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Loss of hypothermic and anti-pyretic action of paracetamol in cyclooxygenase-1 knockout mice is indicative of inhibition of cyclooxygenase-1 variant enzymes

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Abstract

Paracetamol (acetaminophen), is a centrally-acting antipyretic analgesic drug, which can also lower body temperature. Despite a century of clinical use, its mechanism of pharmacological action has not been completely elucidated. Previously, we demonstrated significant attenuation in the paracetamol induced hypothermia in parallel with its inhibitory action on the synthesis of brain prostaglandin E_2 (PGE_2) in cyclooxygenase-1 (COX-1) knockout mice in comparison to wild-type mice. The above reported pharmacological actions by paracetamol were completely retained in COX-2 knockout mice. We thus concluded that the mechanism of hypothermic action of paracetamol is dependent on inhibition of a COX-1 gene-derived enzyme. In the current investigation, we provide further support for this notion by demonstrating that the paracetamol-induced hypothermia is not mediated through inhibition of COX-1 as neither the COX-1 selective inhibitor, SC560, nor the COX-1/COX-2 dual inhibitor, indomethacin, induced hypothermia at pharmacologically active doses in mice. In addition, using a COX-2-dependent and PGE_2-mediated model of endotoxin-induced fever, paracetamol induced anti-pyretic and hypothermic actions in COX-1 wild-type mice. These effects were fully or partially attenuated in COX-1 knockout mice after prophylactic or therapeutic administration, respectively. Therapeutically-administered paracetamol also reduced hypothalamic PGE_2 biosynthesis in febrile COX-1 wild-type mice, but not in febrile COX-1 knockout mice. In conclusion, we provide further evidence which suggests that the hypothermic and now anti-pyretic actions of paracetamol are mediated through inhibition of a COX-1 variant enzyme.

Keywords: Cyclooxygenase, Fever, Hypothermia, Lipopolysaccharide, Paracetamol, Prostaglandin E_2

1. Introduction

Paracetamol (acetaminophen) is an analgesic antipyretic drug with weak anti-inflammatory actions; commonly used for the treatment of acute pain and fever in adults and children (Roberts and Morrow, 2001). Despite its widespread clinical use for over a century, the totality of the mechanism of pharmacological actions of paracetamol has not been satisfactorily explained.

The mechanism of action of aspirin and other non-steroidal anti-inflammatory drugs (NSAIDs) is dependent on inhibition of cyclooxygenase (COX) activity (Vane 1971). Unlike NSAIDs, paracetamol was shown to reduce prostaglandin E_2 (PGE_2) synthesis more potently in the brain than in peripheral (e.g. spleen) tissues (Flower and Vane 1972) suggesting specificity of action. Several *in vivo* studies confirmed this hypothesis (Ayoub et al. 2004; Ayoub et al. 2006; Feldberg et al., 1972; Muth-Selbach et al. 1999; Yaksh and Malmberg, 1993).

Paracetamol produced weak inhibition of COX-1 and COX-2 activities in *in vitro* inhibitory assays (Mitchell et al. 1993), thus inhibition of these two enzymes could not provide a satisfactory explanation for the reduction of CNS PGE_2 synthesis by paracetamol. From *in vitro* experiments, it was shown that paracetamol worked as a reducing agent to inhibit COX activities and that this inhibitory action was therefore dependent on the intracellular lipid hydroperoxide tone. (Boutaud et al., 2002; Hanel and Lands, 1982; Oulellet and Percival, 2001).

In 2002, Simmons and colleagues identified a novel cyclo-oxygenase species, COX-3, in canine tissues as a new catalytically active splice variant of COX-1, which unlike COX-1, retained intron-1 in its mRNA and protein sequences. Paracetamol potently inhibited COX-3 activity in a cell based assay, but not COX-1 or COX-2 (Chandrasekharan et al. 2002). As COX-3 was shown to be highly expressed in the CNS, it was therefore thought that it could provide the long sought after target for the pharmacological actions of paracetamol. However,

Retention of intron-1 in rodent and human COX-1 mRNA was shown to result in an out-of-frame sequence that terminated translation, producing a truncated catalytically inactive protein (Chandrasekharan et al. 2002; Dinchuk et al. 2003; Schwab et al. 2003). Despite this, COX-3 protein has been detected in rodent and human tissues (Ayoub et al. 2006; Qin et al. 2005; Shaftel et al. 2004; Snipes et al. 2005). It has been suggested that removal of one or more nucleotides from the intron-1 mRNA sequence prior to translation could lead to an in-frame sequence (Qin et al. 2005).

Previously, we provided evidence that the hypothermic action of paracetamol in normothermic mice is dependent on inhibition of a COX-1 gene-derived protein (Ayoub et al., 2004). This conclusion is derived from the demonstration that the brain PGE_2-dependent paracetamol-induced hypothermia was significantly attenuated in COX-$1^{-/-}$ mice, and was completely retained in COX-$2^{-/-}$, in comparison to the respective littermate wild-type controls. The two likely targets for the paracetamol-induced hypothermia are either COX-1 or its variant COX-3.

In the current study, we provide evidence that the target for the paracetamol-induced hypothermia in normothermic mice is not COX-1 as the COX-1 selective inhibitor SC560 and dual inhibitor indomethacin both failed to induce hypothermia at pharmacologically active doses. We also investigated the mechanism of antipyretic action of paracetamol, and we provide evidence that, unlike NSAIDs, the antipyretic action of paracetamol is not dependent on inhibition of COX-2, but on a COX-1 gene-derived protein.

2. Materials and methods

2.1. Animals

Male C57BL/6J mice (20±2g) were supplied from Harlan UK (Bicester, UK). COX-$1^{-/-}$, COX-$2^{-/-}$ (Langenbach et al. 1995; Morham et al. 1995) and littermate wild-type mice (COX-$1^{+/+}$ and COX-$2^{+/+}$) were from stocks bred at Bart's 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.

2.2. Reagents

Paracetamol (Sigma, Poole, UK) was dissolved in 12.5% (v/v) 1,2-propanediol. 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%. Indomethacin was dissolved in a 5% solution of sodium bicarbonate. Lipopolysaccharide (LPS) from *Escherichia coli* (serotype 0111:B4) purified by trichloroacetic acid extraction was purchased from Sigma and constituted in pyrogen-free saline (PFS).

2.3. Measurement of body temperature

To measure core body temperature of freely moving mice, temperature-sensitive transponders (Plexx B.V. AB Elst, The Netherlands) were implanted subcutaneously under light isoflurane anaesthesia. Mice were immediately placed under warming lamps to aid with recovery from the anaesthetic with a piece of tissue paper placed between the light source and mice to protect their eyes from direct light exposure. Implantation of the temperature-sensitive transponders was done one week prior to the experiment. Temperature measurements were

made using a temperature-sensitive scanner held 3cm above the back of the animals. Seven days prior to the experiment, the body temperature of mice was measured and recorded on a daily basis to familiarize (condition) mice to the temperature scanner in order to reduce stress and also to monitor their body temperature.

2.4. Induction of fever and drug administration

At 8:00am the animals were transferred to a warm air system (Vet Tech Solutions Limited, UK) set to the murine thermoneutral zone of 30^{o}C. Prior to the start of the experiments, body temperatures were measured twice in order to screen for any abnormally high or low body readings. At 9:00am LPS (see results section for doses used) was administered intraperitoneally. Paracetamol at a dose of 200mg/kg was administered subcutaneously either prophylactically (0.5h prior to LPS) or therapeutically (2h after LPS). In each experiment the time-profile of body temperature, usually up to 5h was determined. In other experiments, normothermic mice housed at standard 22^{o}C were treated intraperitoneally with either 300mg/kg paracetamol, 15mg/kg SC560, 15mg/kg celecoxib or 5 - 10mg/kg indomethacin (subcutaneously) in order to determine whether these drugs induce hypothermia.

2.5. Measurement of hypothalamic PGE_2

2.5.1. Dissection of hypothalamus

At the end of the experiments the mice were killed by cervical dislocation and the brains were quickly removed from the skull, placed on ice and immediately washed with cool 10μM indomethacin (constituted in 5% w/v biocarbonate buffer). Whole brains or hypothalamic tissues were removed and snap-frozen in liquid nitrogen. All tissues were stored at -80^{o}C ready for PGE_2 extraction and subsequent measurement.

2.5.2. PGE_2 extraction and measurement

The same procedures as previously published (Ayoub et al. 2004; Ayoub et al. 2006; Ayoub et al. 2008) were followed. Briefly, hypothalamic tissues or whole brains were pulverized with a nitrogen bomb (Biospec Products, Bartlesville, OK). One millilitre of 15% (v/v) ethanol (diluted in distilled water and then acidified to pH 3) was added to each tissue sample. The tissue homogenates were left at 4°C for 10 min and then spun at 375*g* for 10min at 4°C. C-18 Sep-Pak cartridges (Waters, Milford, MA, USA) were conditioned with 4ml of ethanol followed by 4ml of distilled water. The supernatant from tissue homogenates were then applied to the columns at a flow rate of 5 ml/min. The columns were then washed in 4ml of distilled water followed by 4ml of 15% ethanol in distilled water. The samples were finally eluted with 2 ml of ethyl acetate. The samples were dried in a Speed-Vac and then stored at -80°C.

Measurement of hypothalamic PGE_2 was performed using a commercial enzyme immunoassay kit from Cayman Chemicals (Ann Arbor, MI, USA), 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 with a standard PGE_2 curve (15.6-2,000pg/ml).

2.6 Quantification of paracetamol in plasma

After the subcutaneous administration of 200mg/kg paracetamol to wild-type C57BL/6J mice, plasma was collected from mice at 0.5, 1, 2, 3 and 4h and the concentration of paracetamol was measured using a colorimetric method (Cambridge Life Science). This method relies on the enzymatic conversion of paracetamol by aryl acyl amidohydrolase to acetate and 4-aminophenol. 4-aminophenol then reacts with o-cresol in the presence of ammoniacal copper sulphate to give a blue indophenol dye which is measured at 630nm. The

paracetamol concentrations were measured against standards of 0.02-5mmol/L (Campbell et al., 1983; Slater 1987; Smith et al., 1991).

2.7. Statistical analysis

The results were analysed using Graph Pad Prism 3.0 (San Diego, CA, USA) and, after checking that it was normally distributed, expressed and presented graphically as mean±S.E.M. Statistical analysis was performed using unpaired Students t-test to compare different treatments at the same time point. A P-value of <0.05 was considered to be statistically significant.

3. Results

3.1. Selective inhibition of COX-1 or COX-2 does not result in hypothermia

At pharmacologically active doses, the selective COX-1 and COX-2 inhibitors, SC560 (15mg/kg) and celecoxib (15mg/kg), respectively (Smith et al. 1998) administered intraperitoneally failed to induce hypothermia in normothermic mice housed at 22ºC ambient temperature over a 4h observation period (Fig. 1A). As a positive control, 300mg/kg paracetamol induced significant hypothermia ($P<0.05$), 0.5h (34.13ºC) and 1h (33.0ºC) after administration, resulting in 2.5 and 3.63ºC decreases in core body temperature, respectively. Paracetamol was given at a pharmacological dose of 300mg/kg (Crawley et al. 2008; Dalmann et al. 2015; Kanashiro et al. 2009; Muth-Selbach et al. 1999) for consistency with our previously published research (Ayoub et al. 2004).

At the same pharmacological dose of 15mg/kg, SC560 significantly ($P<0.01$) reduced brain PGE_2 synthesis in comparison to vehicle treated mice resulting in 76% reduction in PGE_2 synthesis (Fig. 1B) demonstrating that the absence of a hypothermic effect with this compound is not related to pharmacokinetic limitations.

3.2. LPS-induced fever is PGE_2-mediated and COX-2-dependent

To study the anti-pyretic mechanism of paracetamol, we developed and characterised a model of pyrexia in mice. We observed that 10µg/kg LPS (i.p.) resulted in a significant increase of core body temperature in mice housed at 30ºC ambient temperature from 0.5h (0.75ºC increase) to 4h (1.18ºC increase) post-administration (Fig. 2A). Administration of 100µg/kg LPS (i.p.) did not cause any significant changes in body temperature whilst, paradoxically, 1000µg/kg LPS (i.p.) resulted in an initial increase of body temperature at 0.5h, immediately followed by persistent hypothermia. This is consistent with previously published research in which 1000µg/kg LPS produced polyphasic changes in body temperature (Rudaya et al. 2005; Steiner et al. 2009). Based on the fact that 10µg/kg LPS gave a consistent mono-phasic

increase in body temperature (fever), this dose of LPS was used in subsequent experiments for the induction of fever in mice.

Administration of 10µg/kg LPS to mice housed at 30ºC ambient temperature significantly increased ($P<0.05$) the hypothalamic levels of PGE_2, 2 and 4h post-administration, in comparison to PFS-treated mice; with 61% and 53% increases, respectively (Fig. 2B). No statistically significant difference in the hypothalamic concentrations of PGE_2 between LPS and PFS-treated mice at 0h (immediately after injection) was found (Fig. 2B). The increases in hypothalamic PGE_2 synthesis induced by 10µg/kg LPS are temporally consistent with the induction of fever.

Intraperitoneal administration of 10µg/kg LPS to COX-$1^{-/-}$ and littermate COX-$1^{+/+}$ mice induced comparable pyrexia when compared to genetically matched COX-$1^{-/-}$ and COX-$1^{+/+}$ mice treated with PFS (Fig. 3A). There was some difference in the degree of diurnal body temperature change between COX-$1^{+/+}$ and COX-$1^{-/-}$ treated with PFS. This is mostly due to a small drop of temperature in PFS-treated COX-$1^{-/-}$ mice and not a decrease of temperature in PFS-treated COX-$1^{+/+}$ mice, as supported by observing the natural circadian changes in body temperature in COX-$1^{+/+}$ and COX-$1^{-/-}$ mice from other experiments where the body temperature at the start of the experiments was approximately 37.0ºC and dropped to approximately 36.0ºC (Figs. 4 and 5). Despite this, there was still a significant increase in the body temperature of COX-$1^{-/-}$ mice treated with 10µg/kg LPS compared to COX-$1^{-/-}$ mice treated with PFS ($P<0.05$; Fig. 3A).

COX-$2^{+/+}$ mice developed statistically significant pyrexia (1-5h) to 10µg/kg LPS when compared to COX-$2^{+/+}$ mice treated with PFS with increases in body temperature ranging from 0.65-1.3ºC (Fig. 3B). On the other hand, COX-$2^{-/-}$ mice treated with 10µg/kg LPS intraperitoneally failed to develop pyrexia when compared to COX-$2^{+/+}$ mice receiving the same treatment (Fig. 3B).

These data demonstrate that 10µg/kg LPS induced a COX-2 and not COX-1-mediated fever, which is associated with increased production of hypothalamic PGE_2 synthesis. These findings are consistent with previously published investigation on the mechanism of LPS-induced fever (Cao et al. 1999; Matsumura et al. 1998; Ootsuka et al. 2008; Saha et al. 2005; Steiner et al. 2005; Ushikubi et al. 1998).

3.3. The anti-pyretic action of paracetamol is dependent on inhibition of a COX-1 gene-derived protein

COX-$1^{-/-}$ but not COX-$2^{-/-}$ mice were used to investigate the mechanism of anti-pyretic action of paracetamol for two reasons. Firstly, COX-$2^{-/-}$ mice failed to respond with a febrile reaction in response to LPS and secondly, our previous research (Ayoub et al. 2004) demonstrated dependency by paracetamol on a COX-1 gene-derived enzyme for the induction of hypothermia.

Prophylactic treatment of COX-$1^{+/+}$ mice with 200mg/kg paracetamol 0.5h before challenge with 10µg/kg LPS, resulted in reversible and statistically significant ($P<0.01$ at 0h and $P<0.05$ at 1h and 2h post-LPS administration) hypothermia in comparison to COX-$1^{+/+}$ mice treated with vehicle and LPS with 1.76ºC, 1.16ºC and 0.54ºC hypothermia at 0h, 1h and 2h of LPS administration, respectively (Fig. 4A). Consistent with the results presented in fig. 3, LPS induced statistically significant fever in COX-$1^{+/+}$ mice ($P<0.01$, $P<0.001$ and $P<0.05$ after 1h, 2h and 3h of LPS administration, respectively) housed at 30ºC ambient temperature in comparison to COX-$1^{+/+}$ mice treated with PFS (Fig. 4A).

On the other hand, prophylactic administration of 200mg/kg paracetamol to COX-$1^{-/-}$ mice challenged with LPS, did not induce a hypothermic or anti-pyretic effect in comparison to COX-$1^{-/-}$ mice treated with vehicle and LPS (Fig. 4B). In fact the pyrexia observed in paracetamol treated COX-$1^{-/-}$ mice persisted longer than in non-paracetamol treated mice at 4-5h post-LPS administration. LPS induced statistically significant ($P<0.05$ and $P<0.01$)

pyrexia in both the paracetamol treated and vehicle treated mice in comparison to PFS treated mice (Fig. 4B).

In fig. 4A, after the paracetamol-induced hypothermia has subsided at 1.5h post-paracetamol administration, COX-$1^{+/+}$ mice went on to develop a febrile response to LPS. Therefore it can be assumed that paracetamol is able to induce hypothermia only in the absence of LPS. In order to test this assumption, in fig. 5A 200mg/kg paracetamol was administered therapeutically to COX-$1^{+/+}$ mice housed at 30ºC ambient temperature 2h after the administration of LPS, hence well beyond the establishment of a COX-2-mediated fever. In these animals paracetamol reduced the LPS-induced febrile response and indeed, similar to the data presented in fig. 4A, it also was able to significantly induce hypothermia ($P<0.001$ at 1, 2 and 3h after paracetamol administration. The maximum hypothermia observed with paracetamol was 3h after paracetamol administration with the body temperature falling to 31.1ºC. It is not clear why therapeutically administered paracetamol (2h after LPS) is able to induce a more substantial hypothermia in comparison to prophylactically administered paracetamol. Following on from the subcutaneous administration of paracetamol, the plasma concentration peaked at 0.5h at a concentration of 0.7mmol/L (700µM), which then started to steadily decline from 1h (0.56mmol/L) onwards reaching a concentration of 0.025mmo/L at 4h (Fig. 4C). The paracetamol plasma profile follows temporally follows its effect on body temperature in COX-$1^{+/+}$ mice (Fig. 4A).

In comparison, the substantial hypothermia induced with therapeutically administered 200mg/kg paracetamol in COX-$1^{+/+}$ mice (32.8 - 31.1ºC hypothermia), there was a moderate reduction in febrile body temperature with therapeutically administered paracetamol (2h post-LPS) in COX-$1^{-/-}$ mice with body temperatures ranging from 35.8 to 35.93ºC (Fig. 5B). In COX-$1^{-/-}$ mice, paracetamol non-significantly reduced the febrile body temperature by around 0.67ºC after 1h of administration (Fig. 5B). This is in comparison to the significant reduction

of febrile body temperature in COX-$1^{+/+}$ mice compared to LPS treated mice (4.56ºC after 1h of paracetamol administration). There was no statistically significant difference in the body temperatures between COX-$1^{+/+}$ and COX-$1^{-/-}$ mice treated with LPS after 3h of LPS administration (1h post-paracetamol administration; figs. 5A and B). Hence the febrile responses in COX-$1^{+/+}$ and COX-$1^{-/-}$ mice 1h post-paracetamol administration was negligible. The loss of paracetamol induced hypothermia in febrile COX-$1^{-/-}$ mice in comparison to COX-$1^{+/+}$ mice correlated with a loss of the effect of the same dose of paracetamol on hypothalamic PGE_2 synthesis in COX-$1^{-/-}$ mice, again, in comparison to COX-$1^{+/+}$ mice at 1h after paracetamol administration (Fig. 5C).

In the data presented in figs. 4 and 5, non-LPS challenged paracetamol treated mice were not included as availability of sufficient numbers of littermate COX-$1^{-/-}$ mice was a problem. Equally, from previous data presented in fig. 1A and previously published by us (Ayoub et al. 2004) the effect of paracetamol given to non-febrile mice on normothermic body temperature has already been demonstrated.

3.4. Indomethacin is antipyretic, but does not induce hypothermia

To compare the hypothermic and antipyretic profile of paracetamol (as reported in the current study and previously: Ayoub et al. 2004) with an NSAID, indomethacin was selected as a non-selective dual COX-1/COX-2 inhibitor (Mitchell et al. 1993). At pharmacological doses of 5 and 10mg/kg (Crawford et al. 1979; Masferrer et al. 1994) indomethacin administered subcutaneously failed to induce hypothermia over a 5h period (Fig. 6A), despite resulting in a statistically significant inhibition ($P<0.05$) in the synthesis of brain PGE_2 measured at 1h after administration (Fig. 6B). Despite the lack of hypothermia in normothermic mice housed at 22ºC ambient temperature (Fig. 6A), 5mg/kg indomethacin (administered subcutaneously) resulted in a statistically significant drop in the febrile response ($P<0.05$) induced by 10µg/ml LPS in mice housed at 30ºC ambient temperature at 4h and in a statistically non-significant

decrease at 1, 2 and 3h post-LPS administration (Fig. 6C). Indomethacin was administered prophylactically 0.5h before LPS. Compared to PFS, LPS resulted in statistically significant increases in core body temperature from 0.5-4h post administration and with increase of body temperatures ranging from 0.8-1.44ºC (Fig. 6C).

4. Discussion

Previously we demonstrated significant attenuation in brain PGE_2-mediated paracetamol-induced hypothermia in COX-$1^{-/-}$, but not COX-$2^{-/-}$ mice, leading us to conclude that this action of paracetamol is dependent on the inhibition of a centrally expressed COX-1 gene-derived protein (Ayoub et al. 2004). With the identification of COX-3 as a splice variant of COX-1 (Chandrasekharan et al. 2002), which is present in mouse tissues at the expected molecular size (Ayoub et al. 2006; Shaftel et al. 2004), there are two possible targets for the paracetamol-induced hypothermia; either COX-1 or a COX-1 variant protein. Since the selective COX-1 inhibitor SC560 and the dual COX-1/COX-2 inhibitor indomethacin at pharmacologically active doses (Fig. 1B; Crawford et al. 1979; Masferrer et al. 1994; Smith et al. 1998), failed to induce hypothermia, we conclude that the target for the paracetamol-induced hypothermia is not COX-1 and is likely to be a variant of COX-1.

Contrary to the generally accepted notion, Brennies (2006) reported that SC560 was equipotent as an inhibitor of COX-1 and COX-2 in cultured cells. These findings do not alter our conclusion that inhibition of a COX-1 variant enzyme mediates the paracetamol-induced hypothermia because whether SC560 is COX-1 selective or is non-selective for COX-1 and COX-2, does not indicate potential inhibition of COX-1 variant proteins. In fact, if SC560 does inhibit both COX-1 and COX-2 in vivo, this gives more support to our theory that the paracetamol-induced hypothermia is not mediated by inhibition of either enzymes. In addition, indomethacin, a dual COX-1 and COX-2 inhibitor, was non-hypothermic, but is anti-pyretic through inhibition of COX-2 at pharmacological doses.

Since paracetamol is commonly used for the treatment of fever, we also investigated the mechanism of antipyretic action of paracetamol. As demonstrated by others (Abe et al. 2001; Li et al. 2001) and confirmed in the current study, intraperitoneally-administered LPS induces

COX-2-dependent and hypothalamic PGE_2-mediated pyrexia. Since paracetamol has previously been shown to induce hypothermia in mice after 0.5h of administration (Ayoub et al. 2004), it was expected that prophylactic paracetamol would result in hypothermia prior to LPS treatment. Consistent with our previous study (Ayoub et al. 2004), we also find that this hypothermic action is also abolished in febrile COX-$1^{-/-}$ mice compared to littermate wild-type controls. The evidence to support the notion that paracetamol induces an anti-fever effect through inhibition of a COX-1 gene-derived enzyme is provided by the finding that paracetamol administered therapeutically induced potent hypothermic and anti-pyretic actions in COX-$1^{+/+}$ mice with established pyrexia and that this effect was partly lost in COX-$1^{-/-}$ mice. The induction of hypothermia by therapeutically administered paracetamol in febrile mice was also observed by Li and colleagues (2008). Moreover the loss of hypothermia in febrile COX-$1^{-/-}$ mice was also associated with loss in the reduction of hypothalamic PGE_2 synthesis by paracetamol. We therefore, make the assumption that paracetamol reduces body temperature through the induction of hypothermia through inhibition of a constitutively expressed COX-1 variant enzyme (Ayoub et al. 2004; Satinoff, 1972), whilst NSAIDs induce antipyretic actions through inhibition of the inducible COX-2 enzyme expressed in hypothalamic endothelial cells (Li et al. 2001).

In contrast, Li et al. (2008) showed that the hypothermic and anti-pyretic actions of paracetamol were not attenuated in COX-$1^{-/-}$ mice in comparison to wild-type mice. These authors used mice housed at 23ºC ambient temperature, which is well below the well-established 30ºC thermoneutral temperature for mice (Fraifeld et al. 1995; Kozak et al. 1998); a key requirement for the reliable induction of fever in mice (Rudaya et al. 2005; Steiner et al. 2009). The anti-pyretic activity of paracetamol reported by Li et al (2008) cannot be attributed to inhibition of inducible COX-2 protein as it was observed 1h following on from LPS administration, which is insufficient time for the induction of COX-2 (Ryseck et al. 1992). However when we tested the anti-pyretic action of paracetamol 2h after LPS

administration, the anti-pyretic action of paracetamol was maintained in COX-$1^{+/+}$ mice and partly attenuated in COX-$1^{-/-}$ mice. The discrepancy between our results and those of Li and colleagues (2008) could be due to phenotypic differences between the COX-$1^{-/-}$ mice used in Li's study and those used in the current study. For example, Ballou et al. (2000) reported a significantly lower number of writhing counts in COX-$1^{-/-}$ mice in comparison to wild-type mice, whereas we found no such difference (Ayoub et al., 2006).

It has been argued by several groups that both the antipyretic and analgesic target for paracetamol is COX-2. Hinz and colleagues in 2007 demonstrated a 4.4 fold selectivity by paracetamol for the inhibition of human COX-2 (IC_{50} = 25.8µmol/L) over COX-1 (IC_{50} = 113.7µmol/L) and argued that the weak inhibitory effect by paracetamol on COX-2 activity during inflammation and hence its weak anti-inflammatory activity is dictated by its lower potency for COX inhibition under elevated intracellular lipid hydroperoxide tone (Hanel and Lands 1982). It is noteworthy that to date the link between the lipid hydroperoxide tone and the inhibition of COX enzymes by paracetamol has only been demonstrated in vitro (Boutaud et al. 2002; Lucas et al. 2005; Ouellet and Percival 2001) and not shown *in vivo*. In fact scavenging intracellular lipid hydroperoxides did not reverse the lack of inhibition by paracetamol of the endotoxin-induced COX-2 activity in macrophages (Ayoub et al. 2011a). Since COX-$2^{-/-}$ mice fail to develop fever to LPS, Engstrom Ruud et al. (2013) went on to use COX-$2^{+/-}$ mice to study the mechanism of anti-pyretic action of paracetamol. At a dose which is non-hypothermic in COX-$2^{+/+}$ mice, 50mg/kg paracetamol actually reduced the LPS-induced pyrexia in COX-$2^{+/-}$ mice. It is not clear how losing one allele of the COX-2 gene would render paracetamol more effective at reducing fever and how it can be concluded from these results that COX-2 is the anti-pyretic target for paracetamol. In support of the notion that the antipyretic action of paracetamol is mediated through inhibition of a constitutively expressed enzyme, it was shown that prophylactically administered paracetamol produced the

same decrease in body temperature as therapeutically administered paracetamol in children in randomised controlled trials (Yalçin et al., 2008; Prymula et al., 2009). Induction of hypothermia with paracetamol and the concomitant reduction in brain PGE_2 synthesis (Ayoub et al. 2004) suggest a role for PGE_2 in the maintenance of normothermia. Evidence for such a function for PGE_2 is scarce, however Oka and colleagues (2004) showed administration of EP1, EP3 and EP4 receptor agonists in the absence of LPS fever to induce an increase in body temperature and have suggested a counter-regulatory role for the EP4 receptor.

Paracetamol is regarded generally speaking as a centrally-acting analgesic (Ayoub et al. 2006; Muth-Selbach et al. 1999; Yaksh and Malmberg 1993). The same notion applies to the temperature lowering effects of paracetamol (Ayoub et al. 2004; Feldberg et al. 1972; Massey et al. 1982). Paracetamol-induced hypothermia is temporally correlated with reduction in brain PGE_2 synthesis (Ayoub et al., 2004; Feldberg et al. 1973; Kanashiro et al. 2008; Mirrasekhian et al., 2018). Intracerebroventricular administration of paracetamol resulted in profound hypothermia (Clark and Alderdice, 1972) and anti-pyretic actions (Crawford et al. 1979; Massey et al. 1982). Massey et al (1982) identified that the central hypothermic action of paracetamol was mediated predominantly by the parent compound and not its reactive metabolite *N*-acetyl-*p*-benzoquinone imine (NAPQI). The same authors also reported that 500mg/kg of orally administered paracetamol to result a plasma concentration of 113µg/ml (747µM) after 1.5h. We showed that 200mg/kg paracetamol administered orally to produce a peak plasma paracetamol concentrations of 210µM after 1h (unpublished observation), which is within a similar range reported by Hinz et al. (2007) in humans given the standard therapeutic oral dose of 1000mg (104.8µM). We also showed that subcutaneously administered 200mg/kg paracetamol in mice to result in a plasma concentration within the high micromolar concentration range. These findings justify the use of 200mg/kg dose of

paracetamol in mice as a therapeutic pharmacological dose. Indeed, doses between 100-300mg/kg of paracetamol are considered sub-toxic and have been extensively used for investigations on the mechanism of action of this drug (Pini et al. 1996). The therapeutic dose for paracetamol in humans is 1000mg, whereas the therapeutic doses for celecoxib and indomethacin are in the range of 100-200mg. When calculating the doses used for all three drugs given to humans on a body weight basis, the differences in doses between celecoxib and indomethacin compared to paracetamol used in mice in the current study, are comparable to dose differences in humans.

The study by Högetätt and colleague (2005) provided evidence of the metabolism of paracetamol into N-acyl phenolamine (AM404) in the brain concluding that the pharmacological actions of paracetamol, including hypothermia, may be mediated through AM404. Indeed AM404 induces hypothermia and analgesia (Borsani et al. 2007; Mitchell et al. 2007; Rawls et al. 2006); either through activation of endocannabinoids or the transient receptor potential vanilloid-1 (TRPV1) channel (Beltramo et al. 1997; De Petrocellis et al. 2000). However, inhibition of the conversion of paracetamol to AM404 by inhibition of fatty acid amido hydrolase (FAAH) did not prevent the development of hypothermia induced by paracetamol and paracetamol induced comparable hypothermia in $FAAH^{-/-}$ mice to wild-type mice (Ayoub 2011b). Recently we have detected AM404 in the cerebrospinal fluid of patients given paracetamol systemically (Sharma et al. 2017), however we do not know whether AM404 was pharmacologically active in these patients.

5. Conclusion

In conclusion, we provide further supportive evidence that the hypothermic action of paracetamol and also the antipyretic action of this drug are likely to be mediated through

inhibition of a centrally expressed (mostly likely in the hypothalamus) COX-1 gene-derived protein.

Acknowledgements

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Figure legends

Fig. 1: Selective inhibition of COX-1 with SC560 and COX-2 with celecoxib does not result in hypothermia. A: Male C57BL/6 mice were treated with 15mg/kg SC560, 15mg/kg celecoxib or 300mg/kg paracetamol intraperitoneally and their body temperatures monitored for 4h. B: SC560 at the same non-hypothermic dose resulted in statistically significant reduction in brain levels of PGE_2 after 1h treatment, *P<0.05 vehicle versus paracetamol, n=4.

Fig. 2: Intraperitoneally administered 10µg/kg LPS induces consistent PGE_2 dependent pyrexia. A: dose-response to LPS (10-1000µg/kg) for the induction of pyrexia in male C57BL/6 mice (expressed as change in core body temperature relative to zero time-point for the same treatment group). B: in comparison to pyrogen-free saline (PFS) treated mice, 10µg/kg LPS resulted in significant increases in hypothalamic levels of PGE_2 after 2 and 4h, n=3-4.

Fig. 3: Intraperitoneally administered 10µg/kg LPS induced COX-2, but not COX-1-dependent pyrexia. Time-profile of LPS-induced pyrexia in COX-$1^{-/-}$ (A) and COX-$2^{-/-}$ (B) mice compared to littermate wild-type control mice. A: *P<0.05 and **P<0.01 COX-$1^{+/+}$ PFS versus COX-$1^{+/+}$ LPS; #P<0.05 COX-$1^{-/-}$ PFS versus COX-$1^{-/-}$ LPS. B: *P<0.05, **P<0.01 and ***P<0.001 COX-$2^{+/+}$ PFS versus COX-$2^{+/+}$ LPS; n=3-4.

Fig. 4: The antipyretic effect of prophylactically administered paracetamol correlates with the paracetamol plasma concentration and is abolished in COX-1 knockout mice. The antipyretic effect of 200mg/kg paracetamol administered subcutaneously 0.5h prior to 10µg/kg LPS was examined in COX-$1^{+/+}$ (A) and COX-$1^{-/-}$ (B) mice. A: *P<0.05, **P<0.01 and ***P<0.001 vehicle and PFS versus vehicle and LPS; #P<0.05 and ##P<0.01 vehicle and

LPS versus paracetamol and LPS. B: *P<0.05 and **P<0.01 vehicle and PFS versus vehicle and LPS; ##P<0.01 vehicle and LPS versus paracetamol and LPS; n=3-5. C: the paracetamol plasma concentration in C57BL/6J mice peaked at 0.5h, which correlated with the peak of hypothermia in COX1+/+ mice.

Fig. 5: The antipyretic and inhibitory effect of therapeutically administered paracetamol on hypothalamic PGE_2 synthesis was abolished in COX-1 knockout mice. The antipyretic effect of 200mg/kg paracetamol administered subcutaneously 2h after 10µg/kg LPS was examined in COX-$1^{+/+}$ (A) and COX-$1^{-/-}$ (B) mice. Panel C shows comparisons of the effect of therapeutically administered 200mg/kg paracetamol on hypothalamic PGE_2 levels 1h after paracetamol administration. A: *P<0.05, **P<0.01 and ***P<0.001 PFS and vehicle versus LPS and vehicle; ##P<0.01 and ###P<0.001 LPS and vehicle versus LPS and paracetamol. B: *P<0.05 and **P<0.01 PFS and vehicle versus LPS and vehicle; n=4-5.

Fig. 6: Indomethacin reduced brain PGE_2 synthesis, but did not induce hypothermia in wild-type mice, while reducing the LPS-induced fever. Indomethacin was administered subcutaneously at 5 and 10mg/kg to male C57BL/6 mice housed at 22ºC and their body temperature was monitored for 5h (A) and brain PGE_2 synthesis was compared between mice treated with vehicle and 5mg/kg indomethacin 1h after administration (B). Indomethacin (5mg/kg) administered subcutaneously 0.5h before LPS significantly reduced the febrile response induced by intraperitoneally administered 10µg/ml LPS at 0.5, 2, 3 and 4h post-LPS administration. B: *P<0.05 vehicle versus 5mg/kg indomethacin. C: **P<0.01 and ***P<0.001 vehicle and PFS versus vehicle and LPS; #P<0.05 vehicle and LPS versus indomethacin and LPS; n=3-6.

Figure 1

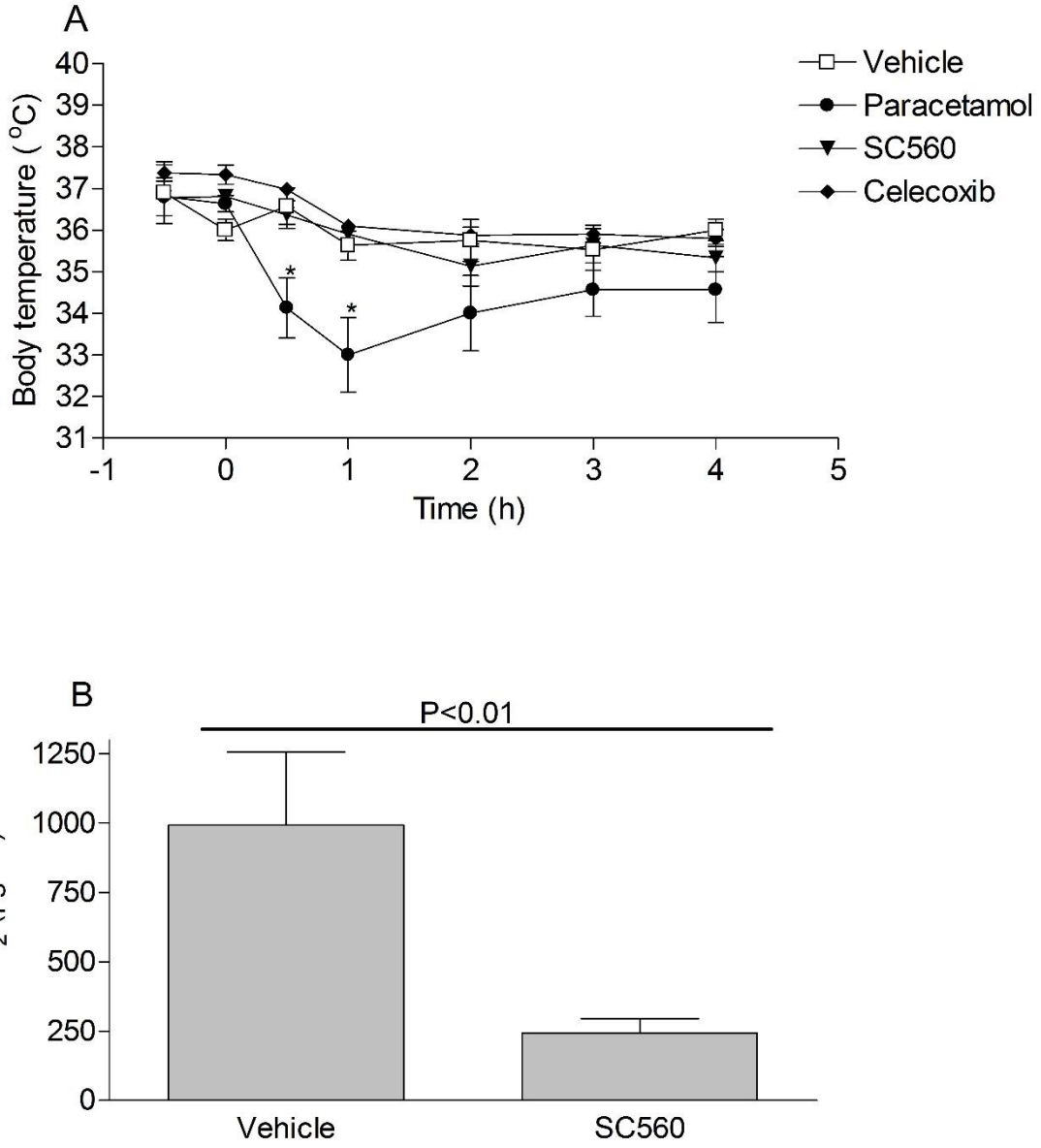

A
Body temperature (°C)
40
39
38
37
36
35
34
33
32
31
-1
0
1
2
3
4
5
Time (h)
*
*
Vehicle
Paracetamol
SC560
Celecoxib
B
P<0.01
PGE2 (pg/ml)
1250
1000
750
500
250
0
Vehicle
SC560

Figure 2

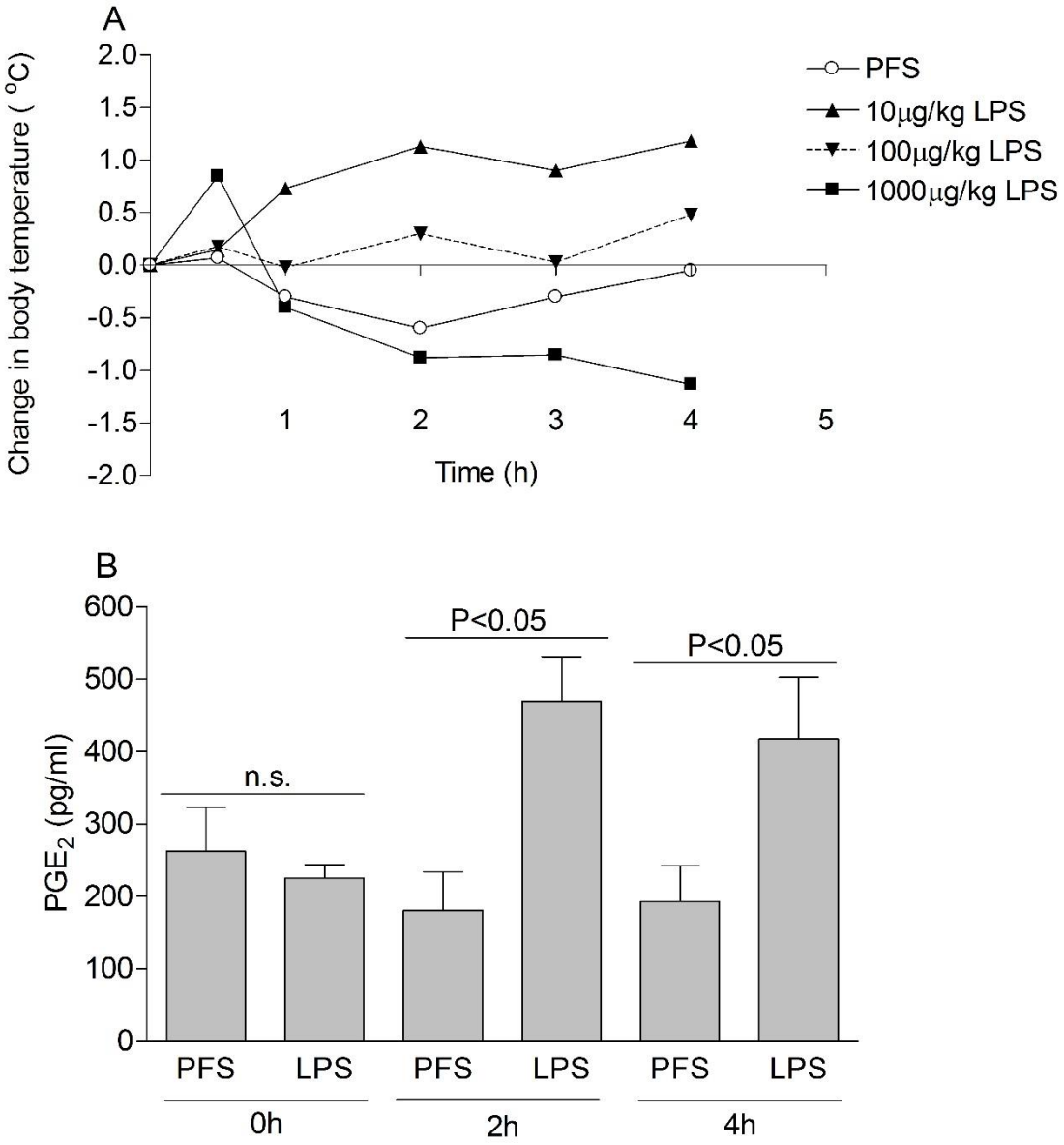

A
Change in body temperature (°C)
2.0
1.5
1.0
0.5
0.0
-0.5
-1.0
-1.5
-2.0
1
2
3
4
5
Time (h)
PFS
10μg/kg LPS
100μg/kg LPS
1000μg/kg LPS
B
PGE2 (pg/ml)
600
500
400
300
200
100
0
n.s.
P<0.05
P<0.05
PFS
LPS
PFS
LPS
PFS
LPS
0h
2h
4h

Figure 3

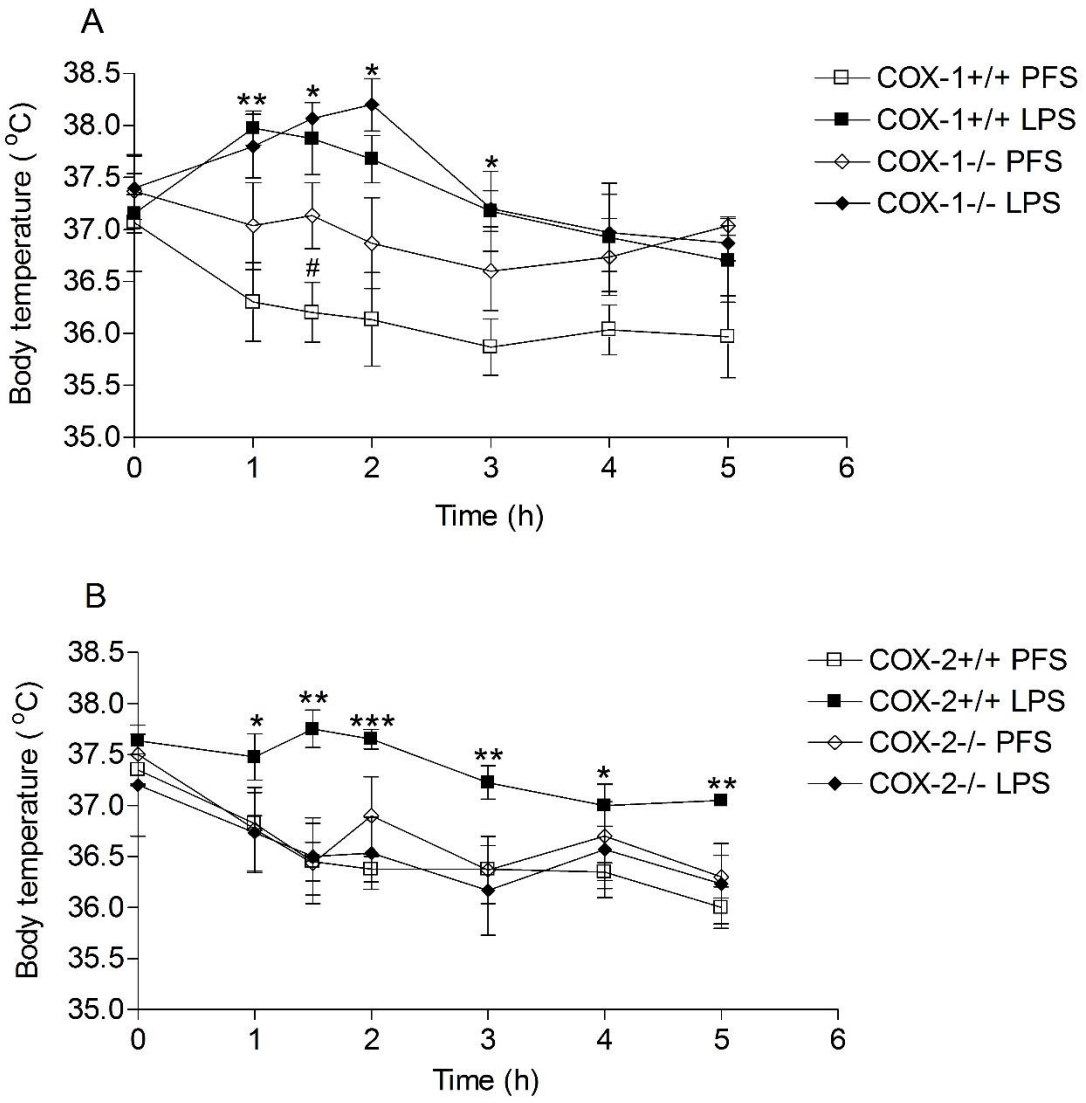

A
Body temperature (°C)
38.5
38.0
37.5
37.0
36.5
36.0
35.5
35.0
0
1
2
3
4
5
6
Time (h)
**
*
*
*
#
COX-1+/+ PFS
COX-1+/+ LPS
COX-1-/- PFS
COX-1-/- LPS
B
Body temperature (°C)
38.5
38.0
37.5
37.0
36.5
36.0
35.5
35.0
0
1
2
3
4
5
6
Time (h)
*
**
***
**
*
**
COX-2+/+ PFS
COX-2+/+ LPS
COX-2-/- PFS
COX-2-/- LPS

Figure 4

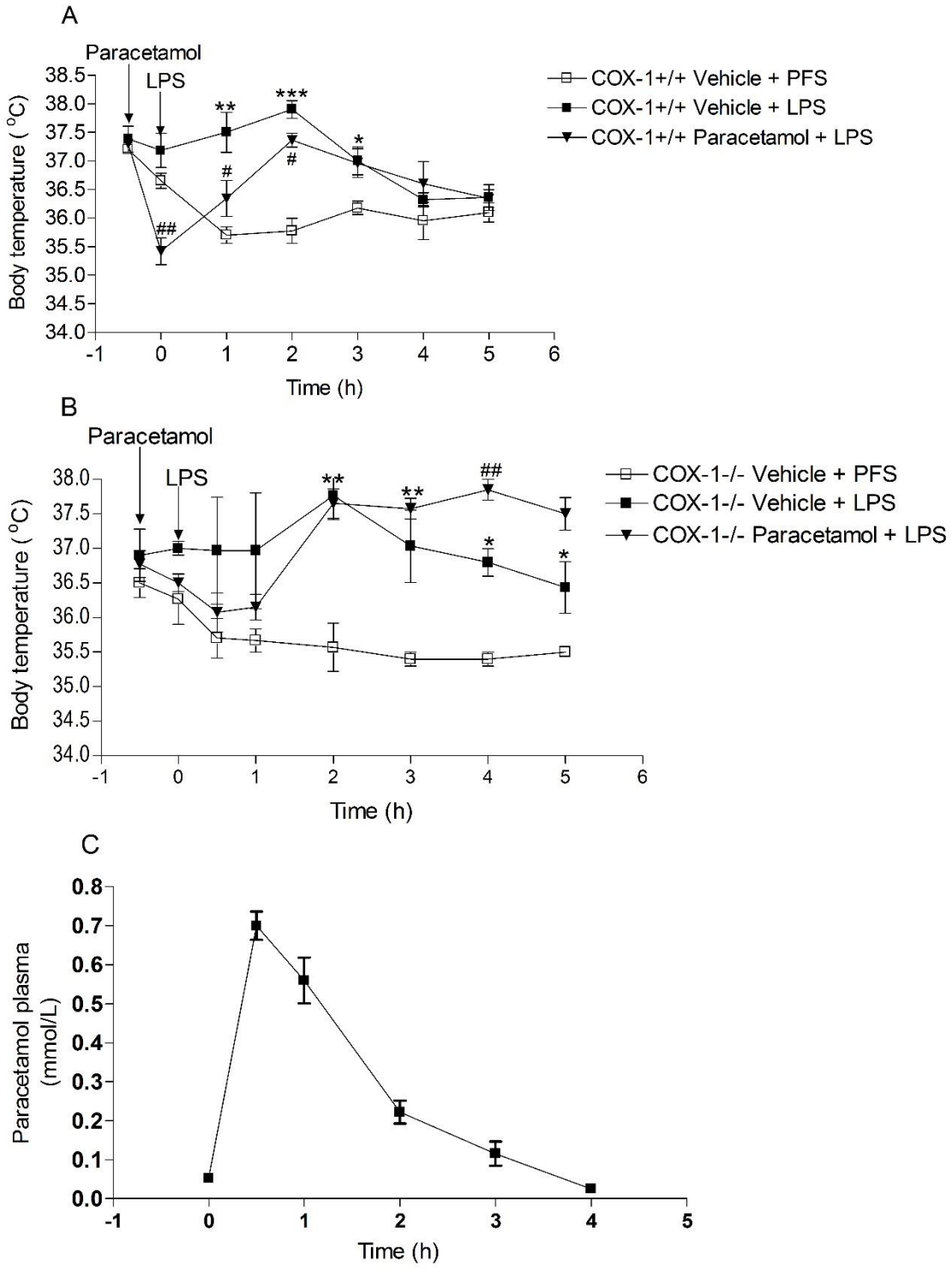

A
Paracetamol
LPS
Body temperature (°C)
Time (h)
COX-1+/+ Vehicle + PFS
COX-1+/+ Vehicle + LPS
COX-1+/+ Paracetamol + LPS
B
Paracetamol
LPS
Body temperature (°C)
Time (h)
COX-1-/- Vehicle + PFS
COX-1-/- Vehicle + LPS
COX-1-/- Paracetamol + LPS
C
Paracetamol plasma (mmol/L)
Time (h)

Figure 5

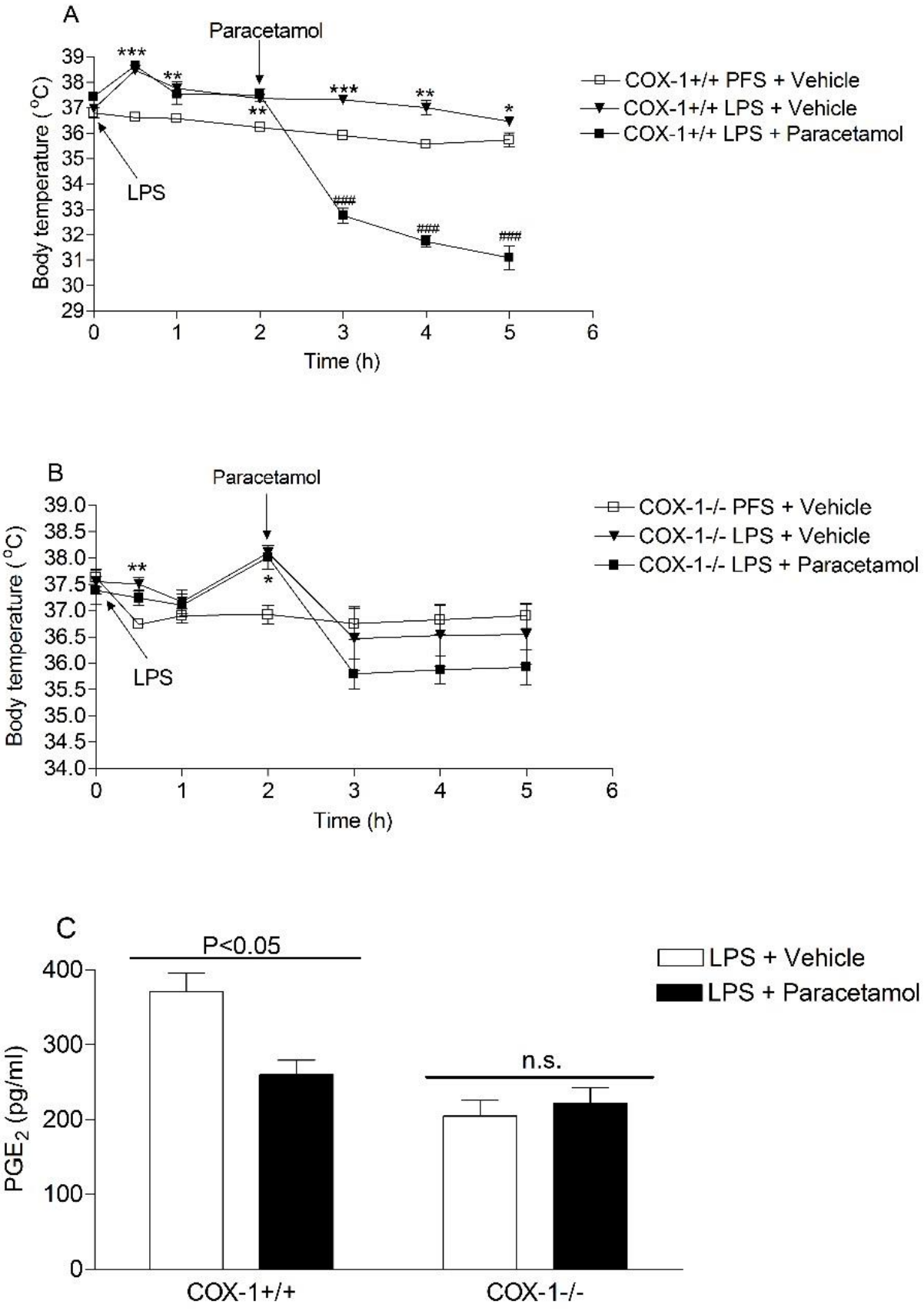
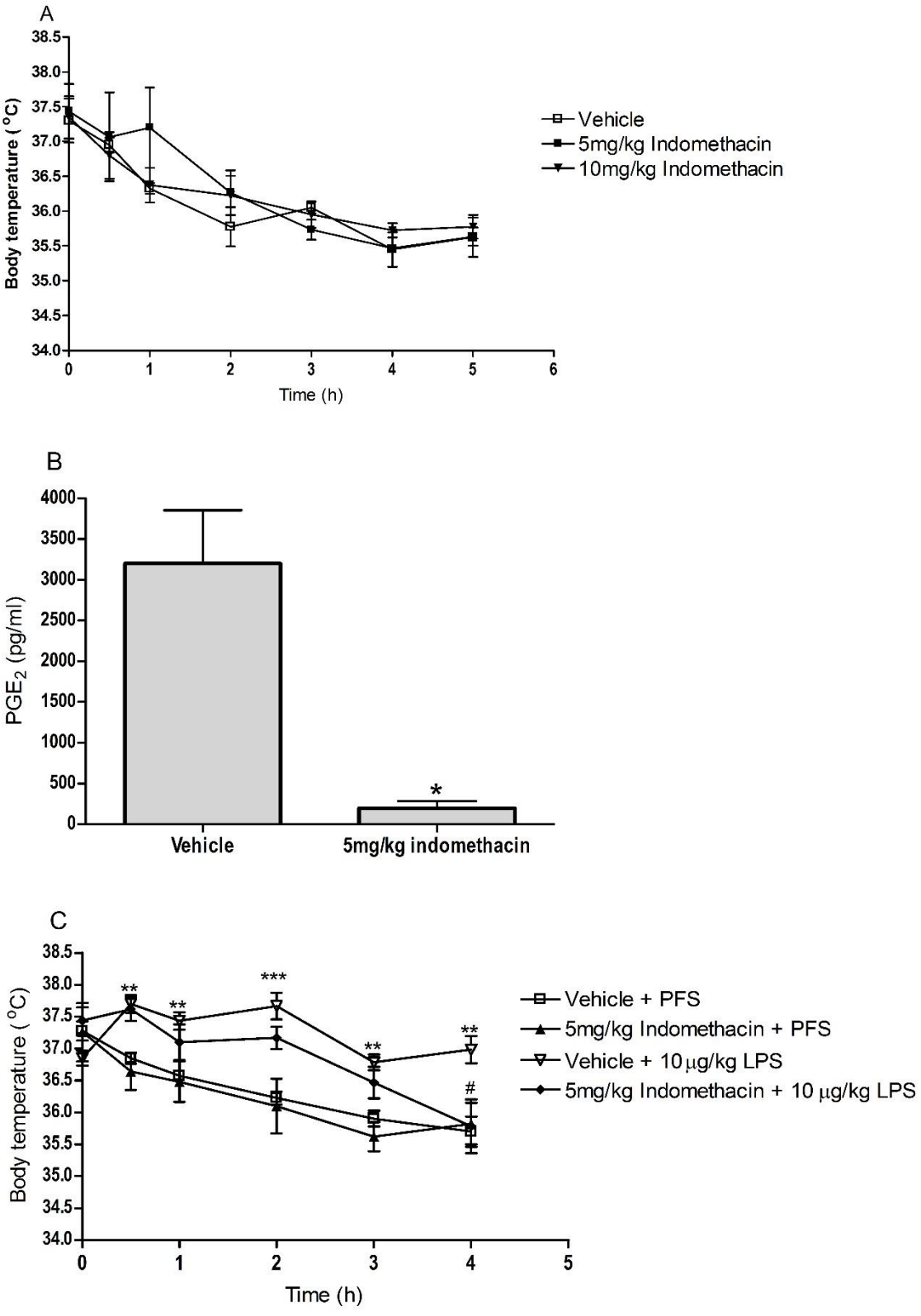

A
Paracetamol
LPS
Body temperature (°C)
Time (h)
COX-1+/+ PFS + Vehicle
COX-1+/+ LPS + Vehicle
COX-1+/+ LPS + Paracetamol
B
Paracetamol
LPS
Body temperature (°C)
Time (h)
COX-1-/- PFS + Vehicle
COX-1-/- LPS + Vehicle
COX-1-/- LPS + Paracetamol
C
P<0.05
n.s.
PGE2 (pg/ml)
COX-1+/+
COX-1-/-
LPS + Vehicle
LPS + Paracetamol

Figure 6


A
Body temperature (°C)
Time (h)
Vehicle
5mg/kg Indomethacin
10mg/kg Indomethacin
B
PGE2 (pg/ml)
Vehicle
5mg/kg indomethacin
C
Body temperature (°C)
Time (h)
Vehicle + PFS
5mg/kg Indomethacin + PFS
Vehicle + 10 μg/kg LPS
5mg/kg Indomethacin + 10 μg/kg LPS