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The involvement of a cyclooxygenase 1 gene-derived protein in the antinociceptive action of paracetamol in mice

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Abstract

Paracetamol is a widely used analgesic and antipyretic with weak anti-inflammatory properties. Experimental evidence suggests that inhibition of prostaglandin biosynthesis contributes to its pharmacological actions. Three cyclooxygenase (COX) isoenzymes are involved in prostaglandin biosynthesis, COX-1, COX-2 and a recently discovered splice-variant of COX-1, COX-3. Our aim was to identify the relative roles for these enzymes in the antinociceptive action of paracetamol in mice. We compared the antinociceptive action of paracetamol with the non-selective non-steroid anti-inflammatory drug, diclofenac and studied paracetamol antinociception in COX-1 and COX-2 knockout mice.

Paracetamol (100–400 mg/kg) inhibited both acetic acid- and iloprost-induced writhing responses. In contrast, diclofenac (10–100 mg/kg) inhibited only acetic acid-induced writhing. Only diclofenac reduced peripheral prostaglandin biosynthesis whereas both drugs reduced central prostaglandin production. Prostaglandin E_2 (PGE_2) concentrations were reduced in different brain regions by administration of paracetamol. COX-1, COX-2 and COX-3 enzyme proteins were expressed in the same brain regions. The effects of paracetamol on writhing responses and on brain PGE_2 levels were reduced in COX-1, but not COX-2, knockout mice. The selective COX-3 inhibitors, aminopyrine and antipyrine also reduced writhing responses and brain PGE_2 biosynthesis. These results suggest that the antinociceptive action of paracetamol may be mediated by inhibition of COX-3.

Keywords: Paracetamol; Diclofenac; Aminopyrine; Prostaglandin; Cyclooxygenase-3; Writhing; (Cyclooxygenase knockout mice)

1. Introduction

Three isoforms of the prostaglandin synthesising enzyme, cyclooxygenase (COX), have been characterised, COX-1, COX-2 and COX-3 (DeWitt and Smith, 1988; Xie et al., 1991; Chandrasekharan et al., 2002). COX-1, generally expressed constitutively, is responsible for the synthesis of prostaglandins involved in the regulation of physiological functions (Crofford, 1997). Thus, COX-1 is regarded as a 'house-keeping' enzyme, unlike COX-2, which is induced by various pro-inflammatory stimuli (Jones et al., 1993) and in pathological conditions such as inflammation, pain and fever (Vane et al., 1994; Yamamoto and Nozaki-Taguchi, 1996; Cao et al., 1996). COX-3 is a splice-variant of COX-1 that retains the intron-1 gene sequence at the mRNA level which encodes a 30 aa sequence inserted into the N-terminal hydrophobic signal peptide of the enzyme protein. In the dog, COX-3 protein was expressed predominantly in the central nervous system (CNS) and in the heart (Chandrasekharan et al., 2002). Paracetamol selectively inhibited this COX-3 protein in preference to COX-1 and COX-2. In the canine, COX-3 mRNA intron-1 is within frame, but in humans and rodents there is a frameshift mutation in intron-1 of the COX-3 transcript. The mechanism for conversion of COX-3 mRNA to active enzyme protein in mice is therefore not clear as yet. However, in rat tissues, an approximately 64 kDa protein was detected using antibody against the intron-1 moiety of a COX-1 variant protein (Snipes

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et al., 2005). Also, an intron-retained COX-1 protein was identified in human tissues (Qin et al., 2005). Although this human COX-1 variant (named COX-1b_2) synthesised prostaglandin $F_{2\alpha}$ from arachidonic acid, its activity was not selectively inhibited by paracetamol (Qin et al., 2005).

Prostaglandin E_2 (PGE_2), is a potent hyperalgesic mediator at the site of injury (Ferreira et al., 1973), in the spinal cord (Yaksh and Malmberg, 1993; Malmberg and Yaksh, 1994) and in the brain (Hori et al., 2000; Abe et al., 2001). An increased release of PGE_2 into the splenic vein of an anaesthetised dog was detected after a nociceptive injection of bradykinin into the spleen (Ferreira et al., 1973). Subsequently, PGE_2 release from spinal cord slices in vitro was reported after stimulation with capsaicin or K^+(60 mM) (Malmberg and Yaksh, 1994; Dirig et al., 1997). PGE_2 was also collected by microdialysis with a probe implanted in the spinal cord of rats in vivo. The concentrations of PGE_2 in the dialysate increased after an injection of formalin into the hind paw of the rat (Malmberg and Yaksh, 1995; Muth-Selbach et al., 1999; Tegeder et al., 2001) or after an intraspinal injection of substance P (Yaksh et al., 2001). A hyperalgesic, systemic injection of bacterial lipopolysaccharide (Abe et al., 2001) raised the levels of PGE_2 in the preoptic area of the rat hypothalamus and PGE_2 concentrations in mouse brains fell after systemic administration of cyclooxygenase inhibitors (Ferrari et al., 1990).

Paracetamol is a widely used analgesic and antipyretic with weak anti-inflammatory effects (Clissold, 1986). Although its mode of pharmacological action has not been fully elucidated, paracetamol is generally believed to act centrally rather than by a peripheral action (Clissold, 1986). The initial report of Flower and Vane (1972), describing an inhibitory effect of paracetamol on COX activity in the CNS is now supported by a number of other studies (Malmberg and Yaksh, 1994; Muth-Selbach et al., 1999; Ferrari et al., 1990). For example, in mice, antinociceptive doses of paracetamol attenuated the ex vivo synthesis of brain PGE_2 in a dose-related manner (Ferrari et al., 1990) and in human volunteers, paracetamol raised the threshold to painful electrical stimulation of the sural nerve (Piletta et al., 1991). In addition, in the formalin test in the rat, antinociceptive doses of paracetamol reduced flinching behaviour and release of PGE_2 from the spinal cord (Muth-Selbach et al., 1999). In spite of its potent inhibition of the biosynthesis of prostaglandins in the CNS in vivo (Muth-Selbach et al., 1999; Ferrari et al., 1990; Flower and Vane, 1972; Grèen et al., 1989), paracetamol was shown to be a weak inhibitor of both COX-1 and COX-2 in vitro (Warner et al., 1999). It has been suggested that the recently discovered COX-1 variant, namely COX-3, may be the target for the antinociceptive action of paracetamol, which inhibits the activity of COX-3 at therapeutic concentrations in vitro (Chandrasekharan et al., 2002).

2. Materials and methods

2.1. Animals

Female C57Bl/6 mice (Harlan-Olac Ltd, UK) were maintained under 12 h/12 h light/dark cycle at 22±1 °C. Food and water were provided ad libitum. The animals were acclimatized to the experimental room prior to testing. Experimental procedures were conducted in accordance with the United Kingdom Home Office Guidelines. The C57Bl/6 strain of mice is moderately sensitive to acetic acid-induced writhing and has a greater sensitivity to paracetamol than to most other NSAIDs (Lariviere et al., 2001).

COX-1 and COX-2 knockout mice were kindly donated by Professor I. Bjarnason of King's College, London and were of the same strain as reported by Morham et al. (1995) and Langenbach et al. (1995). Groups composed of both male and female knockout animals were used, after a pilot study had established that there was no sex difference in their responses to acetic acid.

2.2. The writhing test and collection of tissues

Writhing responses were elicited in female C57Bl/6 mice (21.3±0.25 g) and male and female COX-1 or COX-2 knockout mice by the i.p. administration of either acetic acid (0.1 ml/10 g body weight of 0.6% (*v*/*v*) acetic acid in physiological saline) or iloprost (0.25 μg/10 g body weight in 0.1 ml saline). The degree of nociception was assessed by counting the number of writhing responses (abdominal constrictions accompanied by stretching of the hind limbs) during a 20 min period in freely moving mice (Collier et al., 1968). For assessment of antinociceptive activity, animals were pre-treated by s.c. injection, with either paracetamol (100–400 mg/kg), diclofenac sodium (10–100 mg/kg), aminopyrine (50 mg/kg) or antipyrine (100 mg/kg). Paracetamol and diclofenac sodium were administered 30 min prior to acetic acid or iloprost since a preliminary study established that this was the correct time interval for the optimum effect of paracetamol. The pre-treatment time for aminopyrine and antipyrine was 1 h, as used in previous publications (Hachisu et al., 1985; Ishikawa et al., 1989).

At the end of the 20 min observation period, the animals receiving acetic acid were sacrificed and the peritoneal cavity washed out with 1 ml of physiological saline. Whole brain and spinal cord tissues were removed from animals responding both to acetic acid and iloprost, and immediately snap-frozen in liquid nitrogen.

2.3. Extraction of prostaglandins from brain and spinal cord tissues

Prostaglandin extraction was performed as described by Powell (1980) with some modifications. Briefly, frozen brain or spinal cord tissues were pulverised with a nitrogen bomb (Biospec Products, Bartlesville, OK) and 1 ml of 15% (*v*/*v*) ethanol (pH 3) was added to each tissue sample. The tissue homogenates were kept at 4 °C for 10 min and then spun at 375 g for 10 min also at 4 °C. C-18 Sep-Pak columns (Waters Inc, USA) were conditioned with 4 ml ethanol followed by 4 ml distilled water at a flow rate of 5–10 ml/min. The supernatant from homogenates was then applied to the columns at a flow rate of 5 ml/min and washed with 4 ml distilled water followed by 4 ml of 15% (*v*/*v*) ethanol. The samples were then eluted with

2 ml of ethyl acetate at a flow rate of 5 ml/min. The samples were freeze-dried overnight and stored at −80 °C ready for prostaglandin estimations.

2.4. PGE_2 and 6-ketoPGF$_{1\alpha}$ enzyme immunoassays

Commercial enzyme immunoassay (EIA) kits were used for the measurement of prostaglandins. PGE_2 and 6-keto-prostaglandin $F_{1\alpha}$ (6-ketoPGF$_{1\alpha}$, the stable breakdown product of prostaglandin I_2) were measured in the peritoneal washings from animals responding to acetic acid. PGE_2 was measured in the spinal cord and brain tissues, including whole brain, cerebral cortex, mid-brain, brain stem and cerebellum, from animals responding to acetic acid and iloprost. Assays were performed according to the manufacturers' instructions. The samples were reconstituted in assay buffer. The limits of detection for PGE_2 and 6-ketoPGF$_{1\alpha}$, were 0.05 and 0.01 ng/ml respectively.

2.5. Western blotting

Pulverised dissected brain tissues were reconstituted in a protease inhibitory cocktail solution containing 4-(2-aminoethyl)benzenesulphonylflouride (AEBSF, 104 mM), aprotinin (0.08 mM), leupeptin (2.1 mM), bestatin (3.6 mM), pepstatin A (1.5 mM) and E-64 (1.4 mM) in 50 mM Tris buffer (pH 7.4). The cells were lysed by sonication for 20 s in an ultrasonic processor (Jencons, Buzzard). Following the determination of protein concentration using Bradford reagent (Biorad), the samples were added to ×2 Laemmli buffer followed by boiling.

Twenty micrograms of protein was separated by electrophoresis on 10% sodium dodecyl sulphate-polyacrylamide (SDS) gels at 180 V. The separated proteins were then transferred electrophoretically to nitrocellulose sheets at 100 V for 1 h.

The blots were blocked overnight at 4 °C in 5% (*w*/*v*) low fat milk and 0.2% (*w*/*v*) bovine serum albumin (BSA). Immunoblotting was performed by incubating the blots for 1 h in the presence of anti-COX-1 (0.2 μg/ml), anti-COX-2 (2 μg/ml) or anti-COX-3 (2 μg/ml) antibodies. For the detection of COX-3, the blots were incubated with goat anti-rabbit horseradish peroxidase (HRP) conjugated secondary antibody and with goat anti-mouse HRP-conjugated secondary antibody for the detection of COX-1 and COX-2. Finally, protein bands were visualised using ECL plus reagent (Amersham, UK) and developed on X-ray films. The COX-3 antibody was raised against the first 12 amino acids on the N-terminus which encode for intron-1 in mouse cells. Preliminary control experiments showed that this COX-3 antibody does not cross-react with COX-1 or COX-2 and that the bands seen for COX-3 are not due to secondary antibody staining.

2.6. Drugs

Drugs and reagents used were of the highest purity. Paracetamol (acetaminophen; 4-acetamidophenol), diclofenac sodium (2-[(2,6-dichlorophenyl)amino]benzeneacetate sodium), aminopyrine (4-(dimethylamino)-1,2-dihydro-1,5-dimethyl-2-phenyl-3H-pyrazol-3-one; amidopyrine) and antipyrine (1,2-dihydro-1,5-dimethyl-2-phenyl-3H-pyrazol-3-one; phenazone) were obtained from Sigma (Poole, UK). Paracetamol was dissolved in 12.5% (*v*/*v*) 1,2-propanediol. Diclofenac sodium, aminopyrine and antipyrine were dissolved in physiological saline. Glacial acetic acid was obtained from VWR (Poole, U.K). Iloprost was kindly donated by Schering Aktiengesellschaft (Berlin, Germany). PGE_2 and 6-ketoPGF$_{1\alpha}$ EIA kits were purchased from Amersham (Buckinghamshire, U.K). The following were also used: monoclonal anti-COX-1 antibody (Santa Cruz, CA, U.S.A., sc-19998), monoclonal anti-COX-2 antibody (Transduction Laboratories, KY, U.S.A., C22420/L2), polyclonal anti-COX-3 antibody (Alpha Diagnostics, TX, U.S.A., COX31-A), goat anti-mouse IgG HRP (Santa Cruz, CA, U.S.A.), goat anti-rabbit IgG HRP (Santa Cruz, CA, U.S.A.).

2.7. Statistical analysis of results

The results were analysed by analysis of variance (ANOVA) followed by post hoc Dunnett's test to compare vehicle with drug treated groups and expressed as mean±S.E.M. $P<0.05$ was considered to be significant. ID_{50} values and 95% confidence intervals (CI) were estimated using Prism 4, GraphPad Software Inc., San Diego, CA, U.S.A.

3. Results

3.1. Comparison of the effect of paracetamol and diclofenac on nociception and on peripheral and central prostaglandin biosynthesis

Paracetamol (100–400 mg/kg) and diclofenac (10–100 mg/kg) dose-dependently reduced acetic acid-induced writhing (Fig. 1A and B). The ID_{50} values calculated for paracetamol and diclofenac were 172.0 (CI: 158.0–188.0) mg/kg and 16.0 (CI: 8.0–32.0) mg/kg, respectively. Paracetamol (100–250 mg/kg) reduced iloprost-induced writhing (Fig. 2A), but there was no significant reduction of iloprost-induced writhing after diclofenac up to a dose of 100 mg/kg (Fig. 2B). The ID_{50} value calculated for paracetamol in the iloprost-induced writhing model was 149.0 (CI: 79.9–277.9) mg/kg.

Measurement of the levels of prostaglandins in the peritoneal cavity, released in response to acetic acid, was carried out in order to determine whether paracetamol and diclofenac had a peripheral site of antinociceptive action in this model. Estimations of the levels of prostaglandin I_2 (PGI_2; measured as its stable breakdown product, 6-keto-PGF$_{1\alpha}$) and PGE_2, showed that paracetamol had no effect on peripheral prostaglandin production (Fig. 3A and C). On the other hand, diclofenac reduced the levels of 6-keto-PGF$_{1\alpha}$ and PGE_2 released into the peritoneal cavity by acetic acid in a dose-dependent manner (Fig. 3B and D).

Administration of paracetamol in the acetic acid-induced writhing test resulted in the reduction of the central levels of PGE_2. Thus, the concentrations of PGE_2 in the spinal cord and whole brain tissues of mice writhing to acetic acid were reduced (Fig. 4A and C). However, although diclofenac failed to

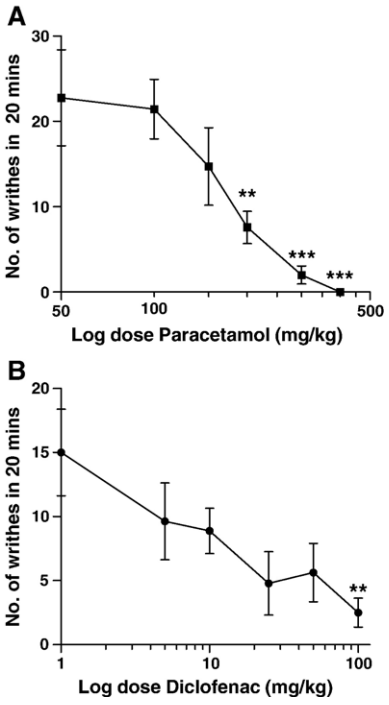

Fig. 1. The effect of paracetamol and diclofenac on acetic acid-induced writhing in C57Bl/6 mice. The number of writhing responses produced in 20 min after i.p. injection of 0.6% acetic acid was reduced dose-dependently by paracetamol (100–400 mg/kg; panel A) or diclofenac sodium (10–100 mg/kg; panel B) injected s.c. 30 min before. Each point represents the mean+S.E.M. **=$P<0.01$; ***=$P<0.001$ compared to vehicle. $N=6-10$ animals per group.

produce a significant reduction in the levels of PGE_2 in spinal cord tissues (Fig. 4B), at the highest dose it produced a significant reduction of PGE_2 concentration in the whole brain (Fig. 4D).

All three enzyme proteins, COX-1, COX-2 and COX-3, were shown by Western blotting to be expressed constitutively in brain tissues dissected into the cerebral cortex, midbrain, brainstem and cerebellum (Fig. 5). The effect of paracetamol (200 mg/kg) on the levels of PGE_2 in the same brain regions was also examined during acetic acid-induced writhing. Administration of paracetamol reduced the concentration of PGE_2 in all brain regions (Fig. 6). Thus, the inhibition of the biosynthesis of PGE_2 by paracetamol correlated with the expression of COX-3 enzyme protein.

3.2. The effect of 200 mg/kg paracetamol on the writhing response and on PGE_2 levels in the CNS in COX-1 and COX-2 knockout mice

Paracetamol (200 mg/kg) reduced the writhing responses in wild type littermate controls, and *COX-1* heterozygous knockout mice, but not in *COX-1* homozygous knockout mice (Fig. 7A). The reduction in writhing responses with paracetamol in wild type and *COX-1* heterozygous knockout animals was approximately 50%, whereas in *COX-1* homozygous gene-deleted animals only a 20% reduction was achieved which was not significantly different from control. In *COX-2* gene-ablated mice, 200 mg/kg paracetamol produced a reduction in writhing responses in both heterozygous and homozygous knockout animals as well as in wild type littermate controls (Fig. 8A). COX-2 homozygous knockout animals demonstrated hypersensitivity to acetic acid with an exaggerated number of writhing responses.

The spinal cord tissues of *COX-1* heterozygous and *COX-1* homozygous gene-deleted mice were shown to have approximately half the concentrations of PGE_2 of the wild type littermates (Fig. 7B). Also, in the spinal cords of *COX-1* heterozygous and *COX-1* homozygous knockout animals, the reduction of PGE_2 levels shown for wild type littermates with 200 mg/kg paracetamol was abolished (Fig. 7B). On the other hand, the effect of 200 mg/kg paracetamol on PGE_2 levels in spinal cords of *COX-2* knockout mice was not altered, as significant reduction was seen both in the *COX-2* heterozygous and *COX-2* homozygous knockout groups (Fig. 8B).

Paracetamol (200 mg/kg) produced a significant reduction in PGE_2 concentrations in the whole brains of wild type mice and in *COX-1* heterozygous knockout animals (Fig. 7C). In whole brain tissues of *COX-1* homozygous knockout mice writhing to acetic acid, however, the effect of paracetamol in reducing the levels of PGE_2 was abolished (Fig. 7C). The level of PGE_2 in the vehicle-treated *COX-1* homozygous gene-deleted animals was approximately 20% of that of the wild-type group, which

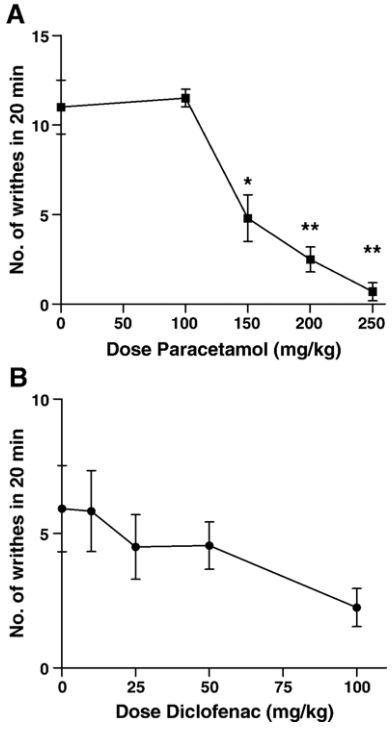

Fig. 2. The effect of paracetamol and diclofenac on iloprost-induced writhing in C57Bl/6 mice. The number of writhing responses produced in 20 min after i.p. injection of 0.25 μg iloprost/10 g body weight in 0.1 ml saline was reduced by paracetamol (100–250 mg/kg; panel A), but not by diclofenac sodium (10–100 mg/kg; panel B) injected s.c. 30 min before. Each point represents the mean +S.E.M. *=$P<0.05$; **=$P<0.01$ compared to vehicle. $N=6–10$ animals per group.

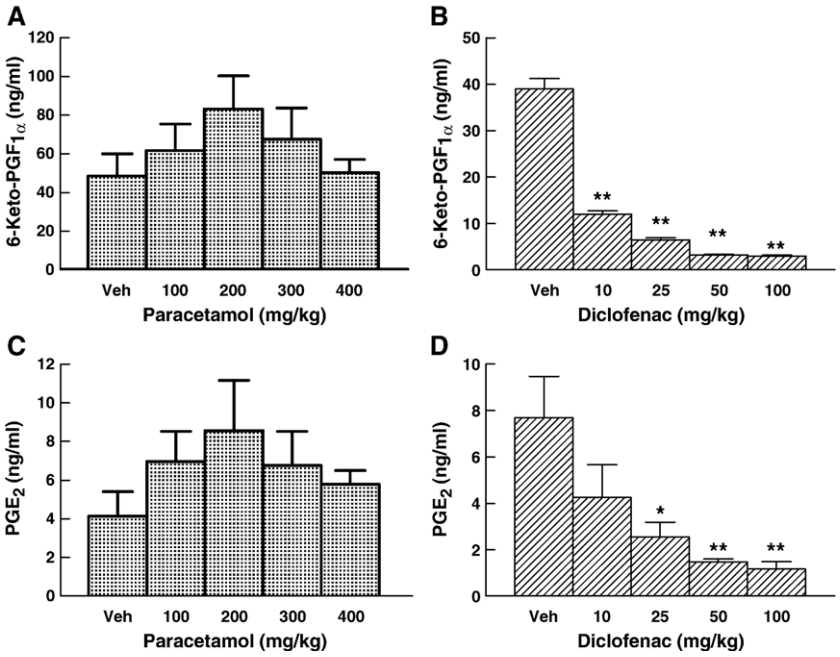

Fig. 3. Prostaglandin concentrations in peritoneal washings of mice injected with paracetamol or diclofenac. Concentrations of prostaglandin I_2 (measured as the stable metabolite, 6-keto-$PGF_{1\alpha}$; panels A and B) and of prostaglandin E_2 (PGE_2; panels C and D) in peritoneal washings were not altered in mice injected s.c. with increasing doses of paracetamol (panels A and C). Concentrations of prostaglandin I_2 and prostaglandin E_2 in peritoneal washings were reduced dose-dependently in mice injected s.c. with diclofenac sodium (panels B and D). Data represent mean+S.E.M. Veh=vehicle. *=$P<0.05$; **=$P<0.01$ compared to vehicle. N=6–10 animals per group.

may indicate that most of the PGE_2 in the CNS is synthesised by *COX-1* gene-derived proteins. Similarly to the effect seen in spinal cord tissues, 200 mg/kg paracetamol significantly reduced the levels of PGE_2 in brain tissues from *COX-2* heterozygous and *COX-2* homozygous knockout mice as well as those of their wild type littermates (Fig. 8C).

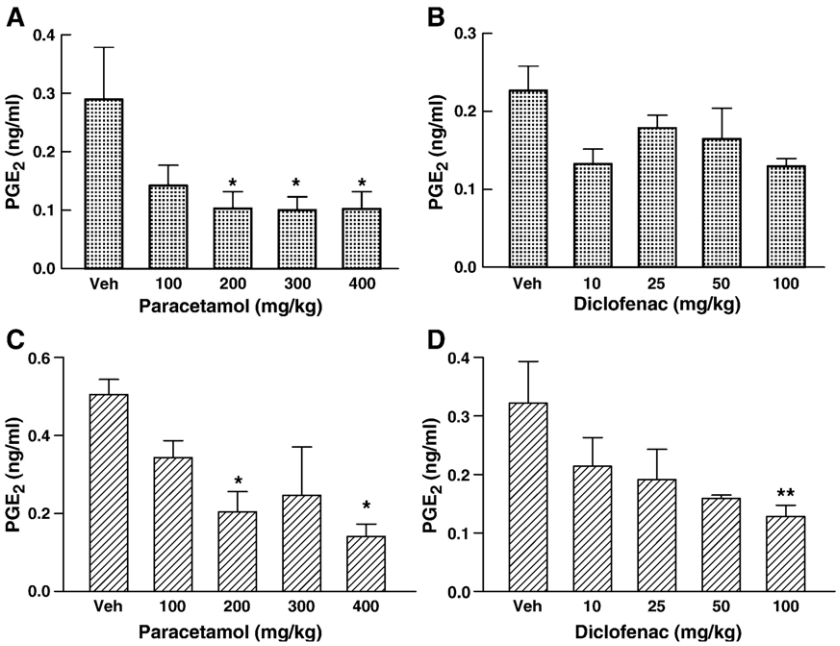

Fig. 4. Prostaglandin E_2 concentrations in spinal cord and brain of mice injected with paracetamol or diclofenac sodium. Concentrations of prostaglandin E_2 (PGE_2) in extracts of spinal cord (panels A and B) and brain (panels C and D) were reduced in mice injected s.c. with increasing doses of paracetamol (panels A and C). Concentrations of PGE_2 in extracts of spinal cord were not reduced by diclofenac sodium (panel B). However, concentrations of PGE_2 in extracts of brain were reduced by the highest dose of diclofenac sodium (panel D). Data represent mean+S.E.M. Veh=vehicle. *=$P<0.05$; **=$P<0.01$ compared to vehicle. N=6–10 animals per group.

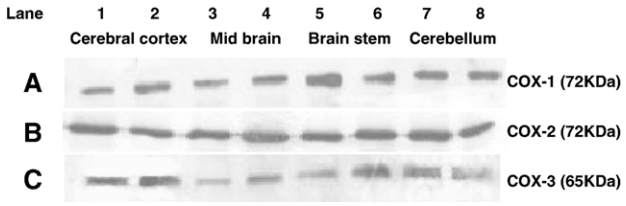

Fig. 5. Demonstration of COX isoenzyme proteins in different regions of mouse brains. Western blots of cyclooxygenase-1 (COX-1; A), cyclooxygenase-2 (COX-2; B) and cyclooxygenase-3 (COX-3; C) from different brain regions of C57Bl/6 mice. Lanes 1 and 2=cerebral cortex; lanes 3 and 4=midbrain; lanes 5 and 6=brain stem; lanes 7 and 8=cerebellum.

3.3. The effect of selective COX-3 inhibitors on the iloprost-induced writhing response and on brain PGE_2 concentrations

Both aminopyrine and antipyrine have been shown to inhibit COX-3 selectively (Chandrasekharan et al., 2002). The IC_{50} values for the inhibition of COX-3 in vitro by aminopyrine and antipyrine were 688 and 863 μM, respectively, while no inhibition of COX-1 or COX-2 was demonstrated with doses of <1000 μM. Thus, based on these results, both drugs were considered to be selective COX-3 inhibitors.

The antinociceptive effect of 50 mg/kg aminopyrine and 100 mg/kg antipyrine was tested on centrally mediated writhing induced by the i.p. administration of iloprost. Both drugs produced a statistically significant reduction in the writhing response ($P<0.01$ and $P<0.05$, respectively; Fig. 9). The reduction of iloprost-induced writhing was accompanied by a reduction in the biosynthesis of brain PGE_2 by both aminopyrine and antipyrine ($P<0.05$; Fig. 9).

4. Discussion

In this study, the mechanism of the antinociceptive action of paracetamol was investigated using the mouse writhing model of acute nociception (Collier et al., 1968). This model is sensitive to the antinociceptive action of non-steroid antiinflammatoty drugs (NSAIDs) including paracetamol, which reduce writhing responses to i.p. injection of acetic acid (Collier et al., 1968) or of iloprost (Akarsu et al., 1989). It has also been used to study the different components of pain pathways (Gyires and Knoll, 1975). The role of prostaglandins in mediating nociception in this model has been clearly demonstrated in peripheral tissues. Murata and colleagues (1997) showed that prostaglandin I_2 (PGI_2) and to a lesser extent, PGE_2 were released from peritoneal cells to activate peripheral nociceptors. Mainly PGI_2, but also some PGE_2 were released by irritant substances injected i.p. (Doherty et al., 1987; Berkenkopf and Weichman, 1988) which stimulated IP receptors (Murata et al., 1997) on nociceptive sensory nerve fibres.

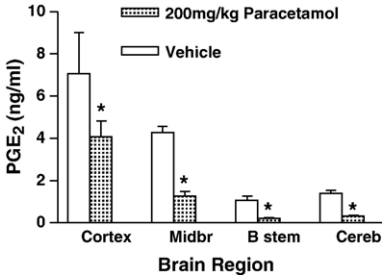

Fig. 6. Concentrations of prostaglandin E_2 (PGE_2) in different brain regions of C57Bl/6 mice. Subcutaneous injection of 200 mg/kg paracetamol caused a reduction in PGE_2 concentration 50 min later in all regions of the brains studied. Cortex=cerebral cortex; midr=midbrain; bstem=brain stem; cereb=cerebellum. *=$P<0.05$. N=5–8 animals per group.

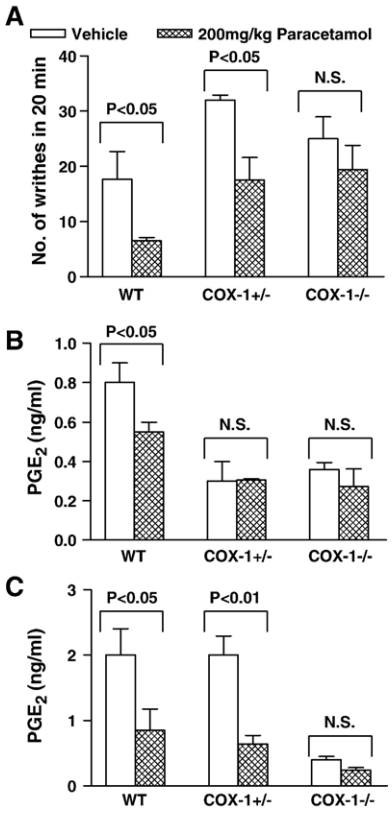

Fig. 7. The effect of 200 mg/kg paracetamol on writhing responses and prostaglandin E_2 concentrations in spinal cords and brains of COX-1 knockout mice. The number of writhing responses (panel A), prostaglandin E_2 (PGE_2) concentrations in extracts of spinal cord (panel B) and PGE_2 concentrations in extracts of whole brain (panel C) were measured in wild type mice (WT), COX-1 heterozygous knockout mice ($COX\text{-}1^{+/-}$) and COX-1 homozygous knockout mice ($COX\text{-}1^{-/-}$) injected s.c. with 200 mg/kg paracetamol. Data represent mean+S.E.M. Significance levels of differences between control and test measurements are indicated in the figures. N=5–6 animals per group.

In the present study, both paracetamol and diclofenac, dose-dependently reduced the nociceptive responses to acetic acid, but only paracetamol reduced the nociceptive response to the stable PGI_2 analogue, iloprost (Akarsu et al., 1989). Iloprost stimulates IP receptors directly so its action is not reduced by inhibition of COX in peripheral tissues. Inhibition of iloprost-induced writhing thus indicates that paracetamol has a central antinociceptive action. After injection of acetic acid, PGI_2 and PGE_2 levels in the peritoneal fluid were not reduced by paracetamol, but were dose-dependently reduced by diclofenac (Fig. 3), indicating a peripheral site of action for diclofenac and a central action for paracetamol. PGE_2 levels in the spinal cords and brains of mice responding to an i.p. injection of acetic acid

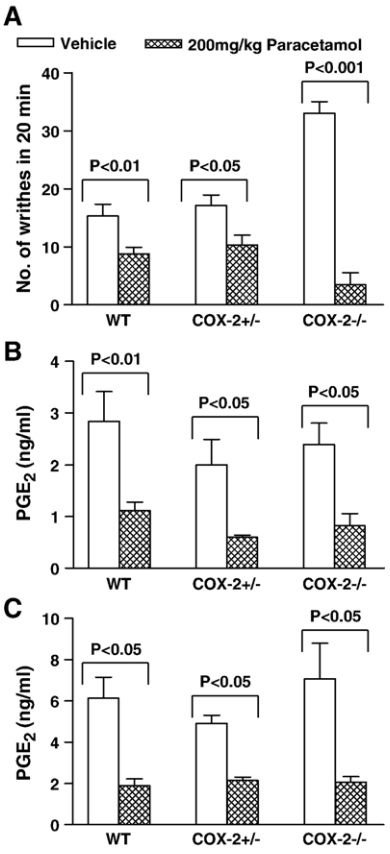

Fig. 8. The effect of 200 mg/kg paracetamol on writhing responses and prostaglandin E_2 concentrations in spinal cords and brains of COX-2 knockout mice. The number of writhing responses (panel A), prostaglandin E_2 (PGE_2) concentrations in extracts of spinal cord (panel B) and PGE_2 concentrations in extracts of whole brain (panel C) were measured in wild type mice (WT), COX-2 heterozygous knockout mice ($COX\text{-}2^{+/-}$) and COX-2 homozygous knockout mice ($COX\text{-}2^{-/-}$) injected s.c. with 200 mg/kg paracetamol. Data represent mean+S.E.M. Significance levels of differences between control and test measurements are indicated in the figures. $N=5$–6 animals per group.

were reduced after injection of paracetamol (Fig. 4A and C) but only a reduction in the brain was seen at the highest dose of diclofenac (Fig. 4D). Whole brain PGE_2 levels were also reduced when paracetamol was given to mice responding to iloprost (unpublished data). Thus, the antinociceptive action of paracetamol is a central effect and that of diclofenac is largely peripheral.

The most likely target for the antinociceptive action of paracetamol is the recently discovered variant of COX-1, named COX-3, which is present mainly in the CNS and heart and, unlike COX-1 and COX-2 (Warner et al., 1999), is potently inhibited by paracetamol in vitro (Chandrasekharan et al., 2002). COX-3 protein was expressed in all brain regions where paracetamol reduced PGE_2 biosynthesis. In addition, the analgesic antipyretic drugs, aminopyrine and antipyrine, which are also weak inhibitors of COX-1 and COX-2, but potently inhibit canine COX-3 (Chandrasekharan et al., 2002), reduced iloprost-induced writhing and central PGE_2 biosynthesis at pharmacologically active doses (Ishikawa et al., 1989; Hachisu et al., 1985). These drugs have been shown to easily penetrate the blood–brain barrier (van Bree et al., 1989).

The antinociceptive effect of paracetamol in the writhing model was dependent on the inhibition of a *COX-1* gene-derived protein. Inhibition of the writhing response and reduction of central PGE_2 concentrations was significantly less in *COX-1* homozygous gene-deleted than in wild type mice, whereas *COX-2* homozygous knockout animals behaved similarly to their wild type controls. This is consistent with the inhibition by paracetamol of the COX-1 variant protein, COX-3 (Chandrasekharan et al., 2002). *COX-2* homozygous knockout mice exhibited increased responsiveness to acetic acid writhing (see Fig. 8A). In these mice, COX-3 mRNA (Ayoub et al., 2004) and COX-3 protein (unpublished observation) are over-expressed.

As already mentioned, fully functional COX-3 protein was originally identified in canine tissues. The mRNA for COX-3 in human, mouse and rat cells is out of reading frame, hence translation into a fully functional protein should not be possible (Chandrasekharan et al., 2002; Dinchuk et al., 2003). Despite this, using Western blotting, COX-3 protein was detected in human (Chandrasekharan et al., 2002), mouse (Fig. 5) and rat tissues (unpublished observations). COX-3 protein was also detected with immunocytochemistry in mouse tissues (Dou et al., 2004; unpublished observations) and in rat tissues where incubation of the primary anti-COX-3 antibody with a blocking peptide produced a marked reduction in the immunocytochemical staining (unpublished observations). Thus, it is possible that human, mouse and rat cells are able to overcome the shift in reading frame of COX-3 mRNA by a mechanism that still needs to be defined.

Ballou et al. (2000) showed that *COX-1* homozygous knockout mice had a reduced writhing response to acetic acid compared to wild-type mice, whereas in the present study both COX-1 and COX-2 homozygous knockout animals exhibited writhing responses equal to their wild-type littermate controls. This discrepancy may be due to the different genetic backgrounds used to develop these knockout animals. Ballou et al. (2000) backcrossed C57Bl/6 mice with DBA1 mice for six generations, whereas the *COX-1* knockout mice used in the present experiments were developed by backcrossing C57Bl/6 mice only (Morham et al., 1995). Analysis of mouse strain

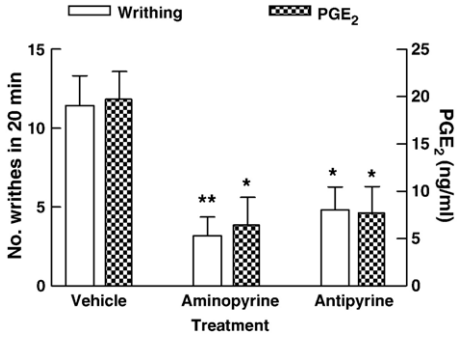

Fig. 9. The effect of aminopyrine and antipyrine on writhing responses to iloprost and on brain prostaglandin E_2 concentrations. The number of writhing responses induced by iloprost and prostaglandin E_2 (PGE_2) concentrations in extracts of whole brain of C57Bl/6 mice injected s.c. with 50 mg/kg aminopyrine or 100 mg/kg antipyrine were reduced. Data represent mean+S.E.M. *=$P<0.05$; **=$P<0.01$. $N=5$–6 animals per group.

sensitivity to acetic acid writhing has shown that C57Bl/6 is one of the most responsive strains (Lariviere et al., 2001). Thus, it is possible that the accumulation of other genes from the C57Bl/6 strain renders our *COX-1* knockout mice more responsive than those of Ballou et al. (2000) despite the loss of COX-1 protein. However, this does not exclude the involvement of protein products from the *COX-1* gene in mediating the writhing response in wild-type mice. The *COX-1* knockout mice in the present study responded to acetic acid either by producing prostaglandins from COX-2 or by utilising non-prostaglandin mediator pathways. COX-2 is an unlikely source of prostaglandins for the induction of writhing as selective COX-2 inhibitors do not reduce the writhing response in wild type mice (Jett et al., 1999) or in COX-1 knockout mice (unpublished observations). Moreover, prostaglandins were not detected in the peritoneal fluid of *COX-1* homozygous gene-deleted mice after i.p. injection of acetic acid (unpublished observations).

Our results demonstrate, like those of others (Dirig et al., 1997; Tegeder et al., 2001) that *COX-1* gene products in the CNS play a greater role than those of COX-2 in mediating some types of acute pain. Thus, COX-1 and its splice variant, COX-3, synthesise PGE_2 in acute nociception, whereas COX-2 is involved in inflammatory hyperalgesia (Beiche et al., 1996; Samad et al., 2001). Inhibition of COX activity in the CNS provides an additional site of action for some NSAIDs (Yaksh et al., 2001), in addition to their anti-inflammatory effects in peripheral tissues (Tomlinson et al., 1994). That a centrally-expressed COX-1 variant is involved in mediating pain sensation, provides a pharmacological target for the analgesic effect of paracetamol.

Our results show that the antinociceptive effect of paracetamol in mice is mediated by a central but not a peripheral action which depends on the presence of a functional *COX-1* gene. This central antinociceptive effect of paracetamol is mimicked by other selective COX-3 inhibitors. We propose that paracetamol-induced antinociception is mediated by inhibition of COX-3 in the CNS of the mouse and may constitute the mechanism of action of paracetamol in humans.

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