JOURNAL OF PHYSIOLOGY AND PHARMACOLOGY 2000, 51, 4, 609–618

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PARACETAMOL-INHIBITABLE COX-2

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Although paracetamol potently reduces pain and fever, its mechanism of action has so far not been satisfactorily explained. It inhibits both COX-1 and COX-2 weakly *in vitro*, but reduces prostaglandin synthesis markedly *in vivo*. In mouse macrophage J774.2 cells, COX-2 induced for 48 hr with high concentrations of NSAIDs is more sensitive to inhibition with paracetamol than endotoxin-induced COX-2. In the rat pleurisy model of inflammation, a second peak of COX-2 protein appears 48 hr after administration of the inflammatory stimulus, during the resolution phase of the inflammatory process. Inhibition of the activity of this late-appearing COX-2 with indomethacin or a selective COX-2 inhibitor, delays resolution and the inflammation is prolonged. Cultured lung fibroblasts also express COX-2 activity after stimulation with IL-1β which is highly sensitive to inhibition with paracetamol. Thus, evidence is accumulating for the existence of a COX-2 variant or a new COX enzyme which can be inhibited with paracetamol.

K e y w o r d s: *paracetamol, COX-1, COX-2, prostaglandins, NSAID-induced variant enzyme.*

INTRODUCTION

Paracetamol was developed as a drug by von Mehring in 1893 (1), but did not become widely used until 1949, when it was identified as the less toxic metabolite of the highly toxic antipyretic drug, phenacetin (2, 3). It is now the most popular over-the-counter analgesic, even outstripping the sales of aspirin. The gastric side effects of paracetamol are less severe than those of other non-steroid anti-inflammatory drugs (NSAIDs) and liver damage is normally seen only with daily doses greater than 10 g, whereas the recommended therapeutic dose for adults is 4 g (4).

In spite of its wide use, the mechanism of action of paracetamol has not been satisfactorily explained. It is only a weak inhibitor of prostaglandin (PG) synthesis *in vitro* and appears to have very little anti-inflammatory activity, although some reduction of tissue swelling following dental surgery has been reported (5, 6). Because of its potent analgesic and antipyretic actions, it is

generally regarded as an NSAID. However, it lacks the other typical actions of NSAIDs such as the anti-platelet activity and gastrotoxicity.

The elucidation of the structure and cloning of the gene for COX-1 (7—9) was followed by the discovery in 1991 of the gene encoding for the second COX enzyme (COX-2) which could be induced with bacterial lipopolysaccharide (LPS), cytokines or mitogens (10—12). Investigations of the relative sensitivity of the two enzymes to paracetamol found that although the drug was a weak inhibitor of both enzymes, COX-1 was marginally more sensitive to its inhibitory action than COX-2 (13). In view of the low sensitivity of both COX-1 and COX-2 to paracetamol, the existence of a new, so far unknown isoform of COX has been postulated and provisionally named COX-3 (14).

Antipyretic and analgesic actions of paracetamol

Paracetamol has been frequently recommended as the drug of choice for treating febrile children (15—18) and used as a standard for comparison in trials of new antipyretic drugs (24). Earlier reports demonstrated suppression of fever in conscious cats and rats (20—23) as well as in rabbits rendered febrile by interleukin-1 (IL-1) (24).

Recent evidence in COX-2 knockout mice strongly suggests that PGs synthesised by COX-2 mediate the febrile response (25). Matsumura *et al.* (26) have postulated that fever results from induction of COX-2 by endotoxin in endothelial cells of hypothalamic blood vessels and PGs formed by this enzyme penetrate into the organum vasculosum laminae terminalis to produce fever. Thus, paracetamol may inhibit the enzyme in endothelial cells or exert its antipyretic action centrally. However, a peripheral site for the antipyretic action has also been proposed. This postulates that endotoxin increases formation of PGs in the central nervous system by stimulating receptors on sensory fibres of the vagus nerve (27). Sectioning of the vagus below the diaphragm in rats or guinea pigs prevents endotoxin-induced fever, induction of IL-1β and increase in hypothalamic PGE_2 levels (28, 29). Perhaps this vagal sensory mechanism contributes to the genesis of fever but does not account entirely for the febrile response.

Extensive clinical trials have demonstrated that paracetamol is a safe and effective analgesic for the relief of mild to moderate pain associated with oral surgery, episiotomy, post-partum pain, cancer, osteoarthritis, dysmenorrhea and headache (30, 31). The early experiments of Lim *et al.* (32) on the cross-perfused dog spleen, indicated a peripheral rather than a central site for the analgesic action of paracetamol. It was subsequently generally accepted that the analgesic action of all NSAIDs was due to inhibition of PG formation at peripheral sites and that the only animal models which demonstrated their effect were the mouse and rat abdominal constriction tests (33, 34, 35). More recent human and animal tests (36, 37), have provided evidence for a central

analgesic action of paracetamol. Perhaps low doses of paracetamol have a peripheral analgesic action by inhibiting COX-1 and high doses act centrally by suppressing a paracetamol-sensitive COX-2.

Paracetamol lacks anti-thrombotic and ulcerogenic activity

The meta-analysis of Cryer and Feldman (38) demonstrated a significant correlation between the anti-platelet action and the ulcerogenic effect of a number of NSAIDs. These two actions appear to run in parallel and paracetamol typically neither inhibits aggregation of platelets or damages the stomach mucosa. Thus, the COX-1 enzymes in platelets and in stomach mucosa are similar in their lack of sensitivity to inhibition with paracetamol.

In a comparative study with aspirin, paracetamol did not alter template bleeding time or platelet function in healthy volunteers or in patients with haemophilia (39). Similar doses of aspirin, on the other hand, prolonged bleeding time and impaired platelet aggregation.

The absence of gastrotoxicity of paracetamol has been extensively reported both in human and animal studies. Comparison with aspirin demonstrated that paracetamol caused no gastric mucosal damage or faecal occult blood loss in normal volunteers or patients with rheumatoid arthritis (40—44). However, even 300 mg aspirin four time daily produced a mean blood loss of 4.5 ml a day compared to a normal loss of about 0.5 ml per day (45). The study of Konturek *et al.* (46) compared the inhibition of PGE_2 generation by human gastric mucosa with 2.5 g aspirin or paracetamol. While aspirin reduced PGE_2 formation by more than 60%, paracetamol barely lowered the PG production. Similarly, prostacyclin synthesis by homogenates of rat stomach mucosa was stimulated by concentrations of paracetamol below 1mM (47).

Inhibition of COX-1 by paracetamol

Tissue-specific inhibition of PG production was first described by Flower and Vane (48) who found that paracetamol was approximately ten times more potent in inhibiting PG synthesis by rabbit brain than by dog or rabbit spleen. Other animal studies demonstrated stimulation of PG production or no effect on COX-1 of stomach enzyme preparations (49—51) and inhibition of PG synthesis by COX-1 enzyme in preparations of brain and kidney *in vitro* (49, 52). In humans, orally administered paracetamol reduced the synthesis of prostacyclin, measured by the urinary excretion of the inactive metabolite, 2,3-dinor-6-keto-$PGF_{1\alpha}$ in healthy volunteers (53) and in a group of pregnant women (54). Urinary excretion of the thromboxane metabolite, 2,3-dinor-TXB_2, was not affected in either case. Excretion of the major urinary metabolite of PGE_2 was reduced in volunteers receiving 3 g daily of paracetamol for two consecutive days (55).

We have tested the COX-1 enzyme in homogenates of mouse brain, spleen and stomach mucosa for its sensitivity to paracetamol and found that ten times the concentration was needed to inhibit stomach mucosa enzyme than the enzyme of brain or spleen (56). Ex vivo studies of COX-1 activity in homogenates of rabbit tissues after intravenous administration of 100 mg/kg of paracetamol showed that the activity of the enzyme in stomach mucosa was hardly reduced compared to the almost complete inhibition of COX-1 in brain and spleen (unpublished observations). An interesting observation was that COX-1 in homogenates of rabbit lungs was ten times more sensitive to inhibition with paracetamol than the enzymes of brain or spleen.

An anti-cancer action of paracetamol associated with COX-1 inhibition has been reported. Human ovarian adenocarcinomas overexpress COX-1 in contrast to most tumours which overproduce COX-2 (57). A significant inverse association was found between the use of paracetamol and ovarian cancer risk, whereas the apparent inverse association with aspirin use was not significant (58). In a prospective study, women who reported using paracetamol daily had a 45% lower death rate from ovarian cancer than women reporting no use (59). Thus, ovarian tumours are unusual in overexpressing COX-1 and in their susceptibility to inhibition by paracetamol.

Inhibition of COX-2 by paracetamol

Paracetamol in low concentrations stimulates and in high concentrations inhibits COX-2 induced with LPS in homogenates of J774.2 mouse macrophages. However, when the cofactors, glutathione and adrenaline are included in the reaction mixture, paracetamol only inhibits PG production (56). Thus, in the absence of cofactors, paracetamol itself appears to behave as a cofactor, stimulating COX-2 activity to maximum levels. This dual action of paracetamol had been demonstrated by Robak *et al.* (60) on COX-1 of ram seminal vesicle microsomes and by McDonald-Gibson and Collier (61) in homogenates of bull seminal vesicles.

We have recently reported (62) that COX-2 can be induced in cultured J774.2 mouse macrophages by incubation for 48 hrs with high concentrations of NSAIDs (*Fig. 1*). 0.5 mM diclofenac is particularly effective and induces an enzyme which is sensitive to inhibition by paracetamol in concentrations which do not inhibit LPS-induced COX-2 (*Fig. 2*). The diclofenac-induced enzyme is also inhibited by low concentrations of other NSAIDs, such as tolfenamic acid or flurbiprofen, but not by aspirin. The COX-2 upregulated with diclofenac differs from COX-2 induced with LPS, since it is not attached to a cell membrane but remains free in the cytosol. For this reason, its active site may also have different properties from the LPS-induced enzyme. It is possible that a similar variant of COX-2 is the target for the antipyretic and analgesic actions of paracetamol.

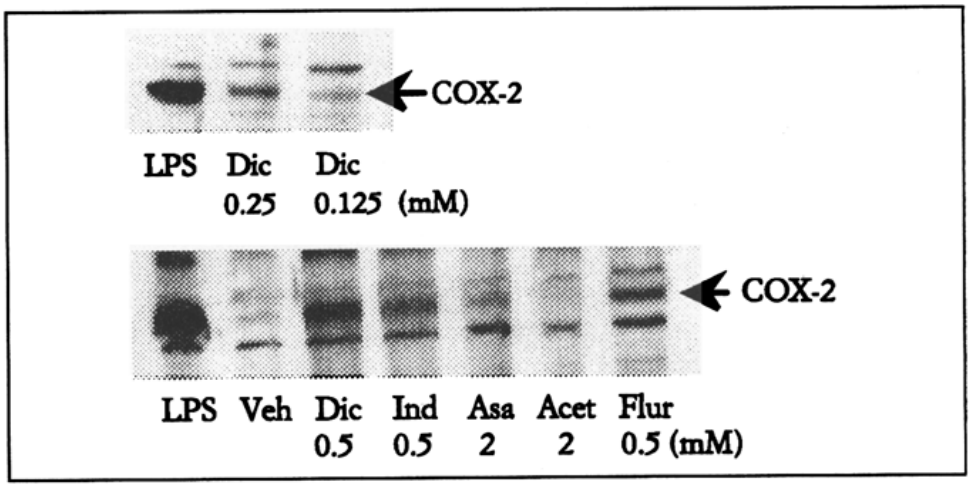

Fig. 1. Induction of COX-2 expression by NSAIDs. J774.2 cells were treated with LPS (1 μg/ml for 12 h), vehicle or NSAIDs (for 48 h) at the doses indicated. Cellular proteins were electrophoresed and probed by Western blot with isoenzyme-specific anti-COX-2 sera. COX-2 was induced 10- to 30- fold by 0.5 mM indomethacin (Ind 0.5), 0.5 mM flurbiprofen (Flur 0.5), and 0.5 mM diclofenac (Dic 0.5) but was not detectably induced by 2 mM paracetamol (Acet 2) and was only marginally induced by 2 mM aspirin (Asa 2). Induction of COX-2 by diclofenac was dose dependent (compare upper and lower panels). (Reproduced with permission from Simmons DL *et al. Proc Natl Acad Sci USA* 1999; 96: 3275—3280).

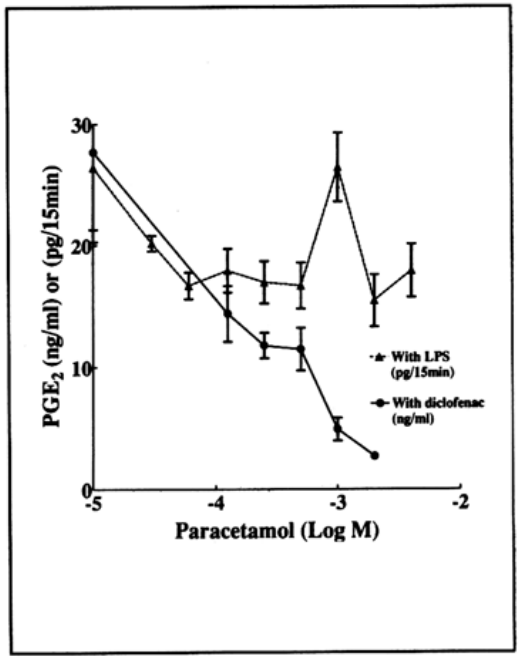

Fig. 2. Sensitivity of diclofenac-induced COX-2 but not LPS-induced COX-2 to inhibition by paracetamol. J774.2 cells were treated with 0.5 mM diclofenac for 48 h to induce COX-2 and apoptosis (circles). After this treatment, diclofenac was removed by washing and the cells were exposed to paracetamol for 30 min at the doses shown. After paracetamol treatment, cells were exposed to 30 μM arachidonic acid for 15 min and PGE_2 released was measured as ng of PGE_2/ml of media. J774.2 cells were treated with LPS (1 μg/ml for 12 h) to induce COX-2 (triangles). Cells were treated with paracetamol and PGE_2 release was measured as pg of PGE_2/15 min. (Reproduced with permission from Simmons DL *et al. Proc Natl Acad Sci USA* 1999; 96: 3275—3280).

Another putative COX-2 enzyme which requires 48 hrs to develop its peak of activity, becomes upregulated in the rat carrageenin pleurisy model of inflammation (63). In this model, COX-2 is not only induced in the initial stages of the inflammatory response but also during the resolution phase (*Fig. 3*). The first peak of COX-2 activity appears 6 hrs after the injection of carrageenin. However, 48 hrs after the start of inflammation, COX-2 protein increases for a second time during the resolution phase. Whereas the early-appearing enzyme synthesizes largely PGE_2, the products of the late enzyme are mainly PGD_2 and its metabolite, 15-deoxy$\Delta^{12-14}PGJ_2$ (15-deoxy) (*Fig. 4*). Inhibition of the synthesis of the late-appearing prostanoids with indomethacin or NS 398, a selective COX-2 inhibitor, prevents the resolution of inflammation, so that pleural exudate volume and numbers of migratory cells remain high. A possible explanation is that the cyclopentenone prostaglandin, 15-deoxy, inhibits IκB kinase and thus reduces the activity of NFκB (64, 65) which leads to resolution of the inflammation.

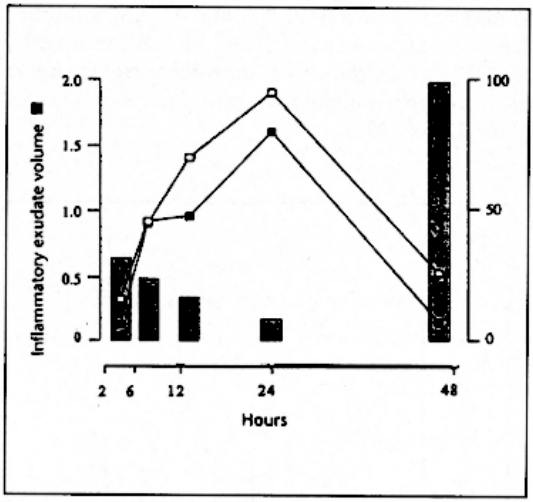

Fig. 3. Correlation between inflammation and COX-2 expression in rat carrageenin-induced pleurisy. Vertical bars indicate COX-2 protein expression measured in arbitrary units. Total inflammatory cell number, multiplied by 10^6, is shown on the right vertical axis (open squares). Inflammatory exudate volume (ml) is shown on the left vertical axis (closed squares). (Adapted with permission from Gilroy *et al. Nature Medicine* 1999; 5: 698—701).

It is not known which cells in the pleural space form this late-induced COX-2 or whether the enzyme can be inhibited by paracetamol. However, the COX-2 in cultured lung fibroblasts from COX-1 knockout mice is upregulated by IL-1β and the resulting COX-2 activity is sensitive to inhibition with paracetamol in concentrations as low as 1 μM (unpublished observation). This or a similar enzyme may have a role in the resolution of carrageenin-induced pleural inflammation.

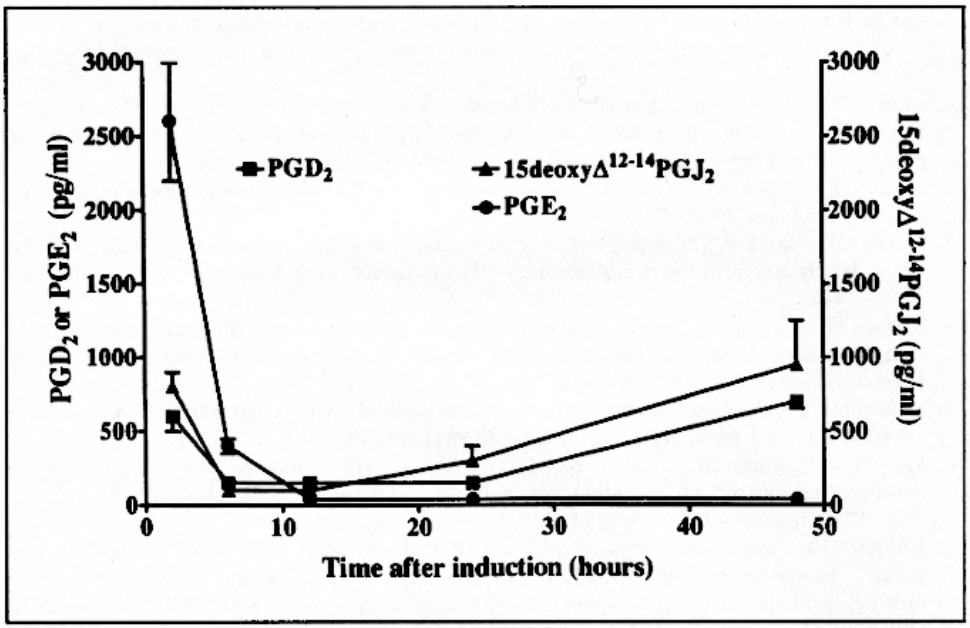

Fig. 4. Time course of changes in concentrations of prostaglandins in inflammatory exudate. PGE_2 (pg/ml exudate; closed circles), PGD_2 (pg/ml exudate; closed squares) and 15-deoxy$\Delta^{12\text{-}14}PGJ_2$ (pg/ml exudate; closed triangles). Horizontal axis shows time after start of inflammation (h). (Adapted with permission from Gilroy *et al. Nature Medicine* 1999; 5: 698—701).

CONCLUSIONS

The COX-2 enzyme induced with diclofenac for 48 hrs in J774.2 macrophages is more sensitive to inhibition with paracetamol than LPS-induced COX-2. This NSAID-induced variant enzyme may exist free in the cytoplasm, without the attachment to a cell membrane. An alternate form of COX-2 is also induced after 48 hrs in the rat pleurisy model of inflammation and may be important in the resolution of inflammation. COX-2, upregulated with IL-1β in cultured lung fibroblasts from COX-1 knockout mice, is dose-dependently inhibited with low concentrations of paracetamol. This may be the same enzyme which is involved in the resolution of inflammation.

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Received: October 3, 2000
Accepted: October 18, 2000

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