

Drug Metabolism and Disposition

Paracetamol-induced hypothermia is independent of cannabinoids and transient
receptor potential vanilloid-1 and is not mediated by AM404

Samir S Ayoub, Gareth Pryce, Michael P Seed, Christopher Bolton, Roderick J Flower and
David Baker

Centre for Biochemical Pharmacology (SSA, RJF), Centre for Experimental Medicine and
Rheumatology (MPS), William Harvey Research Institute, Barts and the London School of
Medicine and Dentistry, Queen Mary University of London, London EC1M 6BQ, United
Kingdom.

Centre for Neuroscience, Institute of Cell and Molecular Science, Barts and the London
School of Medicine and Dentistry, Queen Mary University of London, London, E1 2AT,
United Kingdom E1 2AT (GP, CB, DB).

Running title: Paracetamol-induced hypothermia and AM404

Corresponding author: Samir S Ayoub, Centre for Biochemical Pharmacology, William Harvey Research Institute, Barts and the London School of Medicine and Dentistry, Queen Mary University of London, London EC1M 6BQ, United Kingdom.uk, Tel: +44(0)207 882 5817; Fax: +44(0)207 882 6076; Email: s.s.ayoub@qmul.ac.uk

Number of text pages = 28

Number of tables = 0

Number of figures = 7

Number of references = 40

Number of words in abstract = 225

Number of words in introduction = 498

Number of words in discussion = 1,180

Abbreviations: AM404, N-(4-hydroxyphenyl)arachidonamide; CB₁R, cannabinoid receptor-1; COX, cyclooxygenase; FAAH, fatty acid amide hydrolase; PGE₂, prostaglandin E₂; TRPV1, transient receptor potential vanilloid-1

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, AM404, which activates the cannabinoid (CB) and transient receptor potential vanilloid-1 (TRPV1) systems, mediates the paracetamol-induced hypothermia. The hypothermic response to 300mg/kg paracetamol in CB₁ receptor (CB₁R) and TRPV1 knockout mice was compared to wild-type mice. Hypothermia induced by paracetamol was also investigated in animals pre-treated with the CB₁R or TRPV1 antagonists AM251 or SB366791, respectively.

In CB₁R or TRPV1 knockout mice paracetamol induced hypothermia to the same extent as in wild-type mice. In addition, in C57BL/6 mice pre-treated 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. Additionally, 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 (Denes et al., 2002; Dippel et al., 2001; Kasner et al., 2002; Richardson and Sills, 2004; Tröglauer et al., 2002) 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; Richardson et al., 2004; Tröglauer et al., 2002), 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 -2 enzymes (Mitchell et al., 1993). However, it significantly reduces central nervous system (CNS) prostaglandin synthesis (Flower and Vane 1972; Feldberg et al., 1972; Ayoub et al., 2006), indicating inhibition of a COX activity. Recently, we 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, while 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 E₂ (PGE₂) synthesis (Ayoub et al., 2004).

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 metabolised through a two step pathway involving brain fatty acid amide hydrolase (FAAH) activity into N-(4-hydroxyphenyl)arachidonamide (AM404). AM404 activates the endocannabinoid system by increasing the synaptic availability of the endocannabinoid anandamide, by inhibition of the uptake of anandamide into pre-synaptic neurones resulting in reduction of its degradation by FAAH (Beltramo et al., 1997). Anandamide produces anti-nociceptive and hypothermic actions, both mediated through the CB₁ receptor (CB₁R; Howlett, 1995). Potent hypothermia has been reported after the administration of selective CB₁R 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 a n 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 and colleagues 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.

Methods

Animals

Male C57BL/6 mice (20±2g) were supplied from Harlan UK (Bicester, UK). COX-1, COX-2 (Langenbach et al., 1995; Morham et al., 1995), Biozzi ABH and ABH mice lacking the CB₁R 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/12-h light/dark cycle at 22°C±1. Food and water were provided *ad libitum*. Experimental procedures were conducted in accordance with the United Kingdom Home Office Guidelines.

Chemicals

Paracetamol (Sigma, Poole UK) was dissolved in 12.5% (v/v) 1,2-propanediol. WIN55,212-2, AM251, SB366791, AM404, anandamide (Tocris, Bristol, UK), capsaicin, URB597, (Sigma), SC560 and celecoxib (kind gifts from Schering Aktiengesellschaft, Berlin, Germany) were initially dissolved in 100% dimethyl sulphoxide (DMSO) then diluted to the appropriate doses in a solution containing 10% cremophor oil, 10% ethanol and 80% saline reducing the concentration of DMSO to 0.1%.

Temperature measurement and administration of drugs

Body temperature was measured using a thermocouple probe placed under the hindlimb as previously described (Brooks et al., 2002). The animals were pre-conditioned to the temperature probe by taking temperature measurements three days before the experiment and twice on the day of the experiment prior to drug administration in order to reduce handling-induced temperature changes associated with stress. In each experiment the time-profile of

hypothermia, usually up to 5h was determined. The ambient temperature was set to $22\pm 1^{\circ}\text{C}$ during the entire duration of the experiments.

Experimental objective 1: To determine whether activation of CB₁R or TRPV1 is involved in the paracetamol-induced hypothermia, the time-profile of the hypothermic response of 300mg/kg paracetamol (intraperitoneally, i.p.) was determined in CB₁R and TRPV1 knockout mice and was compared to their wild-type littermate controls. In a different experiment, C57BL/6 mice were pre-treated with 5mg/kg of AM251 (CB₁R antagonist; i.p.) then treated 1h after with either 20mg/kg of the CB₁R/CB₂R agonist WIN55-212,2 (i.p.) or 300mg/kg paracetamol (i.p.). Another group of C57BL/6 mice were treated with 2 mg/kg of the TRPV1 antagonist SB366791 (i.p.) then treated 0.5h after with either 1mg/kg of the TRPV1 agonist capsaicin (subcutaneously, s.c.) or 300mg/kg paracetamol (i.p.).

Experimental Objective 2: To address whether the induction of hypothermia by the CB₁R or TRPV1 agonists 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 20mg/kg WIN55-212,2 (i.p.) and 1mg/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, 40mg/kg AM404 was administered to C57BL/6 and the body temperature was measured over 5h and compared to vehicle treated animals. In a different experiment the activity of FAAH was inhibited in C57BL/6 mice with 0.3mg/kg URB597 (i.p.) for 30min, animals were then treated with 300mg/kg paracetamol (i.p.) and the body temperature monitored over 5h. Two additional groups of mice were included, one treated with 5mg/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 300mg/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 (200mg/kg; i.p.) and WIN55-212,2 (5mg/kg; i.p.) would induce additive hypothermia, the two compounds were administered to C57BL/6 mice and the body temperature monitored for 5h.

Prostaglandin extraction and measurement

For measurement of PGE₂ 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; Ayoub et al., 2006). Briefly, frozen brain tissues were pulverised with a nitrogen bomb. One millilitre of 15% (v/v) ethanol in distilled water (pH 3) was added to pulverised tissues and samples were stored at 4°C for 10min and then spun at 375g for 10min at 4°C. C-18 Sep-Pak columns (Waters, Milford, MA, USA) were conditioned with 4ml ethanol followed by 4ml distilled water at a flow rate of 5-10ml/min. The supernatants from homogenates were then applied to the columns at a flow rate of 5ml/min. The columns were then washed with 4ml of distilled water followed by 4ml of 15% (v/v) ethanol in distilled water. The samples were eluted with 2ml of ethyl acetate at a flow rate of 5ml/min. The samples were dried and then stored at -80°C ready for prostaglandin measurement. Measurement of brain PGE₂ was performed using a commercial enzyme immunoassay kit from GE Healthcare (Chalfont, Bucks, UK), according to the manufacturer's instructions. The concentration of PGE₂ in the samples was determined by comparing the calculated percentage binding of PGE₂ in the samples with a standard PGE₂ curve (0.05-6.4 ng/ml).

Statistical analysis

The results were analysed using Graph Pad Prism 3.0 (San Diego, CA, USA), expressed and presented graphically as mean \pm s.e.m. Statistical analysis was performed using two-way ANOVA with *posthoc* Bonferroni test to compare temperature changes between the different treatment groups. For comparison of the effect of drugs on the synthesis of PGE₂ 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 300mg/kg of paracetamol was previously used to investigate the mechanism of paracetamol-induced hypothermia (Ayoub et al., 2004). Although high, this dose is within the pharmacological sub-toxic range in mice (Muth-selbach et al., 1999; Vaquero et al., 2007). In CB₁R knockout mice 300mg/kg paracetamol (i.p.) resulted in a significant hypothermic response within 1h 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 PGE₂ concentration of CB₁R knockout mice were also compared 1h after 300mg/kg paracetamol or vehicle treatments. Paracetamol reduced brain PGE₂ levels in CB₁R 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 pre-treated C57BL/6 mice with 5mg/kg of the CB₁R antagonist AM251 for 1h followed by 300mg/kg paracetamol. AM251 did not prevent the development of hypothermia by paracetamol (Fig 1C). To demonstrate antagonism of CB₁R with AM251 we showed that AM251 inhibited the development of the hypothermic response induced by the CB₁/CB₂ receptor agonist WIN55,212-2 (20mg/kg; $P < 0.001$, two-way ANOVA; fig 1C). The dose of WIN55,212-2 used here is within the calculated ED₅₀ dose for hypothermia, which is 33mg/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 others (Boctor et al., 2007) and us (data not shown).

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 20mg/kg WIN55-212,2 induced a hypothermic response ($p < 0.05$, two-way ANOVA), of about 8°C, which was similar to that seen in their wild-type littermate controls in both the initial (0.5-1h) and resolving phases (2-5h; Fig 2A and B; $P < 0.05$). Similar to paracetamol, the peak of hypothermia with WIN55-212,2 occurred 1h after administration.

TRPV1 is not involved in the induction of hypothermia by paracetamol

In order to investigate whether TRPV1 is involved in the induction of hypothermia by paracetamol, TRPV1 knockout mice were treated with 300mg/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 pre-treated with 2mg/kg of the selective TRPV1 antagonist SB366791, 30min after the animals were treated with either 300mg/kg paracetamol or 1mg/kg of the TRPV1 agonist capsaicin. SB366791 reversed the capsaicin-induced hypothermia by about 2°C ($P < 0.05$; two-way ANOVA). This reduction was observed 30min and 1h 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).

Conversely we wanted to determine whether the TRPV1-induced hypothermia was dependent on the inhibition of COX activity. Capsaicin was administered to COX-1 and COX-2 knockout mice. In both COX-1 and COX-2 knockout mice, 1mg/kg capsaicin induced statistically significant ($P < 0.05$; two-way ANOVA) hypothermia which was not statistically different from that observed in their wild-type littermate controls (Fig 4A 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 mice (data not shown) at a top dose of 40mg/kg (Fig 5) or at 10 and 20mg/kg (data not shown).

In order to investigate the involvement of AM404 in mediating the paracetamol-induced hypothermia, mice deficient of FAAH were treated with 300mg/kg paracetamol and their body temperature monitored over 5h. 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. Pre-treatment of mice with 0.3mg/kg URB597 (30min) did not prevent the development of hypothermia by 300mg/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 5mg/kg anandamide-induced hypothermia was investigated. As a result of increased synaptic accumulation of anandamide, URB597 potentiated the anandamide-induced hypothermia 2h post-anandamide administration ($P < 0.05$;

Fig 6B, two-way ANOVA). 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 co-administration of lower doses of paracetamol (200mg/kg) and WIN55-212,2 (5mg/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°C and 9.25°C after 0.5h and 1h, respectively compared to vehicle treated mice (Figure 7; $P < 0.05$; two-way ANOVA).

Discussion

Högestätt and colleagues (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). Prior to that AM404 has been shown to induce analgesia (La Rana et al., 2006; Borsani et al., 2007; Mitchell et al., 2007) and 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 to inhibit 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 hypothesised to mediate the pharmacological actions of paracetamol through the activation of cannabinoids and/or TRPV1 channel (Högestätt et al., 2005). This hypothesis has been supported by recent studies that showed that antagonism of CB₁R 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 CB₁R knockout mice and the selective CB₁R antagonist AM251, the current study demonstrated that CB₁R is not involved in mediating the paracetamol-induced hypothermia as administration of the drug to CB₁R knockout mice resulted in a hypothermic response, similar to wild-type mice. In addition, 5mg/kg AM251 administered 1h 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). Conversely, the cannabinoid-induced hypothermia is not dependent on the inhibition of COX-1 or COX-2 activities, hence activation of CB₁R and inhibition of COX activity to induce hypothermia are not inter-dependent phenomena. This finding is further supported by the demonstration that co-administration 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. Conversely, 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.

Since 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 hence 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 1.5°C drop in body temperature with AM404 in rats 45min after administration. From the results of Rawls and colleagues (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 PGE₂ 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 CNS PGE₂ by paracetamol is supported by other studies (Ayoub et al., 2006; Malmberg and Yaksh, 1994; Muth-Selbach et al., 1999).

The induction of hypothermia for therapeutic purposes has been in clinical practice for many years. The thus termed “therapeutic hypothermia” provides neuroprotection for patients following a cardiac arrest, stroke, spinal cord or head injuries (Cheung et al., 2006; den Hertog et al., 2009; Jiang, and Yang 2007). Hypothermia protects the brain 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 oedema 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 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 35-32°C, employ the use of cooling blankets attached to a cooling device, 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 are impractical for use in the pre-hospital 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 hypothesise 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. Based on the present results that the induction of hypothermia by paracetamol and cannabinoids are not interlinked mechanistically provides support for this hypothesis. Indeed, we found that co-administration of paracetamol with WIN55-212,2, at low doses, resulted in supra-additive hypothermia in mice (Figure 7).

The efficacy and safety of using combinational therapeutic hypothermia induced with paracetamol and a clinically approved cannabinoid agonist on the pre-hospital care of patients with stroke or cardiac arrest need to be tested by setting up phase two clinical trials. We also propose that in the case that the combination of paracetamol with a cannabinoid agonists fails to produce sufficient hypothermia in humans, we propose that as 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 pre-clinical development in order to establish their

efficacy and safety before any clinical trials can be conducted. An understanding on the structure-function relationship for existing hypothermic agents may assist in the discovery of new hypothermic agents as certain chemical moieties may be responsible for the hypothermic actions of existing hypothermic drugs that include paracetamol and cannabinoid CB₁R agonists.

As a 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.

Acknowledgements

The TRPV1 knockout mice were a kind gift from Professor Amrita Ahluwalia and Dr Catherine Panayiotou, Centre for Clinical Pharmacology, William Harvey Research Institute, London.

Authorship Contributions

Participated in research design: Ayoub, Baker

Conducted experiments: Ayoub, Pryce, Baker

Performed data analysis: Ayoub

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

References

Ayoub SS, Botting RM, Goorha S, Colville-Nash PR, Willoughby DA and Ballou LR (2004) Acetaminophen-induced hypothermia in mice is mediated by a prostaglandin endoperoxide synthase 1 gene-derived protein. *Proc Natl Acad Sci USA* **101**: 11165-11169.

Ayoub SS, Colville-Nash PR, Willoughby DA and Botting RM (2006). The involvement of a cyclooxygenase 1 gene-derived protein in the antinociceptive action of paracetamol in mice. *Eur J Pharmacol* **538**: 57-65.

Beltramo M, Stella N, Calignano A, Lin SY, Makriyannis A and Piomelli D (1997). Functional role of high-affinity anandamide transport, as revealed by selective inhibition. *Science* **277**: 1094-1097.

Bilsland, LG, Dick JR, Pryce G, Petrosino S, Di Marzo Y, Baker D and Greensmith L (2006). Increasing cannabinoid levels by pharmacological and genetic manipulation delay disease progression in SOD1 mice. *FASEB J* **20**: 1003-5.

Boctor SY, Martinez JL, Koek W and France CP (2007). The cannabinoid CB1 receptor antagonist AM251 does not modify the methamphetamine reinstatement of responding. *Eur. J. Pharmacol.* **571**: 39-43

Borsani E, Labanca M, Bianchi R and Rodella LF (2007). AM404 decreases Fos-immunoreactivity in the spinal cord in a model of inflammatory pain. *Brain Res* **1152**: 87-94.

Brooks JW, Pryce G, Bisogno T, Jaggar SI, Hankey DJ, Brown P, Bridges D, Ledent C, Bifulco M, Rice AS, Di Marzo V and Baker D (2002) Arvanil-induced inhibition of spasticity and persistent pain: evidence for therapeutic sites of action different from the vanilloid VR1 receptor and cannabinoid CB(1)/CB(2) receptors. *Eur J Pharmacol* **439**: 83-92.

Cheung, K.W., Green, R.S., Magee, K.D. 2006. Systematic review of randomized controlled trials of therapeutic hypothermia as a neuroprotectant in post cardiac arrest patients. *CJEM* **8**, 329-37

Corley G and Rawls SM (2009). Opioids, cannabinoid CB1 and NOP do not mediate APAP-induced hypothermia in rats. *Pharmacol. Biochem. Behav.* **92**: 503-507

Denes E, Amaniou M, Rogez JP, Weinbreck P and Merle L (2002). Acetaminophen-induced hypothermia, an AIDS related side-effect? About 4 cases. *Ann Med Interne* **153**: 411-3.

den Hertog HM, van der Worp HB, van Gemert HM, Algra A, Kappelle LJ, van Gijn J, Koudstaal PJ and Dippel DW; PAIS Investigators (2009). The Paracetamol (Acetaminophen) In Stroke (PAIS) trial: a multicentre, randomised, placebo-controlled, phase III trial. *Lancet Neurol.* **8**: 434-40

De Petrocellis L, Bisogno T, Davis JB, Pertwee RG and Di Marzo V (2000). Overlap between the ligand recognition properties of the anandamide transporter and the VR1 vanilloid receptor: inhibitors of anandamide uptake with negligible capsaicin-like activity. *FEBS Lett* **483**: 52-56.

Ding Z, Cowan A and Rawls SM (2005). Capsaicin evokes hypothermia independent of cannabinoid CB1 and CB2 receptors. *Brain Res.* **1065**: 147-151

Dippel DW, van Breda EJ, van Gemert HM, van der Worp HB, Meijer RJ, Kappelle LJ and Koudstaal PJ (2001). Effect of paracetamol (acetaminophen) on body temperature in acute ischemic stroke: a double-blind, randomized phase II clinical trial. *Stroke* **32**: 1607-12

Fegley D, Kathuria S, Mercier R, Li C, Goutopoulos A, Makriyannis A and Piomelli D (2004). Anandamide transport is independent of fatty-acid amide hydrolase activity and is blocked by the hydrolysis-resistant inhibitor AM1172. *Proc. Natl. Acad. Sci. USA* **101**: 8756-8761

Feldberg W, Gupta KP, Milton AS and Wendlandt S (1972). Effect of bacterial pyrogen and antipyretics on prostaglandin activity in cerebrospinal fluid of unanaesthetized cats. *Br J Pharmacol* **46**: 550P-551P.

Flower RJ and Vane JR (1972). Inhibition of prostaglandin synthetase in brain explains the anti-pyretic activity of paracetamol (4-acetamidophenol). *Nature* **240**: 410-411.

Froehler MT and Geocadin RG (2007). Hypothermia for neuroprotection after cardiac arrest: Mechanisms, clinical trials and patient care. *J Neurol Sci.* **261**: 118-26

Hoedemaekers CW, Ezzahti M, Gerritsen A and van der Hoeven JG (2007). Comparison of different cooling methods to induce and maintain normo- and hypothermia in ICU patients: a prospective intervention study. *Critical Care* **1**: R91

Hogestatt ED, Jonsson BA, Ermund A, Andersson DA, Bjork H, Alexander JP, Cravatt BF, Basbaum AI and Zygmunt PM (2005). Conversion of acetaminophen to the bioactive N-acetylphenolamine AM404 via fatty acid amide hydrolase-dependent arachidonic acid conjugation in the nervous system. *J Biol Chem* **280**: 31405-31412.

Howlett AC (1995). Pharmacology of cannabinoid receptors. *Annu Rev Pharmacol Toxicol* **35**: 607-634

Jiang, JY and Yang XF (2007). Current status of cerebral protection with mild-to-moderate hypothermia after traumatic brain injury. *Curr Opin Crit Care* **13**: 153-5

Kasner SE, Wein T, Piriyaawat P, Villar-Cordova CE, Chalela JA, Krieger DW, Morgenstern LB, Kimmel SE and Grotta JC (2002). Acetaminophen for altering body temperature in acute stroke: a randomized clinical trial. *Stroke* **33**: 130-4.

La Rana G, Russo R, Campolongo P, Bortolato M, Mangieri RA, Cuomo V, Iacono A, Raso GM, Meli R, Piomelli D and Calignano A (2006). Modulation of neuropathic and inflammatory pain by the endocannabinoid transport inhibitor AM404 [N-(4-hydroxyphenyl)-eicosa-5,8,11,14-tetraenamide]. *J Pharmacol Exp Ther* **317**: 1365-1371.

Langenbach R, Morham SG, Tiano HF, Loftin CD, Ghanayem BI, Chulada PC, Mahler JF, Lee CA, Goulding EH, Kluckman KD, Kim HS and Smithies O (1995). Prostaglandin synthase 1 gene disruption in mice reduces arachidonic acid-induced inflammation and indomethacin-induced gastric ulceration. *Cell* **83**: 483-492.

Mallet C, Daulhac L, Bonnefont J, Ledent C, Etienne M, Chapuy E, Libert F and Eschalier A (2008). Endocannabinoid and serotonergic systems are needed for acetaminophen-induced analgesia. *Pain* **139**: 190-200.

Mitchell JA, Akarasereenont P, Thiemermann C, Flower RJ and Vane JR (1993). Selectivity of nonsteroidal antiinflammatory drugs as inhibitors of constitutive and inducible cyclooxygenase. *Proc Natl Acad Sci USA* **90**: 11693-11697.

Mitchell VA, Greenwood R, Jayamanne A and Vaughan CW (2007). Actions of the endocannabinoid transport inhibitor AM404 in neuropathic and inflammatory pain models. *Clin Exp Pharmacol Physiol* **34**: 1186-1190.

Morham SG, Langenbach R, Loftin CD, Tiano HF, Vouloumanos N, Jennette JC, Mahler JF, Kluckman KD, Ledford A, Lee CA and Smithies O (1995). Prostaglandin synthase 2 gene disruption causes severe renal pathology in the mouse. *Cell* **83**: 473-482.

Muth-Selbach US, Tegeder I, Brune K and Geisslinger G (1999). Acetaminophen inhibits spinal prostaglandin E2 release after peripheral noxious stimulation. *Anesthesiology* **91**: 231-9.

Piomelli D, Tarzia G, Duranti A, Tontini A, Mor M, Compton TR, Dasse O, Monaghan EP, Parrott JA and Putman D (2006). Pharmacological profile of the selective FAAH inhibitor KDS-4103 (URB597). *CNS Drug Rev.* **12**: 21-38

Pryce G, Giovannoni G and Baker D (2003). Mifepristone or inhibition of 11beta-hydroxylase activity potentiates the sedating effects of the cannabinoid receptor-1 agonist Delta(9)-tetrahydrocannabinol in mice. *Neurosci Lett* **341**: 164-166.

Rawls SM, Ding Z and Cowan A (2006). Role of TRPV1 and cannabinoid CB1 receptors in AM 404-evoked hypothermia in rats. *Pharmacol Biochem Behav* **83**: 508-516.

Richardson J and Sills J (2004). Hypothermia following fever. *Arch Dis Child* **89**,1177

Schwab, J.M., Beiter, T., Linder, J.U., Laufer, S., Schulz, J.E., Meyermann, R., Schluesener, H.J., 2003. COX-3-a virtual pain target in humans? *FASEB J* **17**: 2174-2175.

Sexton A, McDonald M, Cayla C, Thiemermann C and Ahluwalia A (2007). 12-Lipoxygenase-derived eicosanoids protect against myocardial ischemia/reperfusion injury via activation of neuronal TRPV1. *FASEB J* **21**: 2695-2703

Sim-Selley LJ and Martin BR (2002). Effect of Chronic Administration of R-(+)-[2,3-Dihydro-5-methyl-3-[(morpholinyl)methyl]pyrrolo[1,2,3-de]-1,4-benzoxazinyl]-(1-naphthalenyl)methanone Mesylate (WIN55,212-2) or Δ^9 -Tetrahydrocannabinol on Cannabinoid Receptor Adaptation in Mice. *J. Pharmacol. Exp Ther* **303**: 36-44

TrÃ©luyer JM, Tonnelier S, d'Athis P, Leclerc B, Jolivet-Landreau I and Pons G (2001). Antipyretic efficacy of an initial 30-mg/kg loading dose of acetaminophen versus a 15-mg/kg maintenance dose. *Pediatrics* **108**: E73.

Vaquero J, Belanger M, James L, Herrero R, Desjardins P, Cote J, Blei AT and Butterworth RF (2007). Mild hypothermia attenuates liver injury and improves survival in mice with acetaminophen toxicity. *Gastroenterol.***132**: 372-83.

Varga A, Nemeth J, Szabo A, McDougall JJ, Zhang C, Elekes K, Pinter E, Szolcsanyi J and Helyes Z (2005). Effects of the novel TRPV1 receptor antagonist SB366791 in vitro and in vivo in the rat. *Neurosci. Lett* **385**: 137-142

Wilkinson D and McDougall R (2007). Primary trauma care. *Anaesthesia* **62(Suppl1)**: 61-64

Footnotes

The authors would like to acknowledge the financial support provided by the Research Advisory Board of Barts and the London Hospitals. SS Ayoub is funded by the Leverhulme Trust and William Harvey Research Foundation.

Figure legends

Figure 1: Paracetamol-induced hypothermia in CB₁R knockout (CB₁R^{-/-}) mice and in wild-type mice pre-treated with the CB₁R antagonist AM251: A: time-profile of the hypothermic response of 300 mg/kg paracetamol in CB₁R^{-/-} mice. Paracetamol or vehicle was administered i.p. at time point zero and the temperature of mice measured at 0.5 and 1h. *P<0.05 vehicle-treated CB₁R^{-/-} versus paracetamol-treated CB₁R^{-/-} mice (two-way ANOVA with *posthoc* Bonferroni test). B: comparison of the levels of PGE₂ in brain tissues of CB₁R^{-/-} mice with or without paracetamol administration (1h post-administration). Brain tissues were dissected and PGE₂ measured using enzyme immunoassay after extraction with C18 Sep-Pak columns. C: mice were pre-treated 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 (i.p.) 1h after. The body temperature was monitored over 5h. **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 *posthoc* Bonferroni test), n=5-6.

Figure 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 were administered i.p. at time point zero and the body temperature of mice measured over 5h. 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 and ##P<0.01 vehicle-treated COX-2 knockout versus WIN55-212,2-treated COX-2 knockout (two-way ANOVA with *posthoc* Bonferroni test), n=5.

Figure 3: Paracetamol-induced hypothermia in TRPV1 knockout (TRPV1^{-/-}) mice and in wild-type mice pre-treated with the TRPV1 antagonist SB366791. A: 300 mg/kg paracetamol or vehicle was administered i.p. at time point zero and the body temperature of mice monitored over 5h. #P<0.05 vehicle-treated TRPV1 knockout versus paracetamol-treated TRPV1 knockout (two-way ANOVA with *posthoc* Bonferroni test). B: mice were pre-treated with 2 mg/kg SB366791 (i.p.) followed by treatment with 300 mg/kg paracetamol (i.p.) or 1 mg/kg capsaicin (s.c.) 0.5h after. The body temperature was monitored over 5h, n=5-6.

Figure 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 s.c. at time point zero and the body temperature of mice measured over 5h. A: **P<0.01 vehicle-treated COX-1 wild-type (COX-1^{+/+}) versus capsaicin-treated COX-1 wild-type mice; ##P<0.01 and ###P<0.001 vehicle-treated COX-1 knockout versus capsaicin-treated COX-1 knockout. Panel 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 *posthoc* Bonferroni test), n=5

Figure 5: AM404 did not induce hypothermia in C57BL/6. Time-profile of the body temperature of mice over 4h following the intraperitoneal administration of 40 mg/kg of AM404, n=5.

Figure 6: Paracetamol-induced hypothermia in FAAH knockout (FAAH^{-/-}) mice and in wild-type mice pre-treated with the FAAH inhibitor URB597 to increase the synaptic

concentration of anandamide. A: 300mg/kg paracetamol or vehicle was administered i.p. at time point zero and the body temperature of mice monitored over 5h. * $P < 0.05$ 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 *posthoc* Bonferroni test). B: C57BL/6 mice were pre-treated with 0.3 mg/kg URB597 (i.p.) then were treated with either 5 mg/kg anandamide (i.p.) or 300mg/kg paracetamol (i.p.), 30min after. * $P < 0.05$ vehicle and anandamide versus URB597 and anandamide; # $P < 0.05$, ## $P < 0.01$ vehicle and vehicle versus vehicle and paracetamol (two-way ANOVA with *posthoc* Bonferroni test), n=5-6

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

Figure 1

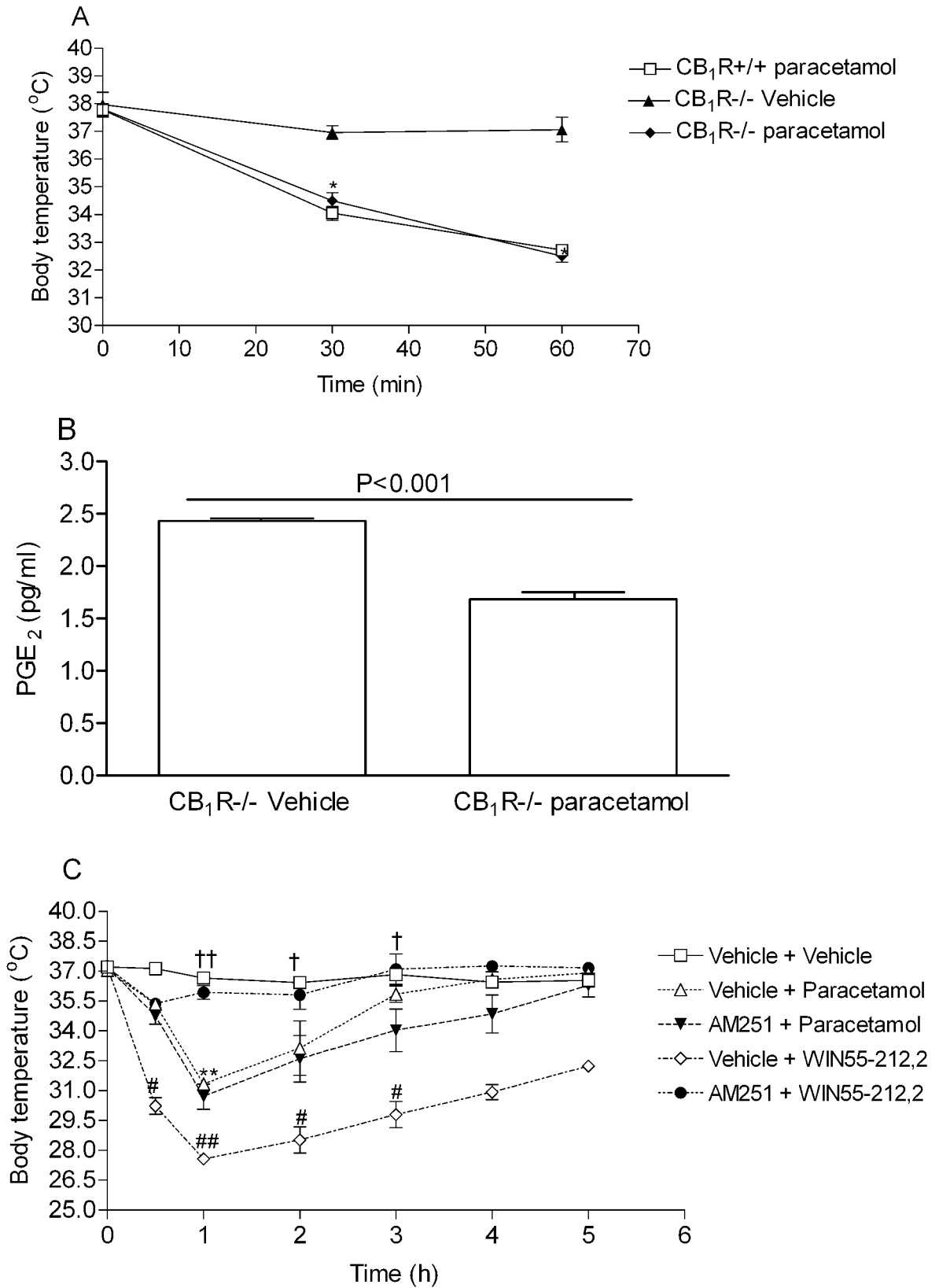


Figure 2

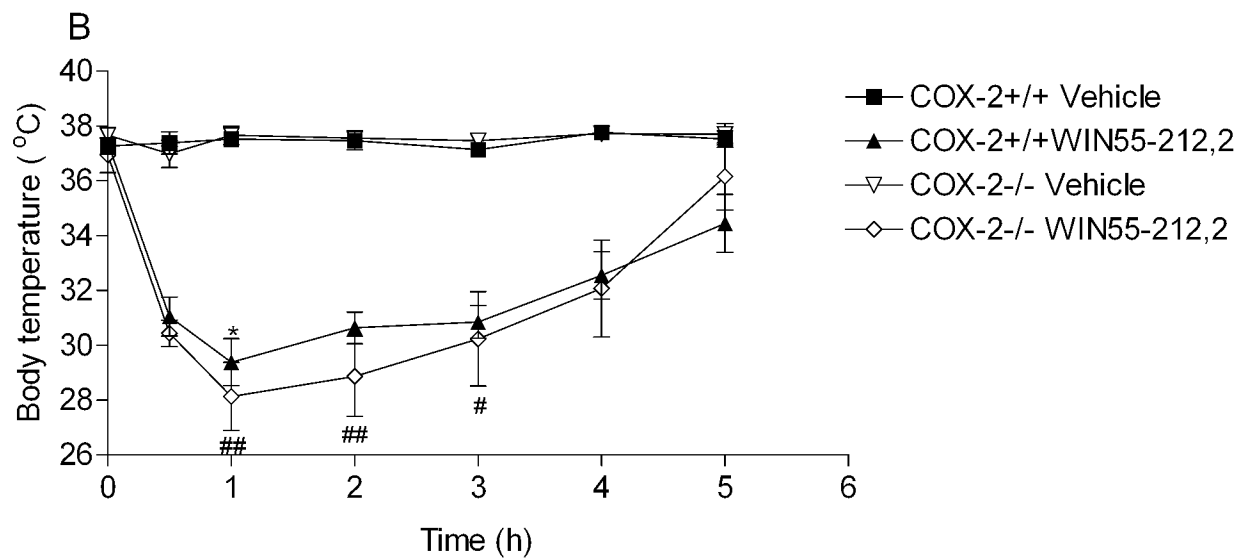
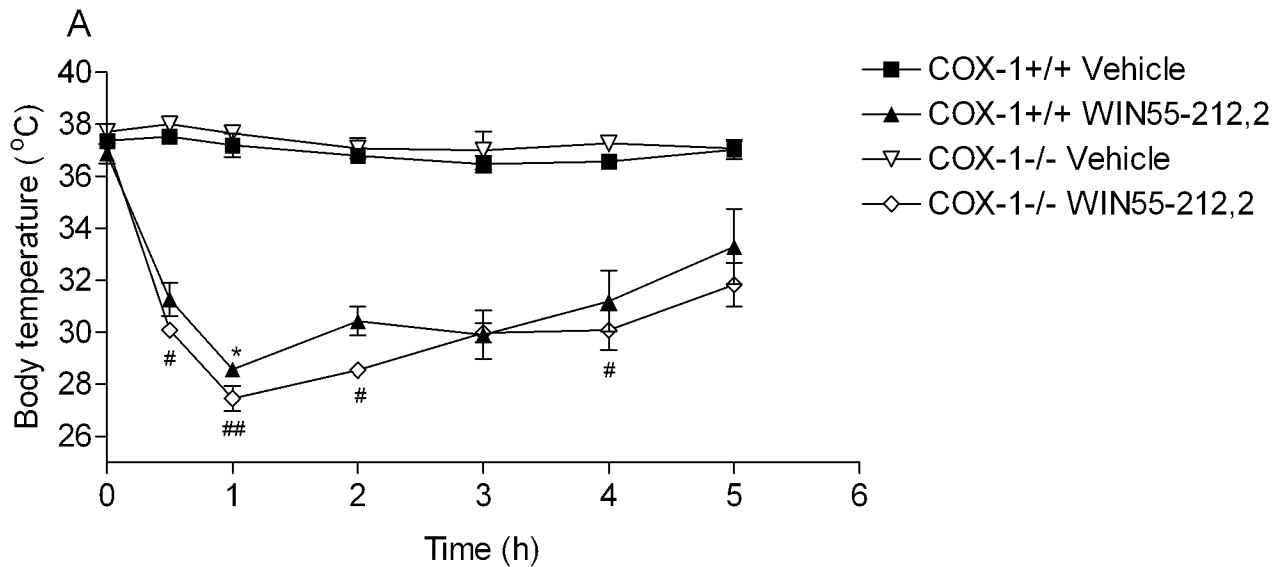


Figure 3

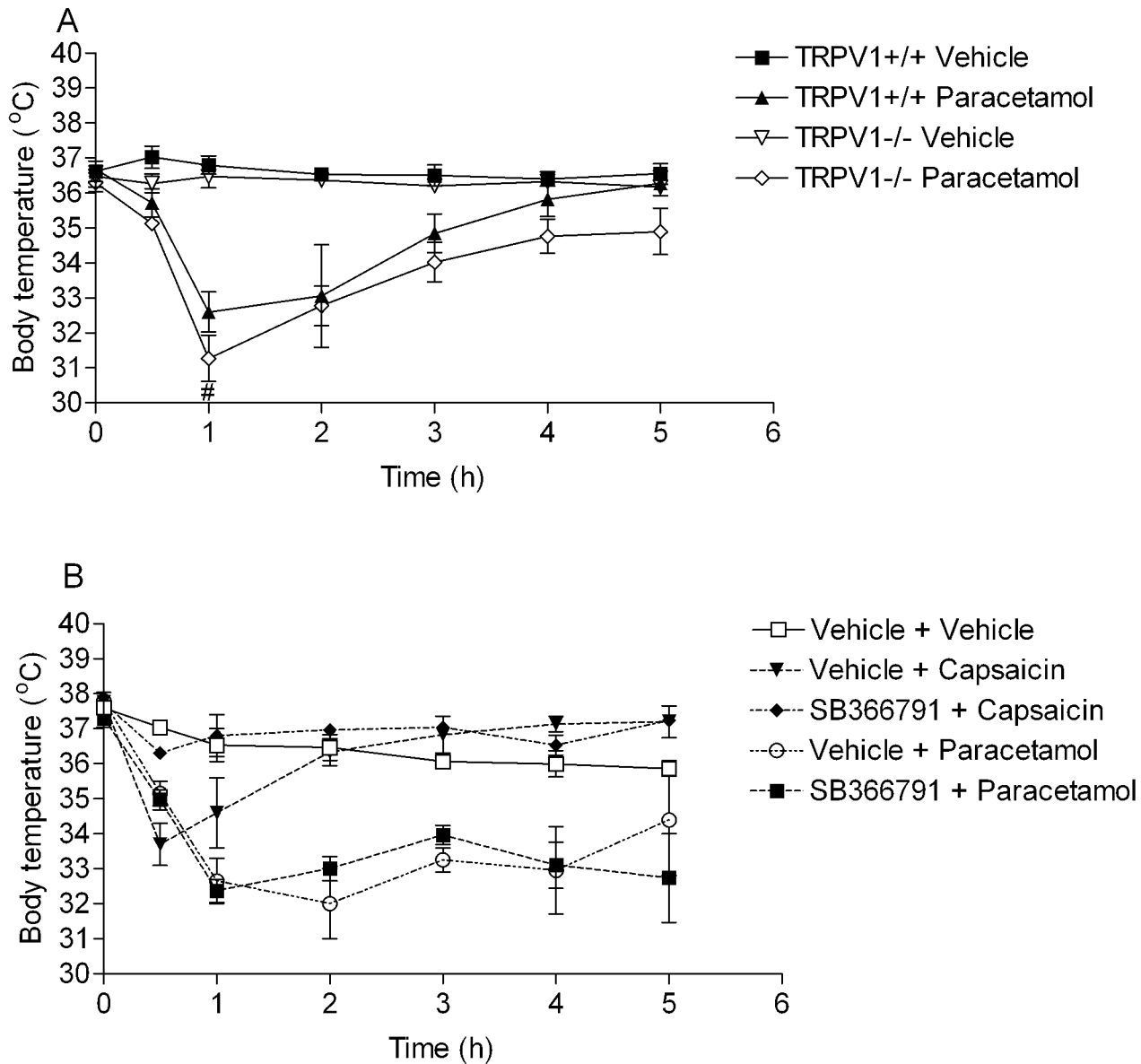


Figure 4

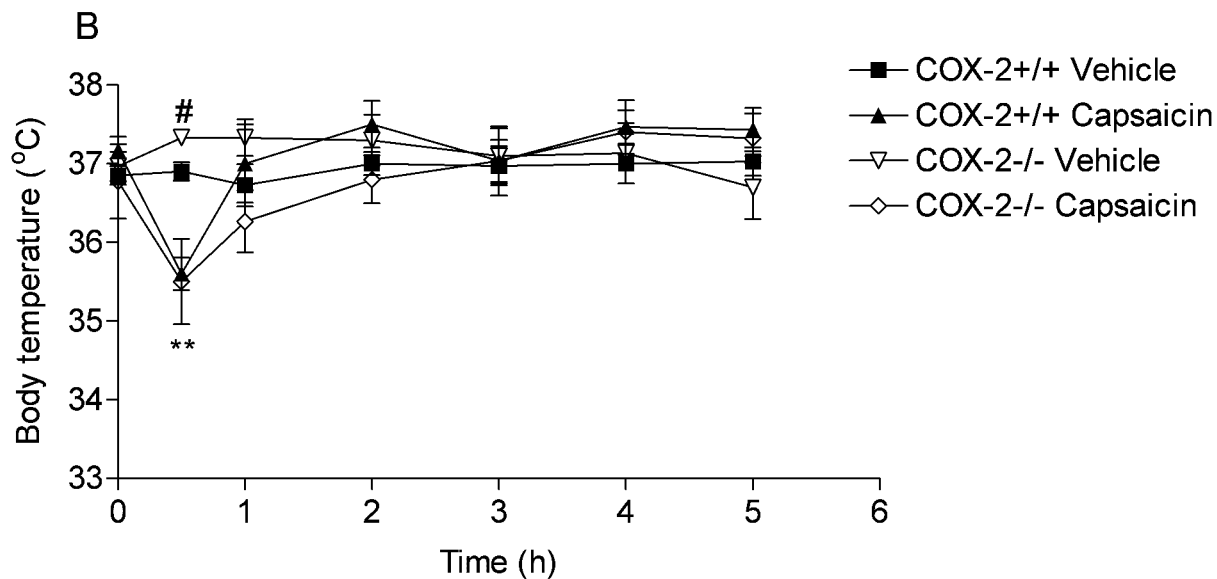
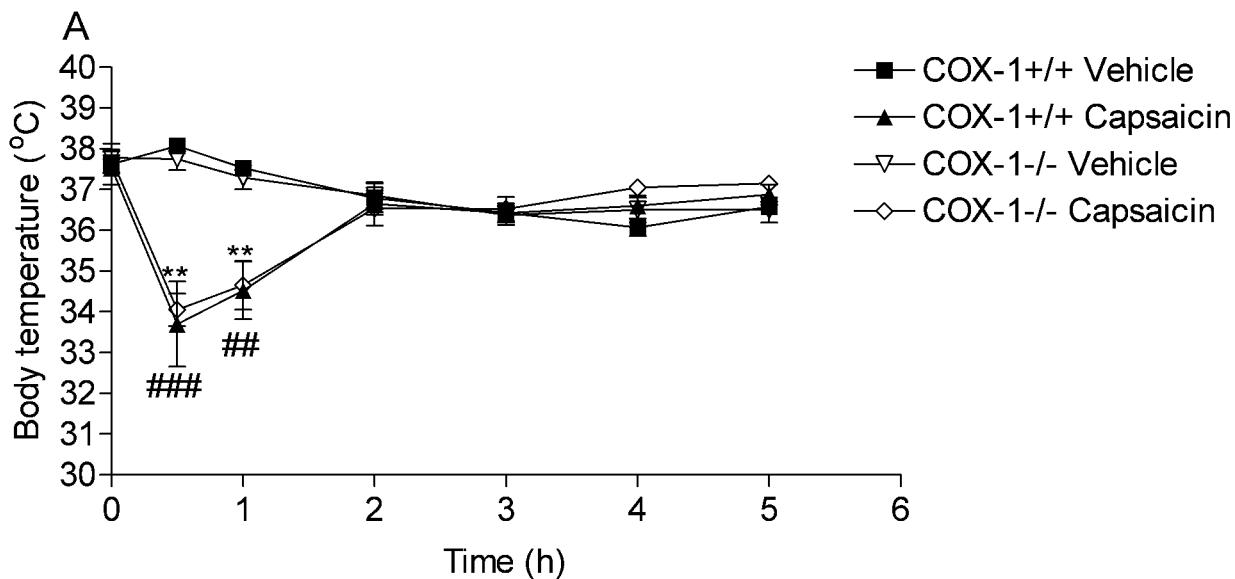


Figure 5

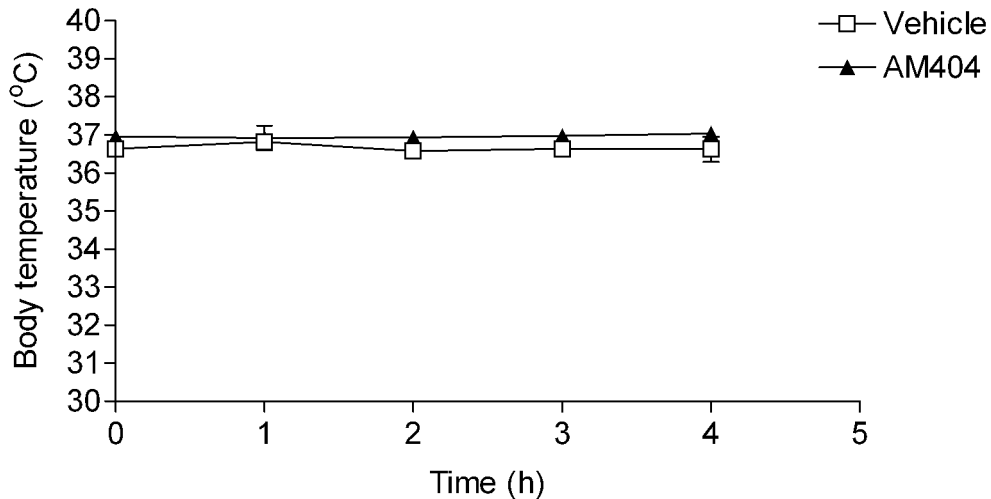


Figure 6

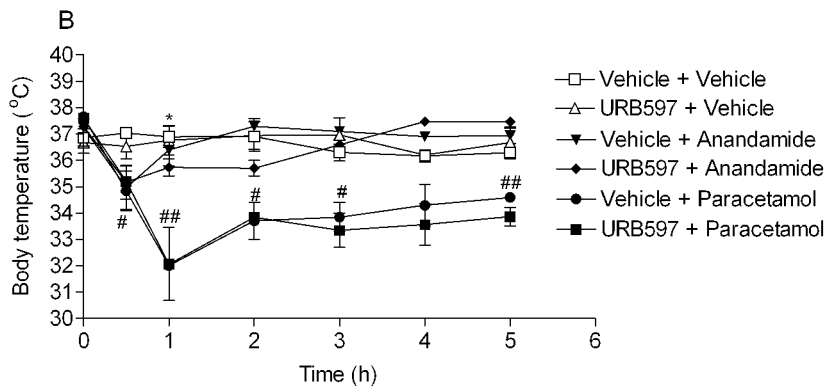
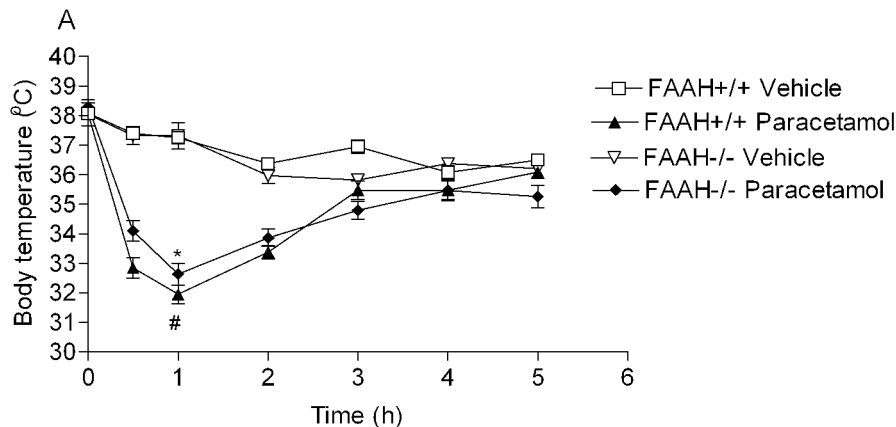


Figure 7

