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Paracetamol-Induced Hypothermia Is Independent of Cannabinoids and Transient Receptor Potential Vanilloid-1 and Is Not Mediated by AM404

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ABSTRACT:

In recent years, there has been increasing interest in hypothermia induced by paracetamol for therapeutic purposes, which, in some instances, has been reported as a side effect. Understanding the mechanism by which paracetamol induces hypothermia is therefore an important question. In this study, we investigated whether the novel metabolite of paracetamol, *N*-(4-hydroxyphenyl)arachidonylamide (AM404), which activates the cannabinoid (CB) and transient receptor potential vanilloid-1 (TRPV1) systems, mediates the paracetamol-induced hypothermia. The hypothermic response to 300 mg/kg paracetamol in CB_1 receptor (CB_1R) and TRPV1 knockout mice was compared to wild-type mice. Hypothermia induced by paracetamol was also investigated in animals pretreated with the CB_1R or TRPV1 antagonist 1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-*N*-1-piperdinyl-1*H*-pyrazole-3-carboxamide trifluoroacetate salt (AM251) or 4′-chloro-3-methoxycinnamanilide (SB366791), respectively. In CB_1R or TRPV1 knockout mice, paracetamol induced hypothermia to the same extent as in wild-type mice. In addition, in C57BL/6 mice pretreated with AM251 or SB366791, paracetamol induced hypothermia to the same extent as in control mice. AM404 failed to induce hypothermia at pharmacological doses. Inhibition of fatty acid amide hydrolase (FAAH), which is involved in the metabolism of paracetamol to AM404, did not prevent the development of hypothermia with paracetamol. Paracetamol also induced hypothermia in FAAH knockout mice to the same extent as wild-type mice. We conclude that paracetamol induces hypothermia independent of cannabinoids and TRPV1 and that AM404 does not mediate this response. In addition, potential therapeutic value of combinational drug-induced hypothermia is supported by experimental evidence.

Introduction

Paracetamol (acetaminophen) is an analgesic antipyretic drug that has been in clinical use for reducing elevated body temperature (fever) for over a century. In addition to antipyretic actions, paracetamol has also been shown to possess hypothermic actions in humans (Dippel et al., 2001; Denes et al., 2002; Kasner et al., 2002; Tréluyer et al., 2002; Richardson and Sills, 2004) and in experimental animals (Ayoub et al., 2004). In some cases, hypothermia induced by paracetamol in patients has been reported as a self-resolving, reversible, unwanted effect (Denes et al., 2002; Tréluyer et al., 2002; Richardson et al., 2004), whereas in other cases, it has been induced for therapeutic purposes such as the acute management of stroke (Dippel et al., 2001; Kasner et al., 2002).

The mechanism of pharmacological actions of paracetamol has not been fully elucidated. The compound weakly inhibits activities of cyclooxygenase-1 (COX-1) and COX-2 enzymes (Mitchell et al., 1993). However, it significantly reduces central nervous system prostaglandin synthesis (Feldberg et al., 1972; Flower and Vane, 1972; Ayoub et al., 2006), indicating inhibition of a COX activity. We recently demonstrated that the hypothermic action of paracetamol in normothermic mice is dependent on the inhibition of a COX-1-derived protein. We demonstrated significant reduction in the paracetamol-induced hypothermia in COX-1 knockout mice compared to their littermate controls, whereas COX-2 knockout mice developed hypothermia after paracetamol administration to the same extent as their wild-type littermate controls. The reduction of paracetamol-induced hypothermia in COX-1 knockout mice was accompanied by reduction in the paracetamol-induced inhibition of brain prostaglandin E_2 (PGE_2) synthesis (Ayoub et al., 2004).

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ABBREVIATIONS: COX, cyclooxygenase; AM404, *N*-(4-hydroxyphenyl)arachidonylamide; CB_1R, cannabinoid receptor-1; FAAH, fatty acid amide hydrolase; PGE_2, prostaglandin E_2; TRPV1, transient receptor potential vanilloid-1; WIN55-212,2, (*R*)-(+)-[2,3-dihydro-5-methyl-3[(4-morpholinyl) methyl]pyrrolo [1,2,3-de]-1,4-benzoaxzinyl]-(1-naphthalenyl)methanone mesylate salt; AM251, *N*-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide; SB366791, 4′-chloro-3-methoxycinnamanilide; URB597, cyclohexyl carbamic acid 3′-carbamoyl-biphenyl-3-yl ester; SC560, 5-(4-chloro-phenyl)-1-(4-methoxyphenyl)-3-trifluoromethylpyrazole; ANOVA, analysis of variance.

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More recently, it has been proposed that the pharmacological actions of paracetamol are mediated through interactions with the cannabinoids and/or the transient receptor potential vanilloid-1 (TRPV1) channel (Högestätt et al., 2005). This hypothesis is based on the demonstration that paracetamol is metabolized through a two-step pathway involving brain fatty acid amide hydrolase (FAAH) activity into *N*-(4-hydroxyphenyl)arachidonylamide (AM404). AM404 activates the endocannabinoid system by increasing the synaptic availability of the endocannabinoid anandamide, by inhibition of the uptake of anandamide into presynaptic neurons resulting in reduction of its degradation by FAAH (Beltramo et al., 1997). Anandamide produces antinociceptive and hypothermic actions, both mediated through the CB_1 receptor (CB_1R) (Howlett, 1995). Potent hypothermia has been reported after the administration of selective CB_1R agonists (Pryce et al., 2003). Activation of neuronal TRPV1 channels by selective agonists such as capsaicin has also been shown to result in the development of hypothermia (Varga et al., 2005). AM404 is an agonist of TRPV1 (De Petrocellis et al., 2000) and is also able to induce hypothermia in rats in a TRPV1-dependent manner (Rawls et al., 2006). More recently, Mallet et al. (2008) demonstrated that the analgesic action of paracetamol in experimental pain was dependent on activation of the cannabinoid system by AM404 derived from paracetamol.

In the present study, we sought to investigate whether AM404 mediates the hypothermic actions of paracetamol in a manner dependent on the activation of the cannabinoid and/or TRPV1 systems.

Materials and Methods

Animals. Male C57BL/6 mice (20 ± 2 g) were supplied from Harlan UK Limited (Bicester, Oxon, UK). COX-1, COX-2 (Langenbach et al., 1995; Morham et al., 1995), Biozzi ABH, and ABH mice lacking the CB_1R or FAAH (Brooks et al., 2002; Pryce et al., 2003; Bilsland et al., 2006), and TRPV1 knockout mice (Sexton et al., 2007) were from stocks bred at Barts and the London School of Medicine and Dentistry. All strains of mice were maintained under a 12-h light/dark cycle at 22 ± 1°C. Food and water were provided ad libitum. Experimental procedures were conducted in accordance with the UK Home Office guidelines.

Chemicals. Paracetamol (Sigma Chemical, Poole, Dorset, UK) was dissolved in 12.5% (v/v) 1,2-propanediol. WIN55-212,2 *N*-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide (AM251), 4′-chloro-3-methoxycinnamanilide (SB366791), AM404, anandamide (Tocris Bioscience, Bristol, UK), capsaicin, cyclohexyl carbamic acid 3′-carbamoyl-biphenyl-3-yl ester (URB597) (Sigma Chemical), 5-(4-chloro-phenyl)-1-(4-methoxyphenyl)-3-trifluoromethylpyrazole (SC560), and celecoxib (kind gifts from Schering Aktiengesellschaft, Berlin, Germany) were initially dissolved in 100% dimethyl sulfoxide and then diluted to the appropriate doses in a solution containing 10% cremophor oil, 10% ethanol, and 80% saline, reducing the concentration of dimethyl sulfoxide to 0.1%.

Temperature Measurement and Administration of Drugs. Body temperature was measured using a thermocouple probe placed under the hindlimb as described previously (Brooks et al., 2002). The animals were preconditioned to the temperature probe by taking temperature measurements 3 days before the experiment and twice on the day of the experiment before drug administration to reduce handling-induced temperature changes associated with stress. In each experiment, the time profile of hypothermia, usually up to 5 h, was determined. The ambient temperature was set to 22 ± 1°C during the entire duration of the experiments.

Experimental Objective 1. To determine whether activation of CB_1R or TRPV1 is involved in the paracetamol-induced hypothermia, the time profile of the hypothermic response of 300 mg/kg paracetamol i.p. was determined in CB_1R and TRPV1 knockout mice and was compared to their wild-type littermate controls. In a different experiment, C57BL/6 mice were pretreated with 5 mg/kg AM251 (CB_1R antagonist; intraperitoneally) and treated 1 h later with either 20 mg/kg WIN55-212,2 i.p. (CB_1R/CB_2R agonist) or 300 mg/kg paracetamol i.p. Another group of C57BL/6 mice was treated with 2 mg/kg SB366791 i.p. (TRPV1 antagonist) and treated 30 min later with either 1 mg/kg capsaicin s.c. (TRPV1 agonist) or 300 mg/kg paracetamol i.p.

Experimental Objective 2. To address whether the induction of hypothermia by the CB_1R or TRPV1 agonist WIN55-212,2 or capsaicin, respectively, is mediated by the inhibition of COX-1 or COX-2, the time profile of the hypothermic responses of 20 mg/kg WIN55-212,2 i.p. and 1 mg/kg capsaicin s.c. were determined in COX-1 and COX-2 knockout mice and compared to their wild-type littermate controls.

Experimental Objective 3. To determine whether AM404 is involved in mediating the hypothermic action of paracetamol, 40 mg/kg AM404 was administered to C57BL/6 mice, and the body temperature was measured over 5 h and compared to vehicle-treated animals. In a different experiment, the activity of FAAH was inhibited in C57BL/6 mice with 0.3 mg/kg URB597 i.p. for 30 min; animals were then treated with 300 mg/kg paracetamol i.p., and the body temperature was monitored over 5 h. Two additional groups of mice were included, one treated with 5 mg/kg anandamide i.p. with URB597 and the other without URB597. This was used as a positive control to confirm inhibition of FAAH. The hypothermic response induced by 300 mg/kg paracetamol was also compared between wild-type and FAAH knockout mice.

Experimental Objective 4. To determine whether the combination of lower doses of paracetamol (200 mg/kg i.p.) and WIN55-212,2 (5 mg/kg i.p.) would induce additive hypothermia, the two compounds were administered to C57BL/6 mice, and the body temperature was monitored for 5 h.

Prostaglandin Extraction and Measurement. For measurement of PGE_2 concentrations, whole brains were removed from the skull, immediately washed with 10 μg/ml indomethacin, and snap-frozen in liquid nitrogen. Prostaglandins were extracted using a protocol described previously (Ayoub et al., 2004, 2006). In brief, frozen brain tissues were pulverised with a nitrogen bomb. One milliliter of 15% (v/v) ethanol in distilled water (pH 3) was added to pulverized tissues, and samples were stored at 4°C for 10 min and spun at 375*g* for 10 min at 4°C. C-18 Sep-Pak columns (Waters, Milford, MA) were conditioned with 4 ml of ethanol followed by 4 ml of distilled water at a flow rate of 5 to 10 ml/min. The supernatants from homogenates were then applied to the columns at a flow rate of 5 ml/min. The columns were then washed with 4 ml of distilled water followed by 4 ml of 15% (v/v) ethanol in distilled water. The samples were eluted with 2 ml of ethyl acetate at a flow rate of 5 ml/min. The samples were dried and stored at −80°C ready for prostaglandin measurement. Measurement of brain PGE_2 was performed using a commercial enzyme immunoassay kit from GE Healthcare (Chalfont St. Giles, Buckinghamshire,, UK), according to the manufacturer's instructions. The concentration of PGE_2 in the samples was determined by comparing the calculated percentage binding of PGE_2 in the samples to a standard PGE_2 curve (0.05–6.4 ng/ml).

Statistical Analysis. The results were analyzed using GraphPad Prism 3.0 (GraphPad Software Inc., San Diego, CA), expressed and presented graphically as mean ± S.E.M. Statistical analysis was performed using two-way ANOVA with the post hoc Bonferroni test to compare temperature changes between the different treatment groups. For comparison of the effect of drugs on the synthesis of PGE_2, the unpaired *t* test was used. A P value of <0.05 was considered statistically significant.

Results

The Cannabinoid System Is Not Involved in the Induction of Hypothermia by Paracetamol. A dose of 300 mg/kg paracetamol was previously used to investigate the mechanism of paracetamol-induced hypothermia (Ayoub et al., 2004). Although high, this dose is within the pharmacological subtoxic range in mice (Muth-Selbach et al., 1999; Vaquero et al., 2007). In CB_1R knockout mice, 300 mg/kg paracetamol i.p. resulted in a significant hypothermic response within 1 h of administration ($P < 0.05$, two-way ANOVA). This hypothermic effect was not different from that seen in wild-type mice treated with the same dose of paracetamol (Fig. 1A). A wild-type vehicle-treated group was not currently undertaken, but based on previous experiments in our laboratory, these mice do not display significant temperature changes when treated with the same vehicle used here (Brooks et al., 2002; Pryce et al., 2003). The brain PGE_2 concentration of CB_1R

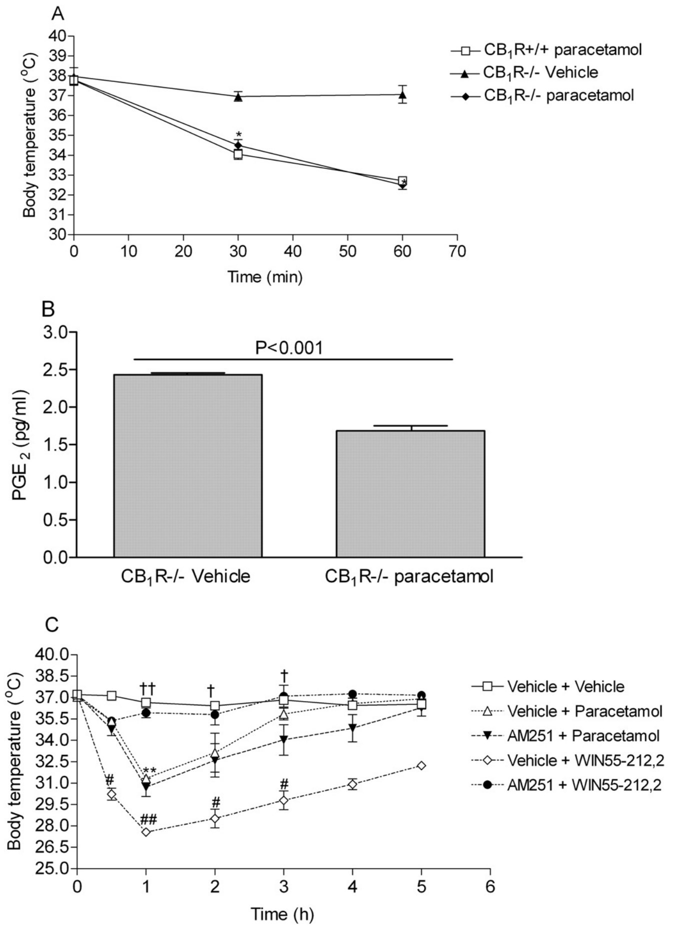

FIG. 1. Paracetamol-induced hypothermia in CB_1R knockout ($CB_1R-/-$) mice and in wild-type mice pretreated with the CB_1R antagonist AM251. A, time profile of the hypothermic response of 300 mg/kg paracetamol in $CB_1R-/-$ mice. Paracetamol or vehicle was administered intraperitoneally at time point 0, and the temperature of mice was measured at 0.5 and 1 h. ∗, $P < 0.05$, vehicle-treated $CB_1R-/-$ versus paracetamol-treated $CB_1R-/-$ mice (two-way ANOVA with post hoc Bonferroni test). B, comparison of the levels of PGE_2 in brain tissues of $CB_1R-/-$ mice with or without paracetamol administration (1 h after administration). Brain tissues were dissected, and PGE_2 was measured using enzyme immunoassay after extraction with C18 Sep-Pak columns. C, mice were pretreated with 5 mg/kg AM251 i.p. followed by treatment with 300 mg/kg paracetamol i.p., 20 mg/kg WIN55-212,2 i.p., or vehicle (intraperitoneally) 1 h later. The body temperature was monitored over 5 h. ∗∗, $P < 0.01$, vehicle versus paracetamol; #, $P < 0.05$; ##, $P < 0.01$, vehicle versus WIN55-212,2; †, $P < 0.05$; ††, $P < 0.01$, WIN55-212,2 versus WIN55-212,2 with AM251 (two-way ANOVA with post hoc Bonferroni test); n = 5–6.

knockout mice was also compared 1 h after 300 mg/kg paracetamol or vehicle treatments. Paracetamol reduced brain PGE_2 levels in CB_1R knockout mice compared to vehicle-treated mice ($P < 0.001$; Fig. 1B). Using a different experimental approach to determine whether paracetamol produces its hypothermic action by activation of cannabinoids, we pretreated C57BL/6 mice with 5 mg/kg of the CB_1R antagonist AM251 for 1 h followed by 300 mg/kg paracetamol. AM251 did not prevent the development of hypothermia by paracetamol (Fig. 1C). To demonstrate antagonism of CB_1R with AM251, we showed that AM251 inhibited the development of the hypothermic response induced by the CB_1/CB_2 receptor agonist WIN55-212,2 (20 mg/kg; $P < 0.001$, two-way ANOVA; Fig. 1C). The dose of WIN55-212,2 used here is within the calculated ED_{50} dose for hypothermia, which is 33 mg/kg (Sim-Selley and Martin, 2002). Administered on its own, at the same dose used above, AM251 did not affect body temperature as demonstrated by our results (data not shown) and other studies (Boctor et al., 2007).

To determine whether the cannabinoid-induced hypothermia is mediated by inhibition of a COX activity, WIN55-212,2 was administered to COX-1 and COX-2 knockout mice. In both COX-1 and COX-2 knockout mice, 20 mg/kg WIN55-212,2 induced a hypothermic response ($P < 0.05$, two-way ANOVA), of approximately 8°C, that was similar to that seen in their wild-type littermate controls in both the initial (0.5–1 h) and resolving phases (2–5 h; Fig. 2, A and B; $P < 0.05$). Similar to paracetamol, the peak of hypothermia with WIN55-212,2 occurred 1 h after administration.

TRPV1 Is Not Involved in the Induction of Hypothermia by Paracetamol. To investigate whether TRPV1 is involved in the induction of hypothermia by paracetamol, TRPV1 knockout mice were treated with 300 mg/kg paracetamol. The hypothermic response to paracetamol in TRPV1 knockout mice was similar to that seen in wild-type mice treated with the same dose of paracetamol (Fig. 3A) with a statistically significant drop in body temperature in paracetamol-treated mice compared to vehicle in both the wild-type and TRPV1 knockout mice ($P < 0.05$, two way ANOVA).

In a different experiment, C57BL/6 mice were pretreated with 2 mg/kg of the selective TRPV1 antagonist SB366791, 30 min after the animals were treated with either 300 mg/kg paracetamol or 1 mg/kg of the TRPV1 agonist capsaicin. SB366791 reversed the capsaicin-induced hypothermia by approximately 2°C ($P < 0.05$, two-way ANOVA). This reduction was observed 30 min and 1 h after capsaicin treatment (Fig. 3B). On the other hand, mice treated with SB366791 and paracetamol developed hypothermia to the same extent as animals treated with paracetamol alone. SB366791 administered alone did not affect the body temperature of mice (data not shown). The experimental design, doses, and routes of administration for SB366791 and capsaicin have been devised from previously published studies on hypothermia (Ding et al., 2005; Varga et al., 2005; Rawls et al., 2006).

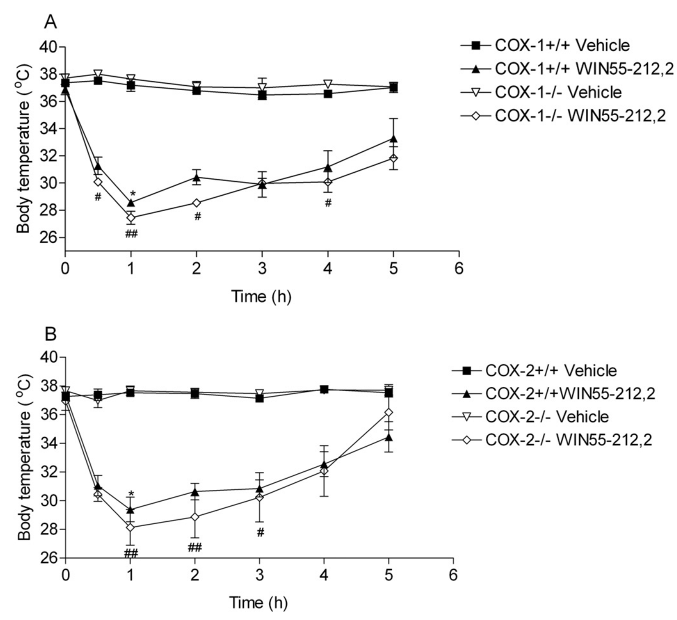

FIG. 2. Time profile of the hypothermic response of 20 mg/kg WIN55-212,2 in COX-1 (COX-1−/−; A, and COX-2 (COX-2−/−; B, knockout mice. WIN55-212,2 or vehicle was administered intraperitoneally at time point 0, and the body temperature of mice was measured over 5 h. A, ∗, $P < 0.05$, vehicle-treated COX-1 wild-type (COX-1+/+) versus WIN55-212,2-treated COX-1 wild-type mice; #, $P < 0.05$; ##, $P < 0.01$, vehicle-treated COX-1 knockout versus WIN55-212,2-treated COX-1 knockout. B, ∗, $P < 0.05$, vehicle-treated COX-2 wild-type (COX-2+/+) versus WIN55-212,2-treated COX-2 wild-type; #, $P < 0.05$; ##, $P < 0.01$, vehicle-treated COX-2 knockout versus WIN55-212,2-treated COX-2 knockout (two-way ANOVA with post hoc Bonferroni test); n = 5.

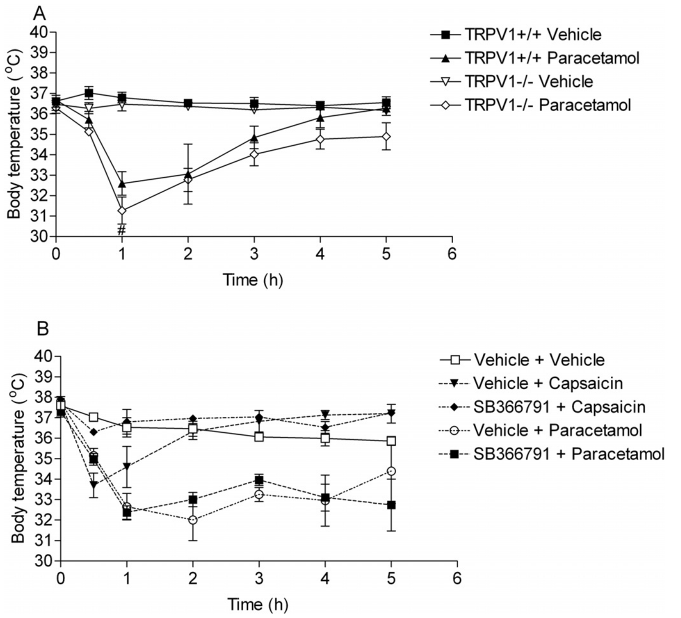

FIG. 3. Paracetamol-induced hypothermia in TRPV1 knockout (TRPV1−/−) mice and in wild-type mice pretreated with the TRPV1 antagonist SB366791. A, a concentration of 300 mg/kg or vehicle was administered intraperitoneally at time point 0, and the body temperature of mice was monitored over 5 h. #, $P < 0.05$, vehicle-treated TRPV1 knockout versus paracetamol-treated TRPV1 knockout (two-way ANOVA with post hoc Bonferroni test). B, mice were pretreated with 2 mg/kg SB366791 i.p. followed by treatment with 300 mg/kg paracetamol i.p. or 1 mg/kg capsaicin s.c. 30 min later. The body temperature was monitored over 5 h; $n = 5$–6.

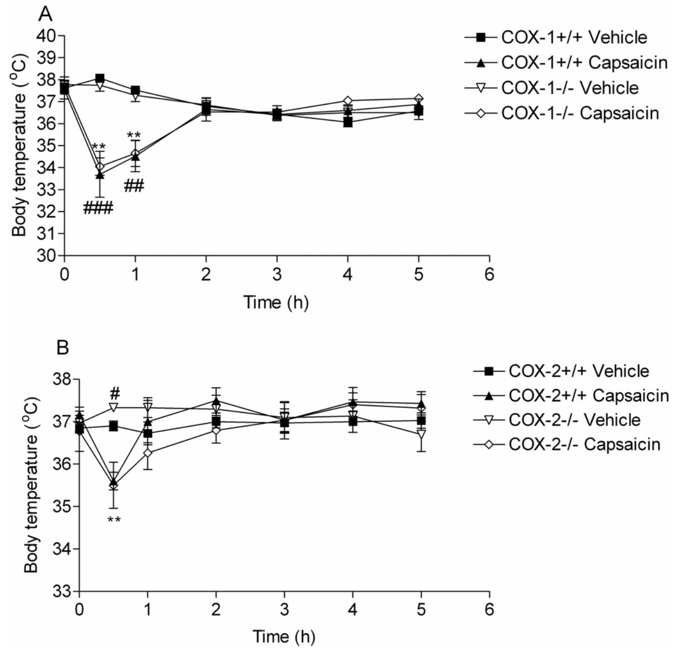

FIG. 4. Time profile of the hypothermic response of 1 mg/kg capsaicin in COX-1 (COX-1−/−; A, and COX-2 (COX-2−/−; B, knockout mice. Capsaicin or vehicle was administered subcutaneously at time point 0, and the body temperature of mice was measured over 5 h. A, **, $P < 0.01$, vehicle-treated COX-1 wild-type (COX-1+/+) versus capsaicin-treated COX-1 wild-type mice; ##, $P < 0.01$; ###, $P < 0.001$, vehicle-treated COX-1 knockout versus capsaicin-treated COX-1 knockout. B, **, $P < 0.01$, vehicle-treated COX-2 wild-type (COX-2+/+) versus capsaicin-treated COX-2 wild-type mice; #, $P < 0.05$, vehicle-treated COX-2 knockout versus capsaicin-treated COX-2 knockout (two-way ANOVA with post hoc Bonferroni test); $n = 5$.

In contrast, we wanted to determine whether the TRPV1-induced hypothermia was dependent on the inhibition of COX activity. Capasicin was administered to COX-1 and COX-2 knockout mice. In both COX-1 and COX-2 knockout mice, 1 mg/kg capsaicin induced statistically significant ($P < 0.05$, two-way ANOVA) hypothermia that was not statistically different from that observed in their wild-type littermate controls (Fig. 4, A and B).

AM404 Does Not Mediate the Paracetamol-Induced Hypothermia. AM404 administered exogenously did not induce hypothermia in either C57BL/6 (Fig. 5) or DBA1 (data not shown) mice at a top dose of 40 mg/kg (Fig. 5) or at 10 and 20 mg/kg (data not shown).

To investigate the involvement of AM404 in mediating the paracetamol-induced hypothermia, mice deficient in FAAH were treated with 300 mg/kg paracetamol, and their body temperature was monitored over 5 h. The rationale here was to block the conversion of paracetamol to AM404, which has previously been shown to be mediated through FAAH in the brain (Hogestatt et al., 2005). One hour after administration, paracetamol induced hypothermia to the same extents in both wild-type and FAAH knockout mice ($P < 0.001$, two-way ANOVA; Fig. 6A).

We also used URB597, a selective inhibitor of FAAH activity, to examine the effect of inhibition of the conversion of paracetamol into AM404 on the development of hypothermia induced by paracetamol. Pretreatment of mice with 0.3 mg/kg URB597 (30 min) did not prevent the development of hypothermia by 300 mg/kg paracetamol, and URB597 alone did not induce hypothermia (Fig. 6B). As a control to demonstrate that URB597 inhibited brain FAAH activity, the effect of URB597 on 5 mg/kg anandamide-induced hypothermia was investigated. As a result of increased synaptic accumulation of anandamide, URB597 potentiated the anandamide-induced hypothermia 2 h after anandamide administration ($P < 0.05$, two-way ANOVA; Fig. 6B). The dose of anandamide used in the present study is within the pharmacological range (Fegley et al., 2004).

Combinational Hypothermia Induced by Lower Doses of Paracetamol and WIN55-212,2. The coadministration of lower doses of paracetamol (200 mg/kg) and WIN55-212,2 (5 mg/kg), compared to those used in the previous experiments, resulted in supra-additive hypothermia in C57BL/6 mice with drops in body temperatures by 5.75 and 9.25°C after 0.5 and 1 h, respectively, compared to vehicle-treated mice ($P < 0.05$, two-way ANOVA; Fig. 7).

Discussion

Högestätt et al. (2005) have shown that the intermediate paracetamol metabolite, *p*-aminophenol, is converted in the brain into the novel metabolite AM404 through the action of FAAH (Högestätt et al., 2005). Before that, AM404 has been shown to induce analgesia (La Rana et al., 2006; Borsani et al., 2007; Mitchell et al., 2007) and

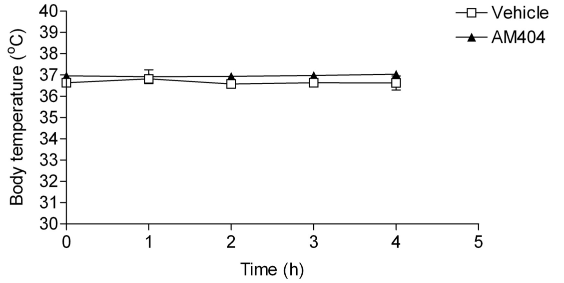

FIG. 5. AM404 did not induce hypothermia in C57BL/6. The time profile of the body temperature of mice over 4 h after the intraperitoneal administration of 40 mg/kg AM404 is shown; $n = 5$.

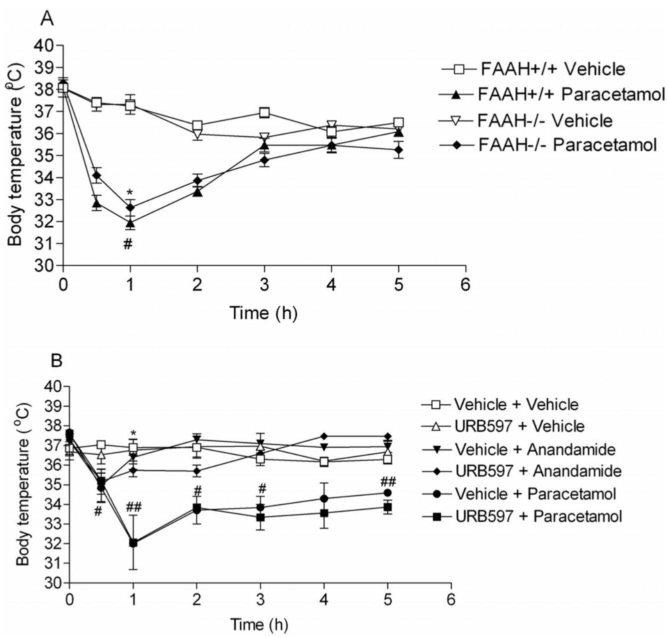

FIG. 6. Paracetamol-induced hypothermia in FAAH knockout (FAAH−/−) mice and in wild-type mice pretreated with the FAAH inhibitor URB597 to increase the synaptic concentration of anandamide. A, a concentration of 300 mg/kg paracetamol or vehicle was administered intraperitoneally at time point 0, and the body temperature of mice was monitored over 5 h. *, $P < 0.05$, vehicle-treated FAAH wild-type (FAAH+/+) versus paracetamol-treated FAAH wild-type; #, $P < 0.05$, vehicle-treated FAAH knockout versus paracetamol-treated FAAH knockout (two-way ANOVA with post hoc Bonferroni test). B, C57BL/6 mice were pretreated with 0.3 mg/kg URB597 i.p. and were treated with either 5 mg/kg anandamide i.p. or 300 mg/kg paracetamol i.p., 30 min later. *, $P < 0.05$, vehicle and anandamide versus URB597 and anandamide; #, $P < 0.05$; ##, $P < 0.01$, vehicle and vehicle versus vehicle and paracetamol (two-way ANOVA with post hoc Bonferroni test); $n = 5$–6.

hypothermia at high doses in rats (Rawls et al., 2006). AM404 also acts as an activator of the TRPV1 channel (De Petrocellis et al., 2000) and inhibits COX-1 and COX-2 activities (Högestätt et al., 2005). Furthermore, this metabolite has been shown to inhibit the cellular uptake of anandamide, thereby preventing its degradation by FAAH (Beltramo et al., 1997). Therefore, AM404 has been hypothesized to mediate the pharmacological actions of paracetamol through the activation of cannabinoids and/or the TRPV1 channel (Högestätt et al., 2005). This hypothesis has been supported by recent studies that showed that antagonism of CB_1R inhibited the analgesic action of paracetamol and that inhibition of FAAH, to prevent the formation of AM404, resulted in the loss of the paracetamol-induced analgesia (Mallet et al., 2008).

Using CB_1R knockout mice and the selective CB_1R antagonist AM251, the current study demonstrated that CB_1R is not involved in mediating the paracetamol-induced hypothermia as administration of the drug to CB_1R knockout mice resulted in a hypothermic response, similar to wild-type mice. In addition, 5 mg/kg AM251 administered 1 h before paracetamol did not affect the drug's hypothermic action, while completely preventing the development of hypothermia induced by WIN55-212,2. These results confirm and extend recent studies (Mallet et al., 2008; Corley and Rawls, 2009). In contrast, the cannabinoid-induced hypothermia is not dependent on the inhibition of COX-1 or COX-2 activities; hence, activation of CB_1R and inhibition of COX activity to induce hypothermia are not interdependent phenomena. This finding is further supported by the demonstration that coadministration of paracetamol and WIN55-212,2 induce supra-additive hypothermia.

We also present evidence that activation of the TRPV1 channel is not involved in mediating the paracetamol-induced hypothermia, as TRPV1 knockout mice developed hypothermia to the same extent as wild-type mice, and that the TRPV1 antagonist SB366791 did not inhibit the development of hypothermia induced by paracetamol. In contrast, the TRPV1 agonist, capsaicin, induced hypothermia in COX-1 and COX-2 knockout mice to the same extent as their wild-type littermate controls, which indicates that hypothermia induced by activation of the TRPV1 channel does not involve inhibition of COX activity.

Because the inhibition of FAAH activity with URB597 (Piomelli et al., 2006), which is thought to inhibit the formation of AM404 from paracetamol, does not inhibit the development of hypothermia induced by paracetamol, we conclude that AM404 does not mediate the paracetamol-induced hypothermia. Conclusive support for this is provided by the finding that paracetamol was capable of the induction of hypothermia in FAAH knockout mice. Indeed, the failure by AM404 at analgesic doses (10–40 mg/kg) to induce hypothermia in mice provides further support that AM404 does not mediate the paracetamol-induced hypothermia. AM404 at the doses used in this study has been reported to possess central effects and therefore is able to cross the blood-brain barrier (Rawls et al., 2006). This conclusion is contrary to previously published work (Rawls et al., 2006) in which the authors demonstrated a 1.5°C drop in body temperature with AM404 in rats 45 min after administration. From the results of Rawls et al. (2006), one would predict that AM404 might contribute to mediation of the initial phase of the paracetamol-induced hypothermia. The discrepancy between our present results and those of Rawls et al. (2006) may be species related.

The mechanism of paracetamol-induced hypothermia remains unexplained. Using COX-1 and COX-2 knockout mice, we provided evidence that the paracetamol-induced hypothermic action is dependent on the inhibition of a COX-1 gene-derived protein (Ayoub et al., 2004). The paracetamol-induced hypothermia and inhibition of brain PGE_2 synthesis was reduced in a gene-dependent manner in COX-1 knockout mice but completely retained in COX-2 knockout mice. Research dating back to the 1970s suggested that paracetamol is a centrally acting drug, through inhibition of COX activity. This hypothesis was reached by demonstrating potent reduction of prostaglandin biosynthesis in brain tissues but not in peripheral tissues (Flower and Vane, 1972). Reduction of central nervous system PGE_2 by paracetamol is supported by other studies (Malmberg and Yaksh, 1994; Muth-Selbach et al., 1999; Ayoub et al., 2006).

The induction of hypothermia for therapeutic purposes has been in clinical practice for many years. The thus termed "therapeutic hypothermia" provides neuroprotection for patients after a cardiac arrest, stroke, or spinal cord or head injuries (Cheung et al., 2006; Jiang and Yang, 2007; den Hertog et al., 2009). Hypothermia protects the brain

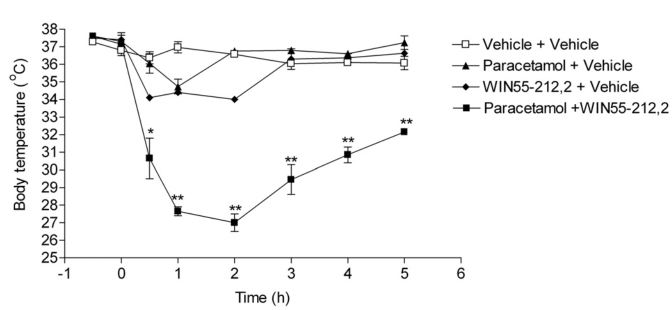

FIG. 7. Coadministration of paracetamol and WIN55-212,2 induced additive hypothermia in C57BL/6. Male C57BL/6 mice were treated with 200 mg/kg paracetamol i.p., 5 mg/kg WIN55-212,2, or paracetamol with WIN55-212,2, and their body temperature was monitored over 5 h. *, $P < 0.05$; **, $P < 0.01$, paracetamol and WIN55-212,2 versus paracetamol or WIN55-212,2 alone (two-way ANOVA with post hoc Bonferroni test); $n = 5$.

through several mechanisms that include reduction in brain metabolic rate, effects on cerebral blood flow, reduction of the critical threshold for oxygen delivery, blockade of excitotoxic mechanisms, calcium antagonism, preservation of protein synthesis, reduction of brain thermopooling, a decrease in edema formation, modulation of the inflammatory response, neuroprotection of the white and gray matter, and modulation of apoptotic cell death (Froehler and Geocadin, 2007). The acute management of these patients is a major challenge and determines the long-term clinical outcome. The first hour after their occurrence is the most critical, defined as the "golden hour" (Wilkinson and McDougall, 2007). The challenge is to stabilize and oxygenate the patient and to transfer the patient to the hospital as quickly as possible. The induction of hypothermia as a means of stabilization of the patient has been shown to dramatically improve outcome and reduce the occurrence of long-term disability.

Current methods used for the induction of therapeutic hypothermia, which is defined as core temperature between 35 and 32°C, apply the use of cooling blankets attached to a cooling devise, which is large in size and expensive. However, as humans are endothermic, we have many physiological mechanisms to resist this "outside-in" cooling. Therefore, existing methods of cooling are slow, inadequate, and impractical for use in the prehospital environment (Hoedemaekers et al., 2007); thus, alternative approaches for the induction of therapeutic hypothermia are needed. To this end, pharmacological agents have been proposed. Paracetamol as a safe and readily available drug has been exploited for this purpose. In a recent clinical trial, paracetamol resulted in a 0.25–0.3°C drop in body temperature of stroke patients with no conclusive improvement in clinical outcomes (den Hertog et al., 2009).

Despite reduction in body temperature of approximately 4°C in mice, paracetamol at therapeutic doses is not expected to consistently produce a similar drop in temperature in humans. We hypothesize that the combination of paracetamol with another hypothermic agent may provide a safe, fast, and effective means for the induction of therapeutic hypothermia. A low dose of a clinically approved cannabinoid agonist is one such option. The present results support the hypothesis that the induction of hypothermia by paracetamol and cannabinoids are not interlinked mechanistically. Indeed, we found that coadministration of paracetamol with WIN55-212,2, at low doses, resulted in supra-additive hypothermia in mice (Fig. 7).

The efficacy and safety of using combinational therapeutic hypothermia induced with paracetamol and a clinically approved cannabinoid agonist on the prehospital care of patients with stroke or cardiac arrest need to be tested by setting up Phase II clinical trials. When the combination of paracetamol with a cannabinoid agonist fails to produce sufficient hypothermia in humans, we propose an alternative approach: new chemical entities that share the same mechanism of hypothermic action as paracetamol, cannabinoids, and TRPV1 agonists but are capable of the induction of more profound hypothermia could be developed.. The drawback of developing new hypothermic agents is that they would have to go through extensive preclinical development to establish their efficacy and safety before any clinical trials can be conducted. An understanding of the structure-function relationship for existing hypothermic agents may assess in the discovery of new hypothermic agents because certain chemical moieties may be responsible for the hypothermic actions of existing hypothermic drugs, which include paracetamol and cannabinoid CB_1R agonists.

In summary, the current study provides clear-cut evidence that AM404 does not mediate the paracetamol-induced hypothermia and that the cannabinoids and TRPV1 are not activated during this hypothermic response.

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Authorship Contributions

Participated in research design: Ayoub and Baker.

Conducted experiments: Ayoub, Pryce, and Baker.

Performed data analysis: Ayoub.

Wrote or contributed to the writing of the manuscript: Ayoub, Pryce, Bolton, and Baker.

Other: Bolton, Seed, and Flower.

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