Peripheral Adenosine A₃ Receptor Activation Causes Regulated Hypothermia in Mice That Is Dependent on Central Histamine H₁ Receptors[§]

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ABSTRACT

Adenosine can induce hypothermia, as previously demonstrated for adenosine A₁ receptor (A₁AR) agonists. Here we use the potent, specific A₃AR agonists MRS5698, MRS5841, and MRS5980 to show that adenosine also induces hypothermia via the A₃AR. The hypothermic effect of A₃AR agonists is independent of A₁AR activation, as the effect was fully intact in mice lacking A₁AR but abolished in mice lacking A₃AR. A₃AR agonist-induced hypothermia was attenuated by mast cell granule depletion, demonstrating that the A₃AR hypothermia is mediated, at least in part, via mast cells. Central agonist dosing had no clear hypothermic effect, whereas peripheral dosing of a non-brain-penetrant agonist caused hypothermia, suggesting that peripheral A₃AR-expressing cells drive the hypothermia. Mast cells release

histamine, and blocking central histamine H₁ (but not H₂ or H₄) receptors prevented the hypothermia. The hypothermia was preceded by hypometabolism and mice with hypothermia preferred a cooler environmental temperature, demonstrating that the hypothermic state is a coordinated physiologic response with a reduced body temperature set point. Importantly, hypothermia is not required for the analgesic effects of A₃AR agonists, which occur with lower agonist doses. These results support a mechanistic model for hypothermia in which A₃AR agonists act on peripheral mast cells, causing histamine release, which stimulates central histamine H₁ receptors to induce hypothermia. This mechanism suggests that A₃AR agonists will probably not be useful for clinical induction of hypothermia.

Introduction

Maintenance of a constant, warm body temperature is an essential component of mammalian physiology. Hypothermia can be caused by cold exposure that overwhelms the animal's ability to generate and conserve body heat. Hypothermia, exemplified by hibernation and torpor, is also a physiologic mechanism used by small mammals to conserve energy (Geiser, 2004; Melvin and Andrews, 2009). Such endogenous hypothermia is actively achieved via disengaging brown adipose tissue, reducing physical activity and vasodilation, and seeking a cool environment (Heller and Ruby, 2004; Lute et al., 2014). Hypothermia can also be elicited pharmacologically by many drugs and neurotransmitters [e.g., Clark and Lipton (1985)]. Finally, hypothermia is used clinically to minimize tissue injury and improve survival, for example,

after neonatal hypoxia or cardiopulmonary resuscitation (Arrich et al., 2012; Azzopardi et al., 2014).

The mouse is an attractive model for studying thermal biology because core body temperature (T_b) changes occur more rapidly and with a much greater magnitude than in larger mammals. Mice have a greater surface area/volume ratio and thus a disproportionately greater energetic cost of staying warm (Hudson and Scott, 1979; Abreu-Vieira et al., 2015). Although the magnitude of T_b fluctuation is less in humans than in mice, it is likely that the neural circuitry controlling T_b is conserved.

Adenosine is a purine nucleoside that is predominantly found intracellularly but also has an extracellular role. Extracellular adenosine comes from intracellular adenosine and hydrolysis of extracellular nucleotides (ATP, ADP, AMP) by ectonucleotidases (Chen et al., 2013). Extracellular adenosine levels can rise dramatically under conditions of cellular injury (Schubert et al., 1997), prolonged wakefulness (Porkka-Heiskanen et al., 1997; Basheer et al., 2004), hypoxia (Van Wylen et al., 1986), metabolic stress (Minor et al., 2001), and high intensity exercise (Dworak et al., 2007). Adenosine signals through four G protein-coupled receptors. The A₁ and A₃ adenosine receptors (ARs)

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ABBREVIATIONS: A₁AR, adenosine A₁ receptor; A₃AR, adenosine A₃ receptor; CNS, central nervous system; H₁R, histamine type 1 receptor; PWT, paw withdrawal threshold; T_b, core body temperature.

inhibit adenylyl cyclase, whereas A_{2A}AR and A_{2B}AR stimulate it.

The ability of adenosine to induce hypothermia has been known for over 80 years (Bennet and Drury, 1931). Following identification of the four receptors, Anderson et al. (1994) used the available ligands to suggest that adenosine analogs cause hypothermia via adenosine A₁ receptor (A₁AR) in the central nervous system. Further evidence for a role for A₁AR includes prevention of AMP hypothermia by an A₁AR antagonist (Muzzi et al., 2013), loss of the hypothermic effect of N⁶-cyclohexyladenosine in A₁AR null (*Adora1*^{-/-}) mice (Johansson et al., 2001), and induction of a hypothermic state with A₁AR agonist (N⁶-cyclohexyladenosine) injection into the nucleus of the solitary tract (Tupone et al., 2013). These observations firmly established that A₁AR activation can cause hypothermia (Fredholm et al., 2011).

There is also evidence that adenosine can induce hypothermia via non-A₁AR mechanisms. For example, hypothermia produced by N⁶-R-phenylisopropyladenosine was only partially attenuated in the *Adora1*^{-/-} mouse (Yang et al., 2007). Also, hypothermia from high dose AMP was attributed to erythrocyte uptake of intact AMP, and this hypothermia was intact in mice lacking any one of the four adenosine receptors (Daniels et al., 2010). Most informative is the observation that N⁶-R-phenylisopropyladenosine-induced hypothermia was attenuated in adenosine A₃ receptor (A₃AR) null (*Adora3*^{-/-}) mice, directly implicating the A₃AR's involvement in hypothermia (Yang et al., 2010). In rodents, the A₃AR is expressed in neutrophils, basophils, mast cells, and other cells mediating inflammatory responses (Borea et al., 2015). Use of *Adora3*^{-/-} mice conclusively demonstrated a role for A₃AR in triggering mouse mast cell degranulation (Salvatore et al., 2000), whereas the A₃AR may not be important in human mast cell degranulation (Auchampach et al., 1997; Gomez et al., 2011; Rudich et al., 2012). The availability of potent, specific mouse A₃AR agonists (Jacobson, 2013) allowed us to investigate the hypothermia induced by A₃AR agonists and the contributing mechanisms.

Materials and Methods

Mice. Male C57BL/6J (Jackson Laboratories, Bar Harbor, ME), *Adora1*^{-/-} [on a mixed background, provided by Dr. Jurgen Schnermann (Sun et al., 2001)], and *Adora3*^{-/-} [on a C57BL/6 background, provided by Dr. Stephen Tilley under a materials transfer agreement with Merck (Salvatore et al., 2000)]. Mice were singly housed at ~21–22°C with a 12-hour light-dark cycle. Chow (NIH-07; Harlan/Teklad, Madison, WI) and water were available ad libitum. Mice were studied ≥7 days after any operation or prior treatment. Studies were approved by the Animal Care and Use Committees of National Institute of Diabetes and Digestive and Kidney Diseases and St. Louis University.

Drugs. Compounds and vehicles were purchased from Sigma-Aldrich (St. Louis, MO) unless synthesized in house. MRS5698, 2-(3,4-difluorophenylethynyl)-N⁶-(3-chlorobenzyl)-(N)-methanocarba-adenosine-5'-methyluronamide (Tosh et al., 2012), was dissolved in 4:1 (v/v) PEG300/warm Solutol HS15, then an equal volume of 10% 2-hydroxypropyl-β-cyclodextrin in water was added during vortexing. MRS5841, N⁶-3-chlorobenzyl-2-(3-sulfophenylethynyl) (N)-methanocarba-adenosine-5'-methyluronamide, was dissolved in saline (Paoletta et al., 2013). MRS5980, (1*S*,2*R*,3*S*,4*R*,5*S*)-4-(2-((5-chlorothiophen-2-yl)ethynyl)-6-(methylamino)-9*H*-purin-9-yl)-2,3-dihydroxy-N-methylbicyclo [3.1.0]hexane-1-carboxamide (Tosh et al., 2014), was dissolved in dimethyl sulfoxide and adjusted to 30% dimethyl sulfoxide/0.5% methylcellulose/water. Other compounds (vehicles) with references for

dose choice are: histamine type 1 receptor (H₁R) antagonist pyrilamine (saline) (Zarrindast et al., 2005; Kitanaka et al., 2007), H₂R antagonist zolantidine (saline) (Kitanaka et al., 2007), and H₄R antagonist JNJ7777120 (10% ethanol in saline) (Cowden et al., 2013). Mice were treated with escalating doses of compound 48/80 in saline on 5 consecutive days as described (Ge et al., 2006). Plasma stability, Caco-2 cell permeability, and parallel artificial membrane permeability at pH 7.4 (Pion PAMPA method) of MRS5698, MRS5841, and MRS5980 were measured by GVK Biosciences, Hyderabad, India.

Body Temperature and Activity Telemetry. Core body temperature and activity were measured continuously by telemetry (Starr Life Sciences, Oakmont, PA) using ER4000 energizer/receivers, G2 E-mitters implanted intraperitoneally, and VitalView software, with data collected each minute. Unless noted otherwise, the average Tb response was calculated using the first 60 minutes after A₃AR agonist dosing. Hypothermia duration is the time of Tb < 35°C during the interval from dosing to 300 minutes. Activity is the sum of counts from 10–60 minutes after A₃AR agonist administration. Inhibitors were dosed 15 minutes before A₃AR agonist.

Central Infusions. Mice were anesthetized with ketamine/xylazine (80/10 mg/kg i.p.). Sterile guide cannulas (5.25 mm, 26 gauge; Plastics One, Roanoke, VA) were unilaterally implanted into the lateral ventricle (coordinates relative to bregma: -0.34 mm anterior, 1.0 mm lateral, and +1.7 mm ventral) and fixed with dental cement (Parkell, Edgewood, NY). Compounds in 5 μl were infused (0.5 μl/min) through a 33-gauge cannula protruding 0.5 mm past the tip of the guide cannula using PE-50 tubing fitted to a 5-μl syringe (Hamilton, Reno, NV). Cannula positions were verified by postmortem histologic analysis.

Antinociception. Paw withdrawal threshold (PWT) in a mouse sciatic nerve chronic constriction injury (CCI)-neuropathic pain model was measured as described (Bennett and Xie, 1988; Chen et al., 2012). The PWT (in grams) on both the constricted and uninjured side were measured before constriction (day 0), at peak mechano-allodynia (day 7), and at the indicated times after drug administration for both the constricted side and the uninjured contralateral paw. ED₅₀ values at 1 hour after drug treatment were calculated from dose-response data curve-fitted using the least sum of square method by a normalized four-parameter, variable-slope nonlinear analysis of the %Reversal of Mechano-allodynia using GraphPad Prism (v5.03; GraphPad Software, Inc., San Diego, CA). The %Reversal of Mechano-allodynia = (PWT_{1h} - PWT_{D7})/(PWT_{D0} - PWT_{D7}) × 100. Chronic constriction injury data are expressed as mean ± S.D. and were analyzed by repeated-measures two-way analysis of variance with Bonferroni correction using GraphPad Prism.

Other Procedures. Indirect calorimetry was performed using an OxyMax/CLAMS (Columbus Instruments, Columbus, OH) at 22°C as described (Lute et al., 2014). Environmental temperature preference was measured using an in-house apparatus by continuous video monitoring of the position of the mouse in a 45-cm thermal gradient, nominally 18–36°C (Lute et al., 2014). Data are reported as mean ± S.E.M. Significance (two-tailed *P* < 0.05) was determined using SigmaPlot using *t* test or analysis of variance followed by post-hoc Holm-Sidak multiple comparison tests.

Results

MRS5698 Causes Hypothermia and Decreased Physical Activity via A₃AR. To understand the role of A₃AR agonism in hypothermia, we used a highly selective agonist, MRS5698 (Tosh et al., 2012). Handling of mice during vehicle treatment caused an increase in activity and body temperature (Tb), which resolved within an hour. In contrast, MRS5698 treatment caused a dose-dependent drop in Tb, ranging from nonsignificant reduction (with 0.1 mg/kg of the rise in Tb seen with vehicle injection) to a profound, prolonged hypothermia at higher doses (0.3 to 30 mg/kg) (Fig. 1A). The

Fig. 1. MRS5698 causes dose-dependent hypothermia and decreased physical activity. (A) Tb response to the indicated MRS5698 dose injected intraperitoneally into C57BL/6J mice. (B) Physical activity response to indicated MRS5698 dose in arbitrary units each minute. The effect of MRS5698 on (C) mean Tb (of 1–60 minutes), (D) duration of hypothermia, and (E) physical activity calculated from the data in (A) and (B). Data are mean $\pm$ S.E.M., $n = 6$ –13/group; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Every tenth S.E.M. is shown in (A). In (B) data are graphed as splines and S.E.M.s were omitted for visual clarity.

mean Tb during the first hour after dosing is a sensitive measure of hypothermia (Fig. 1C). Higher MRS5698 doses progressively reduced the nadir Tb and increased the duration of the hypothermia (Fig. 1D). The hypothermia was accompanied by reduced physical activity (Fig. 1, B and E).

The receptor selectivity of MRS5698 for A_3 AR determined in binding assays (>3000 -fold versus A_1 AR in both mouse and human) was confirmed in vivo using *Adora1* $^{-/-}$ and *Adora3* $^{-/-}$ mice since hypothermic actions attributed to some A_1 AR agonists may be attributable to A_3 AR (Yang et al., 2010) (and unpublished observations). MRS5698 effects on Tb nadir, hypothermia duration, and physical activity were similar in wild-type (WT) and *Adora1* $^{-/-}$ mice (Fig. 2, A and B, and not shown). In contrast, MRS5698-induced hypothermia was completely abolished in *Adora3* $^{-/-}$ mice (Fig. 2, C and D). These

data demonstrate that the hypothermic effects of MRS5698 are produced via A_3 AR, with no contribution from A_1 AR.

A_3 AR Agonist Acts via Mast Cells to Induce Hypothermia. To test for a role of mast cells in A_3 AR agonist-mediated hypothermia, we pretreated mice with multiple ascending doses of compound 48/80, which depletes mast cell granules (Ge et al., 2006). Hypothermia occurred after each dose of compound 48/80, including the last dose (not shown), suggesting that granule depletion may have been partial. One day after the last 48/80 treatment, the hypothermic effect of MRS5698 was attenuated (Fig. 3). These data suggest that mast cells contribute to the hypothermic response to MRS5698.

We next tested the effect of two doses of MRS5698 given 4 hours apart (Supplemental Fig. 1). Mice treated only once

Fig. 2. MRS5698-induced hypothermia is lost in *Adora3* $^{-/-}$ mice. Tb response to MRS5698 (10 mg/kg i.p.) or vehicle in (A, B) *Adora1* $^{-/-}$ (KO) and C57BL/6J (WT) mice and (C, D) *Adora3* $^{-/-}$ (KO) and C57BL/6J (WT) mice. Data are mean $\pm$ S.E.M., $n = 3$ –5/group in a crossover design, * $P < 0.05$, *** $P < 0.001$. Every tenth S.E.M. is shown.

Fig. 3. Pretreatment with compound 48/80 attenuates MRS5698 hypothermia. (A) C57BL/6J mice were pretreated with compound 48/80 (48/80) or saline (sal) for 5 days (see *Materials and Methods*). On the next day, at time 0, mice were treated with MRS5698 (10 mg/kg i.p.) or vehicle. Data are mean $\pm$ S.E.M., $n = 6$ /group, every tenth S.E.M. is shown. (B) Mean Tb (of 1–60 minutes) calculated from the data in (A), * $P < 0.05$, *** $P < 0.001$.

with MRS5698 showed the expected hypothermia and hypoactivity, whereas a second MRS5698 injection did not reduce Tb or activity, indicating that mice have a refractory period after a hypothermic/hypoactive response to A₃AR activation.

A₃AR Agonist Acts Both Outside and Within the Central Nervous System. The hypothermia caused by MRS5698 could occur via peripheral and/or central A₃ARs, because this agonist crosses the blood-brain barrier (Little et al., 2015). Consistent with the in vivo results, MRS5698 has a high intrinsic permeability across an artificial membrane ($P = 67 \pm 21 \times 10^{-6}$ cm/s). It is also a substrate for intestinal transport (Caco-2 efflux ratio = 86) [Table 1; Tosh et al. (2015)].

The effects of intraperitoneal versus intracerebroventricular dosing were compared using MRS5841, a sulfonated A₃AR agonist with selectivity and potency similar to MRS5698 but improved aqueous solubility and restricted central nervous system (CNS) penetration (Paoletta et al., 2013). MRS5841 was not detectably permeable across an artificial membrane, although it is probably a transporter substrate (Caco-2 efflux ratio = 13) (Table 1). MRS5841, given intraperitoneally at 10 mg/kg, produced hypothermia similar to MRS5698, with no effect in *Adora3*^{-/-} mice, confirming the drug's A₃AR selectivity (Fig. 4, A and B). Mice given a 20-fold lower dose of MRS5841 intracerebroventricularly showed no clear hypothermia (Fig. 4, C and D). These data suggest that A₃AR agonists cause hypothermia via binding to receptors outside the central nervous system.

A₃AR-Agonist Hypothermia Is Mediated by Central Histamine H₁ Receptors. Histamine is one of the bioactive molecules released by mast cells (Fozard et al., 1996). To examine the role of histamine receptors in A₃AR agonist-induced hypothermia, we pretreated mice with histamine antagonists. The H₁R antagonist pyrilamine (10 mg/kg i.p.) completely inhibited MRS5698-induced hypothermia (Fig. 5, A and B). In contrast, pretreatment with either the brain-penetrant H₂R antagonist zolantidine (10 mg/kg i.p.)

(Gogas and Hough, 1988) or the brain-penetrant H₄R antagonist JNJ7777120 (10 mg/kg i.p.) (Hsieh et al., 2010) had no effect on MRS5698-induced hypothermia (Fig. 5, C–F). Pretreatment with intracerebroventricular pyrilamine (0.5 mg/kg) also completely inhibited the hypothermic response to MRS5698 (Fig. 5, G and H). The 0.5 mg/kg dose had no effect when given intraperitoneally (data not shown), indicating that the inhibition of hypothermia resulted from inhibition of central, and not peripheral, H₁R. These results suggest that the central H₁Rs are functionally downstream from the A₃AR receptor in the hypothermia response to MRS5698.

A₃AR-Agonist Hypothermia Is a Coordinated Physiologic Response. MRS5698 administration caused a reduction in metabolic rate, which preceded the reduction in Tb (Fig. 6), suggesting that the hypothermia was being actively driven. When hypothermia is the targeted physiologic state with a reduced Tb set point, the animal prefers a cool environment to attain the hypothermia. In contrast, in hypothermia without a reduced Tb set point, the animal prefers a warm environment (Gordon, 2012). Mice treated with MRS5698 chose a cooler place in a thermal gradient and moved less compared with vehicle-treated controls (Fig. 7). This indicates that the hypothermic state is a coordinated physiologic response to A₃AR agonist treatment.

The Analgesic Effects of A₃AR Agonists Are Independent of Hypothermia. A₃AR agonism is an effective treatment for neuropathic pain (Chen et al., 2012; Little et al., 2015). When peak mechano-allodynia develops [day 7 following chronic constriction injury of the sciatic nerve (Bennett and Xie, 1988)], intraperitoneal administration of MRS5698, but not vehicle, rapidly and dose-dependently (0.1–1 mg/kg) reversed the allodynia, with maximal effect within 1 hour (ED₅₀ 0.2 mg/kg; 95% CI = 0.2–0.3) (Fig. 8A). The uninjured contralateral paws were not affected by treatment with MRS5698 (Fig. 8B). The MRS5698 dose sufficient for analgesia is below doses producing hypothermia.

TABLE 1
In vitro parameters of A₃AR agonists

Property, Units		MRS5698 ^a	MRS5841	MRS5980 ^a
Permeability, Caco-2 ^b	P _{app} (A–B, 10 ⁻⁶ cm/s)	0.08	0.10 (60% recovery)	2.20 (81% recovery)
	P _{app} (B–A, 10 ⁻⁶ cm/s)	7.26	1.32 (71% recovery)	35.8 (93% recovery)
	Efflux ratio	86	13	16.3
Permeability, PAMPA ^c pH7.4	P (10 ⁻⁶ cm/s)	67.1 $\pm$ 21.4	0.0 $\pm$ 0.0	46.7 $\pm$ 2.1
Stability, plasma (human)	% remaining at 2 hours	ND	91%	100%
Stability, plasma (mouse)	% remaining at 2 hours	100%	100%	100%
Stability, plasma (rat)	% remaining at 2 hours	100%	100%	97%

ND, not determined; PAMPA, parallel artificial membrane permeability assay.

^aMRS5698 data are from Tosh et al. (2015); some of the MRS5980 data are from Tosh et al. (2014).

^bCaco-2 permeability and plasma stability data are mean of two replicates.

^cPAMPA permeability data are mean $\pm$ S.D. of three replicates.

Fig. 4. Comparison of the hypothermic response to peripheral versus central dosing with A₃AR agonist MRS5841. (A) Tb and (B) mean Tb response to MRS5841 (10 mg/kg) or vehicle injected intraperitoneally into C57BL/6J (WT) or *Adora3*^{-/-} (A₃AR KO) mice. MRS5841 causes hypothermia in wild-type but not *Adora3*^{-/-} mice. (C) Tb and (D) mean Tb response to MRS5841 (0.5 mg/kg = 25 nmol) or vehicle injected intracerebroventricularly into C57BL/6J mice. Data are mean ± S.E.M., *n* = 4–7/group in (A), *n* = 15/group in (C); every tenth S.E.M. is shown, ***P* < 0.01.

We tested whether the antinociceptive effect requires hypothermia. Pyrilamine pretreatment (10 mg/kg i.p.) had no effect on the reversal of neuropathic pain by MRS5698 (3 mg/kg i.p.) (Fig. 9A). Pyrilamine alone also had no effect on neuropathic pain or in the contralateral paws (Fig. 9, A and B).

The relationship between hypothermia and analgesia was also probed with an orally bioavailable A₃AR agonist, MRS5980 (Tosh et al., 2014). Oral MRS5980 caused

hypothermia and reduced activity with a dose of 30 mg/kg, but not 10 mg/kg (Supplemental Fig. 2). Oral MRS5980 rapidly and dose-dependently reversed allodynia, with maximal effect within 1 hour (ED₅₀ 0.23 mg/kg; 95% CI = 0.15–0.35) and no effect in the contralateral paws (Supplemental Fig. 3). The lack of reversal of the analgesic effect of MRS5698 by pyrilamine and the lower doses of MRS5698 and MRS5980 required for analgesia compared with hypothermia

Fig. 5. A₃AR agonist-induced hypothermia is mediated by central histamine H₁ receptors. (A) Tb and (B) mean Tb (of 1–60 minutes) values in response to pretreatment with H₁R antagonist pyrilamine (10 mg/kg i.p.) or saline followed 15 minutes later by MRS5698 (10 mg/kg i.p., at time 0) or vehicle in C57BL/6J mice. (C) Tb and (D) mean Tb in response to pretreatment with H₂R antagonist zolantidine (10 mg/kg i.p.) or saline followed 15 minutes later by MRS5698 (10 mg/kg i.p.) or vehicle. (E) Tb and (F) mean Tb in response to pretreatment with H₄R antagonist JNJ77720 (10 mg/kg i.p.) or vehicle. (G) Tb and (H) mean Tb in response to pretreatment with intracerebroventricular pyrilamine (0.5 mg/kg) or saline followed 15 minutes later by MRS5698 (10 mg/kg i.p.) or vehicle. Data are mean ± S.E.M., *n* = 4/group; every tenth S.E.M. is shown in (A), (C), (E), and (G); **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

Fig. 6. MRS5698 reduces metabolic rate prior to Tb. Effect of MRS5698 (10 mg/kg i.p.) or vehicle dosed to C57BL/6J mice (A) Tb, (B) mean Tb (of 1–60 minutes), (C) total energy expenditure (TEE), and (D) mean TEE (of 1–60 minutes). The TEE falls before Tb (nadir at ~30 minutes versus ~60 minutes for Tb). Parameters were measured every 13 minutes; data are mean $\pm$ S.E.M., $n = 6$ /group; * $P < 0.05$, ** $P < 0.01$.

demonstrate that hypothermia is not required for the anti-nociceptive effects of A₃AR agonists.

Discussion

We show that A₃AR agonists acting peripherally cause hypothermia and that these actions are independent of A₁AR activation. The data support a model, discussed below, in which peripheral A₃AR agonist causes mast cell activation and histamine release leading to activation of central H₁R.

A Site of A₃AR-Agonist Action Is the Mouse Mast Cell. Local extracellular adenosine levels typically increase under extreme conditions such as hypoxia, injury, or high metabolic stress (Van Wylen et al., 1986; Porkka-Heiskanen et al., 1997; Schubert et al., 1997; Minor et al., 2001; Basheer et al., 2004; Dworak et al., 2007). The A₃AR appears to have a modest affinity for adenosine, being tuned for the elevated adenosine levels occurring with extreme physiologic situations (Fredholm, 2014). A₃ARs are present on immune/inflammatory cells, such as mast cells (Hua et al., 2008), basophils, eosinophils, neutrophils, monocytes, macrophages, and microglial cells [reviewed in Borea et al. (2015)]. However, it is notable that mast cell A₃AR level and biology depend on the species and tissue source, with A₃AR not present in some human mast cells (Auchampach et al., 1997; Gomez et al., 2011; Rudich et al., 2012). Adenosine, acting via A₃AR on mast cells, causes histamine release and airway hyper-responsiveness in mice (Tilley et al., 2000, 2003; Zhong

et al., 2003; Hua et al., 2008). In our study, compound 48/80, which causes mast cell granule depletion [but not depletion of histamine from neurons (Metcalf et al., 1997)], attenuated A₃AR agonist-induced hypothermia, implicating mast cells as an initiator of the events. The partial inhibition may be attributable to incomplete degranulation by compound 48/80 or to A₃AR agonist also acting at other cells to initiate hypothermia via histamine release. We conclude that A₃AR agonists are acting on mast cells but cannot rule out a contribution from additional cell types.

Hypothermia was caused by intraperitoneal dosing of MRS5841, an intrinsically impermeant compound that does not penetrate the CNS (Paoletta et al., 2013). In contraposition, although mast cells are found in the brain and account for a considerable fraction of brain histamine content (Tabarean, 2015), dosing MRS5841 centrally did not produce hypothermia. These results suggest that hypothermia is caused by binding to A₃AR receptors outside the blood-brain barrier. It is possible that other A₃AR agonist-induced effects such as reduced locomotion, behavioral depression, and neuroprotection (Fedorova et al., 2003; Yang et al., 2010; Paoletta et al., 2013; Borea et al., 2015) are also initiated via peripheral A₃AR.

Central Histamine Receptors and Hypothermia. Histamine was first shown to cause hypothermia in 1911 (Dale and Laidlaw, 1911). More recent experiments suggested a brain site of action and the involvement of H₁R (Shaw, 1971; Bugajski and Zacny, 1981; Haas et al., 2008). We show that signaling via CNS histamine H₁R is required for A₃AR agonist-mediated hypothermia. It is not typically thought that peripheral histamine crosses the blood-brain barrier [but see Shaw (1971)]. Possible explanations are that histamine acts at a circumventricular region without a functional blood-brain barrier or that histamine itself increases blood-brain barrier permeability (Boertje et al., 1989). The important point is that hypothermia occurs following peripheral A₃AR activation and is dependent on histamine action in the brain. Although we have not determined which neurons carry the relevant H₁Rs, the preoptic area/anterior hypothalamus is a thermoregulatory region previously identified as a site for histamine-triggered hypothermia (Bugajski and Zacny, 1981; Colboc et al., 1982). However, there are also opposing observations—direct injection of an H₁R agonist into the preoptic area produces hyperthermia, not hypothermia (Lundius et al., 2010; Sethi et al., 2012; Tabarean, 2015). Further investigations are needed to untangle the mechanistic details of central histamine action in hypothermia.

A₃AR Hypothermia and Other Forms of Hypothermia. Many drugs cause hypothermia. Mast cell activation, as demonstrated for A₃AR, may be the mechanism for a significant proportion. Examples of signals to mast cells causing hypothermia include activation of the Fc ϵ RI IgE receptor

Fig. 7. Mice treated with MRS5698 seek cooler temperatures. C57BL/6J mice were treated with MRS5698 (3 mg/kg i.p.) or vehicle and placed in a thermal gradient. The mice were monitored by video, and the (A) position within the gradient and the (C) velocity were calculated. The mean (B) position and (D) velocity for each mouse from 6 to 35 minutes after dosing was calculated. Mean $\pm$ S.E.M., $n = 6$ /group, crossover design; * $P < 0.05$.

Fig. 8. Dose-dependent reversal of neuropathic pain by MRS5698. PWT measured in the ipsilateral (A) and contralateral (B) paws before chronic constriction injury (CCI; day 0), after 7 days of constriction and before drug treatment (day 7), and at the indicated times after administration of MRS5698 (●, 0.1; ■, 0.3; ▲, 1 mg/kg i.p.) or vehicle (○, 0.1% dimethyl sulfoxide in saline). Mean $\pm$ S.D., $n = 5$ /group; * $P < 0.05$ day 7 versus day 0, $^{\dagger}P < 0.05$ indicated time versus day 7.

(Finkelman, 2007), neurotensin receptor (Kandasamy et al., 1991), Toll-like receptor 4 (by lipopolysaccharide) (Nautiyal et al., 2009), and Toll-like receptor 7 (Hayashi et al., 2008). Although we did not measure blood pressure, hypotension probably accompanies hypothermia because of the mast cell activation (Hannon et al., 1995). The hypotension may contribute to the hypothermia, but these can be dissociated [hypothermia without hypotension (Lute et al., 2014) and hypothermia without hypotension (Makabe-Kobayashi et al., 2002)].

Other forms of hypothermia are independent of mast cells. Mast cells do not contribute to the reduced body temperature of the inactive circadian phase (Nautiyal et al., 2009) and A_1 AR hypothermia is elicited via a neuronal mechanism (Tupone et al., 2013). Similarly, there is no documented role for mast cells in the hypothermia of torpor or hibernation. It is likely that different mechanisms trigger hypothermia, depending on the instigating stimulus, with the mechanistic details currently unknown for many.

Therapeutic Uses of A_3 AR Agonists. Hypothermia blunts inflammation, such as the “cytokine storm” of sepsis, and reduces metabolic demand. Endogenous hypothermia can be part of a coordinated hypometabolic response to limit tissue damage and increase survival. In clinical medicine, hypothermia is induced to allow hypoperfusion during surgery and in ominous situations such as after neonatal hypoxia (Azzopardi et al., 2014) or cardiopulmonary resuscitation (Debaty et al., 2014). Therapeutic hypothermia can be induced by physical cooling at the surface and internally. Pharmacological induction of hypothermia is an appealing clinical concept, with the dual goal of lowering body temperature while minimizing counter-regulatory responses such as shivering, non-shivering thermogenesis, and increased sympathetic tone. However, A_3 AR agonism is probably not an optimal pharmacologic approach, owing to the unclear role of A_3 AR in primate mast cell activation and to the undesired effects of mast cell activation, such as bronchoconstriction and vascular leakage. The latter effects likely also render H_1 R agonists suboptimal for clinical hypothermia induction.

The clinical utility of A_3 AR agonists is being explored for rheumatoid arthritis and psoriasis, glaucoma and dry eye, pain, and certain cancers (Fishman et al., 2012; Borea et al., 2015). Since hypothermia is anti-inflammatory, does the

hypothermia contribute to A_3 AR efficacy for these indications? We show for two different A_3 AR agonists that antinociception is achieved at doses that are below those eliciting hypothermia. Since hypothermic effects are greatly amplified in small mammals (e.g., mouse) compared with large mammals (e.g., human), antinociceptive A_3 AR agonist doses are unlikely to cause hypothermia at clinically relevant doses (e.g., a 1- to 2-mg dose of IB-MECA, twice daily PO, for treatment of psoriasis or rheumatoid arthritis [Fishman et al., 2012]). Furthermore, H_1 R blockade did not block antinociception but did prevent hypothermia. Thus, hypothermia does not contribute to or interfere with the antinociceptive effect of A_3 AR agonists.

In conclusion, the results support a model in which A_3 AR agonists activate mast cells in the periphery, causing histamine release. The histamine acts on central H_1 R to lower the Tb set point and cause hypothermia. Importantly, hypothermia is not required for the antinociceptive effects of A_3 AR agonists. These results demonstrate that adenosine can cause hypothermia through A_3 AR, in addition to A_1 AR, activation and provide mechanistic detail for the route by which A_3 AR agonism induces hypothermia. The data suggest that A_3 AR agonists are not ideal for clinical induction of hypothermia. We have used a mouse model in which hypothermic effects are amplified in intensity in comparison with larger species. Furthermore, the beneficial use of such agonists in humans at very low doses in treating chronic diseases is not likely to be complicated by or to be dependent on hypothermic effects.

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Authorship Contributions

Participated in research design: Carlin, Salvemini, Gavrilova, Jacobson, Reitman.

Conducted experiments: Carlin, Xiao, Piñol, Chen, Gavrilova.

Contributed new reagents or analytic tools: Tosh, Jacobson.

Performed data analysis: Carlin, Xiao, Salvemini, Gavrilova, Reitman.

Wrote or contributed to the writing of the manuscript: Carlin, Salvemini, Gavrilova, Jacobson, Reitman.

Fig. 9. Pyrilamine does not reverse the analgesic effect of MRS5698. PWT measured in the ipsilateral (A) and contralateral (B) paws before chronic constriction injury (CCI; day 0), after 7 days of constriction and before drug treatment (day 7), and at the indicated times after administration of saline then vehicle (○), saline then MRS5698 (▲), pyrilamine then vehicle (□), or pyrilamine then MRS5698 (■). The pyrilamine (10 mg/kg i.p.) or its vehicle (saline) was given 15 minutes before MRS5698 (3 mg/kg i.p.) or its vehicle (0.1% dimethyl sulfoxide in saline). Mean $\pm$ S.D., $n = 5$ /group; * $P < 0.05$ day 7 versus day 0, $^{\dagger}P < 0.05$ indicated time versus day 7.

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