Pergamon

Neuropharmacology 39 (2000) 2205–2213

NEURO PHARMACOLOGY

www.elsevier.com/locate/neuropharm

Effects of caffeine and paracetamol alone or in combination with acetylsalicylic acid on prostaglandin E_2 synthesis in rat microglial cells

B.L. Fiebich [a,*], K. Lieb [a], M. Hüll [a], B. Aicher [b], J. van Ryn [b], M. Pairet [b], G. Engelhardt [b]

[a] *Department of Psychiatry, University of Freiburg Medical School, Hauptstr. 5, D-79104 Freiburg, Germany*
[b] *Departments of Pulmonary Research and Medical Sciences, Boehringer Ingelheim Pharma KG, Birkendorfer Str. 65, D-88397 Biberach, Germany*

Accepted 20 January 2000

Abstract

Paracetamol has mild analgesic and antipyretic properties and is, along with acetylsalicylic acid, one of the most popular "over the counter" analgesic agents. However, the mechanism underlying its clinical effects is unknown. Another drug whose mechanism of action is unknown is caffeine, which is often used in combination with other analgesics, augmenting their effect. We investigated the inhibitory effect of paracetamol and caffeine on lipopolysaccharide (LPS)-induced cyclooxygenase (COX)- and prostaglandin (PG)E_2-synthesis in primary rat microglial cells and compared it with the effect of acetylsalicylic acid, salicylic acid, and dipyrone. Furthermore, combinations of these drugs were used to investigate a possible synergistic inhibitory effect on PGE_2-synthesis. Both paracetamol (IC_{50}=7.45 μM) and caffeine (IC_{50}=42.5 μM) dose-dependently inhibited microglial PGE_2 synthesis. In combination with acetylsalicylic acid (IC_{50}=3.12 μM), both substances augmented the inhibitory effect of acetylsalicylic acid on LPS-induced PGE_2-synthesis. Whereas paracetamol inhibited only COX enzyme activity, caffeine also inhibited COX-2 protein synthesis. These results are compatible with the view that the clinical activity of paracetamol and caffeine is due to inhibition of COX. Furthermore, these results may help explain the clinical experience of an adjuvant analgesic effect of caffeine and paracetamol when combined with acetylsalicylic acid.

Keywords: Cyclooxygenase; COX; Aspirin; Pain; Salicylic acid; Dipyrone; Acetaminophen

1. Introduction

Prostaglandins (PGs) play an important role in central nervous system (CNS) functioning, including the generation of fever (Stiit, 1991), the transmission of pain (Horiguchi et al., 1986), and the control of the sleep-wake cycle (Hayaishi, 1991). PGs are produced by the enzyme cyclooxygenase (COX), which exists in two isoforms, a constitutive COX-1 and an inducible COX-2. COX-1 is constitutively expressed by a variety of tissues including kidney, stomach, platelets and glial cells (astrocytes/microglia) in the CNS (for review, see Pairet and Engelhardt, 1996; Deininger and Schluesener, 1999). In contrast to COX-1, COX-2 is an enzyme whose activity is mainly induced during inflammatory conditions. In the periphery, COX-2 is expressed in several tissues, including monocytes and macrophages (Pairet and Engelhardt, 1996). In the CNS, COX-2 is constitutively expressed in neurons of the cortex, hippocampus and amygdala in the rat and can be induced by seizures or NMDA-dependent synaptic activity (Yamagata et al., 1993). The distribution of COX-2 immunoreactive neurons in the rat CNS suggests that this isoform may be involved in the regulation of sensory input and the elaboration of behavioral and autonomic responses (Breder et al., 1995). In addition to neuronal

Abbreviations: COX, cyclooxygenase; LPS, lipopolysaccharide; NSAID, nonsteroidal antiinflammatory drug; PG, prostaglandin; SDS, sodium dodecyl sulfate.

* Corresponding author. Tel.: +49-761-270-6501; fax: +49-761-270-6917.

E-mail address: bernd_fiebich@psyallg.ukl.uni-freiburg.de (B.L. Fiebich).

0028-3908/00/$ - see front matter
PII: S0028-3908(00)00045-9

cells, COX-2 is expressed in microglial cells and astrocytes which appear to be an important source of PGs during inflammatory conditions (Bauer et al., 1997; Minghetti and Levi, 1995; O'Banion et al., 1996). COX-2 and subsequent PG synthesis can be induced in cultured microglial cells by different inflammatory stimuli including lipopolysaccharide (LPS) and adenosine (Fiebich et al., 1996a; Minghetti and Levi, 1995). Cultured microglial cells therefore represent a useful model to study the regulation of COX expression and production of PGs in the CNS (Bauer et al., 1997; Fiebich et al., 1996b).

The anti-inflammatory, analgesic and antipyretic actions of nonsteroidal anti-inflammatory drugs (NSAIDs) such as acetylsalicylic acid (aspirin) are thought to be due to an inhibition of COX-2, whereas the unwanted side effects such as irritation of the stomach and toxic effects on the kidney are due to inhibition of the constitutive enzyme, COX-1. The mild analgesic paracetamol (acetaminophen), however, does not fit in with this hypothesis of COX-inhibition. All studies performed to date show that paracetamol is a very weak or ineffective inhibitor of peripheral COX activity as measured in mononuclear cells (Brune et al., 1984; Grossman et al., 1995; Tordjman et al., 1995; Vane and Botting, 1995). Nevertheless, paracetamol has clear analgesic and antipyretic properties and has been shown to intensify the effects of acetylsalicylic acid when the two are given simultaneously (Engelhardt, 1984). The ineffectiveness of paracetamol in inhibiting monocyte/macrophage COX-2 activity led to different hypothesis about its mechanism of action, namely that COX from different regions of the body may show different sensitivity to drugs, that there may be different tissue-specific isoforms of COX, or that there may even be a COX-3 on which paracetamol has a preferential action (Vane, 1994). Due to the clinical observation that paracetamol is analgesic and antipyretic (central actions), but not anti-inflammatory (a peripheral action), Flowers and Vane (1972) proposed that specific inhibition of a COX-isoform in the CNS may explain its activity. However, the underlying mechanisms of such a central action have not been elucidated so far.

Although a direct analgesic effect of caffeine when given alone is still controversial, an adjuvant analgesic effect when given in combination with mild analgesics, especially acetylsalicylic acid is well established. This has been demonstrated in various animal experiments (Engelhardt et al., 1997; Vinegar et al., 1976) and in a number of clinical studies (Forbes et al., 1991; Laska et al., 1984; Migliardi et al., 1994; Schachtel et al., 1991; Ward et al., 1991). The mechanism of this adjuvant effect of caffeine is also not known.

In the present study, we investigated the inhibitory effect of paracetamol and caffeine on LPS-induced COX- and PGE_2-synthesis in primary rat microglial cells. These cells were used to investigate effects of those drugs on COX and PGE_2-synthesis in brain cells to test the hypothesis of a "brain-cell-specific" inhibitory effect of paracetamol and caffeine. The effects of the drugs were compared with the effects of NSAIDs such as acetylsalicylic acid, salicylic acid, and dipyrone, whose inhibitory effect on COX activity has been well-documented in peripheral systems (Mitchell et al., 1993). Furthermore, combinations of these drugs were used to investigate a possible synergistic inhibitory effect on PGE_2-synthesis.

2. Materials and methods

2.1. Materials

Acetylsalicylic acid, dipyrone, salicylic acid, and lipopolysaccharide (LPS) (Escherichia coli) were purchased from Sigma (Deisenhofen, FRG), caffeine from RBI (Biotrend, Köln, FRG). Rabbit polyclonal antibodies recognizing COX-1 or COX-2 protein were purchased from Santa Cruz (Heidelberg, FRG).

2.2. Cell culture

Primary astroglial cell cultures were established from cerebral cortices of one-day neonatal Wistar rats as described previously (Keller et al., 1985). Briefly, forebrains were minced and gently dissociated by repeated pipetting in Hank's balanced salt solution (HBSS). Cells were collected by centrifugation, resuspended in Dulbeccos modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS) and antibiotics and cultured on 10 cm cell culture dishes (Falcon, 5×10^5 cells/plate) in 5% CO_2 at 37°C. Medium was prepared taking extreme care to avoid LPS contamination (Gebicke-Haerter et al., 1989). Floating microglia were harvested from 10 to 14 day old mixed (astrocyte-microglia) primary cultures and re-seeded into 35 mm cell culture dishes in fresh complete medium to give pure microglial cultures (2×10^4 cells/dish). Microglial cultures were washed 1 h after seeding to remove non-adherent cells. The purity of the microglial culture was >98% as previously determined by immunofluorescence and cytochemical analysis (Gebicke-Haerter et al., 1989).

2.3. Determination of PGE_2

Microglial cells were seeded in 24-well cell culture plates and pre-incubated with the test substance for 30 min. Thereafter, cells were treated with LPS (100 ng/ml) for 24 h. Supernatants were harvested, centrifuged at 10 000***g*** for 10 min and levels of PGE_2 in the media were measured by enzyme immunoassay (EIA) (Biotrend, Köln, FRG) according to the manufacturer's

instructions. Standards from 39 to 2500 pg/ml were used, sensitivity of the assay was 36.2 pg/ml.

2.4. Western blotting

Microglial cells were seeded into cell culture flasks overnight before medium was replaced with 10 ml of fresh medium. Cells were then exposed to LPS (100 ng/ml) in the presence or absence of the test substance for 24 h. Cells were then washed with phosphate buffered saline (PBS) and lyzed in 1.3 × SDS (sodium dodecyl sulfate) sample buffer containing 100 μM orthovanadate (Laemmli, 1970). Lysates were homogenized through a 26-gauge-needle and measured for protein content using the bicinchonic acid method. For western blotting, 60 μg of protein for each sample was subjected to 10% SDS-PAGE (polyacrylamide gel electrophoresis) under reducing conditions. The protein was then transferred onto a polyvinylidene fluoride (PVDF) membrane (Millipore) by semi-dry blotting. The membrane was blocked using Rotiblock (Roth, Karlsruhe, FRG) for 1 h at room temperature. It was then incubated with rabbit anti-COX-2 serum (M19, Santa Cruz, 1:1000) or rabbit anti-COX-1 (M20, Santa Cruz, 1:1000) in tris-buffered saline (TBS) containing 0.1% Tween-20 (Merck, Darmstadt, FRG) and 1% bovine serum albumin (BSA) (Sigma) for 2 h. After extensive washing, COX-2 or COX-1 protein was detected with a peroxide-coupled rabbit anti-goat IgG (Santa Cruz) using electrochemical luminescence (ECL) reagents (Amersham). Western blot films were scanned densitometrically using ScanPack 2.0 Software from Biometra. Data were analysed by ANOVA with post hoc Bonferroni tests.

2.5. Data analysis

The concentrations resulting in 50% inhibition (IC_{50}) were calculated as follows. The reduction of PGE_2 induced by different concentrations of the test substance was first corrected for the amount of PGE_2 in non-stimulated cultures. These values were then expressed as the percent of the stimulated group without test substance. The resulting concentration-response relationship was analyzed using linear regression analysis with 95% confidence limits (Fieller, 1944; Linder, 1964: 148–162).

3. Results

The inhibitory effect of different analgesics on PGE_2 synthesis was investigated in primary rat microglial cells. Basal production of PGE_2 in these cells (i.e. unstimulated conditions) was 0.17±0.064 ng (mean ± SD) PGE_2/ml over 24 h. The addition of increasing amounts of bacterial LPS led to a concentration-dependent increase of PGE_2 synthesis in these cells, which was maximal with 100 ng/ml LPS (data not shown; Bauer et al., 1997). At this concentration, which was used in all experiments presented here, PGE_2 release from microglial cells increased approximately 5-fold to 0.89±0.39 ng PGE_2/ml media.

3.1. Dose-dependent inhibitory effects of various analgesics on PGE_2 synthesis in LPS-stimulated microglial cells

The inhibitory effects of acetylsalicylic acid, salicylic acid (i.e. the active metabolite of acetylsalicylic acid in vivo), caffeine, paracetamol, or dipyrone (used as a contraol analgesic) were tested on LPS-induced PGE_2 synthesis in rat microglial cells. Acetylsalicylic acid, salicylic acid and dipyrone inhibited PGE_2 synthesis in LPS-stimulated microglial cells in a concentration-dependent manner (Fig. 1). Acetylsalicylic acid and dipyrone were

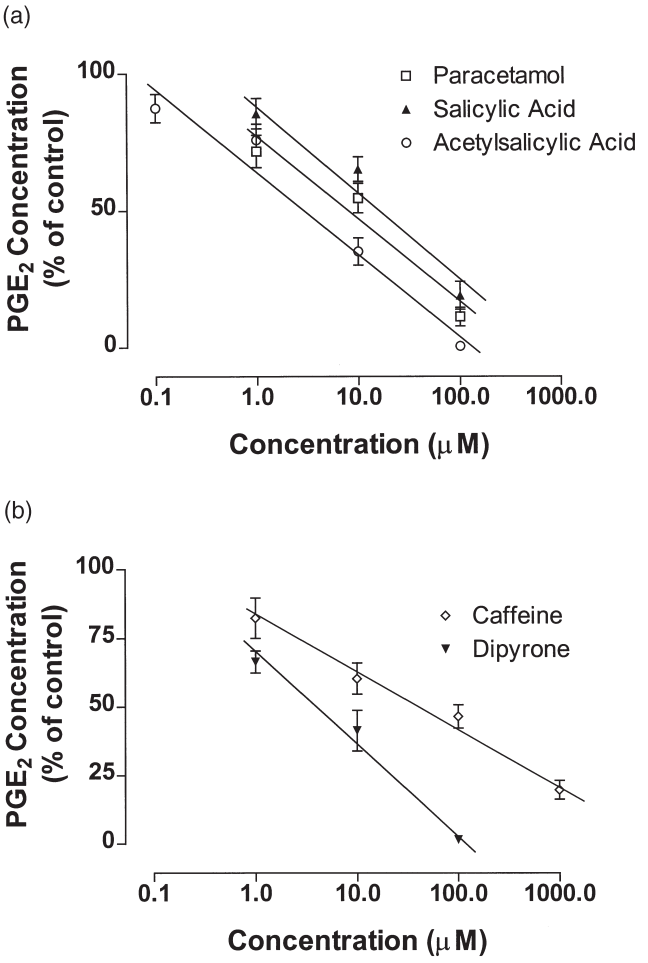

Fig. 1. Dose-dependent inhibitory effect of paracetamol, salicylic acid or acetylsalicylic acid (a) and caffeine or dipyrone (b) on PGE_2 synthesis in LPS-stimulated microglial cells. Cells were pre-incubated with the test substances at the indicated concentrations for 30 min and subsequently treated with LPS (100 ng/ml) for 24 h. PGE_2 in the supernatants was measured by an enzyme immunoassay as described in Materials and methods. Data are expressed as mean ± SE from >6 (a) and >12 (b) replicates per concentration.

the most potent inhibitors under these conditions with an IC_{50} of 3.12 and 3.84 μM, respectively (Table 1). Salicylic acid was 5-times less potent than acetylsalicylic acid (IC_{50} of 15.9 μM, Table 1), giving an order of potency acetylsalicylic acid > dipyrone > salicylic acid. As seen in Fig. 1A, paracetamol also inhibited PGE_2 synthesis in a concentration-dependent manner with an IC_{50} of 7.45 μM (Table 1), indicating that it is 2-times more potent than salicylic acid and half as potent as acetylsalicylic acid. Fig. 1B shows that caffeine inhibited PGE_2 synthesis in LPS-stimulated microglial cells in a concentration-dependent manner also. However, caffeine was only a weak inhibitor of PGE_2 synthesis when given alone (IC_{50} 42.5 μM, Table 1). In addition, the slope of the concentration-activity curve was less steep than that of acetylsalicylic acid and the other mild analgesics, suggesting another mechanism of action.

3.2. Synergistic inhibitory effect of various combinations of analgesics on PGE_2 synthesis in LPS-stimulated microglial cells

When combined with 1 or 3 μM paracetamol, acetylsalicylic acid was a more potent inhibitor of PGE_2 synthesis at all concentrations tested. Thus, in this system, both substances demonstrated a synergistic action (Fig. 2). This was also reflected in the reduction in a change of IC_{50} values from 3.12 μM with acetylsalicylic acid alone to 0.13 μM and 0.034 μM with the addition of 1 μM and 3 μM paracetamol, respectively. In other words, the combination of acetylsalicylic acid and 3 μM paracetamol was 90-times more potent than acetylsalicylic acid alone in inhibiting LPS-induced PGE_2 synthesis.

Also the addition of caffeine at concentrations of 1 and 10 μM to acetylsalicylic acid intensified the inhibitory activity against PGE_2 synthesis in LPS-stimulated microglial cells, particularly at lower concentrations of acetylsalicylic acid (Fig. 3A). However, the slope of the concentration response curve of the combination of acetylsalicylic acid with caffeine, like that of caffeine alone, was less steep than that of acetylsalicylic acid alone (Fig. 3A). The combination of acetylsalicylic acid and 10 μM caffeine was 30-times more potent than acetylsalicylic acid alone in inhibiting LPS-induced

Table 1
Inhibitory effect of different analgesics on LPS-induced PGE_2 synthesis in rat microglial cells in vitro

Compound	IC_{50} (μM)	95% Confidence Intervals
Acetylsalicylic acid	3.12	2.16–4.51
Salicylic acid	15.9	9.91–27.0
Paracetamol	7.45	4.48–11.9
Dipyrone	3.84	2.29–5.86
Caffeine	42.5	22.2–89.3

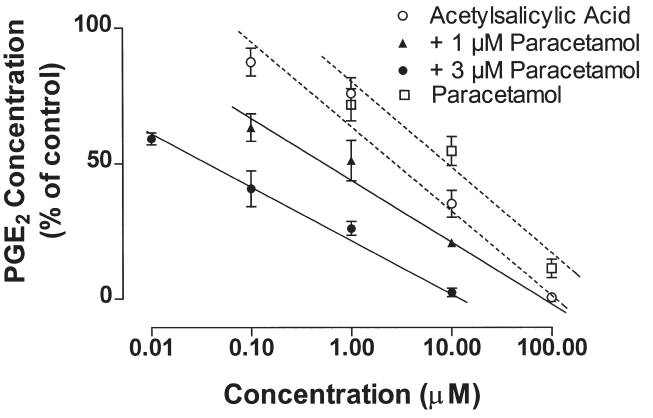

Fig. 2. Synergistic inhibitory effect of paracetamol and acetylsalicylic acid on PGE_2 synthesis in LPS-stimulated microglial cells. Cells were pre-incubated with the test substances at the indicated concentrations for 30 min and subsequently treated with LPS (100 ng/ml) for 24 h. PGE_2 in the supernatants was measured by an enzyme immunoassay as described in Materials and methods. Data are expressed as mean ± SE from 4–6 replicates per concentration.

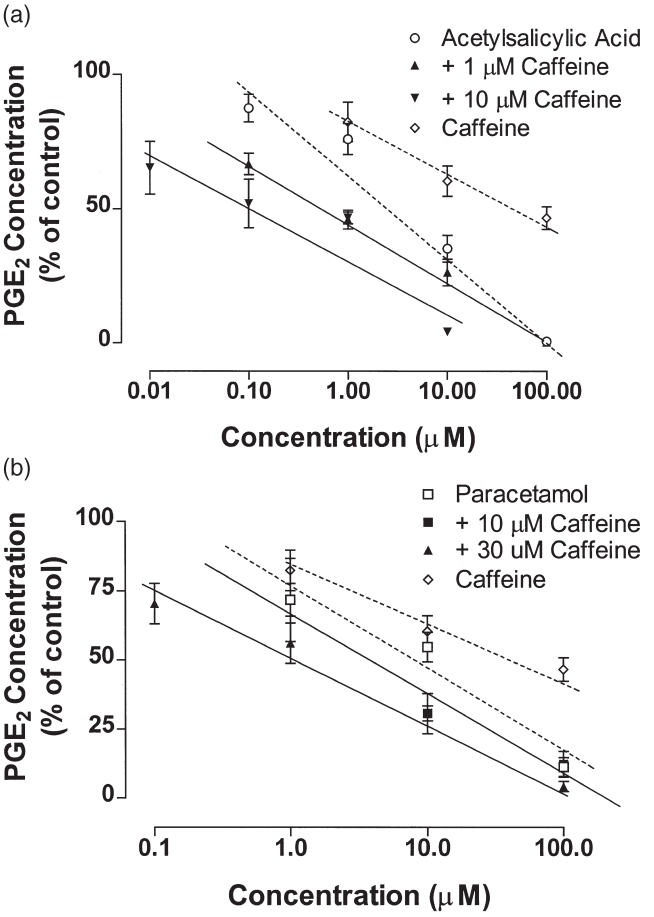

Fig. 3. Synergistic inhibitory effect of caffeine and acetylsalicylic acid (A) or paracetamol (B) on PGE_2 synthesis in LPS-stimulated microglial cells. Cells were pre-incubated with the test substances at the indicated concentrations for 30 min and subsequently treated with LPS (100 ng/ml) for 24 h. PGE_2 in the supernatants was measured by an enzyme immunoassay as described in Materials and methods. Data are expressed as mean ± SE from 8–12 replicates per concentration.

PGE_2 synthesis (Table 2). The combination of caffeine and paracetamol resulted in a weaker additive effect than the combination of caffeine and acetylsalicylic acid (Fig. 3B). The combination of paracetamol and 30 μM caffeine was only about 7-times more potent than paracetamol alone in inhibiting LPS-induced PGE_2 synthesis (Table 2).

3.3. Effect of various analgesics on COX-2 expression synthesis in LPS-stimulated microglial cells

Western blot analysis showed that a protein corresponding to the mass of COX-2 was elevated in samples from LPS-stimulated cultures, and was not detectable in unstimulated microglial cells (Figs. 4 and 5). Two bands at approximately 70 kDa are visible, probably showing different glycosylation forms of COX-2 recognized by the antibody. When LPS-stimulated cultures were preincubated with acetylsalicylic acid, salicylic acid, paracetamol or dipyrone, COX-2 protein levels were not influenced. This was true for all concentrations tested, including those sufficient to almost completely inhibit LPS-induced PGE_2 synthesis (Fig. 4). In contrast, caffeine at concentrations between 10 and 1000 μM reduced the amount of COX-2 protein in a concentration-dependent manner (Fig. 5). Table 3 shows the summary of the COX-2 Western blots when scanned densitometrically, confirming that caffeine significantly inhibited COX-2 protein synthesis, whereas the other analgesics had no significant inhibitory effect on COX-2 levels.

Table 2
Effect of drug combinations on the inhibition of LPS-induced PGE_2 synthesis in rat microglial cells in vitro

Drug combination		IC_{50} (μM)	95% Confidence Intervals
Acetylsalicylic acid	–	3.12	2.16–4.51
Acetylsalicylic acid	0.1 μM Caffeine	4.91	2.19–8.90
Acetylsalicylic acid	1.0 μM Caffeine	0.74	0.37–1.32
Acetylsalicylic acid	10 μM Caffeine	0.12	0.035–0.31
Acetylsalicylic acid	1.0 μM paracetamol	0.13	0.019–0.33
Acetylsalicylic acid	3.0 μM paracetamol	0.034	0.016–0.061
Paracetamol	–	7.45	4.48–11.9
Paracetamol	10 μM Caffeine	4.05	1.11–9.40
Paracetamol	30 μM Caffeine	1.16	0.54–2.23

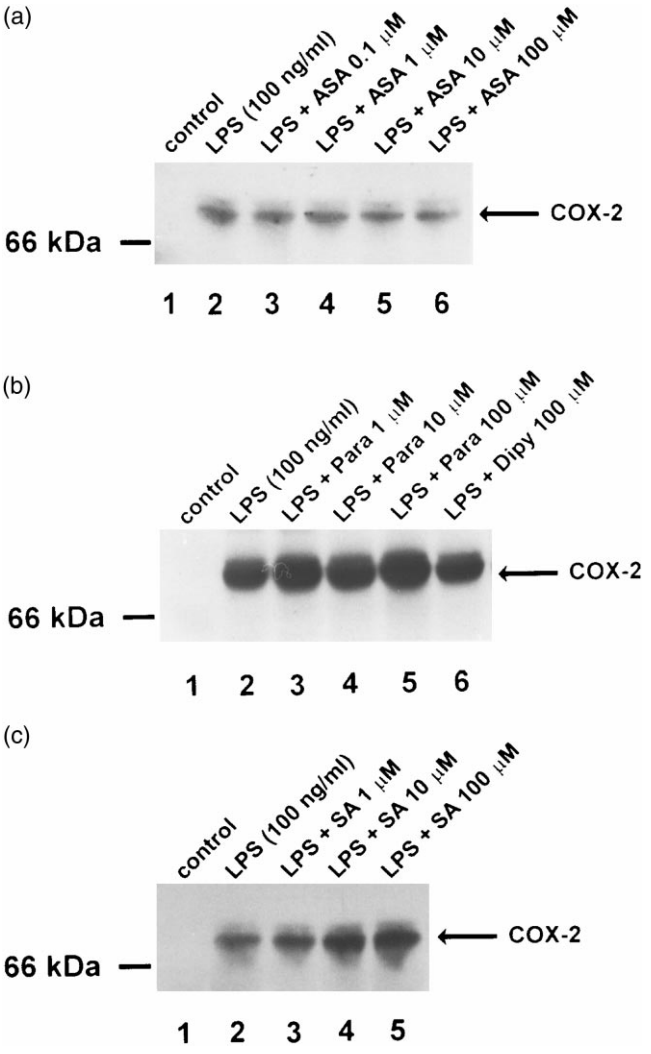

Fig. 4. No effect of acetylsalicylic acid (ASA; a), paracetamol (Para; b), dipyrone (Dipy; b) or salicylic acid (SA; c) on COX-2 synthesis in LPS-stimulated microglial cells. Cells were either left untreated (lane 1) or were stimulated with LPS (100 ng/ml) for 24 h in the absence (lane 2) or presence of different concentrations of acetylsalicylic acid (ASA; a lanes 3–6), paracetamol (Para; b, lanes 3–5), dipyrone (dipy; b, lane 6) or salicylic acid (SA; c, lanes 3–5). Western blotting with an anti-COX-2 antibody was used to detect the amount of COX-2 protein in whole cell lysates as described in Materials and methods.

3.4. COX-1 immunoreactivity is not affected by LPS in microglial cells

In Western blot experiments, COX-1 immunoreactivity was already detected in unstimulated microglial cells. is constitutively expressed in microglial cells (Fig. 6, lane 1). Stimulation with LPS did not increase COX-1 immunoreactivity (Fig. 6, lane B). Since untreated cells only marginally synthesize COX-2 and the treatment with LPS is followed by a strong induction of COX-2 immunoreactivity (Fig. 5 and Table 3), the data suggest that the basal PGE_2 release is due to COX-1 enzyme activity.

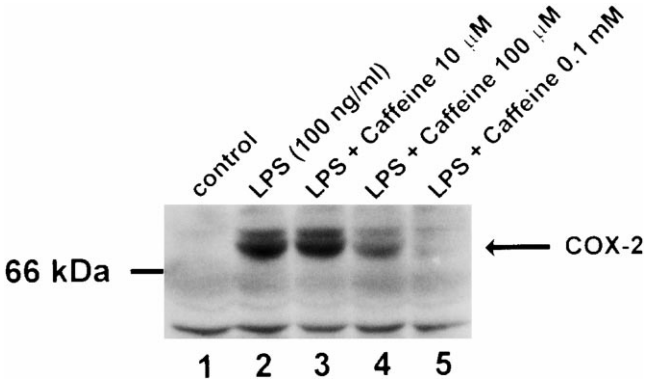

Fig. 5. Dose-dependent inhibitory effect of caffeine on COX-2 synthesis in LPS-stimulated microglial cells. Cells were either left untreated (lane 1) or were stimulated with LPS (100 ng/ml) for 24 h in the absence (lane 2) or presence of different concentrations of caffeine (lanes 3–5). Western blotting with an anti-COX-2 antibody was used to detect the amount of COX-2 protein in whole cell lysates as described in Materials and methods.

Table 3
Effects of different drugs on COX-2 protein synthesis in LPS-stimulated rat microglial cells in vitro. Western blot films from three or four independent experiments were scanned densitometrically. Data are mean values given as percentage of the stimulation by LPS (= 100%). Data were analysed by ANOVA with post hoc Bonferroni tests. * = significantly different from LPS-control

Compound	% of LPS control
Control	< 1
LPS	100
LPS + Acetylsalicylic acid (10^{-4} M)	112.3±14.8
LPS + Salicylic acid (10^{-4} M)	113.8±42.9
LPS + Paracetamol (10^{-4} M)	100.3±43.9
LPS + Caffeine (10^{-5} M)	78.5±19.8
LPS + Caffeine (10^{-4} M)	62.0±10.5 ($p<0.01$*)
LPS + Caffeine (10^{-3} M)	21.3±10.0 ($p<0.001$*)

4. Discussion

Numerous findings in animals and man suggest that aspirin-like drugs have a central analgesic activity in addition to their peripheral analgesic effects (Bannwarth et al., 1995; Bromm et al., 1991; Catania et al., 1991; Devoghel, 1983; Fabbri et al., 1992; Jurna and Brune, 1990). This may be mediated by the inhibition of PG synthesis in the CNS (Beiche et al., 1996; Levy et al., 1998; Malmberg and Yaksh, 1992). In this study, we were interested in potential central mechanisms of paracetamol and caffeine in conjunction with acetylsalicylic acid. Here we present evidence in support of our hypothesis that the central analgesic effect of paracetamol may be due to inhibition of PGE_2 synthesis in microglial cells. Primary rat microglial cells have previously been shown to produce increased amounts of PGE_2 and COX-2 mRNA and protein after stimulation with LPS and thus are a useful in vitro model system to study the COX

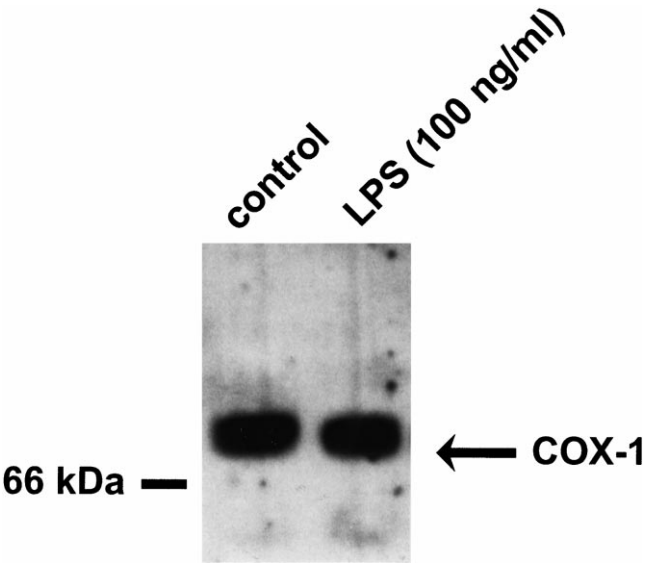

Fig. 6. COX-1 is constitutively expressed in microglial cells. Cells were either left untreated (lane 1) or were stimulated with LPS (100 ng/ml) (lane 2) for 24 h. Western blotting with an anti-COX-1 antibody was used to detect the amount of COX-1 protein in whole cell lysates as described in Materials and methods.

inhibiting activity of NSAIDs in the brain (Bauer et al., 1997; Fiebich et al., 1996b). The forebrain microglia cell culture is a well established and validated model for the investigation of microglial cells in vitro. However, we can not rule out differences to other microglia cell cultures, e.g. adult spinal cord microglia, which have been mainly investigated in vivo (Fu et al., 1999), but are difficult to maintain as cell cultures (Whittmore et al., 1993). Inhibition of COX activity in microglial cells may explain the previous finding that paracetamol, as with acetylsalicylic acid and dipyrone, suppressed the pentetrazol-induced rise in PGE_2 concentration in the rat and mouse brain in a dose-dependent manner (Engelhardt et al., 1996b). Our findings may also provide a cellular mechanism for the proposal of Flowers and Vane (1972) and Vane (1994) that paracetamol acts by inhibition of central COX activity. However, other cell types that may be involved in PG synthesis in the brain, such as astrocytes and neurons, were not investigated in the present study and may also be involved. The expression of COX-2 in spinal cord neurons and the reduction of nociceptive neuronal activity by COX-2 inhibitors and dipyrone may also contribute to the analgesic effects of inhibitors of PG synthesis (Willingale et al., 1997; Neugebauer et al., 1994). The difference in COX-inhibitory activity in microglial cells and in peripheral cells, such as monocytes and macrophages (Vane and Botting, 1995) may explain the specific pharmacological profile of paracetamol, i.e. analgesic and antipyretic activity, but only a weak anti-inflammatory activity as compared to standard NSAIDs (Brune et al., 1984; Grossman et al., 1995; Tordjman et al., 1995). Nevertheless, the data

presented here were obtained in vitro and further studies in vivo are required to investigate the central or peripheral site of action of paracetamol.

COX-1 is expressed constitutively in microglia and its synthesis is not affected by LPS stimulation. In microglial cells, the basal release of PGE_2 is low and increases about fivefold after treatment with LPS. The data from Western blot experiments shown here suggest that the LPS-induced synthesis of PGE_2 is due to the de novo synthesis and activity of COX-2 and that the various analgesics investigated here inhibit microglial PGE_2 release by interfering with COX-2 synthesis or activity. This is also supported by our previous finding that selective inhibitors of COX-2, used in concentrations at which they do not affect COX-1 activity, inhibit the LPS-induced release of PGE_2 in microglia (Bauer et al., 1997). However, an additional inhibitory effect of the compounds tested here on COX-1 cannot be excluded and may also be part of the mechanism by which acetylsalicylic acid, salicylic acid, paracetamol and caffeine inhibit microglial PGE_2 synthesis.

The surprising finding that caffeine inhibited PGE_2 release in stimulated rat microglial cells in vitro contrasts with observations in other systems. Caffeine in concentrations up to 2 mM had no effect on arachidonic acid conversion by an enzyme preparation from bovine brain in vitro, and the addition of 0.5 mM caffeine also had no influence on the inhibitory effect of acetylsalicylic acid or paracetamol in this system (Engelhardt et al., 1997). The lack of effect observed in the bovine brain preparation suggests that caffeine has no direct effect on enzyme activity since only COX activity was measured and not COX expression. In the present study, we measured both induction and activity of the COX-2 enzyme. Unlike paracetamol and NSAIDs, caffeine diminished the amount of COX-2 protein in a concentration-dependent manner. Taken together, these results suggest that caffeine inhibits COX-2 induction rather than COX-2 activity, in contrast to the standard NSAIDs, but we cannot exclude effects of caffeine on the constitutive COX-1 in this in vitro cell model system. In other cell culture models, some NSAIDs have been shown to suppress COX expression. Acetylsalicylic acid and naproxen but not indomethacin reduced IL-1 induced COX-2 expression in endothelial cells (Wu et al., 1991; Zyglewska et al., 1992; Xu et al., 1999), whereas acetylsalicylic acid and naproxen failed to suppress LPS induced COX-2 expression in a human macrophage cell line (Barios-Rodiles et al., 1996). Paracetamol has been shown to reduce the levels COX-2 protein in human umbilical vein endothelilal cells (Wu et al., 1991). Acetylsalicylic acid may fail to suppress COX-2 synthesis in our microglia culture system due to cell type specific differences or because of differences in the involved intracellular signalling pathways induced by IL-1 or LPS.

The difference in caffeine activity in microglial cells and in peripheral cells such as monocytes/macrophages must also be stressed. Although we have no satisfactory explanation for these effects, it could be speculated that caffeine acts on a specific microglial signal transduction cascade leading to COX-2 synthesis, which is not involved in COX-2 regulation in other cells. A parallel can be drawn between these observations and the contrasting effects of caffeine on blood flow and vascular resistance in the CNS and in the periphery. Although caffeine causes a marked increase in cerebrovascular resistance with accompanying decrease in cerebral blood flow, it reduces vascular resistance in peripheral organs such as the kidney at therapeutic doses in man (Ogilvie et al., 1977). These effects on peripheral vascular resistance are thought to be due to the stimulation of the synthesis of vasodilating PGs, such as PGI_2 and PGE_2 (Baer et al., 1983; Caussade and Cloarec, 1993; Engelhardt, 1996a; Naderali and Poyser 1994, 1996; Takeuchi et al., 1981).

The concentrations of caffeine used to inhibit PGE_2 synthesis in activated microglial cells are also therapeutically relevant (Rall, 1985). At these concentrations, caffeine is a non-selective antagonist of adenosine A_1- and A_2-receptors (Fredholm et al., 1994). Adenosine A_{2a} receptors induce intracellular signaling events that cause an upregulation of the COX-2 gene and the release of PGE_2 in rat microglia (Fiebich et al., 1996a). Selective adenosine A_2 receptor antagonism has also been shown to inhibit adenosine-induced COX-2 expression and PGE_2 release in rat microglial cells (Fiebich et al., 1996a). Thus, the inhibitory effect of caffeine on COX-2 activity in stimulated microglial cells may be due to adenosine A_2-receptor antagonism.

Other important findings in this study are that acetylsalicylic acid, paracetamol and caffeine have synergistic effects in the microglia in vitro cell model. Low doses of caffeine and paracetamol significantly decreased the IC_{50} value of acetylsalicylic acid about four to 25 fold in combination with caffeine (1 and 10 μM) and approximately 25 to 90 fold in combination with paracetamol (1 and 3 μM). This might be of clinical relevance since our data probably suggest that the combinations of acetylslalicylic acid with caffeine or paracetamol are much more effective in inhibition of microglial prostaglandin release than acetylsalicylic acid alone. Since high doses of acetylsalicylic acid as well as paracetamol are known to cause severe side effects in the intestines, one could speculate that lower doses of the drugs in combination are as effective as the drugs alone in higher doses, but the safety profile of the combinations is probably much better. Since the data presented here are obtained by an in vitro cell culture model, in vivo studies have to be performed to validate this hypothesis.

The fact that caffeine and paracetamol strongly enhance the effects of acetylsalicylic acid suggests, that these compounds are acting not additively, but syner-

gistically by using different targets in the synthesis pathway of PGE_2. This synergistic effect of the combinations of acetylsalicylic acid with either paracetamol or caffeine may therefore be explained by different mechanisms of COX inhibition. The mechanism of action of aspirin-like drugs on the catalytic site of the COX enzyme has been well-established (Luong et al., 1996). If paracetamol acts on a different site, such as the peroxidation site of COX, it is likely that the combination of acetylsalicylic acid and paracetamol would be more effective than either compound alone. The same is true for the combination of acetylsalicylic acid and caffeine. If we assume that caffeine blocks the synthesis of COX-2 protein, probably at the transcriptional level, it would provide less enzyme for acetylsalicylic acid to inhibit, thereby leading to enhanced COX-2 inhibition.

In conclusion, our results suggest that not only acetylsalicylic acid, salicylic acid, and dipyrone but also paracetamol and caffeine inhibit PGE_2 synthesis in microglial cells and that both paracetamol and caffeine enhance the inhibitory effects of acetylsalicylic acid on microglial PGE_2-synthesis. This supports our hypothesis that inhibition of PGE_2 synthesis in microglial cells can may be a possible explanation for the central analgesic activity of paracetamol and caffeine. This hypothesis, however, has to be further proven investigated in studies using animal models that may overcome the limitations of a cell culture model of brain cells. The observation that paracetamol and caffeine intensify the effect of acetylsalicylic acid in a synergistic manner may help explain the clinical experience of an adjuvant analgesic effect of caffeine when combined with acetylsalicylic acid or paracetamol.

Acknowledgements

We thank Ulrike Götzinger-Berger, Alexander Craig, Brigitte Günter, and Sandra Hess for skillful technical assistance. This work was supported in part by grant Fi 683/1-1 from the Deutsche Forschungsgemeinschaft.

References

Baer, P.G., Armstrong, E.L., Cagen, L.M., 1983. Dissociation of effects of xanthine analogs on renal prostaglandins and renal excretory function in the awake rat. J. Pharmacol. Exp. Ther. 227, 600–604.

Bannwarth, B., Demotes-Mainard, F., Schaeverbeke, T., Labat, L., Dehais, J., 1995. Central analgesic effects of aspirin-like drugs. Fundam. Clin. Pharm. 9, 1–7.

Barios-Rodiles, M., Keller, K., Belley, A., Chadee, K., 1996. Nonsteroidal antiinflammatory drugs inhibit cyclooxygenase-2 enzyme activity but not mRNA expression in human macrophages. Bichem. Biophys. Res. Commun. 225, 896–900.

Bauer, M.K.A., Lieb, K., Schulze-Osthoff, K., Berger, M., Gebicke-Haerter, P.J., Bauer, J., Fiebich, B.L., 1997. Expression and regulation of cyclooxygenase-2 in rat microglia. Eur. J. Biochem. 243, 726–731.

Beiche, F., Scheuerer, S., Brune, K., Geisslinger, G., Goppelt-Struebe, M., 1996. Up-regulation of cyclooxygenase-2 mRNA in the rat spinal cord following peripheral inflammation. FEBS Lett. 390, 165–169.

Breder, C.D., Dewitt, D., Kraig, R.P., 1995. Characterization of inducible cyclooxygenase in rat brain. J. Comp. Neurol. 355, 296–315.

Bromm, B., Rundshagen, I., Scharein, E., 1991. Central analgesic effects of acetylsalicylic acid in healthy men. Arzneimittel-Forschung 41, 1123–1129.

Brune, K., Aehringhaus, U., Peskar, B.A., 1984. Pharmacological control of leukotriene and prostaglandin production from mouse peritoneal macrophages. Agents and Actions 14, 729–734.

Catania, A., Arnold, J., Macaluso, A., Hiltz, M.E., Lipton, J.M., 1991. Inhibition of acute inflammation in the periphery by central action of salicylates. Proc. Natl. Acad. Sci. U.S.A. 88, 8544–8547.

Caussade, F., Cloarec, A., 1993. Effect of tienoxolol, a new diuretic beta-blocking agent, on urinary prostaglandin excretion in the rat. Br. J. Pharmacol. 109, 278–284.

Deininger, M.H., Schluesener, H.J., 1999. Cyclooxygenases-1 and -2 are differentially localized to microglia and endothelium in rat EAE and glioma. J. Neuroimmunol. 95, 202–208.

Devoghel, J.C., 1983. Small intrathecal doses of lysine-acetylsalicylate relieve intractable pain in man. J. Intern. Med. 11, 90–91.

Engelhardt, G., 1984. Animal experimental studies on the question of the interaction of paracetamol and acetylsalicylic acid. Arzneimittel-Forschung 34, 992–1001.

Engelhardt, G., 1996a. Effect of acetylsalicylic acid, paracetamol, caffeine and a combination of these substances on the renal prostaglandin E2, 6-keto-prostaglandin F1 alpha, water, creatinine and electrolyte excretion of the rat. Arzneimittel-Forschung 46, 509–512.

Engelhardt, G., Bogel, R., Schnitzler, C., Utzmann, R., 1996b. Meloxicam: influence on arachidonic acid metabolism, Part II. In vivo findings. Biochem. Pharmacol. 51, 29–38.

Engelhardt, G., Mauz, A.B., Pairet, M., 1997. Role of caffeine in combined analgesic drugs from the point of view of experimental pharmacology. Arzneimittel-Forschung 47, 917–927.

Fabbri, A., Cruccu, G., Sperti, P., Ridolfi, M., Ciampani, T., Leardi, M.G., Ferracuti, S., Bonifacio, V., 1992. Piroxicam-induced analgesia: evidence for a central component which is not opioid mediated. Experientia 48, 1139–1142.

Fiebich, B.L., Biber, K., Lieb, K., Van Calker, D., Berger, M., Bauer, J., Gebicke-Haerter, P.J., 1996a. Cyclooxygenase-2 expression in rat microglia is induced by adenosine A_{2a}-receptors. Glia 18, 152–160.

Fiebich, B.L., Lieb, K., Pairet, M., Trummlitz, G., Engelhardt, G., Roesler, N., Berger, M., Gebicke, H.P., Bauer, J., 1996b. Microglia as a model of NSAID action in the brain. Eur. J. Clin. Pharmacol 50, 541.

Fieller, E.C., 1944. A fundamental formula in the statistics of biological assay, and some applications. Qu. J. Pharm. Pharmacol. 17, 117–123.

Flower, R.J., Vane, J.R., 1972. Inhibition of prostaglandin synthetase in brain explains the anti-pyretic activity of paracetamol (4-acetamidophenol). Nature 240, 410–411.

Forbes, J.A., Beaver, W.T., Jones, K.F., Kehm, C.J., Smith, W.K., Gongloff, C.M., Zeleznock, J.R., Smith, J.W., 1991. Effect of caffeine on ibuprofen analgesia in postoperative oral surgery pain. Clin. Pharmacol. Ther. 49, 674–684.

Fredholm, B.B., Abbracchio, M.P., Burnstock, G., Daly, J.W., Harden, K.T., Jacobson, K.A., Leff, P., Williams, M., 1994. VI. Nomenclature and classification of purinoceptors. Pharmacol. Rev. 46, 143–156.

Fu, K.Y., Light, A.R., Matsushima, G.K., Maixner, W., 1999.

Microglial reaction after subcutaneous formalin injection in rat hind paw. Brain Res. 825, 59–67.
Gebicke-Haerter, P.J., Bauer, J., Schobert, A., Northoff, H., 1989. Lipopolysaccharide-free conditions in primary astrocyte cultures allow growth and isolation of microglial cells. J. Neurosci. 9, 183–194.
Grossman, C.J., Wiseman, J., Lucas, F.S., Trevethick, M.A., Birch, P.J., 1995. Inhibition of constitutive and inducible cyclooxygenase activity in human platelets and mononuclear cells by NSAIDs and Cox 2 inhibitors. Inflamm. Res. 44, 253–257.
Hayaishi, O., 1991. Molecular mechanism of sleep wake regulation: roles of prostaglandins D_2 and E_2. FASEB J. 5, 25–75.
Horiguchi, S., Ueno, R., Hyodo, M., Hayaishi, O., 1986. Alterations in nociception after intracisternal administration of prostaglandin D2, E2 or F2 to conscious mice. Eur. J. Pharmacol. 122, 173–179.
Jurna, I., Brune, K., 1990. Central effect of the non-steroid anti-inflammatory agents, indomethacin, ibuprofen, and diclofenac, determined in C fibre-evoked activity in single neurones of the rat thalamus. Pain 41, 71–80.
Keller, M., Jackisch, R., Seregi, A., Hertting, G., 1985. Comparison of prostanoid forming capacity of neuronal and astroglia cells in primary culture. Neurochem. Int. 7, 655–665.
Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head bacteriophage T4. Nature 227, 680–685.
Laska, E.M., Sunshine, A., Mueller, F., Elvers, W.B., Siegel, C., Rubin, A., 1984. Caffeine as an analgesic adjuvant. JAMA 251, 1711–1718.
Levy, M., Brune, K., Zylber-Katz, E., Cohen, O., Caraco, Y., Geisslinger, G., 1998. Cerebrospinal fluid prostaglandins after systemic dipyrone intake. Clin. Pharmacol. Ther. 64, 117–122.
Linder, A., 1964. Statistische Methoden. Birkhauser Press, Basel.
Luong, C., Miller, A., Barnett, J., Chow, J., Ramesha, C., Browner, M.F., 1996. Flexibility of the NSAID binding site in the structure of human cyclooxygenase-2. Nature Struct. Biol. 3, 927–933.
Malmberg, A.B., Yaksh, T.L., 1992. Hyperalgesia mediated by spinal glutamate or substance P receptor blocked by spinal cyclooxygenase inhibition. Science 257, 1276–1279.
Migliardi, J.R., Armellino, J.J., Friedman, M., Gillings, D.B., Beaver, W.T., 1994. Caffeine as an analgesic adjuvant in tension headache. Clin. Pharmacol. Ther. 56, 576–586.
Minghetti, L., Levi, G., 1995. Induction of prostanoid biosynthesis by bacterial lipopolysaccharide and isoproterenol in rat microglial cultures. J. Neurochem. 65, 2690–2698.
Mitchell, J.A., Akarasereenont, P., Thiemermann, C., Flower, R.J., Vane, J.R., 1993. Selectivity of nonsteroidal antiinflammatory drugs as inhibitors of constitutive and inducible cyclooxygenase. Proc. Natl. Acad. Sci. U.S.A. 90, 11693–11697.
Naderali, E.K., Poyser, N.L., 1994. The effect of caffeine on prostaglandin output from the perfused mesenteric vascular bed of the rat. Prostag. Leukotr. Ess. 51, 415–418.
Naderali, E.K., Poyser, N.L., 1996. Factors controlling prostaglandin production by guinea-pig endometrial cells. J. Reprod. Fertil. 108, 321–328.
Neugebauer, V., Schaible, H.G., He, X., Lucke, T., Gundling, P., Schmidt, R.F., 1994. Electrophysiological evidence for a spinal antinociceptive action of dipyrone. Agents and Action 41, 62–70.
O'Banion, M.K., Miller, J.C., Chang, J.W., Kaplan, M.D., Coleman, P.D., 1996. Interleukin-1β induces prostaglandin G/H synthase-2 (cyclooxygenase-2) in primary murine astrocyte cultures. J. Neurochem. 66, 2532–2540.
Ogilvie, R.I., Fernandez, P.G., Winsberg, F., 1977. Cardiovascular response to increasing theophylline concentrations. Eur. J. Clin. Pharmacol. 12, 409–414.
Pairet, M., Engelhardt, G., 1996. Distinct isoforms (COX-1 and COX-2) of cyclooxygenase: Possible physiological and therapeutic implications. Fundam. Clin. Pharmacol. 10, 1–15.
Rall, T.H., 1985. Central nervous stimulants. The methylxanthines. In: Goodman, A., Gilman, L.S., Rall, T.W., Murad, F. (Eds.), The Pharmacological Basics of Therapeutics. MacMillan Press, New York, NY.
Schachtel, B.P., Fillingim, J.M., Lane, A.C., Thoden, W.R., Baybutt, R.I., 1991. Caffeine as an analgesic adjuvant. A double-blind study comparing aspirin with caffeine to aspirin and placebo in patients with sore throat. Arch. Intern. Med. 151, 733–737.
Stiit, J.T., 1991. Differential sensitivity in the sites of feverproduction by prostaglandin-E_1 within the hypothalamus of the rat. J. Physiol. 432, 99–110.
Takeuchi, K., Kogo, H., Aizawa, Y., 1981. Effect of theophylline on prostaglandin E release from renal medullary tissue in rat. Yakugaku Zasshi 101, 945–950.
Tordjman, C., Coge, F., Andre, N., Rique, H., Spedding, M., Bonnet, J., 1995. Characterisation of cyclooxygenase 1 and 2 expression in mouse resident peritoneal macrophages in vitro; interactions of non steroidal anti-inflammatory drugs with COX2. Biochim. Biophys. Acta 1256, 249–256.
Vane, J.R., 1994. Towards a better aspirin. Nature 367, 215–216.
Vane, J.R., Botting, R.M., 1995. New insights into the mode of action of anti-inflammatory drugs. Inflamm. Res. 44, 1–10.
Vinegar, R., Truax, J.F., Selph, J.L., Welch, R.M., White, H.L., Ellis, C.H., 1976. Potentiation of the anti-inflammatory and analgesic activity of aspirin by caffeine in the rat. P. Soc. Exp. Biol. Med. 151, 556–560.
Ward, N., Whitney, C., Avery, D., Dunner, D., 1991. The analgesic effects of caffeine in headache. Pain 44, 151–155.
Willingale, H.L., Gardiner, N.J., McLymont, N., Giblett, S., Grubb, B.D., 1997. Prostanoids synthesized by cyclo-oxygenase isoforms in rat spinal cord and their contribution to the development of neuronal hyperexcitability. Br. J. Pharmacol. 122, 1593–1604.
Whittmore, S.R., Sanon, H.R., Wood, P.M., 1993. Concurrent isolation and characterization of oligodendrocytes, microglia and astrocytes from adult spinal cord. Int. J. Dev. Neurosci. 11, 755–764.
Wu, K.K., Sanduja, R., Tsai, A.-L., Ferhanoglu, B., Loose-Mitchel, D.S., 1991. Aspirin inhibits interleukin-1-induced prostaglandin H synthase expression in cultured endothelial cells. Proc. Natl. Acad. Sci. USA 88, 2384–2387.
Xu, X.M., Sansores-Garcia, L., Chen, X.M., Matijevic-Aleksic, N., Du, M., Wu, K.K., 1999. Supression of inducible cyclooxygenase 2 gene transcription by aspirin and sodium salicylate. Proc. Natl. Acad. Sci. USA 96, 5292–5297.
Yamagata, K., Andreasson, K.I., Kaufmann, W.E., Barnes, C.A., Worley, P.F., 1993. Expression of a mitogen-inducible cyclooxygenase in brain neurons: regulation by synaptic activity and glucocorticoids. Neuron 11, 371–386.
Zyglewska, T., Sanduja, R., Ohashi, K., Loose-Mitchel, D.S., Sanduja, S.K., Wu, K.K., 1992. Inhibition of endothelial cell prostaglandin synthase gene expression by naproxen. Biochim. Biophys. Acta 1131, 78–82.