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Endocannabinoid catabolic enzymes play differential roles in thermal homeostasis in response to environmental or immune challenge

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Abstract

Cannabinoid receptor agonists, such as Δ^9-THC, the primary active constituent of *Cannabis sativa*, have anti-pyrogenic effects in a variety of assays. Recently, attention has turned to the endogenous cannabinoid system and how endocannabinoids, including 2-arachidonoyglycerol (2-AG) and anandamide, regulate multiple homeostatic processes, including thermoregulation. Inhibiting endocannabinoid catabolic enzymes, monoacylglycerol lipase (MAGL) or fatty acid amide hydrolase (FAAH), elevates levels of 2-AG or anandamide *in vivo*, respectively. The purpose of this experiment was to test the hypothesis that endocannabinoid catabolic enzymes function to maintain thermal homeostasis in response to hypothermic challenge. In separate experiments, male C57BL/6J mice were administered a MAGL or FAAH inhibitor, and then challenged with the bacterial endotoxin lipopolysaccharide (LPS; 2 mg/kg i.p.) or a cold (4° C) ambient environment. Systemic LPS administration caused a significant decrease in Tb after 6 h, and this hypothermia persisted for at least 12 h. Similarly, cold environment induced mild hypothermia that resolved within 30 min. JZL184 exacerbated hypothermia induced by either LPS or cold challenge, both of which effects were blocked by rimonabant, but not SR144528, indicating a CB_1 cannabinoid receptor mechanism of action. In contrast, the FAAH inhibitor, PF-3845, had no effect on either LPS-induced or cold-induced hypothermia. These data indicate that unlike direct acting cannabinoid receptor agonists, which elicit profound hypothermic responses on their own, neither MAGL nor FAAH inhibitors affect normal body temperature.

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Participated in research design: Nass, Long, Schlosburg, Cravatt, Lichtman, Kinsey
Conducted experiments: Nass, Kinsey
Performed data analysis: Nass, Kinsey
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However, these endocannabinoid catabolic enzymes play distinct roles in thermoregulation following hypothermic challenges.

Keywords

Cannabinoid; hypothermia; environmental stress; FAAH; MAGL; MGL

Introduction

Thermoregulation is an essential homeostatic process that maintains core body temperature (Tb) in the presence of environmental or physiological stressors (e.g. extreme ambient temperatures) (Romanovsky, 2007). A hypothermic response is elicited when thermoregulation is dysregulated (Wong, 1983). A stressor, such as cold ambient temperature or systemic inflammation, triggers an inhibition of the predominantly warm thermosensitive neurons in the preoptic anterior hypothalamus (POAH), leading to an increased response of the sympathetic nervous system (i.e. vasoconstriction, and increased oxygen consumption, respiratory rate, heart rate, and blood pressure to promote thermogenesis and return the organism to homeostasis (Wong, 1983; Romanovsky, 2007)

In rodents, hypothermia is induced by administration of exogenous cannabinoids such as Δ^9-tetrahydrocannabinol (THC), the primary active constituent of *Cannabis sativa* (Freeman and Martin, 1983). Cannabinoid-induced hypothermia is a well characterized phenomenon (Freeman and Martin, 1983) and is a component of the “tetrad” screening battery for cannabinoid effects (Wiley and Martin, 2003). Pretreatment with a cannabinoid type 1 (i.e., CB_1) receptor antagonist blocks this decrease in core body temperature, indicating that CB_1 is required for cannabinoid-induced hypothermia (Wiley and Martin, 2003). Indeed, CB_1 selective agonists or mixed CB_1/CB_2 agonists induce hypothermia, whereas highly selective CB_2 receptor agonists, such as O-3223, do not induce hypothermia (Kinsey et al., 2011).

In addition to inducing hypothermia, cannabinoids also modulate thermoregulation in response to endotoxin challenge. For example, the non-selective cannabinoid receptor agonist WIN 55,212-2 dose-dependently attenuates fever induced by the gram negative bacterial endotoxin lipopolysaccharide (LPS), in rats. This anti-pyrogenic effect of WIN 55,212-2 is blocked by the selective CB_1 receptor antagonist rimonabant, but not the CB_2 selective antagonist SR144528, indicating the necessity of CB_1 in mediating the anti-pyrogenic effect of WIN 55,212-2 (Benamar et al., 2007). Conversely, the role of endogenous cannabinoids in thermoregulation is not well defined.

Unlike exogenous cannabinoids, manipulation of the endocannabinoid system does not induce hypothermia. For example OL-135, inhibits fatty acid amide hydrolase (FAAH), the primary catabolic enzyme for the endocannabinoid anandamide, thereby increasing brain levels of anandamide but has no effect on body temperature (Lichtman et al., 2004). Similarly, increased brain levels of the other well characterized endocannabinoid 2-arachidonoylglycerol (2-AG) occur after inhibition of its primary catabolic enzyme, monoacylglycerol lipase (MAGL) (Blankman et al., 2007). The highly selective MAGL

inhibitor JZL184 increases brain 2-AG levels, but does not affect body temperature (Long et al., 2009a).

When administered in a vehicle consisting of 4:1 parts polyethylene glycol (PEG300) and Polysorbate 80 (Tween80), JZL184 induced a mild reduction in Tb (Long et al., 2009b). It is noteworthy that the PEG300 vehicle, administered alone, also produced a mild hypothermic response, which was greatly augmented by JZL184 in PEG (Long et al., 2009b). However, the 1:1:18 vehicle is devoid of hypothermic effects and JZL184 did not alter body temperature when administered in 1:1:18 vehicle (Long et al., 2009a). The following studies were designed to determine whether inhibition of MAGL or FAAH disrupts thermoregulation following physical (i.e., cold ambient temperature) or physiological (i.e., endotoxin) challenge. First, we assessed whether the MAGL inhibitor JZL184 or the FAAH inhibitor PF-3845 potentiates LPS-induced hypothermia. Second, we examined whether JZL184 or PF-3845 potentiates hypothermia induced by cold ambient temperature. Finally, we determined the contribution of CB_1 and CB_2 receptors in these assays.

Materials and Methods

Animals

Adult male C57BL/6J mice weighing approximately 25 g at the start of the experiments were singly housed and maintained on a 12:12 light cycle in a temperature (20-22 °C) and humidity controlled facility, with *ad libitum* access to food and water. Mice were randomly assigned to treatment groups. All experimental protocols were approved by the Institutional Animal Care and Use Committees at West Virginia University and Virginia Commonwealth University. Experimenter was blinded to drug treatment conditions.

Endotoxin Challenge

Baseline rectal temperature was recorded using a lubricated rectal thermocouple probe attached to a BAT12 thermometer (Thomas Scientific, Swedesboro, NJ), and after 2 h, Tb was again taken, and the mice were injected with lipopolysaccharide (LPS) dissolved in saline (2 mg/kg, ip) or saline. Tb was taken 2, 4, 6, 8, 12, and 24 h after LPS injection (Benamar et al., 2007). For the endocannabinoid studies, baseline Tb was taken, and then the mice were injected with JZL184 (1, 4, 16, 40 mg/kg, ip), PF-3845 (10 mg/kg, ip), or vehicle. These high doses JZL184 (40 mg/kg) (Kinsey et al., 2009; Long et al., 2009b; Nomura et al., 2011; Kinsey et al., 2013) and PF-3845 (Ahn et al., 2009) are sufficient to fully inhibit MAGL and FAAH, respectively, in mice. In a third study, mice were pretreated with the CB_1 receptor selective antagonist rimonabant (SR141716A, 3 mg/kg, ip) (Rinaldi-Carmona et al., 1994), the CB_2 receptor selective antagonist SR144528 (3 mg/kg, ip) (Rinaldi-Carmona et al., 1998), or vehicle 30 min prior to administration of JZL184, PF-3845, or vehicle.

Cold Challenge

Baseline rectal temperature was recorded, and then the mice were injected (ip) with rimonabant (3 mg/kg), SR144528 (3 mg/kg), or vehicle. Thirty min later, mice were injected ip with JZL184 (40 mg/kg), PF-3845 (10 mg/kg), or vehicle. After 2 h, core Tb was taken,

and the mice were placed in a cold (5 ± 1°C) room for a 4 h duration. Tb was recorded every 60 min. At 4 h, the mice were removed from the cold room and returned to the 20°C laboratory environment. Tb was taken 30, 60, and 120 min after the mice were removed from the cold room.

Drugs

Rimonabant (SR141716; SR1) and SR144528 (SR2) were generously provided by the National Institute on Drug Abuse Drug Supply Program (Bethesda, MD). The MAGL inhibitor JZL184 (Long et al., 2009b) and the FAAH inhibitor PF-3845 (Ahn et al., 2009) were synthesized in the Cravatt laboratory, as described previously. LPS from Escherichia coli 026:B6 were purchased from Sigma-Aldrich (St. Louis, MO). All drugs were dissolved in a vehicle consisting of ethanol, Alkamuls-620 (Rhone-Poulenc, Princeton, NJ), and saline in a ratio of 1:1:18 parts, and LPS was dissolved in normal saline (i.e., 0.9% NaCl). Doses were based on published reports (Ahn et al., 2009; Kinsey et al., 2009; Nomura et al., 2011) and administered at a volume of 10 μl/g body mass. Solutions were warmed to RT prior to injection.

Data Analyses

All data are reported as mean ± SEM. Body temperature data were analyzed using a mixed design analysis of variance (ANOVA), with drug treatment as the between-subjects variable and time as a within-subjects variable. Post hoc comparisons were made using T tests with Bonferroni correction, with the exception of the dose response data, for which Dunnett's test was used to compare each dose to vehicle. Differences were considered statistically significant at $p < 0.05$.

Results

MAGL inhibition potentiates endotoxin-induced hypothermia

Baseline Tb was recorded, and bacterial endotoxin lipopolysaccharide (LPS 0.1, 0.5, 2.0 mg/kg, ip) or vehicle was administered 1 h later. LPS significantly decreased Tb [$F(3,208) = 14.4$; $p < 0.01$; **Figure 1A**]. Post hoc analyses revealed that this decrease in Tb was driven by the 2.0 mg/kg dose, which was used in each of the following studies.

JZL184 (1, 4, 16, or 40 mg/kg, ip) dose-dependently decreased Tb in LPS (2 mg/kg, ip) treated mice [$F(28,224) = 4.49$; $p < 0.01$; **Figure 1B**]. Post hoc analyses revealed that this interaction was driven by JZL184 at 4 mg/kg and higher doses. However, JZL184 (40 mg/kg, ip) had no effect in saline treated mice ($p = 0.94$), indicating that JZL184 *per se* has no effect on Tb. PF-3845 (10 mg/kg, ip) had no effect on Tb in either saline treated mice ($p = 0.97$; **Figure 1C**) or LPS treated mice ($p = 0.46$).

CB_1 mediates MAGL inhibition potentiated endotoxin-induced hypothermia

A separate group of mice was given an ip injection of rimonabant (3 mg/kg), SR144528 (3 mg/kg), or vehicle, and administered (ip) JZL184 (40 mg/kg) or vehicle 30 min later. LPS (2 mg/kg, ip) was injected at 2.5 h. Tb was measured 0, 2, 4, 6, 8, 12, and 24 h post LPS injection. JZL184 again potentiated LPS-induced hypothermia [$F(21,196) = 4.7$; $p < 0.01$;

Figure 2]. The CB_1 selective antagonist rimonabant blocked the JZL184 potentiation of hypothermia, but the CB_2 selective antagonist SR144528 had no effect.

MAGL inhibition potentiates environmentally-induced hypothermia

In control mice, the cold challenge caused a 1°C decrease in Tb. PF-3845 had no effect on Tb, either during the cold challenge or during exposure to ambient temperature ($p = 0.62$; **Figure 3A**). However, JZL184 treatment caused a significant decrease in Tb, as compared with vehicle-treated mice [$F(24,200) = 5.3$; $p < 0.01$; **Figure 3B**]. Post hoc comparisons revealed that pretreatment with rimonabant blocked the JZL184 potentiation of hypothermia. Conversely, SR144528 had no effect on JZL184 potentiation of hypothermia. Notably, pretreatment with either rimonabant ($p = 0.15$) or SR144528 ($p = 0.50$), *per se*, had no effect on Tb (**Figure 3A)**.

Discussion

The goal of the present study was to determine the effects of MAGL and FAAH inhibitors on thermal homeostasis in mice subjected to cold ambient temperature or endotoxin challenge. The selective MAGL inhibitor, JZL184, potentiated hypothermia induced by either cold ambient temperature or LPS injection. Rimonabant, but not SR144528, blocked the exacerbation of these hypothermic responses, indicating the necessity of CB_1 and dispensability of CB_2. Conversely, the selective FAAH inhibitor, PF-3845, did not affect Tb in either assay. Neither enzyme inhibitor affected Tb in control mice under ambient conditions, indicating that inhibition of MAGL or FAAH, *per se*, is insufficient to elicit hypothermia. In contrast, direct CB_1 receptor activation by THC and other CB_1 receptor agonists elicits profound hypothermic responses (Holtzman et al., 1969; Martin et al., 1981; Rawls et al., 2002; Rawls et al., 2004). These data extend previous knowledge by addressing the relative contributions of MAGL and FAAH to the maintenance of thermal homeostasis in the presence of physical and physiological challenges.

Whereas exogenous administration of either anandamide or OL-135 administered alone has no effect on Tb, anandamide induces hypothermia in either FAAH (-/-) mice (Cravatt et al., 2001) or wild type mice pretreated with OL-135 (Lichtman et al., 2004). Similarly, exogenous administration of 2-AG also has no effect on Tb, but induces hypothermia in mice pretreated with the MAGL inhibitor *N*-arachidonyl maleimide (NAM) . However, NAM lacks specificity and inhibits other serine hydrolases, including FAAH (Burston et al., 2008).

Previous reports indicate that the hypothermic effects of exogenous cannabinoids are at least partially mediated by CB_1 receptors in the POAH, the major thermoregulation center in the brain (Rawls et al., 2004). For example, intra-POAH administration of the pan CB_1/CB_2 agonist WIN 55212-2 elicited dose-dependent hypothermia in rats. Rimonabant, but not SR144528, blocked WIN-induced hypothermia, indicating a CB_1 receptor mechanism of action (Rawls et al., 2002). Furthermore, Δ^9-THC administered directly into the POAH of mice elicited dose-dependent hypothermia (Fitton and Pertwee, 1982), and Δ^9-THC-induced hypothermia was blocked by rimonabant (Compton et al., 1996). However, Δ^9-THC infused into the fourth ventricle, which is located near the midbrain, pons, and medulla that are

thought to contribute to thermoregulation, also elicited hypothermic responses (Fitton and Pertwee, 1982; Rawls et al., 2002). Although it is plausible that CB_1 receptors in the POAH modulate hypothermia, confirmation of the specific site of action remains to be determined and warrants future investigation.

Although it is well established that exogenous cannabinoid administration elicits hypothermia through activation of the CB_1 receptor, the mechanism(s) through which CB_1 activation alters body temperature remain(s) to be elucidated (Fitton and Pertwee, 1982). Other systems, including the GABAergic, dopaminergic, and serotonergic systems are involved in the mechanism of cannabinoid-induced hypothermia. For example, pretreatment with bicuculline, a $GABA_A$ receptor antagonist, blocks the hypothermic effects of WIN 55212-2 in rats, while the $GABA_B$ receptor antagonist SCH 50911 does not affect hypothermia, indicating a $GABA_A$ receptor mechanism in cannabinoid-induced hypothermia (Rawls et al., 2004). However, GABA elicits hypothermia via the $GABA_A$ agonist muscimol and $GABA_B$ agonist baclofen is not blocked by rimonabant, indicating that $GABA_A$, but not $CB_{1,}$ mediates GABA-induced hypothermia (Rawls et al., 2004). Pretreatment with the D_2 dopamine receptor antagonists S(-)-sulpirde and S(-)-racloprode blocks Δ^9-THC-induced hypothermia, while the hypothermic effects of Δ^9-THC are potentiated by the D_2 receptor agonists (-)-quinpirole and (+)-bromocriptine, indicating a potential role of D_2 dopamine receptors in cannabinoid-induced hypothermia (Nava et al., 2000). Furthermore, when administered prior to Δ^9-THC the selective serotonin reuptake inhibitor fluoxetine attenuates Δ^9-THC-induced hypothermia, but administration of fluoxetine after Δ^9-THC administration potentiates the Δ^9-THC hypothermic effects (Malone and Taylor, 1998). Taken together with the present data, these data suggest that multiple mechanisms, possibly in the periphery as well as the central nervous system, are involved in the thermoregulatory effects of cannabinoids.

In general, rodents display increased Tb when administered a low dose of LPS or placed in a high ambient temperature environment; whereas a high dose of LPS or low ambient temperature induces hypothermia (Al-Saffar et al., 2013). In rats, low dose, exogenous anandamide (1 g, icv) induces fever (Fraga et al., 2009) or has no effect on Tb (0.07-520 g, icv) (Steiner et al., 2011). Regardless, exogenous anandamide (50 g, icv) appears to potentiate hypothermia induced by LPS in rats (Steiner et al., 2011). LPS causes systemic inflammation, pain, and thermoregulation disruption through activation of Toll-like receptor 4 (TLR4) on innate immune cells (Poltorak et al., 1998). LPS-induced systemic inflammation is mediated by activation of TLR4 on macrophages and monocytes, after interaction with the CD14 protein (Poltorak et al., 1998; Beutler and Rietschel, 2003). These immune cells release proinflammatory cytokines, predominantly IL-1, IL-6, and TNF-α, which recruit other immune cells to infiltrate the infected tissue. However, the proinflammatory cytokine cascade can result in massive tissue damage, hypothermia, and endotoxemia, which can be lethal (Poltorak et al., 1998; Beutler and Rietschel, 2003). Further research is needed to determine whether neural and/or immune mechanisms contribute to disruption of thermoregulation caused by MAGL inhibition in LPS-treated mice.

The present study provides the first *in vivo* evidence that the 2-AG/MAGL system is important in modulating the hypothermic response to hypothermic challenges. It is noteworthy that, in addition to JZL184 potentiating LPS-induced hypothermia in the present study, JZL184 also attenuates LPS-induced neuroinflammation (Nomura et al., 2011), which is of particular clinical relevance for the treatment of neural inflammation and endotoxemia. JZL184 attenuates LPS-induced neuroinflammation through blockage of microglia activation and LPS-induced proinflammatory cytokine release in the brain, both of which contribute to endotoxemia through a CB_1 and CB_2 independent mechanism (Nomura et al., 2011). Thus, JZL184 may potentiate LPS-induced hypothermia by blocking microglial activation and release of proinflammatory cytokines (e.g. TNF-α, IL-1, and IL-6) induced by LPS administration, which affects a downstream thermoregulatory mechanism that is modulated by CB_1. In support of this hypothesis, WIN55,212-2 dose-dependently attenuates LPS-induced fever and blocks LPS-induced increases in IL-6 plasma levels (Benamar et al., 2007). Furthermore, pretreatment with rimonabant, but not SR144528 blocks this increase in IL-6, indicating a CB_1 mechanism of action (Benamar et al., 2007).

The finding in the present study that FAAH inhibition did not affect Tb in mice in the cold ambient temperature or endotoxin challenge was somewhat surprising, because exogenous intracerebroventricular administration of anandamide potentiates LPS-induced hypothermia in rats (Steiner et al., 2011). Disparities between our findings and those of by Steiner et al. (2011) may be due to differences in route of administration (ip vs. icv), target system (exogenous anandamide vs. FAAH inhibition), species (rats vs. mice), or other methodological differences that may have impacted stress levels in the subjects. In addition, the levels of endogenous anandamide were likely lower following PF-3845 administration than were achieved by exogenous anandamide administration.

Why MAGL inhibition potentiates stress-induced hypothermia, whereas FAAH inhibition remains quiescent, is unclear. Two observations from previous research may account for the differential effects observed here between PF-3845 and JZL184. First, *in vitro* data show that 2-AG behaves as a full agonist, whereas anandamide acts as a partial agonist in activating CB_1 receptors (Sugiura et al., 2000). Second, brain 2-AG levels are approximately three orders of magnitude higher than brain anandamide levels (Bisogno et al., 1999; Kinsey et al., 2009). Thus, the increased levels of anandamide produced by FAAH inhibition may be insufficient to stimulate the necessary CB_1 receptors that modulate thermoregulation. Conversely, the high brain levels of 2-AG resulting from MAGL inhibition, combined with the relatively high efficacy of 2-AG at the CB_1 receptor likely contribute to the enhanced hypothermic effects produced by JZL184.

In conclusion, the present findings indicate that pharmacological inhibition of MAGL effectively potentiates hypothermia in mice subjected to the environmental or physiological stress induced by cold ambient temperature or administration of the endotoxin LPS, respectively. Whereas brain 2-AG metabolism by MAGL appears to maintain homeostasis in the face of a hypothermic challenge, the anandamide/FAAH system remains quiescent. Thus, MAGL inhibition leads to a dysregulation of thermogenesis that occurs via a CB_1 receptor mechanism of action in mice subjected to physical or physiological stress. Thermoregulation is a critical component of homeostasis and is critical for survival across

endothermic and ectothermic species. Beyond self-reported coldness (Hollister, 1971) cannabinoids do not induce the overt, statistically significant hypothermia in humans (Karniol et al., 1975; Fant et al., 1998) that is observed in rodents. However, the present study reveals that MAGL protects mice from thermal dysregulation caused by LPS or cold ambient temperature. Given that thermoregulation protects against a range of ailments, from stroke to viral and bacterial infection (Darwazeh and Yan, 2013), the present study implicates an intricate role of the endocannabinoid system in the control of body temperature. These data demonstrate that MAGL inhibition does not alter body temperature *per se*, but augments hypothermic responses caused by LPS or exposure to cold ambient temperature. Thus, MAGL may function as a protective brake from certain hypothermic challenges, by curtailing 2-AG activation of CB_1 receptors.

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Nonstandard Abbreviations

2-AG	2-arachidonoylglycerol
AA	Arachidonic acid
Anandamide	N-arachidonoylethanolamine
CB_1	Cannabinoid receptor type 1
CB_2	Cannabinoid receptor type 2
FAAH	Fatty acid amide hydrolase
JZL184	4-nitrophenyl 4-(dibenzo[d][1,3]dioxol-5-yl(hydroxy)methyl)piperidine-1-carboxylate
MAGL	Monoacylglycerol lipase
T_b	Body temperature
THC	Δ^9-tetrahydrocannabinol
TNFα	Tumor necrosis factor α

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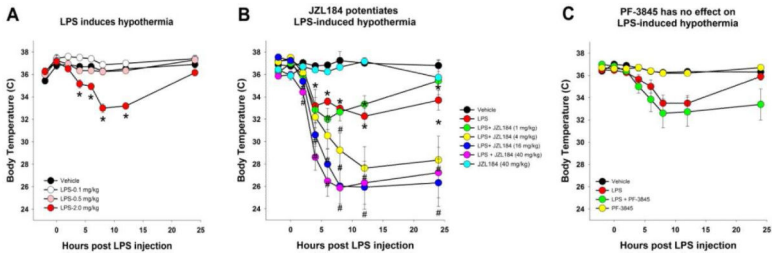

Fig. 1.

Inhibition of MAGL, but not FAAH, exacerbates endotoxin-induced hypothermia. (**A**) LPS induces hypothermia in mice. Baseline Tb was measured, and then the mice were injected with (**B**) the MAGL inhibitor JZL184 (1 - 40 mg/kg), (**C**) the FAAH inhibitor PF-3845 (10 mg/kg), or vehicle. After 2 h, Tb was taken again, and mice were injected with LPS (2 mg/kg). Tb was taken at 2, 4, 6, 8, 12, and 24 h post LPS injection. Data presented as mean ± SEM (n = 7-8). * $p < 0.05$ vs. vehicle; # $p < 0.05$, vs. vehicle and vs. LPS.

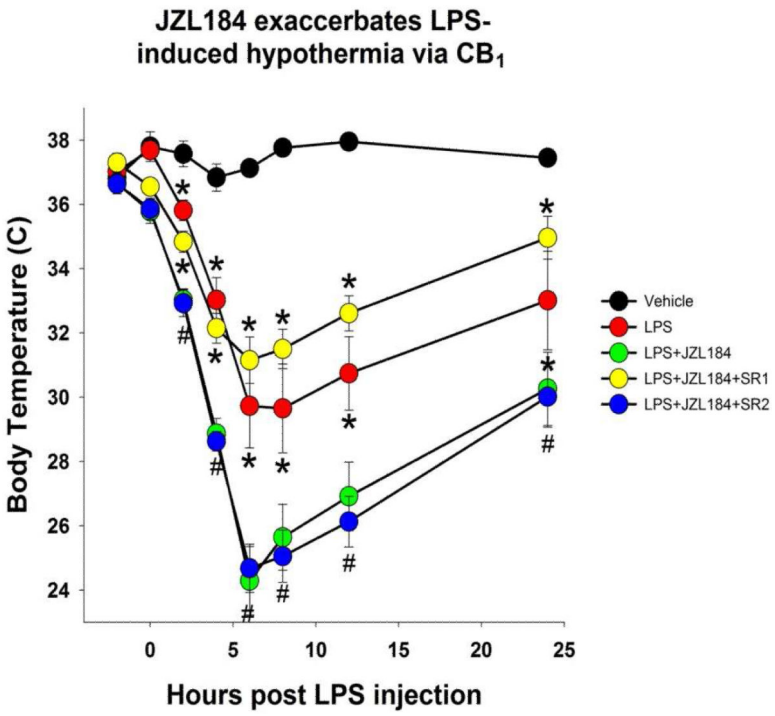

Fig. 2.

The selective MAGL inhibitor JZL184 exacerbates endotoxin-induced hypothermia via a CB_1 receptor-mediated mechanism of action. Baseline Tb was measured, and then the mice were treated the CB_1 antagonist rimonabant (SR1; 3 mg/kg), the CB_2 antagonist SR144528 (SR2; 3 mg/kg). Thirty min later, mice were injected with the MAGL inhibitor JZL184 (40 mg/kg) or vehicle. After 2 h, Tb was taken again, and mice were injected with LPS (2 mg/kg). Tb was taken at 2, 4, 6, 8, 12, and 24 h post LPS injection. Data presented as mean ± SEM (n = 7-8). * $p < 0.05$ vs. vehicle; # $p < 0.05$, vs. vehicle and vs. LPS.

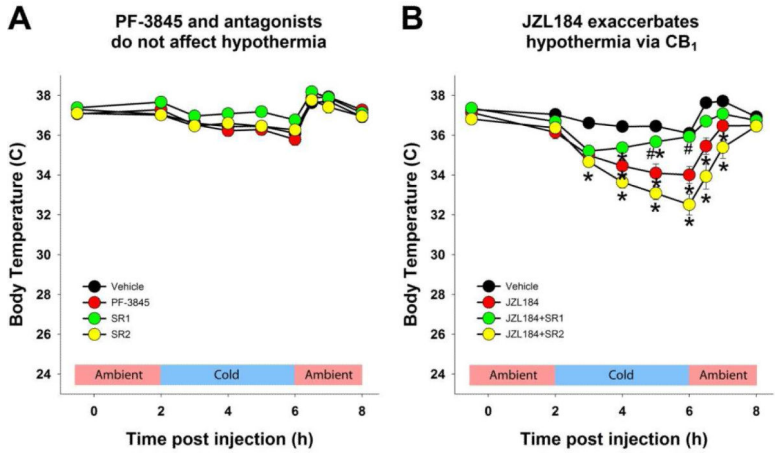

Fig. 3.

The MAGL inhibitor JZL184 potentiates cold-induced hypothermia via the CB_1 receptor. Baseline Tb was measured at -2.5 h, then mice were injected with the CB_1 antagonist rimonabant (SR1), the CB_2 antagonist SR144528 (SR2), or vehicle, followed 30 min later by PF-3845 (10 mg/kg), JZL184 (40 mg/kg), or vehicle. At 0 h, mice were moved into a cold (5 ± 1°C) room. At 4 h, mice were returned to RT (20°C). Data presented as mean ± SEM (n = 6-7). * $p < 0.05$ vs. vehicle; # $p < 0.05$ vs. JZL184.