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European Journal of Pharmacology 508 (2005) 7–13

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NSAIDs increase GM-CSF release by human synoviocytes: comparison with nitric oxide-donating derivatives

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Received 16 September 2004; received in revised form 26 November 2004; accepted 30 November 2004
Available online 28 December 2004

Abstract

Non-steroidal anti-inflammatory drugs (NSAIDs) are used to treat the condition of rheumatoid arthritis, where levels of prostaglandin E_2 (PGE_2) and granulocyte macrophage-colony stimulating factor (GM-CSF) are elevated in the synovial fluid. NO-NSAIDs are a new class of cyclooxygenase (COX)-inhibitors developed by coupling a nitric oxide (NO)-donating moiety to conventional NSAIDs. We show that, in cytokine-treated synoviocytes (from non-rheumatic patients), NO-naproxen and NO-flurbiprofen like their parent compounds concentration-dependently reduce the levels of PGE_2 (an index of COX-2 activity), with a corresponding rise in the release of GM-CSF. Unlike acetylsalicylic acid (ASA), NO-ASA reduces the levels of PGE_2, without increasing GM-CSF release, although cell viability is reduced at the highest concentration (1 mM). The effects of NSAIDs and NO-NSAIDs on GM-CSF release were attributable to the PGE_2 mediated cyclic (c) AMP pathway because PGE_2 reversed the effects of COX blockade. Second, phosphodiesterase inhibitors 3-isobutyl-1-methylxanthine (IBMX) and Ro-201724 (both of which elevate cAMP levels) decreased GM-CSF release, in the presence of PGE_2. Finally, neither sodium nitroprusside nor zaprinast (both of which elevate cGMP levels) affected GM-CSF or PGE_2 release. Our findings demonstrate that GM-CSF is regulated by NSAIDs and NO-NSAIDs via inhibition of COX and appears to be mediated via the cAMP pathway. NO-ASA is the exception, because it does not increase GM-CSF release, although at millimolar concentrations cell viability is reduced.

Keywords: COX (cyclooxygenase); GM-CSF (granulocyte macrophage-colony stimulating factor); NO-NSAIDs (nitric oxide-donating non-steroidal anti-inflammatory drugs); PGE_2 (prostaglandin E_2); Synoviocytes

1. Introduction

Cyclooxygenase (COX)-2 is inducible by a number of cytokines during inflammatory conditions such as rheumatoid arthritis (Kang et al., 1996). Interleukin (IL)-1β or phorbol esters up-regulate the expression of COX-2 in rheumatoid synovial tissue, elevating the local production of prostaglandins (Crofford, 1999; Crofford et al., 1994). In non-vascular tissue, prostaglandin E_2 (PGE_2) is the predominate prostaglandin formed via the activity of COX-2 and its increased production contributes to the progression of rheumatoid arthritis (Hishinuma et al., 1999; Kojima et al., 2002; McCoy et al., 2002). Prostaglandins and in particular PGE_2 stimulate bone resorption which could be a mechanism of bone destruction in rheumatoid synovia (Robinson et al., 1975). PGE_2 has also been identified as a mediator of hyperalgesia and inflammation in arthritis (Kamei et al., 2004; Portanova et al., 1996). In addition,

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doi:10.1016/j.ejphar.2004.11.064

growing evidence suggests that COX-2 and PGE_2 are mediators of angiogenesis, integral in the formation of the inflammatory pannus (Koch, 2003; Woods et al., 2003).

Granulocyte macrophage-colony stimulating factor (GM-CSF) is a pro-inflammatory cytokine released by monocytes and synoviocytes. Like PGE_2, GM-CSF is also elevated in the synovial fluid of patients with rheumatoid arthritis (Bell et al., 1995) and is implicated in the pathophysiology of this disease (Berenbaum et al., 1994; Bischof et al., 2000; Moss and Hamilton, 2000).

Several studies, including those from our own group, have demonstrated a relationship between the pro-inflammatory mediators PGE_2 and GM-CSF. In human lung fibroblasts, pre-treatment with PGE_2 reduces IL-1 induced GM-CSF mRNA and protein expression (Patil and Borch, 1995). Others have shown that exogenous PGE_2 inhibits the release of GM-CSF by human synoviocytes (Agro et al., 1996). Furthermore, GM-CSF release is regulated by endogenous COX-1 and COX-2 in a variety of human cell types (Calatayud et al., 2001; Lazzeri et al., 2001; Stanford et al., 2000b).

Non-steroidal anti-inflammatory drugs (NSAIDs) and selective inhibitors of COX-2 are used to alleviate the symptoms of rheumatoid arthritis by reducing the levels of PGE_2 (Duffy et al., 2003; Emery et al., 1999; Seppala et al., 1990). Nitric oxide (NO)-donating NSAIDs are newly developed COX-inhibitors with reduced gastrointestinal toxicity (Cirino et al., 1996; Fiorucci et al., 2003; Holm et al., 2002; Wallace et al., 1995), a common adverse effect with NSAIDs treatment.

Surprisingly, the relationship between COX activity, NSAID therapy and GM-CSF release in cells present in the arthritic joint, is not completely understood. In view of the emerging importance of CSFs (e.g. GM-CSF) and increasing evidence that COX activity regulates GM-CSF release in several cell types, we have investigated the following in human synoviocytes: (i) A comparison of NO-NSAIDs versus NSAIDs on the expression/activity of COX-2 and release of GM-CSF. (ii) The effect of a typical NO-donor on the activity of COX-2 and GM-CSF release. (iii) The effect of phosphodiesterase (PDE) inhibitors on PGE_2 and GM-CSF release. Thus, in human synoviocytes, we show for the first time that the NO-NSAIDs, NO-naproxen and NO-flurbiprofen inhibit COX-2 activity (PGE_2 release) with an increase in GM-CSF release; whilst NO-acetylsalicyclic acid (NO-ASA) despite inhibiting COX-2 activity, does not elevate GM-CSF release.

2. Materials and methods

After ethical approval, synovium was obtained from the knee joint of 12 patients undergoing routine surgery for mechanical injury. Synovial tissue was minced and following collagenase digestion, cells were resuspended in culture flasks with Dulbecco's modified Eagle medium (DMEM). Cells were grown and cultured to confluence in DMEM supplemented with non-essential amino acids and 20% foetal calf serum (37 °C in 5% CO_2 and 95% O_2). After 2–3 weeks, cells were passaged for use in experiments (passages 2–9), and thus represent populations of human synovial fibroblasts (Richards et al., 1996).

Synoviocytes confluent on 96-well plates were incubated for 24 h in serum-free DMEM and penicillin–streptomycin (100 U/ml). On the next day, cells were treated with DMEM, 10% foetal calf serum, penicillin–streptomycin (100 U/ml) and in the absence or presence of a cytokine mixture of IL-1β, tumour necrosis factor (TNF)-α and IL-6 (all 10 ng/ml) and treated to one of the following protocols (a–d) for 24 h (where COX-2 protein is present and both PGE_2 and GM-CSF release are at maximum). These cytokines were used as they are implicated in the pathogenesis of rheumatoid arthritis. All drugs were added to the medium in 0.1% v/v ratio, except for NO-ASA at the concentration of 1000 μM, a 1% v/v ratio was used. Indomethacin was used to block the endogenous production of PGE_2.

(a) NO-NSAIDs, NSAIDs (1–1000 μM) or DMSO (vehicle).
(b) Sodium nitroprusside (SNP, 0.1–100 μM) or DMSO.
(c) Exogenous PGE_2 (0.01–10 000 nM) in the presence of indomethacin (1 μM).
(d) Zaprinast (selective cGMP PDE5 inhibitor; 100 μM), 3-isobutyl-1-methylxanthine (IBMX; non-selective PDE inhibitor; 10 μM) or RO-201274 (selective cAMP PDE4 inhibitor; 100 μM) or DMSO in the absence or presence of indomethacin (1 μM).

After treatment, the medium was removed and frozen (−80 °C) until assayed. PGE_2, a marker of COX-2 activity was measured by radioimmunoassay using a commercial antibody and tritiated PGE_2, as previously described (Blotman et al., 1980). GM-CSF was determined by a specific enzyme linked immuno-sandwich assay (ELISA), using matched capture and detection antibody pairs against human GM-CSF recombinant protein (R&D systems, Minneapolis, USA). The ELISA was carried out in accordance with the manufacturer's instructions.

2.1. Determination of COX-2 expression by Western blotting

Synoviocytes were cultured and passaged in 10 cm Petri dishes. When confluent, cells were serum-starved for 24 h and then placed into DMEM containing 10% foetal calf serum, penicillin–streptomycin (100 U/ml) and a cytokine mixture of IL-1β, IL-6 and TNF-α (all 10 ng/ml) for a time course of 2–24 h, or with either NO-flurbiprofen (10 μM) or NO-ASA (100 μM) or vehicle (0.1% DMSO) for 8, 12 or 24 h. After treatment, media were aspirated and cells scraped in 250 μl of homogenisation buffer (PBS, 10 mM EDTA, 2 μM leupeptin, 1 μM pepstatin A, 1 mM PMSF, 1% Triton X-

100). The amount of total protein present in each sample was determined by the Bradford colorimetric assay (Bradford, 1976).

Protein extracts were diluted in an equal amount of gel loading buffer (20 mM Tris–HCl pH 8, 2 mM EDTA, 2% SDS, 1% mercaptoethanol, 20% glycerol, 0.02% bromophenol blue) and heated for 5 min at 95 °C. The samples were stored at −80 °C, until assayed. 10% SDS-polyacrylamide gels were prepared and after polymerisation, equal amounts of protein (30 μg) were loaded into each lane of the gels. A rainbow protein molecular weight marker and COX-2 standard (ovine electrophoresis standards, Cayman Chemicals USA) were also loaded. Gels were run at 80 V for 1.5 h and then electrically transferred to nitrocellulose membranes (Hybond-C® extra, Amersham Pharmacia Biotech, UK) using a potential difference of 100 V for 1 h. Following electrotransfer, the blots were placed on an orbital shaker and incubated overnight at 4 °C in blocking solution (5% w/v dried low-fat milk and 0.25% v/v Tween-20 in PBS). Any unspecific bound protein was washed away (3×5 min) with washing buffer (Tween-20, 0.25% v/v in PBS), after blots were probed with anti-COX-2 antibody (murine polyclonal, Cayman Chemicals USA) diluted 1:2000 and then with a horseradish peroxidase conjugated anti-rabbit secondary antibody (Santa Cruz, Biotechnology, California, USA, 1:2000). The blots were developed onto Hyperfilm™ ECL™ (Amersham Biosciences, Bucks, UK) using a Western blotting chemiluminescence luminol reagent kit (Santa Cruz, Biotechnology, California, USA), captured on film and the optical density was determined using the software, Molecular Analyst® (Bio-Rad, Hemel Hempstead, UK).

2.2. Determination of cell viability

Mitochondrial cell respiration, an indicator of cell viability, was assessed by the ability of cells to reduce 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan. At the end of each experiment, cells were incubated with MTT (0.4 mg/ml) at 37 °C for 45–60 min. Free MTT and medium were removed by aspiration. The formazan product present in the cells was solubilised in DMSO (100 μl). The extent of MTT reduction to formazan was quantified by optical density measurements at 550 nm using a Molecular Devices Microplate Reader and expressed as a percentage of respective control cells.

2.3. Materials

NicOx (Sophia Antipolis, France) supplied NO-ASA (NCX-4016), NO-flurbiprofen (HCT-1026) and NO-naproxen (HCT-3012). IBMX, Ro-201724 and zaprinast were purchased from Calbiochem, Noviabiochem (Nottingham, UK). All cytokines were purchased from Boehringer (Mannheim, Germany). The remaining compounds were obtained from Sigma Chemical (Poole, Dorset, UK). For the radioimmunoassay, PGE_2 antiserum was obtained from Sigma Chemical and radiolabelled [^{3}H]PGE_2 was obtained from Amersham Biosciences (Bucks, UK).

2.4. Statistical analysis

All data are presented as means±S.E.M. of cells from patients (assayed in duplicates) and are expressed in absolute values or as percentage changes (to normalise the data). A one-sample *t*-test was used to analyse differences between the vehicle and indicated concentrations of each drug; **P*<0.05. For a comparison of the NO-NSAIDs versus their parent compounds (NSAIDs), a two-way ANOVA and a Bonferroni post test was carried out, •*P*<0.05.

3. Results

In control cells (10 cm Petri dish) treated with vehicle for 24 h, neither COX-2 protein nor levels of PGE_2 or GM-CSF were detectable. Incubation of a cytokine mixture (IL-1β, TNF-α and IL-6) for 2–24 h, resulted in a time-dependent expression of COX-2 protein reaching a maximum at 12 h (Fig. 1A). A sharp increase in PGE_2 release appeared at 8 h, reaching a plateau at 16 h (Fig. 1B). Similarly, GM-CSF release time-dependently increased over 2–24 h (Fig. 1C). None of the NO-NSAIDs had any affect on COX-2 expression at 8, 12 or 24 h (data not shown).

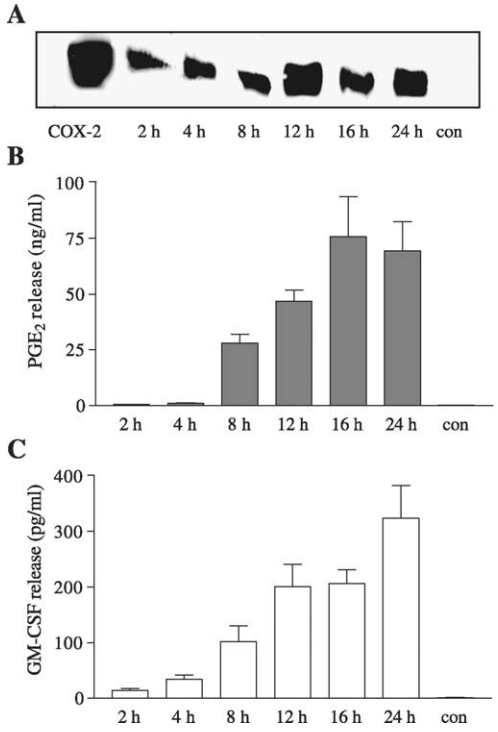

Fig. 1. Effect of cytokines (IL-1β, TNF-α and IL-6) on human synoviocytes for 2–24 h. (A) Western blot showing the expression of COX-2 protein (COX-2 standard=75 ng). (B) PGE_2 release. (C) GM-CSF release. Control cells (con) were treated for 24 h with DMEM and vehicle only. Data represent the means±S.E.M. of cells from four patients, assayed in duplicates.

3.1. Effects of NO-NSAIDs on COX-2 activity (PGE_2 release)

In synoviocytes cultured in 96-well plates, PGE_2 levels were elevated to 16±2 ng/ml (n=20) following 24 h of cytokine treatment. Levels of PGE_2 (an index of COX-2 activity) were reduced by all the NSAIDs and their NO-derivatives in a concentration-dependent manner (Fig. 2A–C). At the highest concentration, all the drugs completely inhibited COX-2 activity (PGE_2 levels). Flurbiprofen was the most potent inhibitor of COX-2 activity, with an apparent IC_{50} of less than 1 μM, whilst NO-ASA was the least potent with an IC_{50} of less than 10 μM. Both NO-ASA (10 and 100 μM) and NO-flurbiprofen (1 and 10 μM) were less potent than their parent compounds, as significant differences on the inhibition of PGE_2 release were observed at the indicated concentrations (Fig. 2A–B, •P<0.05).

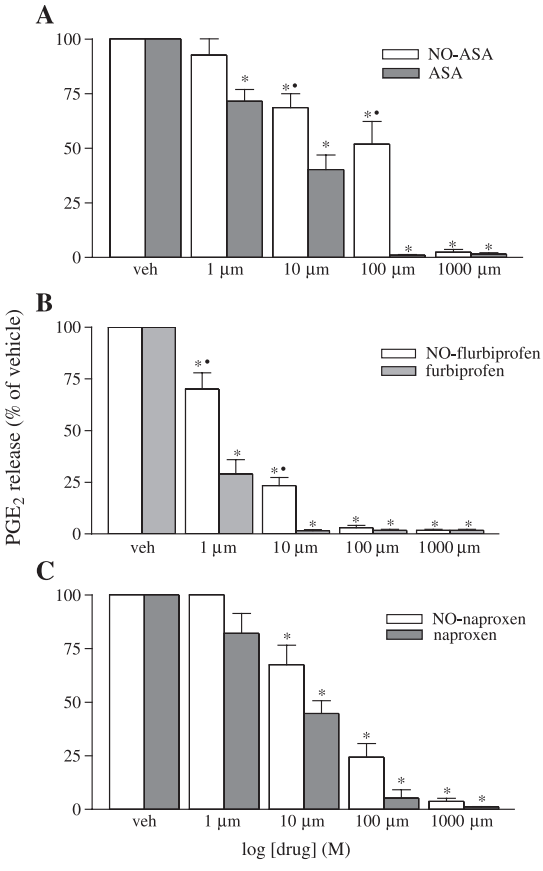

Fig. 2. Effects of (A) NO-ASA vs. ASA, (B) NO-flurbiprofen vs. flurbiprofen and (C) NO-naproxen vs. naproxen on PGE_2 release (index of COX-2 activity) by cytokine-treated synoviocytes (24 h). Data are expressed as percentages of vehicle-treated cells and represent the means±S.E.M. from four to six patients, assayed in duplicates. One-sample t-test comparing indicated concentrations of each drug to the vehicle; *P<0.05. Two-way ANOVA followed by a Bonferroni post-test; •P<0.05.

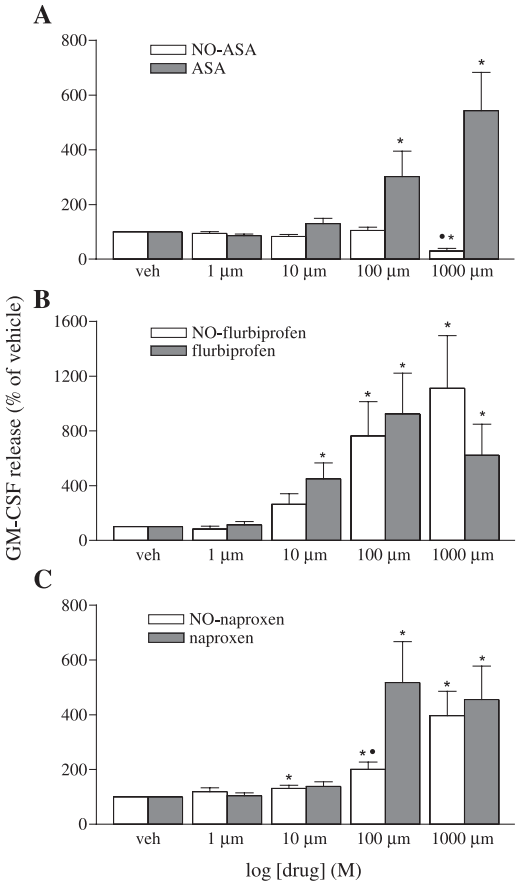

Fig. 3. Effects of (A) NO-ASA vs. ASA, (B) NO-flurbiprofen vs. flurbiprofen and (C) NO-naproxen vs. naproxen on GM-CSF release by cytokine-treated synoviocytes (24 h). Data are expressed as percentages of vehicle-treated cells and represent the means±S.E.M. from four to six patients, assayed in duplicates. One-sample t-test comparing indicated concentrations of each drug to the vehicle; *P<0.05. Two-way ANOVA followed by a Bonferroni post-test; •P<0.05.

3.2. Effects of NO-NSAIDs on GM-CSF release

In the presence of cytokines, GM-CSF levels were 258±29 pg/ml (n=17) in vehicle-treated cells. All the drugs with the exception of NO-ASA increased the levels of GM-CSF in a concentration-dependent manner (Fig. 3A–C). Like their parent compounds, NO-flurbiprofen and NO-naproxen significantly increased GM-CSF levels, at all but one concentration (1 μM). Unlike ASA, NO-ASA did not increase GM-CSF release, even at the highest concentration (1 mM), where COX-2 activity was inhibited by 90% (Fig. 3A, P<0.05). It should be noted, however, that at this concentration NO-ASA induced a modest, but significant reduction in cell viability (MTT assay, Table 1).

3.3. Effect of SNP on COX-2 activity and GM-CSF release

The NO-donor SNP (0.1–100 μM) had no effect on either COX-2 activity (PGE_2 release) or GM-CSF release in cytokine-treated synoviocytes (data not shown).

Table 1
Effects of NO-NSAIDs, NSAIDs and PDE inhibitors on cell viability (MTT assay) in cytokine-treated synoviocytes

Drugs	Optical density (% of vehicle)			
	1 μM	10 μM	100 μM	1000 μM
NO-ASA	113±11	106±9	96±8	66±2[a]
ASA	102±3	124±17	112±15	87±6
NO-flurbiprofen	99±4	102±6	108±17	91±9
flurbiprofen	100±0	125±9	108±2	117±6
NO-naproxen	100±6	96±6	102±5	100±4
naproxen	106±5	101±4	113±5	103±4
SNP	0.1 μM	1 μM	10 μM	100 μM
	95±6	96±9	97±6	88±6

Drugs	IBMX (10 μM)	Ro-201724 (100 μM)	Zaprinast (100 μM)
− indomethacin	104±8	89±6	103±8
+ indomethacin	100±4	89±5	100±4

Data are expressed as a percentages of respective vehicle-treated cells and represent the means±S.E.M. of cells from patients (n=4–6), assayed in duplicates. One-sample t-test comparing concentrations of each drug to the respective vehicles (100%).

[a] P<0.05.

3.4. Effects of cAMP and cGMP elevating drugs

To confirm the inhibitory effects of PGE_2 on GM-CSF release, endogenous PGE_2 was blocked by indomethacin (non-selective COX inhibitor) and exogenous PGE_2 was added to cytokine-treated cells. Under these conditions, exogenous PGE_2 reduced GM-CSF release (Fig. 4). To further characterise the pathway by which PGE_2 mediates its inhibitory effects, synoviocytes were treated with cAMP or cGMP elevating drugs. In the absence of indomethacin, IBMX and Ro-201724 significantly increased PGE_2 levels and simultaneously reduced the levels of GM-CSF. In contrast, zaprinast had no effect on PGE_2 or GM-CSF release (Fig. 5A–B). In the presence of indomethacin, when endogenous PGE_2 synthesis was blocked, GM-CSF release was significantly increased by five- to six-fold. Under these experimental conditions, neither IBMX nor Ro-201724 had any additional affects on GM-CSF release (Fig. 5B).

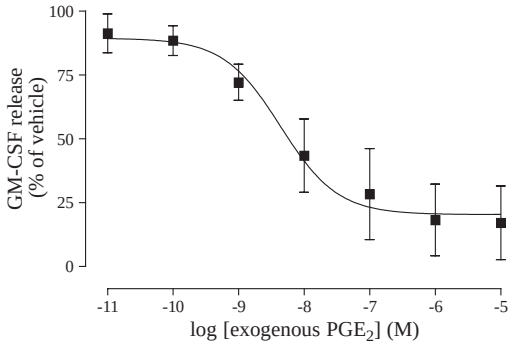

Fig. 4. A dose–response curve of the inhibitory effect of exogenous PGE_2 (0.01–10 000 nM) on GM-CSF release by cytokine-treated synoviocytes (24 h). Endogenous PGE_2 was blocked by indomethacin (1 μM). Data represent the means±S.E.M. of cells from three patients, assayed in duplicates.

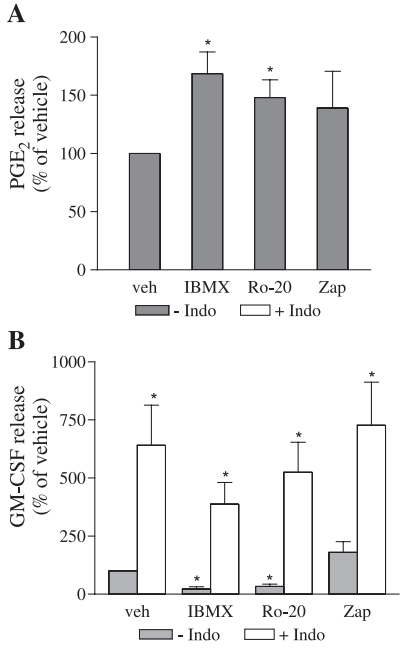

Fig. 5. Effects of IBMX (10 μM), Ro-201724 (Ro-20, 100 μM) or zaprinast (Zap, 100 μM) on (A) PGE_2 and (B) GM-CSF release from cytokine-treated synoviocytes, in the absence and presence of indomethacin (Indo, 1 μM) at 24 h. Data are expressed as percentages of vehicle-treated cells (without indomethacin) and represent the means±S.E.M. from five patients, assayed in duplicates. One-sample t-test, each drug versus vehicle (without indomethacin); *P<0.05.

4. Discussion

Here we show that in human synoviocytes, cytokines (IL-1β, TNF-α and IL-6) increase the expression of COX-2 protein and the release of PGE_2. GM-CSF is also simultaneously released under these experimental conditions. We demonstrate for the first time that in these cells, with the exception of NO-ASA, inhibition of COX-2 activity by either NSAIDs or their NO-derivatives results in increased GM-CSF release.

Prostanoids mediate pain and inflammation in rheumatoid arthritis, therefore COX inhibitors are prescribed to provide symptomatic relief, although they do not modify the course of the disease (Emery, 1996). GM-CSF is thought to contribute to the development of rheumatoid arthritis because it stimulates the proliferation of synoviocytes and in vivo it exacerbates symptoms of acute inflammatory arthritis (Bischof et al., 2000; Moss and Hamilton, 2000; Seitz et al., 1994). More convincingly is the observation that treatment with antibodies directed against GM-CSF reduces inflammation and cartilage destruction in arthritic mice (Cook et al., 2001). It is therefore important to understand how current therapies

including NSAIDs modulate the release of GM-CSF by synoviocytes.

Previously, we have demonstrated in other cell types that NSAIDs and possibly COX-2 selective inhibitors have the common property of increasing GM-CSF release and that this phenomenon is due to a reduction in COX activity (Calatayud et al., 2001; Lazzeri et al., 2001; Stanford et al., 2000b). In human vascular smooth muscle cells, the effects of endogenous COX-2 activity are mimicked by prostacyclin receptor ligands and, to a much lesser extent, PGE_2 (Stanford et al., 2000a). Consequently, we have concluded in the vasculature that prostacyclin and its receptor activation are responsible for COX-2 mediated inhibitory effects on GM-CSF release (Stanford et al., 2000a). By contrast, the same phenomenon in primary cultures of human airway smooth muscle cells is mediated by PGE_2 release and, despite the presence of COX-2 in these cells, appears to be a function of COX-1 activity (Lazzeri et al., 2001). Similarly, in human colon epithelial cells (HT-29) COX-2 and PGE_2 lower endogenous GM-CSF production (Calatayud et al., 2001). In the current study using a key cell type associated with arthritis, namely synoviocytes, we show that a similar phenomenon exists, whereby GM-CSF release is increased by ASA, flurbiprofen and naproxen at concentrations where COX-2 activity is reduced.

NO-NSAIDs are currently under clinical development and so it is of significant interest to assess the effects of NO-NSAIDs not only on COX activity in different systems, but also the related effects on other cellular mediators. First, we determined whether NO-NSAIDs affect the induction of COX-2 protein. NO-NSAIDs have been shown to inhibit the induction of iNOS, in vitro and in vivo, but not that of COX-2 (Cirino et al., 1996; Mariotto et al., 1995a,b). Our findings demonstrate that NO-NSAIDs do not affect the induction of COX-2, despite exhibiting an inhibitory effect on its enzyme activity.

NO-NSAIDs provide all the COX-inhibitory effects of conventional NSAIDs, therefore it seems predictable that they would act similarly to their parent compounds on the release of GM-CSF. Indeed, NO-naproxen and NO-flurbiprofen reduced COX-2 activity and increased GM-CSF release. This effect was most likely attributable to the inhibition of COX-2 and not to the release of a NO specie, as SNP was without any effect. NO-ASA reduced COX-2 activity, but unlike ASA, did not increase GM-CSF release. This trend was apparent at 10^{-4} M, where all other drugs increased GM-CSF release. At the highest concentration, NO-ASA significantly decreased GM-CSF release. This could be due to cellular suffering or, unlike the other NO-NSAIDs or NSAIDs, NO-ASA may have a direct effect on the expression of GM-CSF.

Our findings demonstrate that in human synoviocytes, GM-CSF release is elevated by NSAIDs and their NO-counterparts via the inhibition of COX-2 activity. Furthermore, in the presence of endogenous PGE_2, GM-CSF levels are relatively low. PDE inhibitors which elevate the levels of cAMP reduce further the levels of GM-CSF. This effect was not observed when levels of cGMP were elevated, suggesting that the inverse relationship of PGE_2 and GM-CSF release are mediated through the cAMP pathway. A review of the literature reveals that NO-ASA despite being chemically related to ASA, inhibits a number of pro-inflammatory cytokines and mediators (e.g. caspase-1 and tissue factor) that ASA does not (Fiorucci et al., 2000, 2002; Minuz et al., 2001). NO-ASA is unique and may have some particular properties not possessed by other NO-NSAIDs or indeed classical NSAIDs. This finding is substantiated by our own observations demonstrating that NO-ASA, but not ASA or other NSAIDs, reduce leukotriene release in blood from aspirin-sensitive asthmatics (Gray et al., 2002).

Acknowledgements

This study was funded by a NicOx PhD studentship awarded to P.A. Zacharowski.

A portion of this study has been published in the British Journal of Pharmacology Proceedings Supplement (2000): 133: C37.

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