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

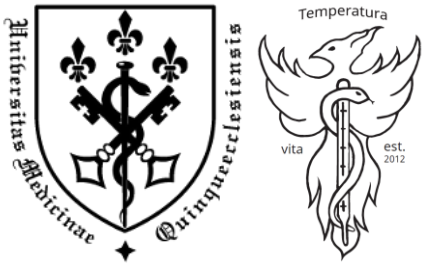
Department of Thermophysiology, Institute for Translational Medicine

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



Pécs, 2022

Introduction

1.1. 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 (47). 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.

1.2. Cholecystokinin (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 (35). 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 (16, 17, 28). In the early 1980s, a role for CCK in the thermoregulation system, which is also part of energy balance (15), was suggested (9), and later it was concluded that activation of the two CCK receptors differently affects body temperature (62). When administered peripherally, CCK caused hypothermia, which was mediated by CCK_1 receptors (43, 60), while the administration of CCK into the central nervous system resulted in fever-like hyperthermia through the activation of CCK_2 receptors (58, 60). The hyperthermic response to CCK suggested a link between central CCK signaling and systemic inflammation-associated fever.

1.3. 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 (14). 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 (54). In the brain [for reviews, see (14, 33)], 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). 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).

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 (61).

1.4. 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) (58-60). Moreover, the first phase of LPS fever was attenuated by a pharmacological antagonist of the CCK_2 receptor (59), while the genetic disruption of the CCK_2 receptor gene suppressed the early and late changes in T_b induced by LPS (63), suggesting that central CCK signaling modulates the fever response. However, COX inhibition with indomethacin did not affect CCK-8-induced hyperthermia (25, 59) and PGE-induced hyperthermia was not influenced by CCK receptor blockers (60), which results question the interaction between the thermal actions of CCK signaling and the COX–PGE pathway.

1.5. 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 (11, 24). 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 (24, 40). 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 (1), 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 (8), decreased sweating (29), and more pronounced skin vasoconstriction (19, 32), consequently in elevated deep T_b (18); that is the same pattern of thermoregulatory effector recruitment which can be observed as part of the cold-defence responses (46).

1.6. 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. (7, 56). 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 (41). Endurance exercise capacity at a high T_a is impaired by heat stress prior to exercise (39), and hyperthermia induces fatigue during short intense activities and prolonged exercise in the heat (37). On the contrary, physical cooling of the body before and during exercise in the warmth improves exercise endurance and reduces cardiovascular strain (20). A recent meta-analysis of 45 studies also concluded that physical cooling improves aerobic and anaerobic exercise performance in hot conditions (10).

1.7. 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 (18, 29), 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 (12, 23, 34, 44, 45, 50, 53, 55, 57), while a decent number of studies found no effect (3-5, 51). 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 (6, 12, 29, 50), whereas other authors found that internally applied menthol is more effective (23, 34, 44, 55, 57), and yet others showed no effect of menthol independently from the application method (3-5, 51, 53).

2. 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 (27);

- 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 (26).

3. Materials and methods

3.1. CCK as an endogenous hyperthermic substance

3.1.1. Animals

Our experiments were performed in 220 adult male Wistar rats (27). The rats were extensively handled and habituated to staying inside wire-mesh cylindrical confiners, as in earlier studies (13, 48). 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.

3.1.2. Surgeries

Each rat was implanted with an icv cannula and with either an intraperitoneal (ip) or an intravenous (iv) catheter in the same anesthesia. The experiments were performed 4-7 days after the surgery. Implantation of the icv cannula was performed as described earlier (2).

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. 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 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.

3.1.3. Thermocouple thermometry

In the thermocouple thermometry setup, the rat was placed in a cylindrical confiner and equipped with a copper-constantan thermocouple 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 connected to a computer. Rats in their confiners were then placed into a temperature-controlled incubator 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 (48). 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. The ip or iv catheter was also connected to a PE-50 extension filled with the drug of interest or saline.

3.1.4. 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.

3.1.5. Immunohistochemistry

The c-Fos staining was performed as in earlier studies (2, 30). 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).

3.1.6. 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. The effects were considered significant when $P < 0.05$. All data are reported as mean ± standard error (SE).

3.2. Menthol as an exogenous hyperthermic substance

Our meta-analysis (26) 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.

3.2.1. 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.

3.2.2. 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. 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, 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).

3.2.3. 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 $P < 0.05$.

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 (21).

4. Results

4.1. CCK as an endogenous hyperthermic substance

4.1.1. 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 (27). As expected, in response to CCK the rats developed a marked elevation in T_b, whereas administration of saline did not cause any effects. 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 ($P < 0.05$).

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%, $P < 0.05$). Importantly, however, we did not detect any significant difference in CCK-induced anorexia between the metamizol- and saline-pretreated rats [ANOVA, $F_{(1,21)} = 0.532$, $P = 0.474$].

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

We hypothesized that blocking CCK-induced hyperthermia with a COX-inhibitor changes the activation of hypothalamic efferent neurons controlling autonomic thermoeffector responses. To test this hypothesis, we measured expression of the inducible transcription factor c-Fos, a marker of neuronal activation in the MPO, DA, and rRPa, which nuclei contribute to the autonomic thermoregulatory responses to cooling and PGE_2. 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, and to study whether the observed changes can be influenced by the inhibition of COX. 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.

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

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. 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.

4.1.4. 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 (14). Previous studies showed that CCK-induced hyperthermia is triggered mainly via CCK_2 receptors in the brain (60, 63), 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. 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.

4.2. Menthol as an exogenous hyperthermic substance

4.2.1. 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 (26).

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). Finally, 17 papers provided eligible data for qualitative and quantitative analyses (3-6, 12, 18, 23, 29, 34, 44, 45, 49-51, 53, 55, 57).

4.2.2. 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 (11), the TS score decreased in the menthol-treated groups as compared to controls in seven studies (3, 5, 6, 23, 49, 50, 57), while two studies reported a slight increase in TS (12, 50). Accordingly, the overall WMD between the menthol-treated and control groups

was − 1.65 (95% CI, − 2.96 to − 0.33; P = 0.014). 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), which indicates that the perceived temperature was more comfortable (i.e., not so hot) after menthol administration compared to controls.

4.2.3. 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). 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). 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).

4.2.4. 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) and TTE tests (WMD = 1.04 min; 95% CI, − 0.47 to 2.55 min).

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. 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). The WMD between the treatment groups was markedly bigger in the higher than in the lower BMI subgroup ($P < 0.001$). 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.

5. Discussion

In our study, we investigated different aspects of hyperthermia. 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 (27), 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.

The hyperthermic effect of central CCK has been known for long (59, 60). It was also shown that it involves the increased activity of the two main autonomic cold-defense effectors: cutaneous vasoconstriction and non-shivering thermogenesis (59, 60). 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 (60). 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 (47). In our study (27), 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 (38) through their inhibitory projections to the DA and

rRPa, from where the sympathoexcitatory drive to brown adipose tissue and skin vessels, respectively, is provided (42). 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 (22).

In our study (27), we provided 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 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.

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 (59) and with mice genetically lacking the CCK_2 receptor (64). 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 (60), 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 (14), our results suggest that a CCK_2 receptor-mediated effect on cells in this region contributes to the development of fever.

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 (52, 65). In the VMH, which is a feeding-related brain region expressing CCK_2 receptors (36), 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* (52) or direct CCK application *in vitro* (31). 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.

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 (26). 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. 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.

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.

6. 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 (27). 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 (26).

7. Acknowledgements

First and foremost, I am grateful to my supervisor, András Garami for his invaluable help, continuous support, and patience during my PhD studies.

I want to thank my program leader Péter Hegyi for providing the inspiring work environment and for the help to learn the methodology of meta-analysis.

I would also like to thank Balázs Gaszner for the cooperation and help in immunohistochemistry and Andrej A. Romanovsky for his professional help.

I am extremely grateful to Alexandra Mikó and Nóra Füredi for their initial inspiration and support and for helping me to master new experimental methods.

I want to express my special thank for the research team at the Department of Thermophysiology – Zoltán Rumbus, Eszter Pákai, Leonardo Kelava, Kata Fekete and Anikó Várnagyné Rózsafi for a productive and cheerful time spent together in the laboratory.

I would also like to extend my thanks to all my colleagues in the Institute for Translational Medicine at the University of Pécs.

8. References

1. **Almeida MC, Hew-Butler T, Soriano RN, Rao S, Wang W, Wang J, Tamayo N, Oliveira DL, Nucci TB, Aryal P, Garami A, Bautista D, Gavva NR, Romanovsky AA**. Pharmacological blockade of the cold receptor TRPM8 attenuates autonomic and behavioral cold defenses and decreases deep body temperature. *J Neurosci* 32: 2086-2099, 2012.
2. **Banki E, Pakai E, Gaszner B, Zsiboras C, Czett A, Bhuddi PRP, Hashimoto H, Toth G, Tamas A, Reglodi D, Garami A**. Characterization of the thermoregulatory response to pituitary adenylate cyclase-activating polypeptide in rodents. *J Mol Neurosci* 54: 543-554, 2014.
3. **Barwood MJ, Corbett J, Thomas K, Twentyman P**. Relieving thermal discomfort: effects of sprayed L-menthol on perception, performance, and time trial cycling in the heat. *Scand J Med Sci Sports* 25: 211-218, 2015.
4. **Barwood MJ, Corbett J, White D, James J**. Early change in thermal perception is not a driver of anticipatory exercise pacing in the heat. *Br J Sports Med* 46: 936-942, 2012.
5. **Barwood MJ, Corbett J, White DK**. Spraying with 0.20% L-menthol does not enhance 5 km running performance in the heat in untrained runners. *J Sports Med Phys Fitness* 54: 595-604, 2014.
6. **Barwood MJ, Kupusarevic J, Goodall S**. Enhancement of exercise capacity in the heat with repeated menthol-spray application. *Int J Sports Physiol Perform* 14: 644-649, 2019.
7. **Best R, Spears IR, Hurst P, Berger NJA**. The development of a menthol solution for use during sport and exercise. *Beverages* 4: 2018.
8. **Bongers CCWG, Hopman MTE, Eijsvogels TMH**. Cooling interventions for athletes: An overview of effectiveness, physiological mechanisms, and practical considerations. *Temperature* 4: 60-78, 2017.
9. **Clark WG, Lipton JM**. Changes in body temperature after administration of amino acids, peptides, dopamine, neuroleptics and related agents: II. *Neurosci Biobehav Rev* 9: 299-371, 1985.
10. **Douzi W, Dugué B, Vinches L, Al Sayed C, Hallé S, Bosquet L, Dupuy O**. Cooling during exercise enhances performances, but the cooled body areas matter: A systematic review with meta-analyses. *Scand J Med Sci Sports* 29: 1660-1676, 2019.
11. **Eccles R**. Menthol and related cooling compounds. *J Pharm Pharmacol* 46: 618-630, 1994.
12. **Flood TR, Waldron M, Jeffries O**. Oral L-menthol reduces thermal sensation, increases work-rate and extends time to exhaustion, in the heat at a fixed rating of perceived exertion. *Eur J Appl Physiol* 117: 1501-1512, 2017.
13. **Garami A, Pakai E, McDonald HA, Reilly RM, Gomtsyan A, Corrigan JJ, Pinter E, Zhu DXD, Lehto SG, Gavva NR, Kym PR, Romanovsky AA**. TRPV1 antagonists that cause hypothermia, instead of hyperthermia, in rodents: compounds' pharmacological profiles, in vivo targets, thermoeffectors recruited and implications for drug development. *Acta Physiol* 223: e13038, 2018.
14. **Garami A, Steiner AA, Romanovsky AA**. Fever and hypothermia in systemic inflammation. *Handb Clin Neurol* 157: 565-597, 2018.
15. **Garami A, Székely M**. Body temperature: its regulation in framework of energy balance. *Temperature* 6: 28-29, 2014.
16. **Gibbs J, Smith GP**. Cholecystokinin and satiety in rats and rhesus monkeys. *Am J Clin Nutr* 30: 758-761, 1977.
17. **Gibbs J, Young RC, Smith GP**. Cholecystokinin elicits satiety in rats with open gastric fistulas. *Nature* 245: 323-325, 1973.

18. **Gillis DJ, House JR, Tipton MJ**. The influence of menthol on thermoregulation and perception during exercise in warm, humid conditions. *Eur J Appl Physiol* 110: 609-618, 2010.
19. **Gillis DJ, Weston N, House JR, Tipton MJ**. Influence of repeated daily menthol exposure on human temperature regulation and perception. *Physiol Behav* 139: 511-518, 2015.
20. **Hasegawa H, Takatori T, Komura T, Yamasaki M**. Combined effects of pre-cooling and water ingestion on thermoregulation and physical capacity during exercise in a hot environment. *J Sports Sci* 24: 3-9, 2006.
21. **Higgins JPT, Altman DG, Gøtzsche PC, Jüni P, Moher D, Oxman AD, Savović J, Schulz KF, Weeks L, Sterne JAC**. The Cochrane Collaboration's tool for assessing risk of bias in randomised trials. *BMJ* 343: d5928, 2011.
22. **Ito M, Matsui T, Taniguchi T, Tsukamoto T, Murayama T, Arima N, Nakata H, Chiba T, Chihara K**. Functional characterization of a human brain cholecystokinin-B receptor. A trophic effect of cholecystokinin and gastrin. *J Biol Chem* 268: 18300-18305, 1993.
23. **Jeffries O, Goldsmith M, Waldron M**. L-Menthol mouth rinse or ice slurry ingestion during the latter stages of exercise in the heat provide a novel stimulus to enhance performance despite elevation in mean body temperature. *Eur J Appl Physiol* 118: 2435-2442, 2018.
24. **Kamatou GPP, Vermaak I, Viljoen AM, Lawrence BM**. Menthol: A simple monoterpene with remarkable biological properties. *Phytochemistry* 96: 15-25, 2013.
25. **Kandasamy SB, Williams BA**. Cholecystokinin-octapeptide-induced hyperthermia in guinea-pigs. *Experientia* 39: 1282-1284, 1983.
26. **Keringer P, Farkas N, Gede N, Hegyi P, Rumbus Z, Lohinai Z, Solymar M, Ruksakiet K, Varga G, Garami A**. Menthol can be safely applied to improve thermal perception during physical exercise: a meta-analysis of randomized controlled trials. *Sci Rep* 10: 13636, 2020.
27. **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. *Am J Physiol Endocrinol Metab* 322: E10-E23, 2022.
28. **Kissileff HR, Pi-Sunyer FX, Thornton J, Smith GP**. C-terminal octapeptide of cholecystokinin decreases food intake in man. *Am J Clin Nutr* 34: 154-160, 1981.
29. **Kounalakis SN, Botonis PG, Koskolou MD, Geladas ND**. The effect of menthol application to the skin on sweating rate response during exercise in swimmers and controls. *Eur J Appl Physiol* 109: 183-189, 2010.
30. **Kovacs LA, Schiessl JA, Nafz AE, Csernus V, Gaszner B**. Both basal and acute restraint stress-induced c-Fos expression is influenced by age in the extended amygdala and brainstem stress centers in male rats. *Front Aging Neurosci* 10: 248, 2018.
31. **Kow LM, Pfaff DW**. CCK-8 stimulation of ventromedial hypothalamic neurons in vitro: a feeding-relevant event? *Peptides* 7: 473-479, 1986.
32. **Lee JY, Nakao K, Bakri I, Tochihara Y**. Body regional influences of l-menthol application on the alleviation of heat strain while wearing firefighter's protective clothing. *Eur J Appl Physiol* 112: 2171-2183, 2012.
33. **Morrison SF, Nakamura K**. Central mechanisms for thermoregulation. *Annu Rev Physiol* 10: 285-308, 2019.
34. **Mundel T, Jones DA**. The effects of swilling an L(-)-menthol solution during exercise in the heat. *Eur J Appl Physiol* 109: 59-65, 2010.
35. **Noble F, Wank SA, Crawley JN, Bradwejn J, Seroogy KB, Hamon M, Roques BP**. International union of pharmacology. XXI. structure, distribution, and functions of cholecystokinin receptors. *Pharmacol Rev* 51: 745, 1999.

36. **Noble F, Wank SA, Crawley JN, Bradwejn J, Seroogy KB, Hamon M, Roques BP**. International union of pharmacology. XXI. structure, distribution, and functions of cholecystokinin receptors. *Pharmacol Rev* 51: 745-781, 1999.
37. **Nybo L**. Hyperthermia and fatigue. *J Appl Physiol* 104: 871-878, 2008.
38. **Osaka T**. Cold-induced thermogenesis mediated by GABA in the preoptic area of anesthetized rats. *Am J Physiol Regul Integr Comp Physiol* 287: R306-R313, 2004.
39. **Otani H, Kaya M, Tamaki A, Watson P**. Separate and combined effects of exposure to heat stress and mental fatigue on endurance exercise capacity in the heat. *Eur J Appl Physiol* 117: 119-129, 2017.
40. **Patel T, Ishiuji Y, Yosipovitch G**. Menthol: A refreshing look at this ancient compound. *J Am Acad Dermatol* 57: 873-878, 2007.
41. **Racinais S, Cocking S, Periard JD**. Sports and environmental temperature: From warming-up to heating-up. *Temperature* 4: 227-257, 2017.
42. **Rathner JA, Madden CJ, Morrison SF**. Central pathway for spontaneous and prostaglandin E2-evoked cutaneous vasoconstriction. *Am J Physiol Regul Integr Comp Physiol* 295: R343-R354, 2008.
43. **Rezayat M, Ravandeh N, Zarrindast MR**. Cholecystokinin and morphine-induced hypothermia. *Eur Neuropsychopharmacol* 9: 219-225, 1999.
44. **Riera F, Trong TT, Sinnapah S, Hue O**. Physical and perceptual cooling with beverages to increase cycle performance in a tropical climate. *PloS one* 9: e103718, 2014.
45. **Rinaldi K, Trong TT, Riera F, Appel K, Hue O**. Immersion with menthol improves recovery between 2 cycling exercises in hot and humid environment. *Appl Physiol Nutr Metab* 43: 902-908, 2018.
46. **Romanovsky AA**. The thermoregulation system and how it works. *Handb Clin Neurol* 156: 3-43, 2018.
47. **Romanovsky AA**. The thermoregulation system and how it works. In: *Handb Clin Neurol*, edited by Romanovsky AAElsevier, 2018, p. 3-43.
48. **Romanovsky AA, Ivanov AI, Shimansky YP**. Selected contribution: ambient temperature for experiments in rats: a new method for determining the zone of thermal neutrality. *J Appl Physiol* 92: 2667-2679, 2002.
49. **Saldaris JM, Landers GJ, Lay BS**. Physical and perceptual cooling: Improving cognitive function, mood disturbance and time to fatigue in the heat. *Scand J Med Sci Sports* 30: 801-811, 2020.
50. **Schlader ZJ, Simmons SE, Stannard SR, Mundel T**. The independent roles of temperature and thermal perception in the control of human thermoregulatory behavior. *Physiol Behav* 103: 217-224, 2011.
51. **Shepherd K, Peart DJ**. Aerobic capacity is not improved following 10-day supplementation with peppermint essential oil. *Appl Physiol Nutr Metab* 42: 558-561, 2017.
52. **Shiraishi T**. CCK as a central satiety factor: behavioral and electrophysiological evidence. *Physiol Behav* 48: 879-885, 1990.
53. **Sonmez G, Çolak M, Sonmez S, Schoenfeld B**. Effects of oral supplementation of mint extract on muscle pain and blood lactate. *Biomed Hum Kinet* 2: 66-69, 2010.
54. **Steiner AA, Ivanov AI, Serrats J, Hosokawa H, Phayre AN, Robbins JR, Roberts JL, Kobayashi S, Matsumura K, Sawchenko PE, Romanovsky AA**. Cellular and molecular bases of the initiation of fever. *PLoS biology* 4: e284-e284, 2006.
55. **Stevens CJ, Bennett KJ, Sculley DV, Callister R, Taylor L, Dascombe BJ**. A comparison of mixed-method cooling interventions on preloaded running performance in the heat. *J Strength Cond Res* 31: 620-629, 2017.

56. **Stevens CJ, Best R**. Menthol: A fresh ergogenic aid for athletic performance. *Sports Med* 47: 1035-1042, 2017.
57. **Stevens CJ, Thoseby B, Sculley DV, Callister R, Taylor L, Dascombe BJ**. Running performance and thermal sensation in the heat are improved with menthol mouth rinse but not ice slurry ingestion. *Scand J Med Sci Sports* 26: 1209-1216, 2016.
58. **Sugimoto N, Simons CT, Romanovsky AA**. Vagotomy does not affect thermal responsiveness to intrabrain prostaglandin E2 and cholecystokinin octapeptide. *Brain Res* 844: 157-163, 1999.
59. **Szekely M, Szelenyi Z, Balasko M**. Cholecystokinin participates in the mediation of fever. *Pflugers Arch* 428: 671-673, 1994.
60. **Szelenyi Z, Bartho L, Szekely M, Romanovsky AA**. Cholecystokinin octapeptide (CCK-8) injected into a cerebral ventricle induces a fever-like thermoregulatory response mediated by type B CCK-receptors in the rat. *Brain Res* 638: 69-77, 1994.
61. **Szelenyi Z, Hummel Z, Szekely M, Petervari E**. CCK-8 and PGE1: central effects on circadian body temperature and activity rhythms in rats. *Physiol Behav* 81: 615-621, 2004.
62. **Szelenyi Z, Szekely M, Hummel Z, Balasko M, Romanovsky AA, Petervari E**. Cholecystokinin: possible mediator of fever and hypothermia. *Front Biosci* 9: 301-308, 2004.
63. **Weiland TJ, Voudouris NJ, Kent S**. CCK(2) receptor nullification attenuates lipopolysaccharide-induced sickness behavior. *Am J Physiol Regul Integr Comp Physiol* 292: 112-123, 2007.
64. **Weiland TJ, Voudouris NJ, Kent S**. CCK(2) receptor nullification attenuates lipopolysaccharide-induced sickness behavior. *Am J Physiol Regul Integr Comp Physiol* 292: R112-R123, 2007.
65. **Willis GL, Hansky J, Smith GC**. Ventricular, paraventricular and circumventricular structures involved in peptide-induced satiety. *Regul Pept* 9: 87-99, 1984.

9. 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])**

Keringer P, Farkas N, Gede N, Hegyi P, Rumbus Z, Lohinai Zs, Solymar M, Ruksakiet K, Varga G, Garami A: Menthol can be safely applied to improve thermal perception during physical exercise: a meta-analysis of randomized controlled trials. Scientific Reports. 2020;10(1):1-12. **(IF 4.379)**

Keringer P, Rumbus Z: The interaction between neurokinin-1 receptors and cyclooxygenase-2 in fever genesis. Temperature. 2019;6(1):4-6.

Other publications, not related to the topic of the PhD thesis:

Janka EA, Varvolgyi T, Sipos Z, Soos A, Hegyi P, Kiss Sz, Dembrovszky F, Csupor D, **Keringer P**, Pecsi D, Varju-Solymar M, Emri G: Predictive performance of serum S100B versus LDH in melanoma patients: a systematic review and meta-analysis. Frontiers in Oncology. 2021; Epub ahead of print. **(IF 5.66 [2020])**

Olah E, Rumbus Z, Kormos V, Tekus V, Pakai E, Wilson HV, Fekete K, Solymar M, Kelava L, **Keringer P**, Gaszner B, Whiteman M, Keeble J, Pinter E, Garami A: The hypothermic effect of hydrogen sulfide is mediated by the transient receptor potential ankyrin-1 channel in mice. Pharmaceuticals. 2021;14(10):992. **(IF 5.863 [2020])**

Kelava L, Nemeth D, Hegyi P, **Keringer P**, Kovacs DK, Balasko M, Solymar M, Pakai E, Rumbus Z, Garami A: Dietary supplementation of transient receptor potential vanilloid-1 channel agonists reduces serum total cholesterol level: a meta-analysis of controlled human trials. Critical Reviews in Food Science and Nutrition. 2021; doi: https://doi.org/10.1080/10408398.2021.1910138. **(IF 11.176 [2020])**

Garai J, Kreko M, Orfi L, Jakus PB, Rumbus Z, **Keringer P**, Garami A, Vamos E, Kovacs D, Vantus VB, Radnai B, Lorand T: Tetralone derivatives are MIF tautomerase inhibitors and attenuate macrophage activation and amplify the hypothermic response in endotoxemic mice. Journal of Enzyme Inhibition and Medicinal Chemistry. 2021;36(1):1357-1369. **(IF 5.051 [2020])**

Sousa-Valente J, Alawi KM, **Keringer P**, Bharde S, Ayaz F, Saleque N, Kodji X, Thapa D, Argunhan F, Brain SD: Examining the role of transient receptor potential canonical 5 (TRPC5) in osteoarthritis. Osteoarthritis and Cartilage Open. 2020;2(4):100119.

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. **(IF 6.429)**