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RESEARCH ARTICLE

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

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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,

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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

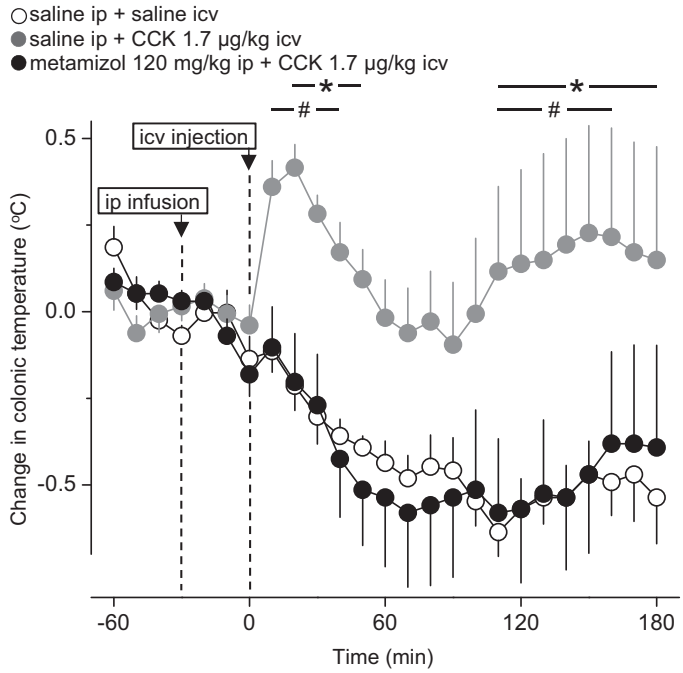

Figure 1. Deep (colonic) body temperature responses of rats to intracerebroventricular (icv) administration of cholecystokinin (CCK; 1.7 μg/kg) or saline after pretreatment at −30 min with an intraperitoneal (ip) infusion of metamizol (120 mg/kg) or saline. For each group, $n = 9$. *$P < 0.05$, icv CCK 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.

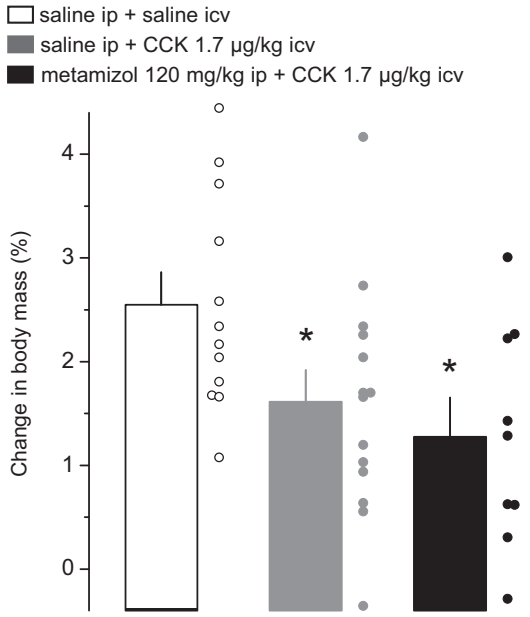

Figure 2. Changes in body mass of rats in response to intraperitoneal (ip) and intracerebroventricular (icv) administration of saline ($n = 12$) and to icv administration of cholecystokinin (CCK; 1.7 μg/kg) after pretreatment at −30 min with an ip infusion of metamizol (120 mg/kg; $n = 9$) or saline ($n = 14$). *$P < 0.05$, 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

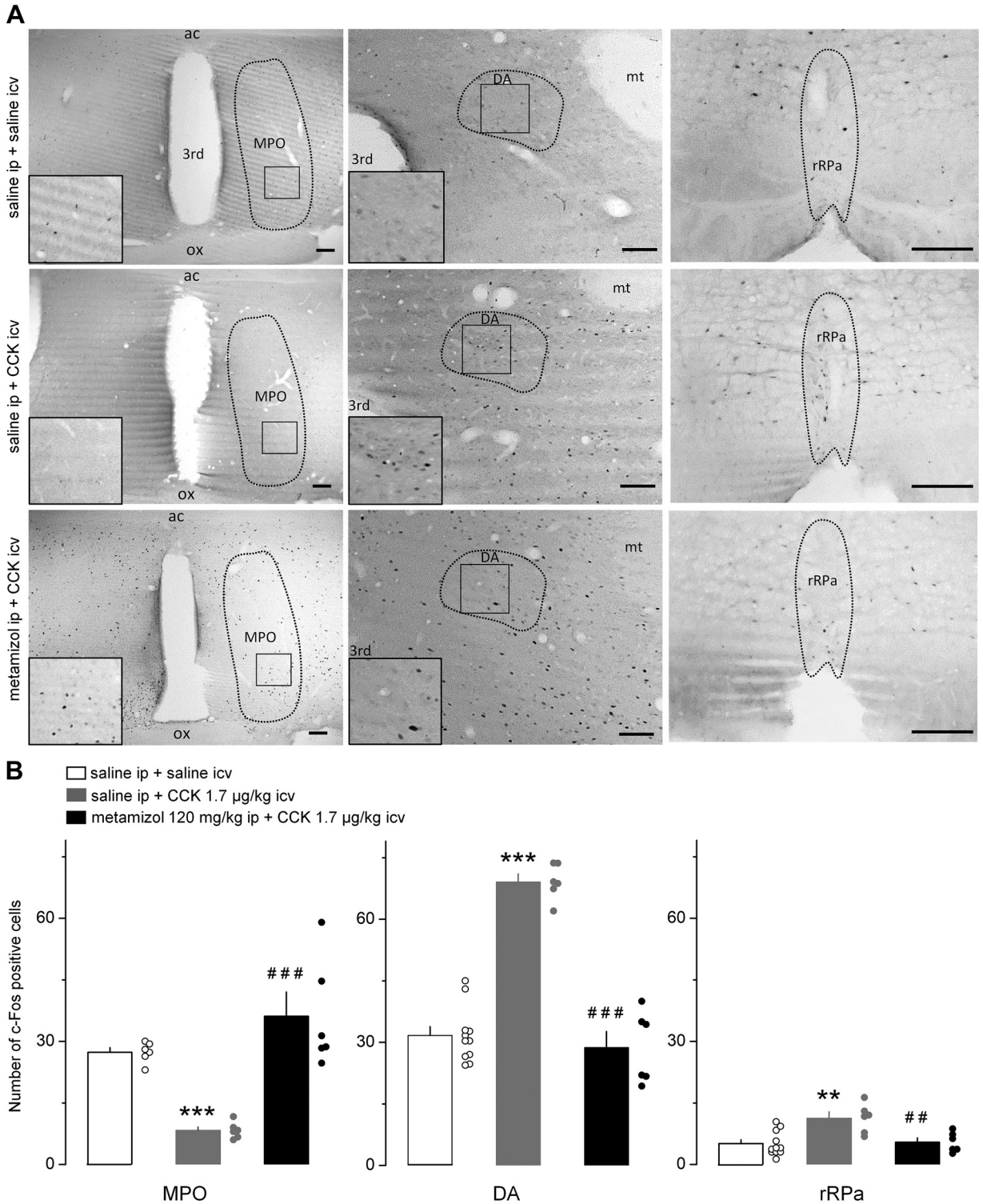

Figure 3. The expression of c-Fos in thermoregulatory nuclei of rats in response to intracerebroventricular (icv) administration of cholecystokinin (CCK; 1.7 µg/kg) or saline after pretreatment at −30 min with an intraperitoneal (ip) infusion of metamizol (120 mg/kg) or saline. *A*: representative photomicrographs 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).

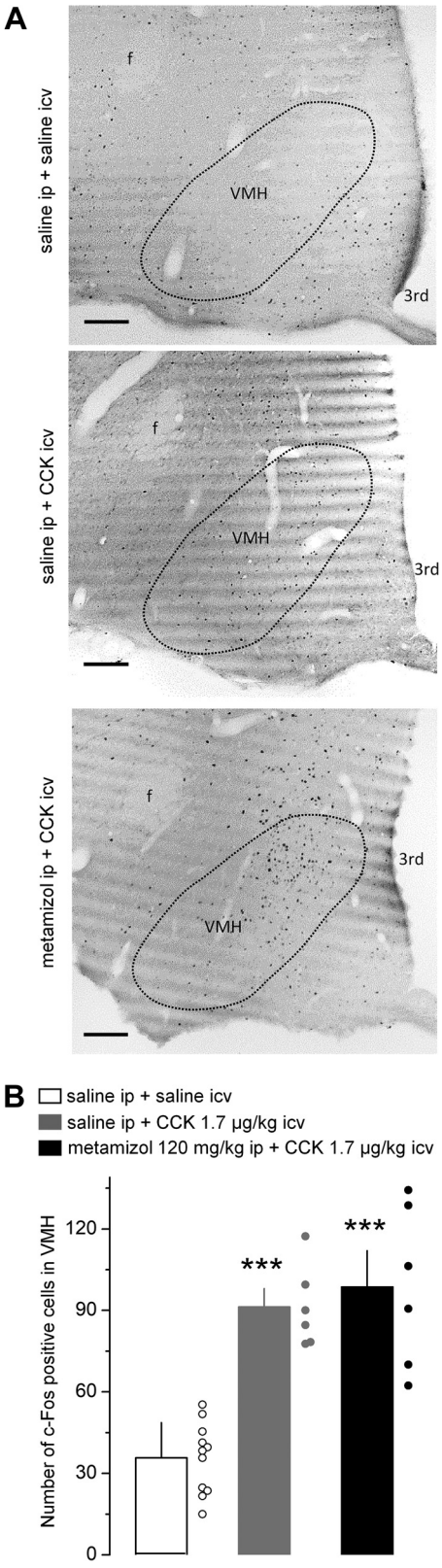

Figure 4. The expression of c-Fos in the ventromedial hypothalamus (VMH) of rats in response to intraperitoneal (ip) and intracerebroventricular (icv) administration of saline (n = 11) and to icv administration of CCK (1.7 µg/kg) after pretreatment at −30 min with an ip infusion of metamizol (120 mg/kg; 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.

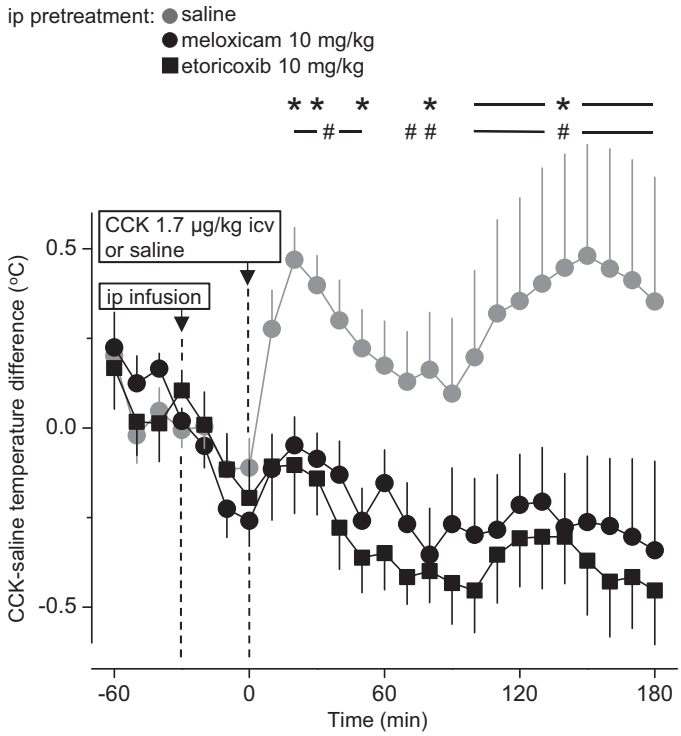

Figure 5. The difference in deep (colonic) body temperature between rats treated intracerebroventricularly (icv) with cholecystokinin (CCK; 1.7 µg/kg) and saline after pretreatment at −30 min with an intraperitoneal (ip) infusion of meloxicam (10 mg/kg; n = 7 and 8, respectively), etoricoxib (10 mg/kg; 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.

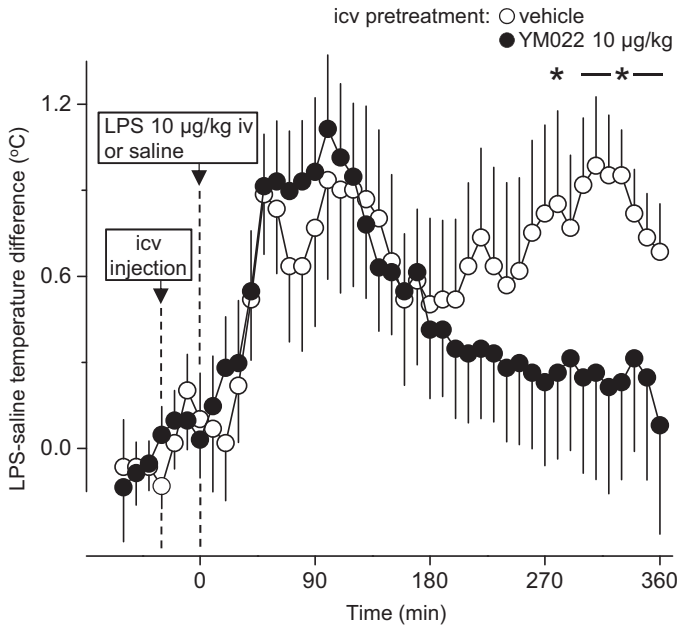

Figure 6. The difference in deep (colonic) body temperature between rats treated intravenously (iv) with lipolysaccharide (LPS; 10 μg/kg) and saline after pretreatment at −30 min with an intracerebroventricular (icv) injection of YM022 (10 μg/kg) or its vehicle. For each group, $n = 6$. *$P < 0.05$, 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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DISCLAIMERS

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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