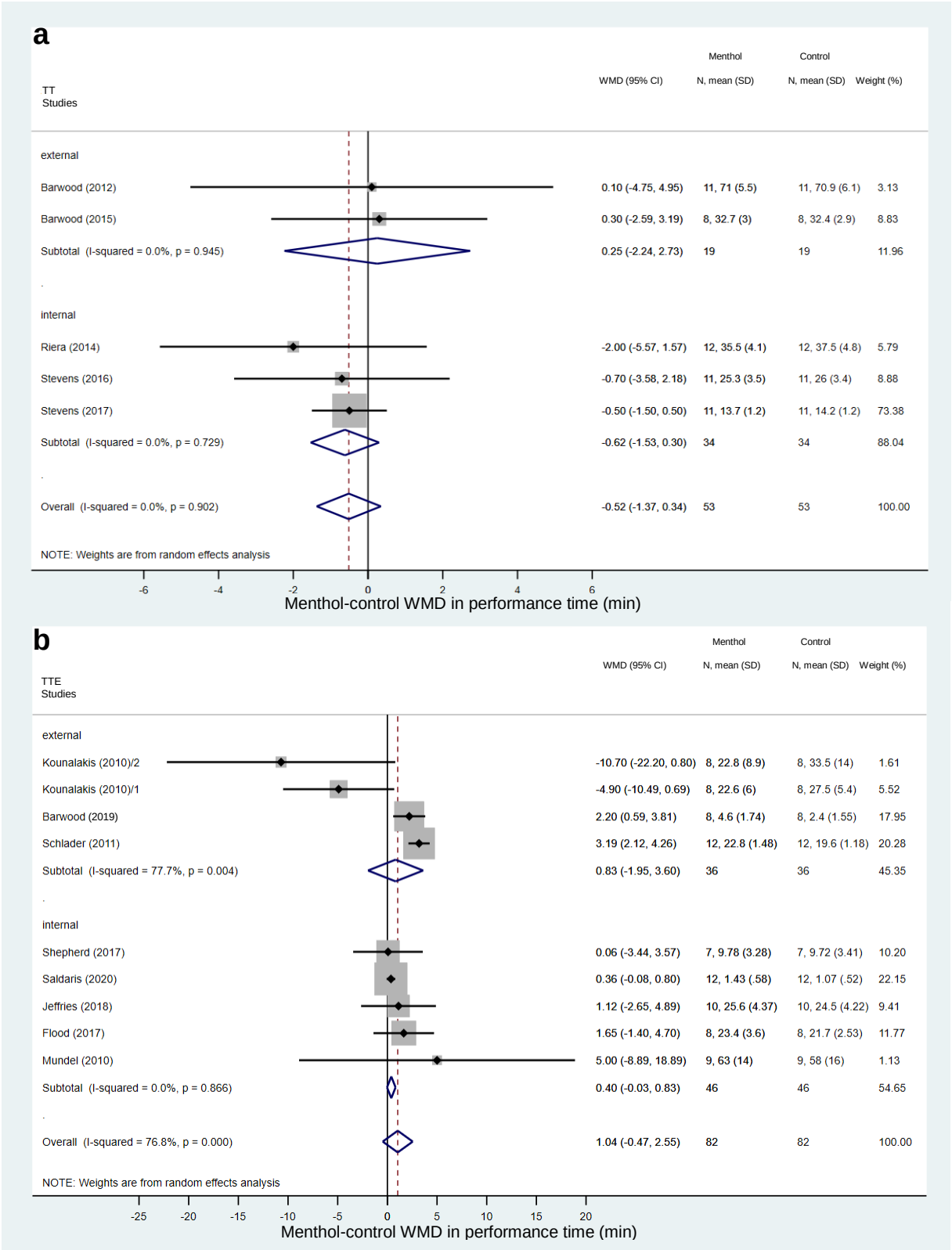


Figure S4. Forest plot of the weighted mean differences (WMDs) for performance time showing the effect of menthol in (**a**) time-trial (TT) and (**b**) time-to-exhaustion (TTE) tests in subgroups of external and internal application.

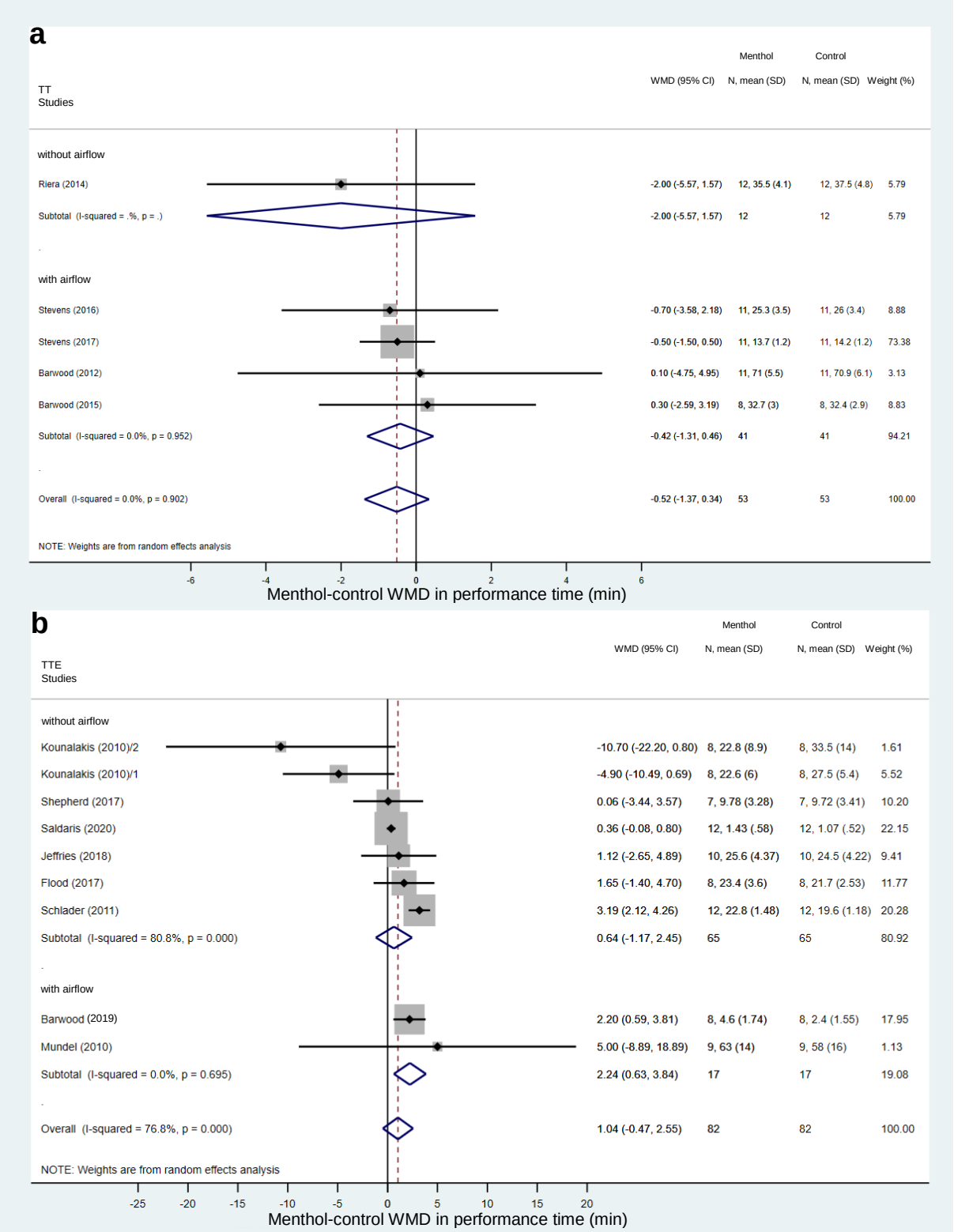


Figure S5. Forest plot of the weighted mean differences (WMDs) for performance time showing the effect of menthol in (**a**) time-trial (TT) and (**b**) time-to-exhaustion (TTE) tests in subgroups without and with airflow.

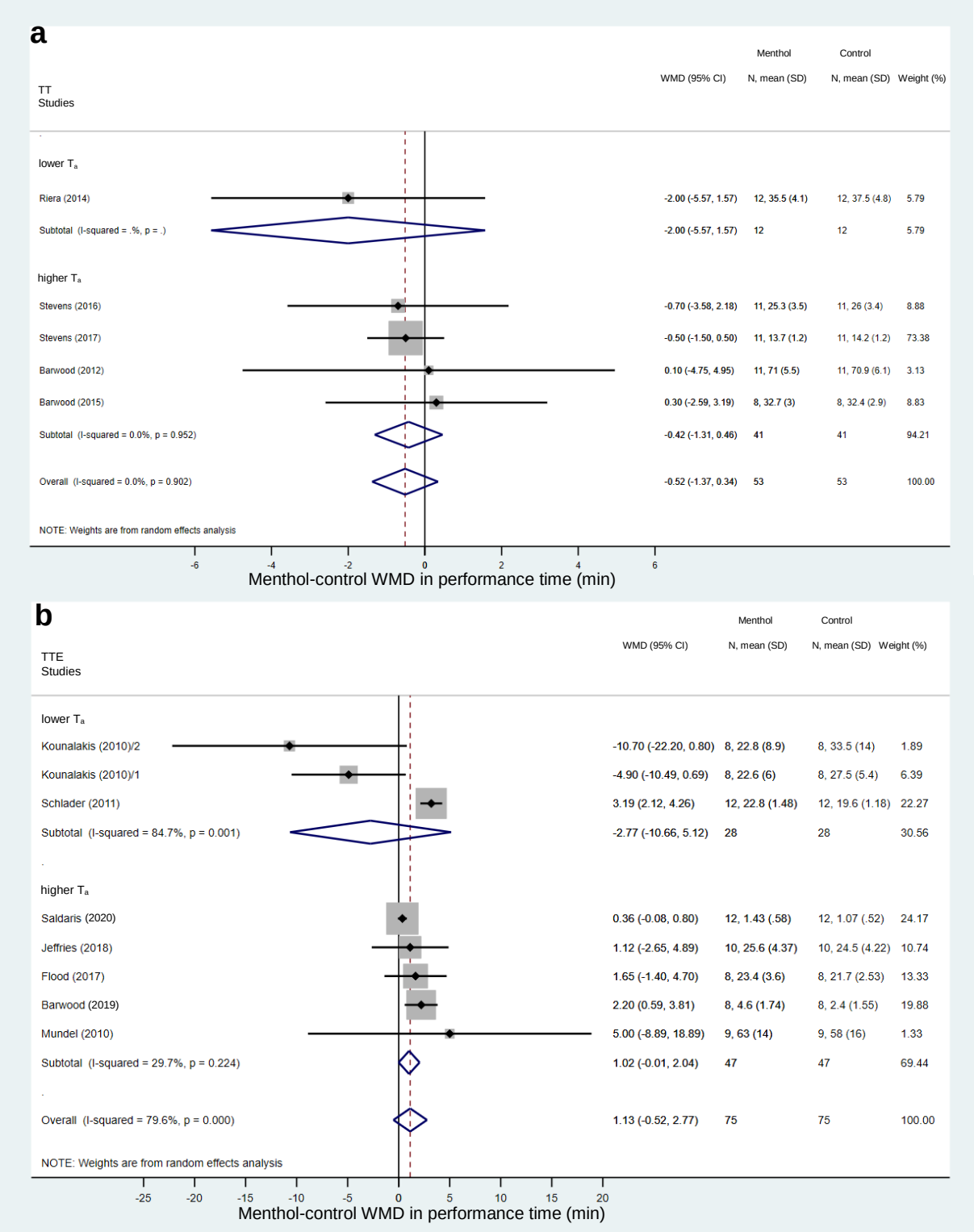


Figure S6. Forest plot of the weighted mean differences (WMDs) for performance time showing the effect of menthol in (**a**) time-trial (TT) and (**b**) time-to-exhaustion (TTE) tests in subgroups of lower (< 31°C) and higher (31°C and above) ambient temperature (T_a).

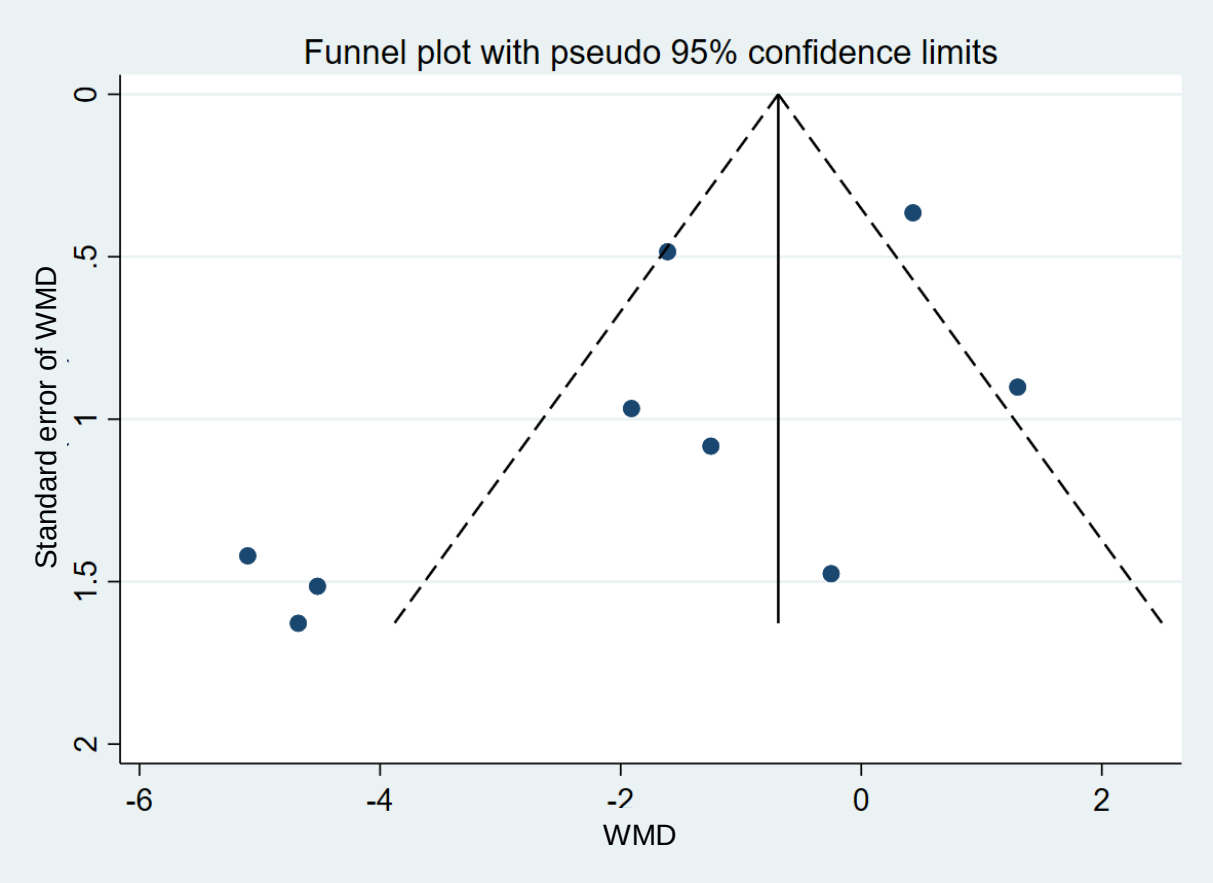


Figure S7. Funnel plot of the studies that were included in the forest plot of menthol's effect on thermal sensation (n = 9, Egger's test: p = 0.073). Here, and in Figures S8-S10, the dots represent results from studies included in the forest plot.

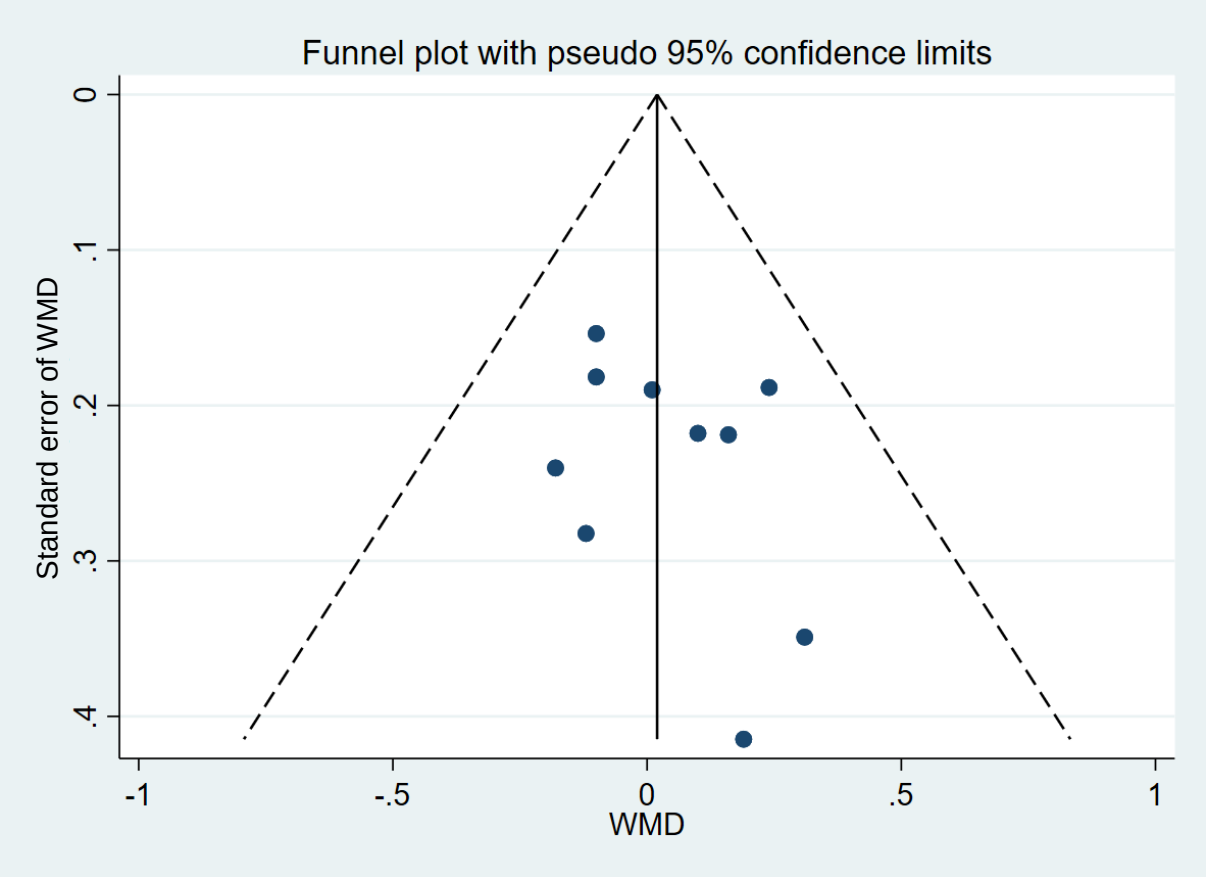


Figure S8. Funnel plot of the studies that were included in the forest plot of menthol's effect on core temperature (n = 10, Egger's test: p = 0.341).

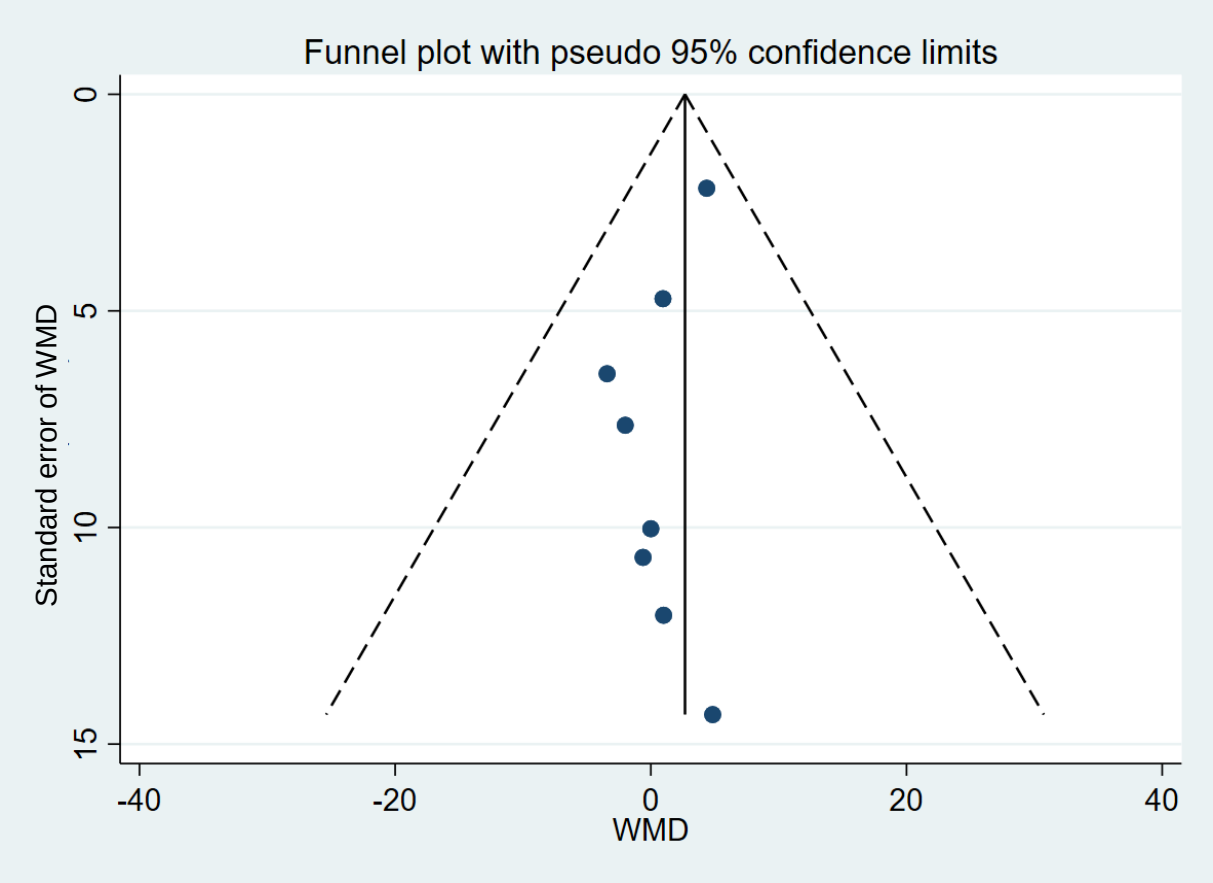


Figure S9. Funnel plot of the studies that were included in the forest plot of menthol's effect on heart rate (n = 8, Egger's test: p = 0.064).

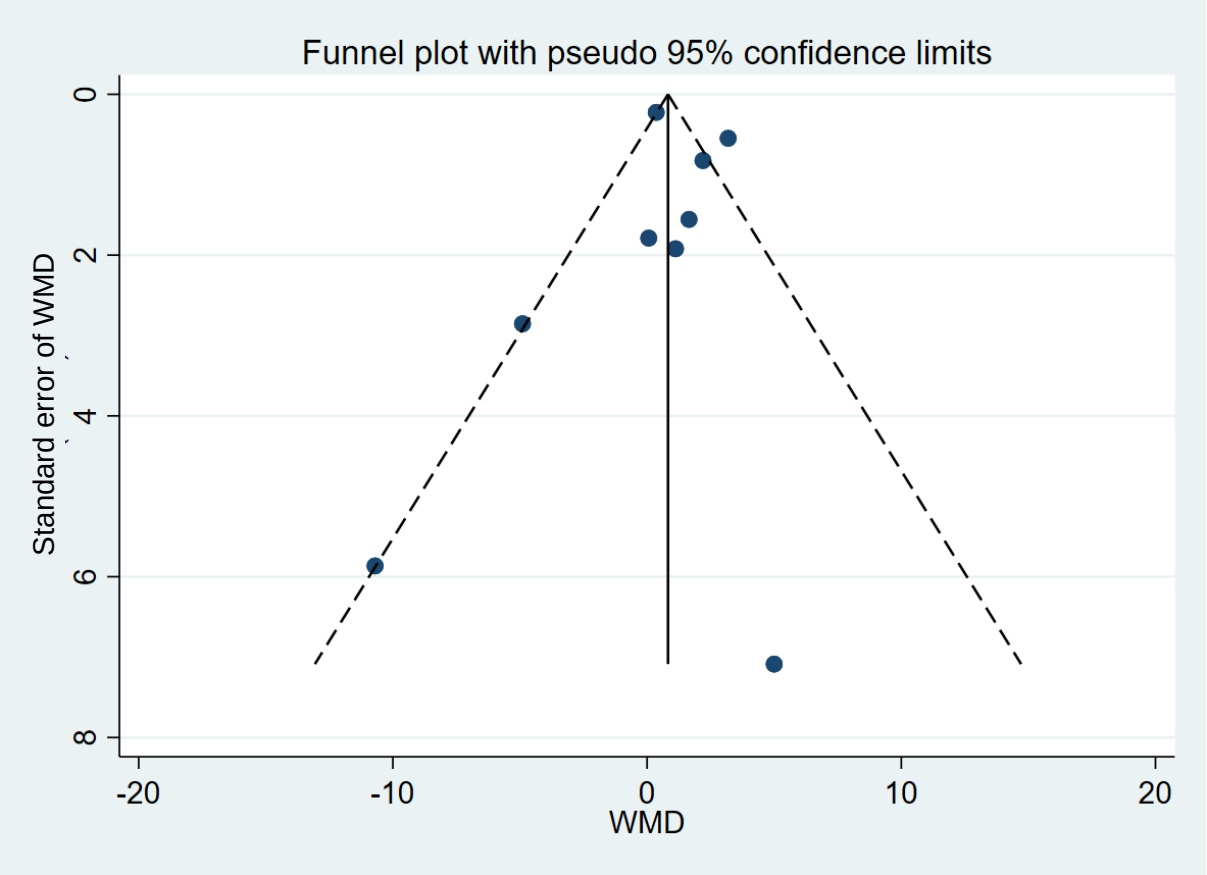


Figure S10. Funnel plot of the studies that were included in the forest plot of menthol's effect on performance time in time-to-exhaustion exercise protocols (n = 9, Egger's test: p = 0.877).

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RESEARCH ARTICLE

The hyperthermic effect of central cholecystokinin is mediated by the cyclooxygenase-2 pathway

Patrik Keringer,[1] Nora Furedi,[2] Balazs Gaszner,[2] Alexandra Miko,[3] Eszter Pakai,[1] Kata Fekete,[1] Emoke Olah,[1] Leonardo Kelava,[1] Andrej A. Romanovsky,[4] Zoltan Rumbus,[1] and Andras Garami[1]

[1]*Department of Thermophysiology, Institute for Translational Medicine, Medical School, University of Pécs, Pécs, Hungary;* [2]*Department of Anatomy, Research Group for Mood Disorders, Centre for Neuroscience, Medical School and Szentagothai Research Centre, University of Pécs, Pécs, Hungary;* [3]*Institute for Translational Medicine, Medical School and Szentagothai Research Centre, University of Pécs, Pécs, Hungary; and* [4]*School of Molecular Sciences, University of Arizona, Tempe, Arizona*

Abstract

Cholecystokinin (CCK) increases core body temperature via CCK_2 receptors when administered intracerebroventricularly (icv). The mechanisms of CCK-induced hyperthermia are unknown, and it is also unknown whether CCK contributes to the fever response to systemic inflammation. We studied the interaction between central CCK signaling and the cyclooxygenase (COX) pathway. Body temperature was measured in adult male Wistar rats pretreated with intraperitoneal infusion of the nonselective COX enzyme inhibitor metamizol (120 mg/kg) or a selective COX-2 inhibitor, meloxicam, or etoricoxib (10 mg/kg for both) and, 30 min later, treated with intracerebroventricular CCK (1.7 μg/kg). In separate experiments, CCK-induced neuronal activation (with and without COX inhibition) was studied in thermoregulation- and feeding-related nuclei with c-Fos immunohistochemistry. CCK increased body temperature by ∼0.4°C from 10 min postinfusion, which was attenuated by metamizol. CCK reduced the number of c-Fos-positive cells in the median preoptic area (by ∼70%) but increased it in the dorsal hypothalamic area and in the rostral raphe pallidus (by ∼50% in both); all these changes were completely blocked with metamizol. In contrast, CCK-induced satiety and neuronal activation in the ventromedial hypothalamus were not influenced by metamizol. CCK-induced hyperthermia was also completely blocked with both selective COX-2 inhibitors studied. Finally, the CCK_2 receptor antagonist YM022 (10 μg/kg icv) attenuated the late phases of fever induced by bacterial lipopolysaccharide (10 μg/kg; intravenously). We conclude that centrally administered CCK causes hyperthermia through changes in the activity of "classical" thermoeffector pathways and that the activation of COX-2 is required for the development of this response.

NEW & NOTEWORTHY An association between central cholecystokinin signaling and the cyclooxygenase-prostaglandin E pathway has been proposed but remained poorly understood. We show that the hyperthermic response to the central administration of cholecystokinin alters the neuronal activity within efferent thermoeffector pathways and that these effects are fully blocked by the inhibition of cyclooxygenase. We also show that the activation of cyclooxygenase-2 is required for the hyperthermic effect of cholecystokinin and that cholecystokinin is a modulator of endotoxin-induced fever.

body temperature; CCK; COX; fever; hyperthermia

INTRODUCTION

Cholecystokinin (CCK), a peptide that serves as a gut hormone and brain neurotransmitter, evokes its effects mainly through two receptors: CCK_1 (formerly, the A type, from "alimentary"), located primarily in the gastrointestinal tract, and CCK_2 (formerly, the B type, from "brain"), expressed predominantly in the central nervous system (1). The contribution of CCK to the regulation of energy balance was well established by the discovery of decreased food intake induced by this peptide in rats, monkeys, and humans (2–4). In the early 1980s, a role for CCK in thermoregulation, which is also part of energy balance (5), was suggested (6), and later it was concluded that activation of the two CCK receptors affects body temperature differently (7). When administered peripherally, CCK causes hypothermia, which is mediated by CCK_1 receptors (8, 9), whereas the administration of CCK into the central nervous system results in hyperthermia through the activation of CCK_2 receptors (9, 10).

In animal models, the administration of bacterial lipopolysaccharide (LPS) in a thermally neutral environment is commonly used to induce fever, which is typically polyphasic [reviewed in Garami et al. (11)]. In rats, the febrile response entails the activation of cold-defense mechanisms,

Correspondence: A. Garami (andras.garami@aok.pte.hu).
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which include both behavioral (warmth seeking) and autonomic (skin vasoconstriction and nonshivering thermogenesis) thermoeffectors. Fever is mediated by the cyclooxygenase (COX)-2–prostaglandin (PG) E_2 pathway, which is activated first in hepatic and pulmonary macrophages and later in brain endothelial cells (12). In the brain (for reviews, see Refs. 11, 13), PGE_2 acts on EP3-expressing, γ-aminobutyric acid (GABA)ergic preoptic hypothalamic neurons, which tonically inhibit both cutaneous vasoconstriction [through projections to the rostral raphe pallidus (rRPa)] and nonshivering thermogenesis in brown adipose tissue [through projections to the dorsal hypothalamic area (DA)]. Hence, PGE_2 reduces the activity of GABAergic preoptic neurons and thereby it disinhibits downstream neural substrates in the rRPa and DA to activate the cold-defense effectors (skin vasoconstriction and thermogenesis).

While endogenous PGE_2 is well known to play a central role in the mediation of LPS fever (11, 14, 15), other pyrogenic substances have been also proposed to be associated with the COX-PGE pathway [e.g., neurokinin-1 signaling (16), kallikrein-kinin system (17), and hydrogen sulfide (18)] or to contribute to fever development by acting through PG-independent mechanisms [e.g., interleukin-8 (19) and platelet-activating factor (20)]. Based on similarities between the thermoregulatory effects of centrally administered CCK and PGE, it was suggested that CCK also participates in the modulation of the febrile response to LPS, but its relation to the COX-PGE pathway remained poorly understood (21).

Similarly to the PGE-induced rise in deep body temperature, skin vasoconstriction and enhanced thermogenesis were also observed to contribute to the hyperthermic response to intracerebroventricularly (icv) administered CCK octapeptide (CCK-8) (9, 10, 22). Moreover, the first phase of LPS fever was attenuated by a pharmacological antagonist of the CCK_2 receptor (22), whereas the genetic disruption of the CCK_2 receptor gene suppressed the early and late changes in body temperature induced by LPS (23), suggesting that central CCK signaling modulates the fever response. It should also be noted that another study using both genetic and pharmacological tools (24) concluded that none of the phases of the febrile response to LPS requires CCK_1 receptors. Furthermore, COX inhibition with indomethacin did not affect CCK-8-induced hyperthermia (22, 25), whereas PGE-induced hyperthermia was not influenced by CCK receptor blockers (9).

The aim of the present study was to investigate whether the hyperthermic and satiety responses to central administration of CCK depend on the COX pathway. To this end, we studied whether COX inhibitors affect the body temperature responses and neuronal activation patterns in thermoregulation-related brain structures in rats treated centrally with CCK.

MATERIALS AND METHODS

Animals

The experiments were performed in 220 adult male Wistar rats. The rats were housed in standard (42.5 × 27.6 × 15.3 cm) plastic cages (model: 1290 D Eurostandard type III; Akronom Ltd. Budapest, Hungary) with wood shaving bedding (Szinkat Ltd., Szada, Hungary) kept in a room with an ambient temperature maintained at 21°C–23°C and humidity at 30%–40%. The room was on a 12/12-h light/dark cycle (lights on at 5:00 AM). Standard rodent chow and tap water were available ad libitum. At the time of the experiments, the rats weighed 300–400 g. The rats were extensively handled and habituated to staying inside wire-mesh cylindrical confiners, as in earlier studies (26, 27). The cylindrical confiner prevented the animal from turning around but allowed for some back-and-forth movements; it was used throughout the thermometry experiments and for substance administration at the beginning of the feeding experiments (see *Thermocouple Thermometry* and *CCK-Induced Anorexia Test*). All procedures were conducted under protocols approved by the Institutional Animal Use and Care Committee of the University of Pecs and followed the directives of the National Ethical Council for Animal Research and those of the European Communities Council (86/609/EEC).

Surgeries

Each rat was implanted with an intracerebroventricular cannula and with either an intraperitoneal (ip) or an intravenous (iv) catheter in the same anesthesia, as described below. Rats were anesthetized with intraperitoneal administration of a ketamine-xylazine cocktail (78 and 13 mg/kg, respectively) and received antibiotic protection intramuscularly (gentamycin, 6.7 mg/kg). During intraperitoneal and intravenous catheter implantation, the rats were heated with a temperature-controlled heating pad (model TMP-5a; Supertech Instruments UK Ltd., London, UK) placed under a surgery board. The experiments were performed 4–7 days after the surgery.

Implantation of the intracerebroventricular cannula was performed, as described earlier (28). In brief, each rat was fixed to a stereotaxic apparatus, the scalp was incised, the periosteum was removed, the skull was cleaned, two supporting microscrews (Fine Science Tools, Heidelberg, Germany) were driven into the skull, and a small hole was drilled in the skull 1 mm posterior from bregma and 1.5 mm lateral from midline. A 22-gauge steel guide cannula was attached to a plastic tube fitted into a stereotaxic manipulator (David Kopf Instruments, Tujunga, CA). The tip of the cannula was placed within the right lateral ventricle (3.8 mm from dura) (29). The cannula was secured to the supporting microscrews with zinc phosphate cement (Adhesor, SpofaDental, Jicin, Czech Republic) and released from the manipulator. The guide cannula was closed by a dummy cannula and covered by an adhesive tape.

For intraperitoneal catheter implantation, a small midline incision was made on the abdominal wall, and then a polyethylene (PE)-50 catheter filled with pyrogen-free saline was inserted into the peritoneal cavity. The internal end of the catheter was fixed to the left side of the abdominal wall with a suture; the free end of the catheter was tunneled under the skin to the nape where it was exteriorized and heat sealed. The surgical wound was sutured in layers. The catheter was flushed with 0.25 mL of saline on the day after the surgery and every other day thereafter.

For intravenous catheterization, a small longitudinal incision was made on the ventral surface of the neck, left to the trachea. The left jugular vein was exposed, cleared from its

surrounding connective tissue, and ligated. A silicone catheter with 0.5 mm inner and 0.9 mm outer diameter filled with heparinized saline (10 U/mL) was passed into the superior vena cava through the jugular vein and secured in place with ligatures. The free end of the catheter was knotted, tunneled under the skin to the nape, and exteriorized. The wound on the ventral surface of the neck was sutured. The intravenous catheters were flushed with heparinized saline (10 U/mL) on the day after the surgery and then every other day.

Thermocouple Thermometry

In the thermocouple thermometry setup, the rat was placed in a cylindrical confiner and equipped with a copper-constantan thermocouple (Omega Engineering, Stamford, CT) to measure colonic temperature (T_c). The colonic thermocouple was inserted 10 cm deep beyond the anal sphincter and was fixed to the base of the tail with a loop of adhesive tape. The thermocouple was plugged into a data logger device (Cole-Palmer, Vernon Hills, IL) connected to a computer. Rats in their confiners were then placed into a temperature-controlled incubator (model BJPX-Newark; Biobase, Jinan, PR China) set to an ambient temperature of ~30°C, which is at the lower end of the thermoneutral zone for rats in this setup and also neutral for adult rats in similar setups (27). A needle injector was fitted into the intracerebroventricular guide cannula and connected to a PE-50 extension, which was passed through a port of the incubator and connected to a 10-μL syringe (model 701 N, Hamilton, Reno, NV). The intraperitoneal or intravenous catheter was also connected to a PE-50 extension filled with the drug of interest or saline.

CCK-Induced Anorexia Test

The anorexigenic response to CCK was tested by measuring the changes in the body mass of the rats after a 24-h food deprivation. On the morning of the experiment, the rat was placed in a restrainer and infused intraperitoneally with metamizol or saline. Thirty minutes later, the rat was injected intracerebroventricularly with CCK or saline and was kept in the restrainer for another 30 min after the injection. Then, the rat was weighed and returned to its home cage, where standard rodent chow was available ad libitum. Three hours later, the rat was weighed again and the difference in body mass between 0 and 3 h was expressed as percentage and also as absolute amount.

Tissue Harvesting

Each rat was implanted with an intracerebroventricular cannula and an intraperitoneal catheter and extensively adapted to the experimental setup. On the day of the experiment, each rat was placed in a confiner and transferred to an incubator chamber (Biobase), which was set to an ambient temperature of 30°C. PE-50 extensions were connected to the intracerebroventricular injector and to the intraperitoneal catheter, as in the thermometry experiments. Rats were left to acclimate for ~2 h; infused intraperitoneally with metamizol or saline and 30 min later administered with CCK or saline intracerebroventricular. Two hours after the intracerebroventricular injection, the rats were anesthetized with a ketamine-xylazine cocktail injected through the extension of the intraperitoneal catheter. Each rat was perfused through the left ventricle with 0.1 M phosphate-buffered saline (PBS), followed by perfusion with 4% paraformaldehyde in 0.2 M Millonig's phosphate buffer, then the entire brain was removed and postfixed in the same fixative for 12 h.

Immunohistochemistry

The c-Fos staining was performed, as in earlier studies (28, 30). Coronal sections (30 μm) were prepared on vibratome (Lancer, Ted Pella Inc., Redding, CA) and stored in antifreeze solution at −20°C. The sections were washed 6 × 10 min in PBS, incubated in 0.5% Triton X-100 (Sigma Chemical, Zwijndrecht, The Netherlands) and, subsequently, in 2% normal goat serum (Jackson Immunoresearch Europe Ltd., Ely, UK) in PBS for 30 min. Then, sections were incubated overnight at room temperature in a rabbit polyclonal c-Fos antiserum (sc-52; Santa Cruz Biotechnology Inc., Santa Cruz, CA) diluted to 1:500 in PBS. Sections were treated with biotinylated goat anti-rabbit IgG (Vectastain Elite ABC Kit, Vector Laboratories, Burlingame, CA) diluted to 1:200 in PBS and with 2% normal goat serum for 2 h. Sections were rinsed in PBS and treated with avidin-biotin complex (Vectastain Elite ABC Kit) in PBS for 1 h. After 3 × 10 min PBS rinses, the immunolabeling was visualized in Tris buffer (pH = 7.6) for 10 min. Finally, preparations were treated with 0.05% diaminobenzidine in the Tris buffer with 0.03% H_2O_2 (Sigma Chemical); the latter reaction was controlled under a stereomicroscope and stopped with PBS. Sections were mounted on gelatin slides, treated with xylene (Merck, Leicester, UK), air-dried, coverslipped with DePex mounting medium (Fluka, Heidelberg, Germany).

The specificity and sensitivity of the primary and secondary antisera were carefully tested earlier in the rat (30). For this study, this was also confirmed: the omission of the primary or secondary serum and their replacement with nonimmune sera prevented the immunolabeling (images not shown).

Microscopy and Morphometry

Preparations were studied with a Nikon Microphot FXA microscope using Nikon PlanApo objective lenses of 4× [numeric aperture (NA): 0.2], 10× (NA: 0.45), and 20× (NA: 0.75) magnification. The regions of interest were photographed with a Spot RT color digital camera using the Spot Advanced Imaging software (Nikon, Tokyo, Japan). In each brain, the cell counts positive for c-Fos were determined in five serial sections, each interspaced by 60 μm in the medial preoptic area (MPO), DA, rRPa, and ventromedial hypothalamus (VMH), according to the atlas by Paxinos and Watson (29). Cell counting was carried out on nonedited digital images using ImageJ software (version 1.37, NIH, Bethesda, MD). Quantitation was performed in a double-blind setup by a colleague who is an expert of rodent neuroanatomy but was blinded to the identity of preparations.

Substance Administration

Sulfated CCK-8 was purchased from Bachem (Bubendorf, Switzerland). A stock solution of CCK-8 (1 μg/μL) in pyrogen-free saline was aliquoted and stored at −20°C. On the day of

the experiment, an aliquot was diluted with saline to a final concentration of 0.1 μg/μL, which was injected intracerebroventricularly (3.4 μL/kg/min for 5 min) to deliver CCK-8 at a total dose of ~1.7 μg/kg. Control rats were infused with saline. The selective CCK_2 receptor antagonist YM022 was purchased from Tocris (Bristol, UK). Aliquots of an ethanolic stock solution of YM022 (6 μg/μL) were stored at −20°C. On the day of experiment, the stock solution was diluted with saline to give a working solution of YM022 at 0.6 μg/μL in 10% ethanol. By infusing this working solution of YM022 into the lateral ventricle (3.3 μL/kg/min for 5 min), a total dose of ~10 μg/kg was delivered intracerebroventricularly. Control rats were infused with the vehicle (10% ethanol in saline).

On the day of the experiment, metamizol (Sanofi, Budapest, Hungary), a nonselective COX inhibitor, and two selective COX-2 inhibitors, viz., meloxicam (Boehringer Ingelheim International GmbH, Ingelheim, Germany) and etoricoxib (Merck & Co., Kenilworth, NJ) were dissolved in saline at concentrations of 120, 10, and 10 mg/mL, respectively. Metamizol (120 mg/kg), meloxicam (10 mg/kg), and etoricoxib (10 mg/kg) were infused through the preimplanted intraperitoneal catheter (0.08 mL/kg/min for 12.5 min).

LPS from *Escherichia coli* 0111:B4 was purchased from Sigma-Aldrich (St. Louis, MO). A stock suspension of LPS (5 mg/mL) in pyrogen-free saline was stored at −20°C. On the day of the experiment, the stock was diluted with saline to a final concentration of 10 μg/mL. The diluted LPS suspension or saline was infused (0.33 mL/kg/min for 3 min) through the extension of the intravenous catheter to deliver LPS at a final dose of 10 μg/kg.

Substances were administered between 9:30 AM and 12:10 PM in the thermometry experiments and between 8:00 and 10:15 AM in the feeding experiments.

Data Processing and Analysis

Changes in T_c were compared by two-way ANOVA, while initial T_c and body mass values, as well as changes in body mass and the numbers of the c-Fos positive cells were compared with one-way ANOVA, as appropriate. ANOVA was followed by the Student–Newman-Keuls post hoc test. Sigmaplot 11.0 (Systat Software, San Jose, CA) was used for statistical analysis. The effects were considered significant when $P < 0.05$. All data are reported as means ± standard error (SE). The difference in the mean temperatures of the drug- and saline-treated rats was calculated ($\Delta_{mean} = mean_{Group\ 1} - mean_{Group\ 2}$), and its standard error was determined according to the formula: $SE_\Delta = \sqrt{[(SE_{Group\ 1})^2 + (SE_{Group\ 2})^2]}$ (31).

RESULTS

Dependence of the Hyperthermic and Anorexic Effects of Centrally Administered CCK on COX Enzymes in Rats

First, we characterized the thermoregulatory effect of CCK administered intracerebroventricularly in rats. As expected, based on previous studies (9, 10, 22, 32, 33), in response to CCK, the rats developed a marked elevation in T_c, whereas administration of saline did not cause any effects (Fig. 1). The hyperthermic response to CCK developed promptly (in less than 10 min) and T_c reached the highest mean increase of 0.4 ± 0.1°C at 20 min ($P = 0.007$), then it gradually decreased

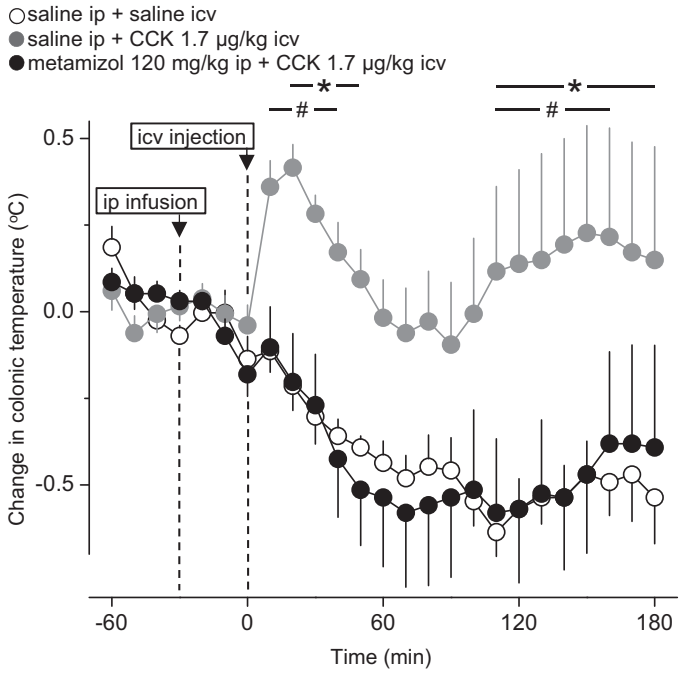

Figure 1. Deep (colonic) body temperature responses of rats to intracerebroventricular (icv) administration of cholecystokinin (CCK; 1.7 μg/kg) or saline after pretreatment at −30 min with an intraperitoneal (ip) infusion of metamizol (120 mg/kg) or saline. For each group, $n = 9$. *$P < 0.05$, icv CCK vs. saline difference in ip saline-pretreated rats; #$P < 0.05$, ip metamizol vs. saline difference in icv CCK-treated rats, as determined by two-way ANOVA followed with Student–Newman–Keuls test. Data are presented as means ± SE. At the time of the icv injection, the values of colonic temperature of the rats in the treatment groups were, respectively, 38.3 ± 0.1°C for saline + saline, 38.3 ± 0.2°C for saline + CCK, and 38.4 ± 0.2°C for metamizol + CCK. These values did not differ statistically from each other.

but remained elevated compared with saline treatment throughout the experiment.

To study the involvement of the COX enzymes in the development of CCK-induced hyperthermia, the rats were treated with the nonselective COX inhibitor metamizol (120 mg/kg ip) 30 min preceding the intracerebroventricular administration of CCK. The effect of the pretreatment was significant on the T_c response in CCK-treated rats [ANOVA, $F_{(1,304)} = 62.994$, $P < 0.001$]. In the intraperitoneal metamizol-pretreated rats, the hyperthermic response to intracerebroventricular CCK was abolished as compared with intraperitoneal saline pretreatment, reaching the level of significance at 10–40 and 110–160 min between the pretreatment groups ($P < 0.05$) (Fig. 1). The T_c of the metamizol-pretreated rats tended to decrease during the experiment after the intracerebroventricular injection of CCK, but it did not differ from the T_c of the control (saline + saline-treated) rats. In both groups, the gradual fall in T_c might have reflected ultradian body temperature rhythms in rats maintained at an ambient temperature of ~30°C, which is at the lower end of the thermoneutral zone.

We also wanted to know whether the inhibition of COX enzymes attenuates the anorexic effect of CCK. For that reason, in another set of experiments, 24-h fasted rats were treated with metamizol or saline before the intracerebroventricular administration of CCK or saline. As expected, in

saline-pretreated rats, the injection of CCK significantly reduced the gain of body mass during 3-h refeeding as compared with intracerebroventricular saline injection (1.6 ± 0.3 vs. 2.6 ± 0.3%, $P < 0.05$) (Fig. 2). The absolute changes in body mass were 9 ± 1, 5 ± 1, and 4 ± 1 g in the saline + saline, saline + CCK, and metamizol + CCK groups, respectively. Importantly, however, we did not detect any significant difference in CCK-induced anorexia between the metamizol- and saline-pretreated rats [ANOVA, $F_{(1,21)} = 0.532$, $P = 0.474$].

CCK-Induced Changes in c-Fos Expression in Thermoregulation- and Feeding-Related Brain Nuclei and Their Dependence on COX Activation

Knowing that CCK-induced hyperthermia entails autonomic thermoeffector responses, i.e., activation of brown fat thermogenesis and cutaneous vasoconstriction (9, 22), we hypothesized that blocking CCK-induced hyperthermia with a COX-inhibitor changes the activation of hypothalamic efferent neurons controlling these responses. To test this hypothesis, we measured expression of the inducible transcription factor c-Fos, a marker of neuronal activation (34) in the MPO, DA, and rRPa (Fig. 3), which nuclei contribute to the autonomic thermoregulatory responses to cooling (35, 36) and PGE_2 (14). We found a significant decrease in the number of c-Fos positive cells in the MPO in response to CCK as compared with intracerebroventricular administration of saline (8.3 ± 0.9 vs. 27.3 ± 1.1, $P < 0.001$), whereas CCK increased c-Fos immunoreactivity expression in the DA (69.1 ± 1.9 vs. 31.9 ± 3.2, $P < 0.001$) and the rRPa (11.3 ± 1.6 vs. 5.1 ± 0.9, $P < 0.01$) when compared with saline. Pretreatment of the rats with intraperitoneal metamizol ip completely reversed the central CCK-induced changes in the number of c-Fos positive cells in the MPO (36.1 ± 5.9, $P < 0.001$), DA (28.7 ± 3.9, $P < 0.001$), and rRPa (5.4 ± 1.07, $P < 0.01$) when compared with intraperitoneal saline pretreatment.

We wanted to confirm that CCK-induced anorexia involves changes in the neuronal activation of the VMH, which harbors neurons involved in the regulation of food intake (37) and to study whether the observed changes can be influenced by the inhibition of COX (Fig. 4). CCK induced an elevation in c-Fos positive cell number in the VMH (91.2 ± 6.7 vs. 35.7 ± 13.0, $P < 0.001$) when compared with intracerebroventricular saline administration. In contrast with our results in thermoregulatory nuclei, the intraperitoneal pretreatment with metamizol had no effect on the CCK-induced neuronal activation in the VMH (98.7 ± 13.3, $P = 0.505$) compared with saline pretreatment.

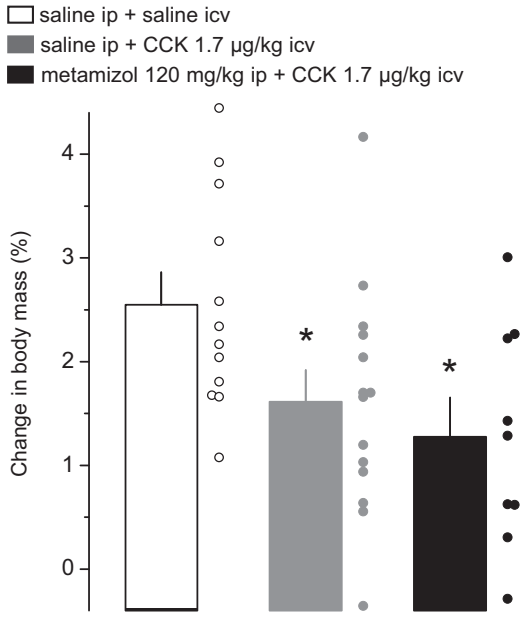

Figure 2. Changes in body mass of rats in response to intraperitoneal (ip) and intracerebroventricular (icv) administration of saline ($n = 12$) and to icv administration of cholecystokinin (CCK; 1.7 μg/kg) after pretreatment at −30 min with an ip infusion of metamizol (120 mg/kg; $n = 9$) or saline ($n = 14$). *$P < 0.05$, icv CCK vs. saline difference as determined by one-way ANOVA followed with Student–Newman–Keuls test. Bars represent group means (± SE), individual data are shown as circles. At the start of the refeeding, the values of body mass of the rats in the treatment groups were, respectively, 340 ± 8 g for saline + saline, 324 ± 8 g for saline + CCK, and 322 ± 9 g for metamizol + CCK. These values did not differ statistically from each other.

Effects of Selective COX-2 Inhibitors on CCK-Induced Hyperthermia

We showed that metamizol blunts the effects of CCK; however, it inhibits both isoforms of COX. In systemic inflammation-associated thermal changes, the two COX isoforms play different roles: COX-2 is essential in the development of fever, whereas COX-1, and not COX-2, is the isoform that mediates the hypothermic response (38). As CCK induced a rise in T_c, we hypothesized that COX-2 is responsible for the mediation of its thermal effect. To test our hypothesis, we studied the effects of two different preferential COX-2 inhibitors, meloxicam and etoricoxib, on CCK-induced hyperthermia (Fig. 5). As expected, the hyperthermic effect of intracerebroventricular administered CCK was significant compared with saline [ANOVA, $F_{(1,285)} = 30.386$, $P < 0.001$] in intraperitoneal saline-pretreated rats. However, when the rats were pretreated with meloxicam or etoricoxib intraperitoneally, the intracerebroventricular injection of CCK did not cause any change in T_c of the rats. As compared with intraperitoneal saline pretreatment, the effect was significant for both etoricoxib [ANOVA, $F_{(1,247)} = 105.804$, $P < 0.001$] and meloxicam [ANOVA, $F_{(1,266)} = 82.613$, $P < 0.001$]. The CCK-induced hyperthermia was attenuated by meloxicam at 20–30, 50, 80, and 100–180 min ($P < 0.05$) and by etoricoxib at 20–50, 70–80, and 100–180 min ($P < 0.05$) during the experiments (Fig. 5). The T_c of the rats pretreated with COX-2 inhibitors tended to decrease after the intracerebroventricular injection of CCK, but it was not statistically different from control (saline + saline-treated) rats. As in Fig. 1, the gradual decrease in all groups might have reflected ultradian body temperature rhythms in rats maintained at an ambient temperature, which is at the lower end of the thermoneutral zone.

Effect of the CCK_2 Antagonist YM022 on LPS-Induced Fever

After we showed that the hyperthermic response to CCK is mediated by COX-2, we wanted to know whether CCK signaling in the central nervous system contributes to LPS-induced fever, which is known to be mediated by COX-2 (11). Previous

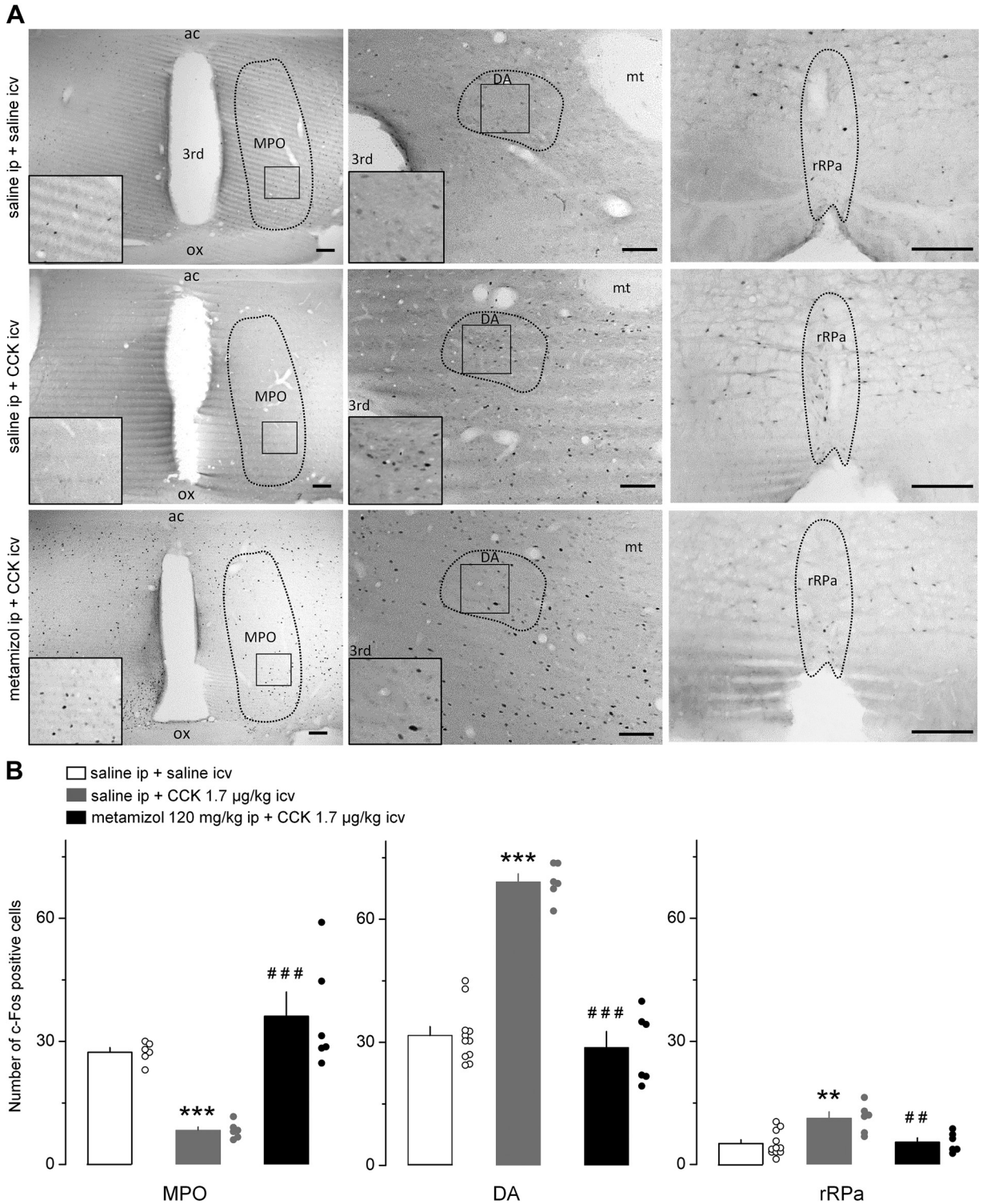

Figure 3. The expression of c-Fos in thermoregulatory nuclei of rats in response to intracerebroventricular (icv) administration of cholecystokinin (CCK; 1.7 µg/kg) or saline after pretreatment at −30 min with an intraperitoneal (ip) infusion of metamizol (120 mg/kg) or saline. *A*: representative photomicrographs of coronal sections from the medial preoptic area (MPO), dorsal hypothalamic area (DA), and rostral raphe pallidus (rRPa) at −0.48, −2.76, and −10.68 mm to Bregma, respectively. The anterior commissure (ac), third ventricle (3rd), optic chiasm (ox), and mamillothalamic tract (mt) are shown as landmarks. Scale bar = 100 µm. *B*: quantitative analyses of c-Fos immunoreactive cells in the MPO, DA, and rRPa. For each group in MPO, $n = 6$. For treatment groups in DA and rRPa, $n = 11$ for saline + saline and $n = 6$ for saline + CCK and for metamizol + CCK. $^{**}P < 0.01$ and $^{***}P < 0.001$, icv CCK vs. saline difference in ip saline-pretreated rats; $\#\#P < 0.01$ and $\#\#\#P < 0.001$, ip metamizol vs. saline difference in icv CCK-treated rats, as determined by one-way ANOVA followed with Student–Newman–Keuls test. Bars represent group means (± SE), individual data are shown as circles.

studies showed that CCK-induced hyperthermia is triggered mainly via CCK_2 receptors (9, 23), thus in our experiments we focused on the role of the CCK_2 receptor in LPS-induced fever. As expected, the intravenous infusion of low-dose LPS in a thermoneutral environment caused a polyphasic febrile response in rats administered intracerebroventricularly with the vehicle of YM022 before LPS; the three phases were peaking at 50–60, 100–120, and 300–330 min (Fig. 6). When the rats were infused intracerebroventricularly with YM022 before the LPS infusion, the first two phases of the fever response to LPS did not differ from what was observed in vehicle-pretreated rats; however, the third febrile phase was markedly attenuated, reaching the level of significance ($P < 0.05$) at 280 and 300–360 min (Fig. 6).

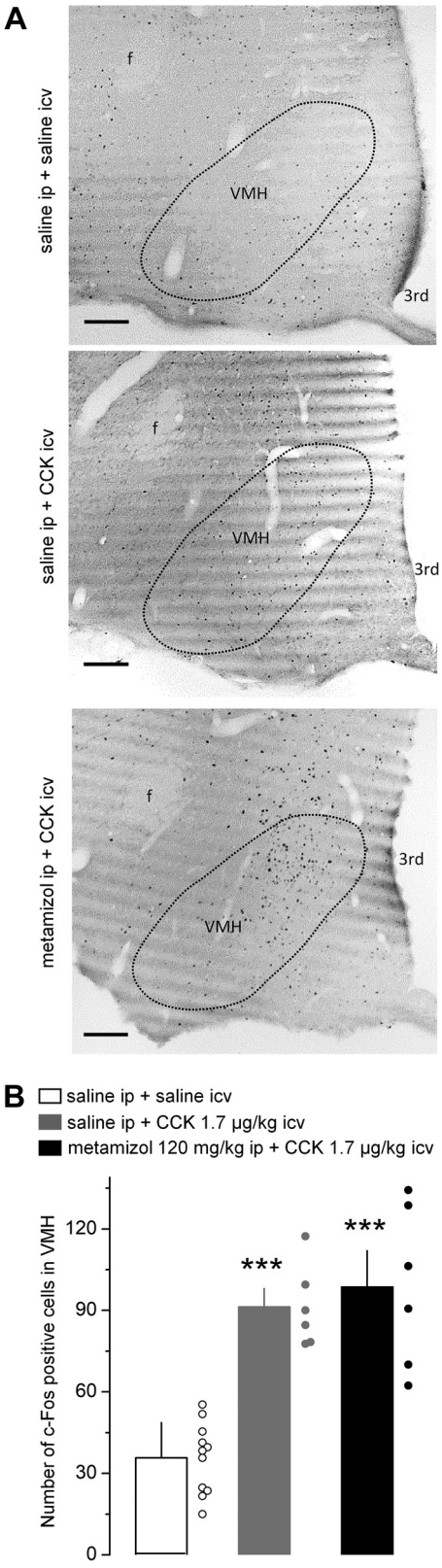

Figure 4. The expression of c-Fos in the ventromedial hypothalamus (VMH) of rats in response to intraperitoneal (ip) and intracerebroventricular (icv) administration of saline ($n = 11$) and to icv administration of CCK (1.7 µg/kg) after pretreatment at −30 min with an ip infusion of metamizol (120 mg/kg; $n = 6$) or saline ($n = 6$). *A*: representative photomicrographs of coronal sections of the VMH at −2.40 mm posterior to Bregma. The third ventricle (3rd) and fornix (f) are shown as landmarks. Scale bar = 100 µm. *B*: quantitative analyses of c-Fos immunoreactive cells in the VMH. ***$P < 0.001$, icv CCK vs. saline difference as determined by one-way ANOVA followed with Student–Newman–Keuls test. Bars represent group means (± SE), individual data are shown as circles.

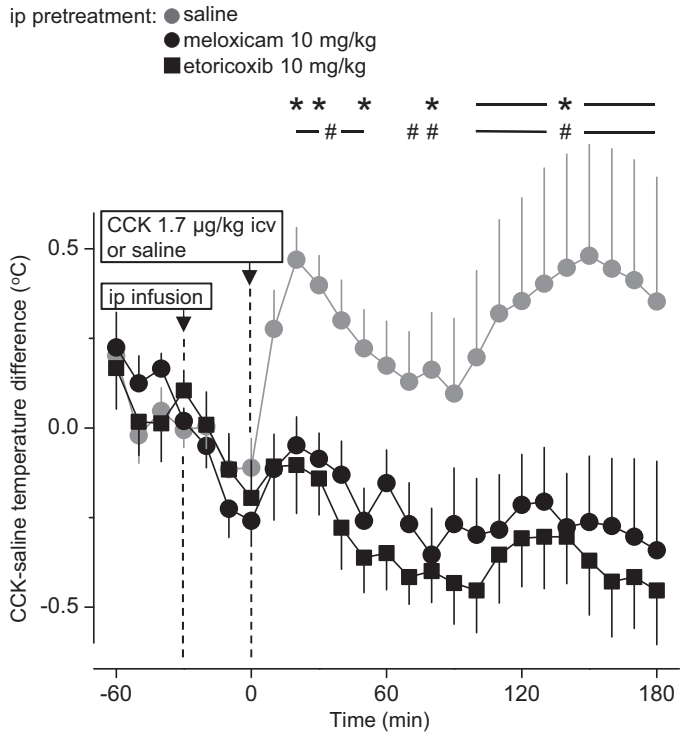

Figure 5. The difference in deep (colonic) body temperature between rats treated intracerebroventricularly (icv) with cholecystokinin (CCK; 1.7 µg/kg) and saline after pretreatment at −30 min with an intraperitoneal (ip) infusion of meloxicam (10 mg/kg; $n = 7$ and 8, respectively), etoricoxib (10 mg/kg; $n = 6$ and 8, respectively), or saline ($n = 9$ and 8, respectively). *$P < 0.05$, ip meloxicam vs. saline difference in icv CCK-treated rats; #$P < 0.05$, ip etoricoxib vs. saline difference in icv CCK-treated rats as determined by two-way ANOVA followed with Student–Newman–Keuls test. Data are presented as means ± SE. At the time of the icv injection, the values of colonic temperature of the rats in the treatment groups were, respectively, 38.5 ± 0.2°C for saline + saline, 38.3 ± 0.2°C for saline + CCK, 38.4 ± 0.1°C for meloxicam + CCK, and 38.5 ± 0.2°C for etoricoxib + CCK. These values did not differ statistically from each other.

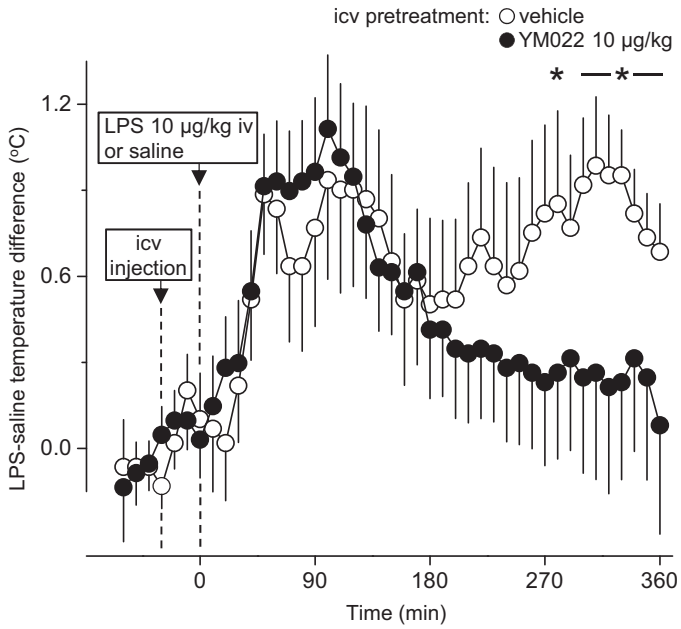

Figure 6. The difference in deep (colonic) body temperature between rats treated intravenously (iv) with lipolysaccharide (LPS; 10 μg/kg) and saline after pretreatment at −30 min with an intracerebroventricular (icv) injection of YM022 (10 μg/kg) or its vehicle. For each group, $n = 6$. *$P < 0.05$, icv YM022 vs. vehicle difference in ip LPS-treated rats, as determined by two-way ANOVA followed with Student–Newman–Keuls test. Data are presented as means ± SE. At the time of the iv injection, the values of colonic temperature of the rats in the treatment groups were, respectively, 38.4 ± 0.1°C for vehicle + saline, 38.4 ± 0.4°C for vehicle + LPS, 38.4 ± 0.4°C for YM022 + saline, and 38.8 ± 0.2°C for YM022 + LPS. These values did not differ statistically from each other.

DISCUSSION

In the present study, for the first time to our knowledge, we show that the hyperthermic response to the intracerebroventricular administration of CCK involves changes in the activity of preoptic (in the MPO), dorsomedial hypothalamic (DA), and raphe (rRPa) neurons within the efferent pathways of autonomic thermoeffectors. Inhibition of COX with nonselective and selective COX-2 inhibitors attenuates these neuronal effects of central CCK. We also show that the pharmacological blockade of CCK_2 receptors reduces the late phase of LPS-induced fever. These findings suggest that interactions between central CCK signaling and the COX pathway are involved in CCK-induced hyperthermia and in the later phases of endotoxin-induced fever. In contrast to the thermoregulatory effects, CCK-induced satiety was not influenced by COX inhibition, indicating that the effects of CCK on food intake are independent from the COX pathway. From this point of view, CCK-induced satiety differs from LPS-induced anorexia, as the latter depends on COX activation (39).

The hyperthermic effect of central CCK has been known for a long time (9, 22). It has been also known that it involves the two main autonomic cold-defense effectors: cutaneous vasoconstriction and nonshivering thermogenesis (9, 10, 22). The centrally induced hyperthermic effect of CCK is mediated by the CCK_2 receptor, which is in contrast with the CCK_1 receptor mediating the hypothermic response to the peripheral CCK administration (9). In a recent study, the importance of CCK-expressing neurons in the lateral parabrachial nucleus was shown, suggesting that these neurons are involved in the transmission of warmth afferent signals from the periphery to the central nervous system, and, consequently, in the recruitment of autonomic heat-defense mechanisms counteracting the elevation of deep body temperature (40). These findings (40) are well in accordance with the body temperature-decreasing effect of peripherally administered CCK, but they do not explain the development of hyperthermia in response to centrally administered CCK. Here, we show that the intracerebroventricular administration of CCK caused changes in the neuronal activation in the MPO, rRPa, and DA, which are well-established brain structures within the efferent pathways of autonomic thermoeffector responses (36, 41–44). In our study, centrally administered CCK decreased the c-Fos immunoreactivity in the MPO but increased it in the DA and the rRPa. The MPO harbors GABAergic neurons, which tonically suppress brown adipose tissue thermogenesis and skin vasoconstriction (45, 46) through their inhibitory projections to the DA and rRPa, from where the sympathoexcitatory drive to brown adipose tissue and skin vessels, respectively, is provided (47, 48). Therefore, our findings are in line with the understanding that CCK reduces the activity of GABAergic neurons in MPO and thereby disinhibits the excitatory DA and rRPa neurons, thus resulting in an increased sympathetic drive to the autonomic cold-defense effectors. A possible explanation for the CCK-induced changes in the hypothalamic neuronal activity could be a direct action of CCK on CCK_2 receptors expressed by these cells. In support of such a scenario, CCK_2 receptors are found in the hypothalamus of adult rats (49–51). However, the level of CCK receptors is lower in the general preoptic area than in other hypothalamic regions (52), and CCK-immunoreactive neurons are restricted to the periventricular and paraventricular hypothalamic nuclei, whereas different preoptic structures have few CCK-immunoreactive cells (53). Moreover, at least one study (54) failed to detect the CCK_2 receptor in both the MPO and dorsomedial hypothalamic nucleus (54). Therefore, an indirect action of CCK on the hypothalamic neurons within the efferent thermoeffector pathways is more plausible.

It was observed that the physiological mechanisms of PGE_1 and CCK-8 hyperthermia are similar in that they both show the same dependence of the effector pattern on the initial body temperature, and both substances increase body temperature to a level that depends on the dose but does not depend on the initial body temperature (9, 55). Furthermore, both PGE_1 and CCK-8, when the hyperthermic response resolves, cause imprecise body temperature regulation in rats (55), which is characteristic of the later febrile phases (56). The imprecise (poikilothermic) type of thermoregulation is further discussed elsewhere (43, 57). Normally, deep body temperature in rats is regulated relatively tightly, due to constant minor adjustments in the skin vasomotor tone seen as frequent changes in the tail-skin temperature (27), but at the end of the hyperthermic response to CCK, pronounced fluctuations in deep body temperature occur due to large waves of tail-skin vasoconstriction and vasodilation repeatedly changing each other (55).

At the time when those phenomenological observations were made, no information was available on whether CCK is involved in the febrile response. Later, multiple interactions

between CCK signaling and the arachidonic acid cascade were found. Activation of the CCK_2 receptor by CCK-8 leads to arachidonic acid production in different cell cultures (58, 59), and a CCK_2 receptor-mediated increase in COX-2 mRNA and protein expression, followed by PGE_2 secretion, was shown in several cell lines (60–62). Accordingly, the contribution of CCK signaling to the mediation of fever was proposed [for review, see Szelenyi et al. (7)]. In the present study, we provide thermophysiological and immunohistochemical evidence for the close interaction between CCK signaling and the COX pathway. First, we showed that the hyperthermic effect of centrally (icv) administered CCK can be completely abolished by nonselective inhibition of COX enzymes with metamizol (also known as dypirone). Then, we demonstrated that the same inhibition also prevented the CCK-induced changes in c-Fos expression observed in neurons within the efferent thermoeffector pathways (i.e., in the MPO, DA, and rRPa). We also found that selective inhibition of COX-2 with two different blockers (viz., meloxicam and etoricoxib) blunted the CCK-induced hyperthermia practically to the same extent as the nonselective COX inhibitor.

At the applied dose of 120 mg/kg, metamizol could be expected to exert maximal inhibition of both COX-1 and COX-2 enzymes, as in humans it elicits nearly complete (94%–97%) COX inhibition already at 14 mg/kg (63). It is also important to note that, at 120 mg/kg dose, metamizol blocked LPS-induced fever, but by itself had no effect on the body temperature in afebrile rats, unlike its effect at higher doses (240–360 mg/kg), at which it caused hypothermia (64). Our findings seem to contradict the earlier report by Szekely et al. (22), which showed that the subcutaneous pretreatment with 10 mg/kg of indomethacin (a nonselective COX inhibitor) had no effect on the hyperthermic response to intracerebroventricular CCK. However, Gamache and Ellis (65) showed that, when indomethacin was injected intraperitoneally to mice at 10 mg/kg, its content in the brain was very low, and it did not reduce brain inflammation, even though it effectively suppressed peripheral inflammation. Indomethacin (10 mg/kg; intramuscularly) also failed to alter arachidonic acid-induced brain edema in another study in rats (66). These results suggest that the applied dose of indomethacin in the study by Szekely et al. (22) was not high enough to efficaciously block COX enzymes in the brain. In contrast to indomethacin, metabolites of metamizol were present in appreciable concentrations in the cerebrospinal fluid of humans after oral administration of metamizol at 14 mg/kg (67). Detectable levels of metamizol metabolites were also found in the brain and spinal cord of mice 2 days after metamizol administration with drinking water (68), as well as in the hypothalamus and cerebrospinal fluid of rats from 0.25 to 8.5 h after the intraperitoneal administration of metamizol at 120 mg/kg (69). Similarly to metamizol, the ability to penetrate the blood-brain barrier was also shown for meloxicam (70, 71) and etoricoxib (72). Both of these drugs inhibit COX-2 more potently than COX-1: the 50% inhibitory concentrations for COX-2 (compared with COX-1) are 2 times lower for meloxicam and 106 times lower for etoricoxib (73). Our finding that these two selective COX-2 inhibitors caused a practically identical to metamizol suppression of CCK-induced hyperthermia exclude the possibility that the observed effects of metamizol were independent of the COX pathway. Moreover, these findings indicate that the activation of COX-2 is required for the hyperthermic response to CCK.

An end product of the COX-2 pathway is PGE_2, which is known as the key mediator of systemic inflammation-associated fever (11, 14, 15, 74). According to the "classical" concepts of febrigenesis, PGE_2 binds to EP3-expressing GABAergic neurons in the preoptic area (including the MPO and the median preoptic nucleus) and suppresses their activity, thereby disinhibiting downstream targets such as the DA and rRPa, which leads to autonomic heat conservation and heat production (11, 13, 14, 46). A recent study by Machado et al. (75) challenged this concept by showing that EP3-expressing glutamatergic neurons in the median preoptic nucleus mediate the febrile response. PGE_2 is thought to inhibit the activity of preoptic neurons (44, 46, 76), and the aforementioned PGE_2-induced changes in the activity of neurons within the efferent thermoeffector pathways are similar to our current immunohistochemistry results observed after the central CCK administration (Fig. 3). Furthermore, the c-Fos expression changes observed in our study were completely blocked by inhibition of COX, thereby supporting the contribution of the COX-2-PGE_2 pathway to CCK-induced hyperthermia.

The rapid onset of the hyperthermic response and the suspected long time lag for COX-2 expression seem mutually contradictory and require explanations. COX-2 is induced by gastrin as an immediate/early gene response in CCK_2 receptor-expressing cell cultures (62). Furthermore, increased COX-2 expression was detected within 30–40 min in the brain of rodents in response to electroconvulsive seizures (77) or systemic administration of LPS (16, 78, 79). The increase in COX-2 expression was even more rapid (~15 min, latency) in human cells incubated with interleukin-1 (80). Expressional upregulation is an established mechanism for activation of COX-2 in systemic inflammation (11, 74), in which it mediates the prompt development of fever. Fever starts as soon as at ~20 min after systemic LPS administration in mice and rabbits (16, 81) and at less than 10 min after systemic interleukin-1 administration in rabbits (81, 82). These findings suggest that the rapid development of CCK-induced hyperthermia could be due to COX-2 upregulation.

It should also be noted that COX-2, while well-known as an inducible enzyme, is also expressed constitutively in the brain, including the hypothalamus (77, 83, 84). Posttranscriptional processes can influence the function of COX-2 by increasing its activity (85) and possibly by slowing down its degradation (86, 87). Moreover, CCK_2 receptors were found to be involved in these processes (88). It is thought that the constitutively expressed COX-2 in the brain modulates synaptic transmission (89), but it may also participate in other physiological functions, including the rapid development of hyperthermia. Further research is needed to determine the exact mechanisms of interactions between CCK and the COX-2 pathway.

Last, we showed that the intracerebroventricular administration of the selective CCK_2 receptor antagonist YM022 attenuated endotoxin-induced fever, which is in harmony with previous results obtained with a different CCK_2 antagonist

(22), as well as with the results obtained in mice genetically lacking the CCK_2 receptor (23). Our findings support and extend the results of previous research. A caveat in knockout models (90–92), including the CCK_2 receptor-deficient mice (92–96), is that compensatory mechanisms for the lack of the gene develop. The observations of the fever response in CCK_2 receptor knockout mice could be confounded by such compensatory mechanisms. Furthermore, in addition to its abundant expression in the central nervous system, the CCK_2 receptor is also expressed in peripheral tissues (e.g., throughout the gastrointestinal tract) (1). Since the CCK_2 receptor is absent in all cells of the knockout mice, it cannot be firmly established whether the observed effect is due to a central or peripheral action. An alternative approach, as suggested, e.g., by Weiland et al. (97), is to study the role of the CCK_2 receptor by using pharmacological antagonists, which do not induce compensatory mechanisms, at least upon acute administration.

In our study, we used YM022, a highly selective and potent CCK_2 antagonist [K_i = 0.26 nM for the CCK_2 receptor and 270 nM for the CCK_1 receptor; 50% inhibitory concentration of 4 nM; rat data (98)]. YM022 causes an extraordinarily long-lasting blockade of CCK_2 receptors; it is biologically effective for days and weeks following the administration of a single dose (99, 100). When we infused YM022 (0.01 mg/kg) into the brain of rats, it attenuated the late phase of the polyphasic LPS fever, but not the early phase. The short-lasting early (first) phase of LPS fever starts within minutes and is thought to be mediated peripherally (16, 74, 101, 102). Because our experiment involved the intrabrain administration of YM022, the antagonist was unlikely to reach peripheral targets before the first phase occurred. Our finding extends the work of Szekely et al. (22), who used a different CCK_2 antagonist, L-365,260 (K_i of 40 nM for the CCK_2 receptor and 14,000 nM for the CCK_1 receptor; see Ref. 98) and showed that, administered subcutaneously, this antagonist attenuated the first phase of LPS fever (the effect that agrees with a peripheral route of administration) but had no effect on the later phases. It should be noted, however, that L-365,260 is a short-lived compound; following the oral administration, its plasma half-life in dogs, monkeys, and rats is only a few tens of minutes (103, 104). With such short dynamics, it is unlikely that the compound could affect the later febrile phases that occur a few hours after LPS administration (74, 105). Since the inhibition of CCK_2 receptors does not attenuate the hyperthermic response to intracerebroventricular PGE (9), CCK is likely to modulate the production of PGE and not the effects of PGE on its receptors.

The later phases of fever are mediated by PGE_2 produced mainly in the preoptic hypothalamus (11), hence our results suggest that a CCK_2 receptor-mediated effect on hypothalamic cells contributes to the development of fever. Upon inflammatory stimulation, PGE_2 can be produced by different cell types in the brain, including endothelial cells (106, 107), perivascular macrophages (108, 109) and microglia (108, 110), astrocytes (111, 112), and neurons (113). Among these cell types, the CCK_2 receptor is abundantly expressed in astrocytes (114–116) and its stimulation with CCK-8 leads to the release of arachidonic acid via diacylglycerol lipase and phospholipase A_2 activation (59, 117). These two enzymes are also involved in the COX-2-mediated synthesis of PGE_2 during the febrile response to systemic inflammation (11). Based on these findings, it can be assumed that the COX-2-PGE_2 pathway functions as a downstream mediator of CCK_2 receptor activation, possibly in astrocytes, but perhaps in other cell types as well. In line with this assumption, COX-2 was suggested as a downstream player of the CCK_2 receptor activation in epithelial, fibroblast, and adenocarcinoma cell lines (118). Alternative possibilities for the interaction between the two systems cannot be excluded, for example, via a direct neuronal mediation, as a CCK_2 receptor-mediated PGE_2 release was detected in the cerebrospinal fluid after microinjection of CCK-8 into the rostral ventromedial medulla (119), which contains sympathetic premotor neurons for autonomic thermoeffectors (13). It is also possible that CCK signaling promotes COX-2 transcription in brain endothelial cells. These cells produce PGE_2 later during the response to LPS and hence play a major role in the maintenance (as opposed to initiation) of fever (106, 107). In line with that possibility, the presence of CCK_2 receptors on endothelial cells was reported in the human umbilical vein (120), porcine coronary arteries (121), and rat pulmonary vessels (122). Furthermore, CCK increased the transport of insulin into the central nervous system in rats, likely by acting directly on CCK_1 receptors on endotheliocytes in brain capillaries (123). It has also to be noted, however, that CCK_2 receptors were not found in the endothelium of bovine cerebral arteries in one study, and that the vascular effects of CCK observed in that study were attributed to CCK_2 receptors on perivascular neuronal endings (124).

It should be also mentioned that metamizol did not influence CCK-induced satiety and neuronal activation in the VMH in the present study. We observed a reduction in the fasting-induced food intake after the central administration of CCK, which is in agreement with earlier findings (125, 126). In the VMH, which is a feeding-related brain region expressing CCK_2 receptors (1), CCK caused an increase in c-Fos expression, which is in line with the previous results showing an increased neuronal activity in the VMH after the intracerebroventricular CCK administration in vivo (125) or after CCK application in vitro (127). Importantly, the inhibition of COX enzymes did not influence CCK-induced satiety and c-Fos expression in VMH in the present study. These results indicate that, in contrast to the hyperthermic effect, the satiety effect of CCK is independent of the COX pathway. The different dependence of the thermal and satiety effects on COX distinguishes the CCK-induced responses from LPS-induced fever and anorexia, as the latter two responses are both dependent on COX-2, even though they are triggered via distinct cell types (39). Similarly to CCK, different mechanisms of the febrile and anorexic effects were found also for other substances, for example, Fortier et al. (128) demonstrated that the viral mimetic polyinosinic:polycytidylic acid induces fever, but not anorexia, through an interleukin-1 and PG-dependent mechanism.

In summary, the present study shows that the hyperthermic response to central CCK depends on the COX-2 pathway, and that central CCK_2 receptors are involved in the maintenance of LPS-induced fever. These findings advance our understanding of the interactions between CCK signaling and the COX pathways in the brain. They also suggest that

the CCK_2 receptor should be evaluated as a potential target in the pharmacological management of fever.

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The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

P.K., N.F., B.G., A.A.R., and A.G. conceived and designed research; P.K., N.F., B.G., A.M., E.P., K.F., E.O., L.K., Z.R., and A.G. performed experiments; P.K., N.F., B.G., A.M., E.P., K.F., E.O., L.K., Z.R., and A.G. analyzed data; P.K., N.F., Z.R., and A.G. interpreted results of experiments; P.K. and B.G. prepared figures; P.K., A.A.R., and A.G. drafted manuscript; B.G., A.M., E.P., L.K., A.A.R., Z.R., and A.G. edited and revised manuscript; P.K., N.F., B.G., A.M., E.P., K.F., E.O., L.K., A.A.R., Z.R., and A.G. approved final version of manuscript.

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