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Pharmacological manipulation of cyclo-oxygenase-2 in the inflamed hydronephrotic kidney

Karen Seibert, Jaime L. Masferrer, Philip Needleman & [1]Daniela Salvemini

Searle Inflammatory Disease Research, 800 N. Lindbergh, St. Louis, MO 63167, U.S.A.

1 Bradykinin (BK, 1 μg) caused a small (2 fold at 6 h) increase in prostaglandin E_2 (PGE_2) in the normal rabbit kidney, perfused *ex vivo*. This was exaggerated (6 fold at 6 h) in the hydronephrotic kidney (HNK). The exaggerated release of PGE_2 was attenuated by cycloheximide, an inhibitor of protein synthesis or by dexamethasone, a steroid known to inhibit the induction of cyclo-oxygenase (COX-2). BK (1 μg) when injected at 6 h of perfusion increased the release of PGE_2 from 90 ± 33 pg ml^{-1} min^{-1} to 3069 ± 946 pg ml^{-1} min^{-1}. This was reduced to 200 ± 30 pg ml^{-1} min^{-1} in kidneys infused with cycloheximide (1 μM) and to 250 ± 40 pg ml^{-1} min^{-1} in kidneys infused with dexamethasone ($n = 8$).

2 When tested on human and murine recombinant COX-1 and COX-2 enzymes, DuP-697 was at least 50 fold more selective for COX-2 than for COX-1.

3 DuP-697 reduced the exaggerated release of PGE_2 elicited by BK in the HNK (e.g., at 6 h of perfusion BK-evoked PGE_2 release decreased from 3069 ± 946 pg ml^{-1} min^{-1} to 187 ± 22 pg ml^{-1} min^{-1} after perfusion with 1 μM DUP-697, $n = 8$).

4 Cycloheximide, dexamethasone or DuP-697 at doses used to inhibit completely the exaggerated release of PGE_2 in the hydronephrotic kidney, failed to inhibit the release of PGE_2 elicited by the injection of BK (1 μg) in the normal contralateral kidney.

5 Indomethacin (1 μM), a non-selective COX-1 and COX-2 inhibitor, completely inhibited PGE_2 release in the normal contralateral as well as in the hydronephrotic kidney.

6 We suggest that renal prostaglandin production in the normal kidney is driven by the activity of constitutive COX-1 while at sites of inflammation, such as the hydronephrotic kidney, there is induction of COX-2 that can be blocked selectively by anti-inflammatory glucocorticoids or selective COX-2 inhibitors.

Keywords: Non-steroidal anti-inflammatory drugs; COX-1; COX-2; prostaglandins; renal inflammation

Introduction

Unilateral ureteral obstruction (hydronephrotic kidney, HNK) results in increased prostaglandin production that is markedly enhanced by stimulation with peptides like bradykinin (BK) and angiotensin (Morrison *et al.*, 1977; Salvemini *et al.*, 1994a). Stimulation of the isolated HNK perfused *ex vivo* reveals a marked induction of prostaglandin E_2 (PGE_2) and thromboxane A_2 (TxA_2) release into the venous effluent that is dependent on new protein synthesis (Morrison *et al.*, 1978). The contralateral (CLK) or normal kidney does not demonstrate this time-dependent increase in prostaglandin release in response to peptide stimulation nor does it respond to cycloheximide with a blockade of PGE_2 release. Cortical microsomes prepared from the HNK possess significantly higher cyclo-oxygenase (COX) activity than those of the normal kidney cortex (Smith & Bell, 1979), the result of inflammatory cell infiltration and activation (Nagle *et al.*, 1973; Okegawa *et al.*, 1983; Lefkowith *et al.*, 1984).

For many years it was thought that COX was a single enzyme; however we demonstrated that *de novo* synthesis of COX occurs in fibroblasts and mononuclear cells *in vitro* as well as *in vivo* following stimulation by inflammatory substances such as interleukin-1, phorbol myristic acid and bacterial endotoxin (Raz *et al.*, 1988; Fu *et al.*, 1990; Masferrer *et al.*, 1992a, b). Furthermore, the synthesis of inducible COX was selectively inhibited by the potent anti-inflammatory glucocorticoid, dexamethasone or by inhibitors of mRNA and protein synthesis with no effect on constitutive COX expression. These data and others suggested that two forms of COX exist, a constitutive form (COX-1) present in tissues such as gut and kidney that produces prostaglandins necessary for normal physiological function (see DeWitt, 1991 for review), and an inducible form that is expressed in inflammatory conditions (Masferrer *et al.*, 1994; Vane *et al.*, 1994). This hypothesis was supported by the isolation of a second COX cDNA (COX-2) that is induced by a number of inflammatory cytokines and which has mRNA expression that is selectively blocked by dexamethasone (Kujubu *et al.*, 1991; Xie *et al.*, 1991; Kujubu & Herschman, 1992; O'Banion *et al.*, 1992; Sirois & Richards, 1992).

Commercially available nonsteroidal anti-inflammatory drugs (NSAIDs) are believed to produce their action through the inhibition of COX activity *in vivo* to block local pro-inflammatory prostaglandin production. These inhibitors of COX inhibit both COX-1 and COX-2 *in vitro* (Meade *et al.*, 1993). Although they are potent anti-inflammatory agents, their therapeutic utility is limited by a high incidence of gastric and renal toxicity linked to the inhibition of prostaglandin production in those tissues as well (Vane, 1971; Allison *et al.*, 1992). Gans *et al.* (1990) reported that DuP-697 was as efficacious as standard NSAIDs, such as indomethacin and piroxicam, in animal models of inflammation, but unlike these drugs, did not cause any gastric or renal toxicity after chronic *in vivo* admininstration (Gans *et al.*, 1990). These data suggested that DuP-697 may selectively inhibit COX-2 at the inflammatory site while sparing constitutive COX-1 in normal tissues.

In the present study we evaluated the relative selectivity of DuP-697 in inhibiting recombinant COX-1 and COX-2 *in vitro*. In addition we have used this compound, as well as dexamethasone and cycloheximide, to examine the relative contribution of COX-1 and COX-2 to the physiological and pathophysiological production of prostaglandins in the CLK as well as in the inflamed HNK.

[1] Author for correspondence.

A preliminary account of part of this study has been presented to the British Pharmacological Society (Salvemini *et al.*, 1994a).

Methods

Ureteral obstruction

Male New Zealand white rabbits (2–3 kg) were obtained form Mohican Valley Rabbitry (Loudenvile, Ohio, U.S.A.) and housed for at least one week before the experiments. All animals were fed a normal rabbit Chow (Ralston Purina Co., St. Louis, MO, U.S.A.) and allowed free access to water. Unilateral ureteral obstruction was performed as previously described (Nishikawa *et al.*, 1977). Briefly, the animals were anaesthetized with ketamine/xylazine (35/3 mg kg^{-1}, i.m.), and a small abdominal incision was made. A silk suture was tied around one of the ureters near the bladder. Penicillin (150,000 iu) was administered (s.c.) postoperatively, and the rabbit allowed to return to dorsal recumbancy before being returned to its cage. Three days after obstruction (Nishikawa *et al.*, 1977) rabbits were anaesthetized again with ketamine/xylazine (35/3 mg kg^{-1}, i.m.), heparinized (250 u kg^{-1}, i.v.), the abdominal cavity opened and the renal arteries cannulated. Following cannulation of the renal arteries, the kidneys were removed, flushed with 30 ml of cold Kreb-Henseleit buffer and perfused at 10 ml min^{-1} with oxygenated (95% O_2/5% CO_2, pH 7.4) Krebs-Henseleit buffer maintained at 37°C,

Ex vivo *kidney perfusion*

Changes in perfusion pressure (measured with a Cobe disposable pressure transducer on a Grass model 7D polygraph) reflect changes in renal resistance. Perfusion of the kidneys at a rate of 10 ml min^{-1} results in pressures of 78 ± 1 mmHg in the CLK and 75 ± 2 mmHg in the HNK ($n=36$). Papaverine (0.1 mM) did not affect basal perfusion pressures (from 75 ± 5 to 78 ± 2 mmHg before and after papaverine respectively, $n=6$, $P<0.1$) indicating that the perfused rabbit kidney lacks intrinsic tone. Perfusion pressure was stable for the duration of the experiments (up to 6 h) thus addition of albumin or other osmotic agents to the buffer system was not required. Likewise, the drugs administered during the perfusion did not alter perfusion pressure (data not shown). No change in kidney weights was observed after perfusion for up to 6 h (from 15 ± 0.1 to 15 ± 1 g in the CLK and from 26 ± 1 to 27 ± 1 in the HNK at the start of, and after 6 h of perfusion respectively, $n=36$, $P<0.1$) suggesting the absence of oedema formation during the experimental protocol.

Drug administration

Bradykinin (BK) was used as a bolus injection of 1 μg to stimulate PGE_2 release from the normal and inflamed kidney (Morrison *et al.*, 1977; Nishikawa *et al.*, 1977). The isolated kidneys were allowed to equilibrate for 30 min prior to experimental manipulations. Kidneys were perfused with indomethacin, dexamethasone or DuP-697 at doses previously shown to exert anti-inflammatory effects and with cycloheximide at a dose shown to block protein synthesis in this model (Morrison *et al.*, 1977; Nishikawa *et al.*, 1977; Gans *et al.*, 1990; Salvemini *et al.*, 1994b). Drugs were infused for 30 min prior to the first BK injection and continued throughout the duration of the experiment. BK was injected as a bolus intra-arterially every hour from 0 to 6 h of perfusion to stimulate PGE_2 release from the kidney. The profile of PGE_2 release by BK revealed the PGE_2 release peaked at the third min after injection and then declined between the fourth and fifth min (Salvemini *et al.*, 1994b). Therefore, in all our experiments we collected the venous effluent for 3 min both before and after every BK injection and assayed for PGE_2 by radioimmunoassay as described by Feben Reigold *et al.* (1981).

COX enzyme activity

The coding regions of human or murine COX-1 or COX-2 were cloned in a BamH1 restriction site of the baculovirus transfer vector pVL1393 (Invitrogen) to generate the baculovirus transfer vectors for COX-1 and COX-2, respectively. Recombinant baculoviruses were isolated as previously described (Gierse *et al.*, 1995). Resulting insect cell pellets expressing COX-1 or COX-2 enzyme were homogenized in Tris/sucrose (50 mM/25%, pH 8.0) containing 1% CHAPS and assayed for COX activity (Gierse *et al.*, 1995). Detergent-solubilized insect cell membranes (2–20 μg total protein) were preincubated in a potassium phosphate buffer (50 mM) containing adrenaline (1 μM), phenol (1 μM) and haeme (1 μM) with the compounds for 10 min prior to the addition of arachidonic acid (10 μM) for 10 min. Reactions were stopped by the addition of 25 μM indomethacin and assayed for PGE_2 production by an ELISA method.

Materials

The composition of the Krebs-Henseleit buffer was (mM): NaCl 120, KCl 4.7, $MgSO_4.7H_2O$ 1.2, $CaCl_2.2H_2O$ 2.5, KH_2PO_4 1.2, $NaHCO_3$ 25, dextrose 10. All drugs were obtained from Sigma (St. Louis, MO, U.S.A.). DuP-697 was synthesized by Dr Len Lee, Monsanto Company, St. Louis, MO, U.S.A.

Statistical analysis

Results are expressed as mean ± s.e.mean for (n) experiments. The results were analysed by a two-way analysis of variance (ANOVA) followed by a least significance procedure to determine the nature of this response. A P value of <0.05 was taken as significant.

Results

Effects of indomethacin on the release of PGE_2 in the CLK and HNK

The unstimulated, basal release of PGE_2 from both the HNK and CLK was low at the initial collection point (CLK: 74 ± 2 pg ml^{-1} min^{-1}, $n=8$ versus HNK: 77 ± 3 pg ml^{-1} min^{-1}, $n=8$); this unstimulated release did not change significantly during the perfusion (Figure 1a and b). BK caused a small increase in PGE_2 production in the CLK which did not change with time. Although there appeared to be a decrease in the amount of PGE_2 released when values obtained after 0 and 1 h of perfusion were compared, this decrease was not significant ($n=8$, $P<0.1$) (Figure 1a and 3a). Hourly stimulation of the HNK with BK also resulted in an increase in prostaglandin release which was time-dependent, reaching a maximum 6 fold increase at 6 h (Figure 1b). The release of PGE_2 in the CLK and HNK elicited by the hourly bolus injection of BK was abolished by infusion of indomethacin (1 μM, $n=8$), a non-selective COX-1 and COX-2 inhibitor (Figure 1a and b).

Effects of dexamethasone and cycloheximide on the release of PGE_2 in the CLK and HNK

Continuous infusion of the HNK with cycloheximide resulted in a profound inhibition of PGE_2 release from the perfused kidney, with a maximal inhibition of 94 ± 2% at the 6 h collection point (Figure 1b). Cycloheximide did not inhibit PGE_2 release in the CLK over 6 h (Figure 1a). As previously described, these data reflect *de novo* COX synthesis in the HNK during perfusion with no change in COX activity in the normal CLK (Morrison *et al.*, 1978). Continuous infusion of the HNK with dexamethasone resulted in similar inhibition of BK-stimulated PGE_2 release (Figure 1b) to that observed with cy-

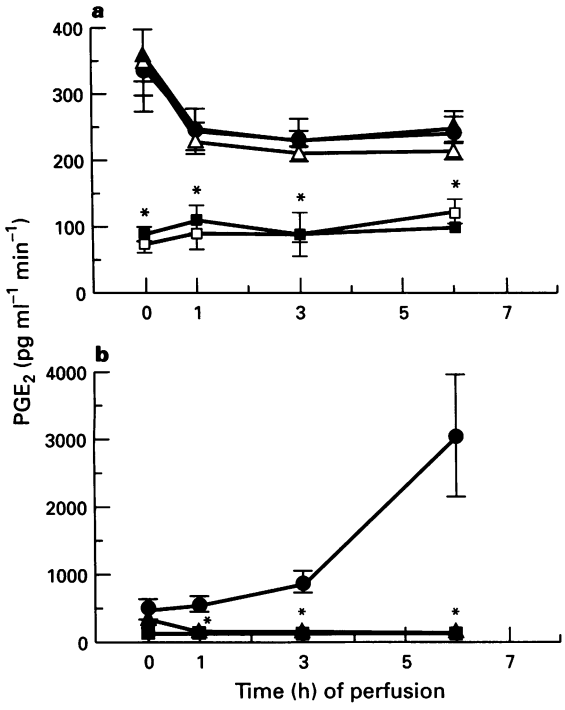

Figure 1 Effect of dexamethasone, cycloheximide, and indomethacin on the release of PGE_2 from the normal contralateral kidney (CLK, a) and hydronephrotic kidney (HNK, b) over time. Bolus injection of bradykinin (BK; 1 μg, ●) increased PGE_2 release above basal levels (□) in the CLK and HNK. This increase was significantly decreased (*$P<0.001$) by perfusion of the kidneys with indomethacin (1 μM, ■) a and b. Perfusion of the HNK (b) but not the CLK (a) with dexamethasone (1 μM, ▲) or cycloheximide (1 μM, △) significantly (*$P<0.001$) inhibited the BK-induced PGE_2 release. Each point is the mean ± s.e.mean for $n=8$ experiments.

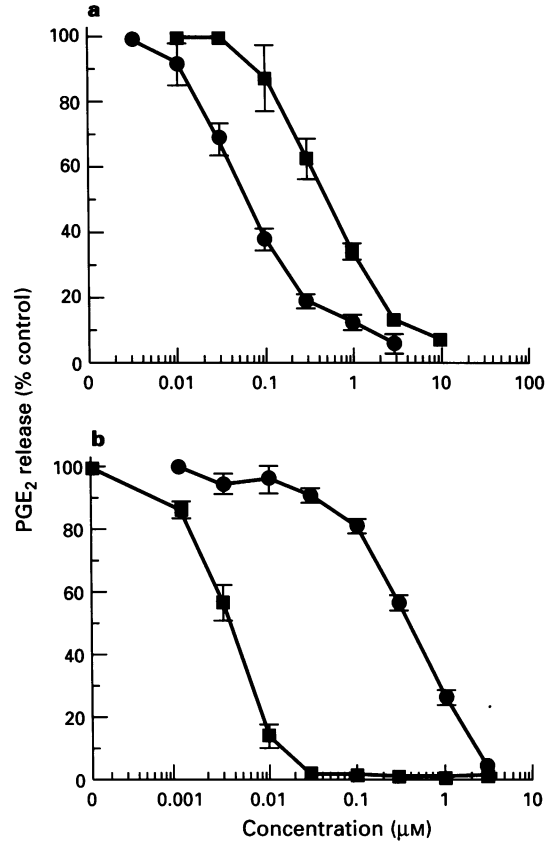

Figure 2 Effect of indomethacin (●) and DuP-697 (■) on murine recombinant COX-1 (a) and COX-2 (b) activity. Each point is the mean ± s.e.mean for $n=10$ experiments.

cloheximide with no effect on prostaglandin production in the CLK (Figure 1a).

Effect of indomethacin and DuP-697 on recombinant COX-1 and COX-2

Indomethacin inhibited both murine recombinant COX-1 (IC_{50} 0.07 ± 0.001 μM, $n=10$) and COX-2 (IC_{50} 0.3 ± 0.02 μM, $n=10$) in a dose-dependent manner (Figure 2a and b). DuP-697 also inhibited both enzymes *in vitro* with 50 fold selectivity for the COX-2 enzyme versus COX-1 (COX-1, IC_{50} 0.5 ± 0.007 μM versus COX-2 IC_{50} 0.006 ± 0.001 μM, $n=10$, $P<0.0005$) (Figure 2a and b). The selectivity of DuP-697 for COX-2 was not limited to the mouse but was also observed with human recombinant enzymes (Gierse *et al.*, 1995). The IC_{50} for DuP-697 on human COX-1 was 0.81 ± 0.003 μM and for COX-2 the IC_{50} was 0.014 ± 0.001 μM ($n=10$, $P<0.0001$). The IC_{50} for indomethacin on human COX-1 was 0.09 ± 0.005 μM and for COX-2 the IC_{50} was 1.03 ± 0.01 μM ($n=10$, $P<0.001$).

Selective inhibition of COX-2 in the perfused HNK by DuP-697

We examined the effect of DuP-697 and indomethacin on prostaglandin release in the perfused HNK compared to CLK. Continuous infusion of DuP-697 (0.1 μM, $n=8$) in the HNK inhibited the BK-stimulated release of PGE_2 in a dose-dependent manner (Figure 3b) but had no effect in the CLK (Figure 3a).

Discussion

It is now known that two isoforms of cyclo-oxygenase mediate prostaglandin production. COX-1 produces physiological le-

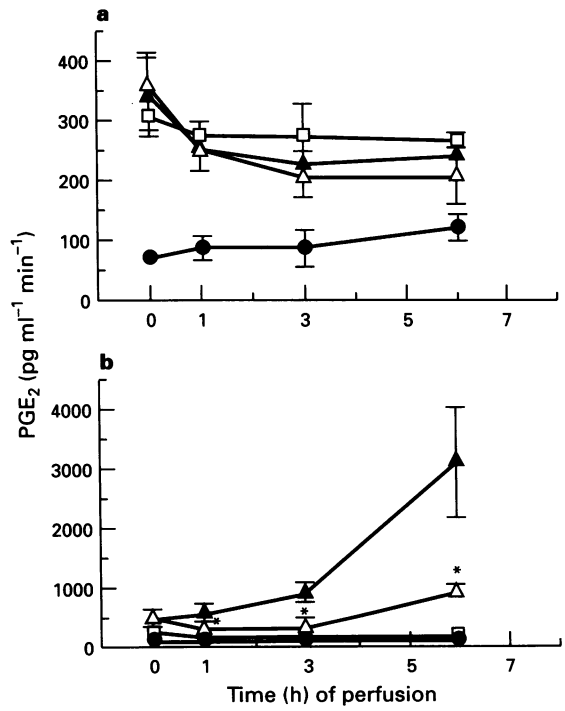

Figure 3 Effect of DuP-697 on the release of PGE_2 from the normal contralateral kidney (CLK, a) and hydronephrotic kidney (HNK, b) over time. Bolus injection of bradykinin (BK; 1 μg, ▲) increased PGE_2 release above basal levels (●) in the CLK and HNK. Perfusion of the kidneys with DuP-697 at 0.1 μM (△) or 1 μM (□) significantly (*$P<0.005$) attenuated the exaggerated release of PGE_2 elicited by BK in the HNK (b); no significant effect was seen in the CLK (a). Each point is the mean ± s.e.mean for $n=8$ experiments. Asterisks represent significant changes of data points for both drug doses when compared to release with BK alone, including the 6 h time point.

vels of prostaglandins and this enzyme is constitutively expressed in most tissues whereas COX-2 is induced under inflammatory conditions (Crofford *et al.*, 1994; Masferrer *et al.*, 1994). Glucocorticoids such as dexamethasone were the first known selective inhibitors of COX-2 (Masferrer *et al.*, 1992a, b). These potent anti-inflammatory drugs inhibit prostaglandin synthesis by blocking the expression of COX-2 while leaving the normal COX-1 expression and activity untouched (Kujubu & Herschman, 1992; Masferrer *et al.*, 1994). Recently, a group of drugs including DuP-697 have been shown to be selective nonsteroidal COX-2 inhibitors (Gans *et al.*, 1990; Masferrer *et al.*, 1994; Gierse *et al.*, 1995). This new class of NSAIDs circumvents some of the most serious side-effects of currently available NSAIDs, namely gastric lesions and renal toxicity which result from the inhibition of COX-1 activity (Masferrer *et al.*, 1994; Seibert *et al.*, 1995). In the present study we used DuP-697, as well as dexamethasone and cycloheximide, to examine the relative contribution of COX-1 and COX-2 to the physiological and pathophysiological production of prostaglandins in the normal kidney, as well as in the inflammed kidney.

We have provided pharmacological evidence which strongly indicates that synthesis of COX-2 accounts for the exaggerated release of prostaglandin in the ureter-obstructed hydronephrotic kidney while the physiological production of PGE_2 in the normal kidney derives from constitutively expressed COX-1 enzyme. Indeed, the exaggerated production of PGE_2 was blocked by cycloheximide, by dexamethasone and by DuP-697. These drugs had no effect in the normal unobstructed kidney in which prostaglandin production was mediated by COX-1 acitivity as PGE_2 release was completely inhibited by indomethacin, a non-selective COX-1 and COX-2 inhibitor.

These observations together provide evidence for a role of the inducible COX-2 in the inflamed HNK. At present we are limited to pharmacological evidence only for the role of COX-2 in the HNK. Thus, we have attempted to visualize COX-2 expression in the HNK using our human, murine and rat probes with no success; unfortunately at present molecular probes and antibodies are not available that cross react in the rabbit.

The therapeutic implications of a selective COX-2 inhibitor are clear. We recently reported that COX-2 mRNA and protein are induced *in vivo* at the inflammatory site following administration of pro-inflammatory stimuli like LPS and carrageenin while COX-1 expression dominates in normal tissues (e.g. renal medulla, stomach) (Masferrer *et al.*, 1994; Seibert *et al.*, 1994). Induction of COX-2 correlated with the production of pro-inflammatory prostaglandins at the sites of inflammation (Masferrer *et al.*, 1994; Vane *et al.*, 1994). Therefore, we postulate that physiological prostaglandin production in the normal kidney is the result of renal COX-1 activity while pro-inflammatory prostaglandins are formed as a consequence of COX-2 induction at the site of inflammation or injury (e.g. ureter obstruction). Traditional NSAIDs, such as indomethacin, derive their therapeutic benefit from inhibition of COX-2 at the site of inflammation but their clinical utility is limited due to prostaglandin-dependent inhibition of renal blood flow (King & Brenner, 1991). Selective inhibitors of COX-2 may therefore provide superior anti-inflammatory activity by inhibiting COX-2, while sparing COX-1 activity and physiological prostaglandin production necessary for renal function.

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