Agents Actions 41: 164–170 (1994)

0065-4299/94/040164-07 $1.50+0.20/0

Effect of leflunomide on constitutive and inducible pathways of cellular eicosanoid generation

K. U. Weithmann, S. Jeske, and V. Schlotte

Hoechst AG, D-65174 Wiesbaden, Germany

Received 30 September 1993; accepted by I. Ahnfelt-Rønne 7 January 1994

Abstract. Leflunomide exerts its effects primarily via the immunomodulating and antiphlogistic activities of its major metabolite A 77 1726. Our investigation in several eicosanoid forming systems revealed that in human white blood cells the metabolite did not cause any alteration on Ca-ionophore stimulated metabolism of membrane bound arachidonic acid to cis-, trans- and epi-LTB_4. Thus, the involved enzyme systems phospholipase A_2, 5-lipoxygenase and LTA_4-hydrolase can be ruled out as a target of the drug. However, in several cellular systems the drug weakly inhibited the generation of 5-HETE and LTB_4 from exogenous arachidonic acid, possibly by interfering with the exogenous substrate's access to the 5-lipoxygenase.

In order to get information about the cyclooxygenase (COX-2) which is inducible in human PMNL by inflammatory mediators via *de novo* protein biosynthesis, we activated the cells with LPS for 18 h. A 77 1726 and indomethacin had no influence on the enzyme activity of the newly induced COX-2. However, both drugs in low concentrations were able to blunt the long term activation process resulting in PGE_2 generation.

In contrast, the prostaglandins generated by constitutive enzymes (COX-1) are probably involved in maintaining vital functions, and their inhibition by indomethacin and other nonsteroidal antiinflammatory drugs (NSAIDs) account for numerous adverse effects, for instance gastric erosion.

Our study revealed that leflunomide and A 77 1726 are not to be regarded as COX-1-inhibitors, and thus cannot be associated with the typical adverse effects of the NSAIDs.

Key words: Antiinflammatory compound – Leflunomide – Indomethacin – Human cells – PMNL – Lipoxygenase – Cyclooxygenase – COX-2

Introduction

Prostaglandins, polyunsaturated hydroxyacids and leukotrienes are metabolites of arachidonic acid which are intimately involved in many biological reactions. The therapeutic mode of action of classical nonsteroidal antiinflammatory drugs (NSAID), such as acetylsalicylic acid or indomethacin, is primarily explained by their inhibitory effect on cyclooxygenase, the key enzyme of the prostaglandin pathway.

Leukotrienes, the products of the 5-lipoxygenase pathway, represent also an important target, since they have been implicated in many inflammatory processes [1–3].

According to recent discoveries there are two forms of cyclooxygenase [4, 5]. Cyclooxygenase-1 (COX-1) was first cloned from sheep vesicular glands [6], and is found as a constitutive enzyme in most tissues including blood platelets [7]. Prostaglandins generated by constitutive pathways may exert cytoprotective effects, and are involved in maintaining vital functions in vascular hemostasis, gastric mucosa and kidney. Thus, inhibition of prostaglandins by the classical cyclooxygenase inhibitors is generally accepted as an explanation of their adverse side effects [8, 9].

COX-2 which shares about 62% aminoacid homology with COX-1, is only expressed after cell activation, especially by mitogenic or inflammatory stimuli [4, 10, 11]. Thus, specific inhibition of COX-2 could represent a superior therapeutic target in inflammatory diseases, without the burden of inhibiting the constitutive cytoprotective prostaglandins.

The new antirheumatic drug leflunomide (*N*-(4-trifluoromethylphenyl)-5-methyl-isoxazol-4-carboxamid, HWA 486) exerts its therapeutic effects primarily via its major metabolite *N*-(4-trifluoromethylphenyl)-2-cyano-3-hydroxy-crotonic acid amide (A 77 1726) [12]. The particular mode of action of this drug is explained by its immunomodulating and antiproliferative effects [13], but also by a significant antiphlogistic activity [14]. The present study deals with the drug's activity in several systems generating cyclooxygenase and lipoxygenase metabolites. Our experimental work concerns also the effects of the drug on the inducible prostaglandin-synthesizing system in human neutrophils originally described by Herrmann et al. [11].

Correspondence to: K. U. Weithmann

Materials and methods

Drugs and reagents

A stock solution of leflunomide in ethanol was prepared and diluted 10 times by volume with water in an ultrasound bath at 37 °C immediately before starting the assay. Aliquots not exceeding 2% (v/v) of the total assay volume were used in inhibition experiments, as indicated. The leflunomide-metabolite A 77 1726 was supplied as the water soluble tris-(2-hydroxyethyl)-ammonium salt by Dr. Kämmerer, Hoechst AG.

Leflunomide is practically stable in aqueous solution at pH values between 7.0 and 8.0 at least for 1 h. Ring opening, resulting in the formation of A 77 1726 could be observed only at pH > 8.0.

Lipopolysaccharide (LPS, salmonella abortus equii) and the Ca-ionophore A 23187 were from Sigma (Deisenhofen, FRG). All cell culture media and supplements were obtained from Gibco (Eggenstein, FRG), and sheep seminal vesicles (SSX) from Paesel (Frankfurt, FRG), whereas Lymphoprep™ was from Dr. Molter (Heidelberg, FRG). Arachidonic acid was purchased from Biodata (Hatboro, PA, USA), and 1-C-14-arachidonic acid as well as all tritiated eicosanoids from NEN (Dreieich, FRG). All other chemicals and biochemicals were of analytical grade. A test kit for the ELISA of PGE_2 was available from Amersham (Braunschweig, FRG).

Drug effects on formation of lipoxygenase products in human white blood cells (WBC)

Preparation of WBC. WBC were prepared according to [15]. Briefly, 40 ml of freshly drawn citrated blood were admixed to 8 ml of PM16-buffer, containing 6% (v/v) dextrane (m.w. = 480 000) and incubated at room temperature for 1 h. The supernatant containing the WBC was removed, diluted 1:1 (v/v) with PM16 and centrifuged for 15 min at 300 × *g*. The precipitate was resuspended in PM16 and adjusted to 10^7 cells/ml (Counter HT, Coulter Electronics, Krefeld, FRG).

Metabolism of endogenous arachidonic acid to LTB_4 in WBC. In a total volume of 0.3 ml WBC suspension, at 37 °C, the reaction tube contained 2 mmol/l $CaCl_2$, 0.5 mmol/l $MgCl_2$ and drug as indicated. After 15 min preincubation the reaction was started by addition of 12.5 µg of the Ca-ionophore A 23187 and 2 µg glutathione. After 5 min the reaction was stopped by addition of 30 µl 0.1 M HCl at 0 °C. After centrifugation for 2 min at 0 °C, aliquots of the supernatant were subjected to HLPC as described in [16], using a C-18 Nucleosil column (5 µm, 100 × 3 mm, Chrompack GmbH, Frankfurt, FRG) and, at a flow rate of 0.7 ml/min, a solvent mixture consisting of 725 ml methanol, 275 ml water and 0.1 ml acetic acid. The formation of cis-, trans- and epi-LTB_4 was followed at its UV-maximum of 278 nm and identified by comparison with the authentic isomeres.

Metabolism of exogenous arachidonic acid to LTB_4 in WBC. The same procedure was followed, however, 0.1 mmol/l arachidonic acid was admixed along with the drug.

Drug effects on formation of eicosanoids from C-14-arachidonic acid in human PMNL and murine bone marrow cells (BMC)

A purified PMNL-preparation was prepared from freshly drawn citrated human blood by the standard procedure, utilizing lymphoprep, as published by Boyum [17]. BMC, type KM 11, were a gift from Dr. R. R. Bartlett, Hoechst AG, Frankfurt/M, FRG. The cell preparations were adjusted to about 2×10^7 cells/l (Coulter Counter). The method of HPLC determination of cellular metabolites of exogenous 1-C-14-archidonic acid, as published by Borgreat and Samuelsson [18], was modified, as briefly described:

0.1 ml of cell suspension were incubated in Dulbeccos phosphate buffered saline (DPBS) at 37 °C with the drugs for 15 min. The incubation was then interrupted by cooling in an ice bath. Calcium ionophore A 23187 (final concentration 7×10^{-5} mol/l) and 1-C-14-arachidonic acid (final concentration 8.4×10^{-5} mol/l, 0.5 µCi) were added, and after a second incubation period of 15 min at 37 °C, terminated by the addition of 0.4 ml methanol. The assay mixture was then extracted with chloroform. The chloroform was evaporated and the residue re-dissolved in a minor amount of methanol/water and analyzed by HPLC and radiomonitoring for the C-14-eicosanoids.

HPLC-conditions were as follows: Column: nucleosil C-18, 5 µm; organic phase 1: 700 ml methanol, 300 ml water, 0.1 ml acetic acid; organic phase 2: methanol. Flow: 1 ml/min 2000 psi. Radiomonitor: LB 503 (Berthold, Wildbad, FRG).

Viability assay (trypan blue exclusion) showed that at least 95% of the cells remained intact during incubation periods.

Table 1. Effects of drugs of cyclooxygenase-associated systems *in vitro*, as indicated. Each experiment was run in quadruple. For experimental details see under 'methods'. SSV, sheep seminal vesicles: PRP, platelet rich plasma.

	Drug (mol/l)	% residual		
		PGE_2-formation		aggregation in human PRP
		in SSV	in rabbit mucosa	
leflunomide				
	1.0×10^{-5}		92±13	
	2.2×10^{-5}			83±5
	3.7×10^{-5}	97±4		
A 77 1726				
	1.0×10^{-5}		108±16	
	1.4×10^{-5}			91±7
	2.2×10^{-5}	112±7		
indomethacin				
	1.0×10^{-5}		12±8	4±3

Drug effects on constitutive prostaglandin formation

Formation of 1-C-14-PGE_2 in rabbit mucosa or sheep seminal vesicles in vitro. The assay was conducted as described in [19, 20]. The total volume of 0.25 ml contained: 10 mmol/l potassium phosphate (pH = 8.0), enzyme system (50 µg homogenized rabbit mucosa [21] plus 0.15 mmol/l epinephrine or 0.125 mg sheep seminal vesicles [22] plus 0.1 mol/l glutathion), and drugs as indicated in Table 1. The reaction was started by the addition of 0.7 to 8 µmol/l 1-C-14-arachidonic acid and terminated after an incubation period of 5 to 15 min at 37 °C by the admixture of 50 µl of 1.3 mol/l citric acid. Then the radioactivity was extracted twice with ethylacetate (1 ml), the combined and dried extract re-dissolved in 0.2 ml ethylacetate/5 µl acetic acid and aliquots separated by TLC (silicagel 60, 110 ml ethylacetate, 100 ml water, 50 ml isooctane, 20 ml acetic acid, upper phase) or by a modified HPLC procedure at a flow rate of 1.5 ml/min, using a C-18 Nucleosil column (5 µm, 125 × 4.6 mm, Bischoff, Leongang, FRG), connected with a pre-column C-18 Nucleosil (5 µm, 20 × 4.6 mm) of the same type. The formed 14-C-eicosanoids were analyzed using a liquid scintillation flow detector LB 503 (Berthold, Wildbad, FRG).

The PGE_2 peak was analyzed by comparison with authentic tritiated eicosanoids, using integrated chromatographics computer software. The solvent systems used were as follows (elution time in

parenthesis): I 725 ml water/275 acetonitrile/1 ml acetic acid (40 min), II 700 ml methanol/300 ml H_2O/1 ml acetic acid (40 min), III pure methanol (20 min).

Arachidonic acid induced aggregation of human platelets was performed turbidometrically in a Born aggregometer (Labor GmbH, Hamburg, FRG). Drugs were preincubated at 37 °C with 0.25 ml samples of freshly prepared citrated human platelet rich plasma (PRP) (2×10^8 platelets/ml) for 10 min, before aggregation was induced by 1 mmol/l arachidonic acid as described in [23].

Stimulation of inducible prostaglandin pathway in human PMNL

The procedure of Herrmann et al. [11] was slightly modified to stimulate cycloheximide-inhibitable generation of PGE_2 in human PMNL by LPS.

2.5×10^6 PMNL/ml culture medium (RPMI 1640, completed with 1 mmol/l sodium pyruvate, 5% FCS (w/v), 2 mmol/l glutamine and each 100 U/ml penicillin/streptomycin) were incubated with 100 µmol/l acetylsalicylic acid for 60 min (37 °C, 5% CO_2), which was subsequently removed by four times washing with medium. 0.25 ml-aliquots (containing 2.5×10^6 PMNL/ml medium) were incubated with 0.1 mg/ml LPS along with the test compound for 18 h (96-well plates, 37 °C, 5% CO_2), and the generation of eicosanoids induced by the admixture of 7×10^{-5} mol/l of the calcium ionophore A 23187. After incubation for 30 min at 37 °C the plates were centrifuged at 400 g for 15 min, and PGE_2 determined in the supernatant, using a commercially available ELISA-kit. Alternatively the test compound was added along with the calcium ionophore.

At least 90% of the cells remained intact during incubation times (trypan blue exclusion assay).

Statistics

Normal distribution was confirmed graphically for the data as described in [24], and the data were shown as means ± SEM. Significance was determined by two-tailed and unpaired Student's *t*-test. *P*-values below 0.05 were considered significant.

Results

As demonstrated in Fig. 1A, generation of LTB_4 in a crude human WBC preparation could be followed by HPLC with spectrophotometrical identification at 278 µm, the wavelength characteristic for conjugated triene systems [16]. It was possible to separate the three formed isomers from each other as well as from the 20-substituted LTB_4-metabolite. The cis-LTB_4 is generated enzymatically by the action of the LTA_4-hydrolase, whereas the epi- and trans-isomers are formed by non-enzymatic hydrolysis.

Fig. 1A shows that the generation of the LTB_4-isomers was not inhibited by A 77 1726 after stimulation of the cells with the Ca-ionophore A 23187. Furthermore, A 77 1726 did not alter the ratio between the peaks corresponding to the LTB_4-isomers. Increasing nonenzymatic hydrolysis would be expected, if A 77 1726 would have blocked LTA_4-hydrolase.

The result of the same experiment, however with the exception that 100 µmol/l exogenous arachidonic acid was added, is shown in Fig. 1B. Under these conditions 100 µmol/l A 77 1726 exerted about 40% reduction of LTB_4 generation.

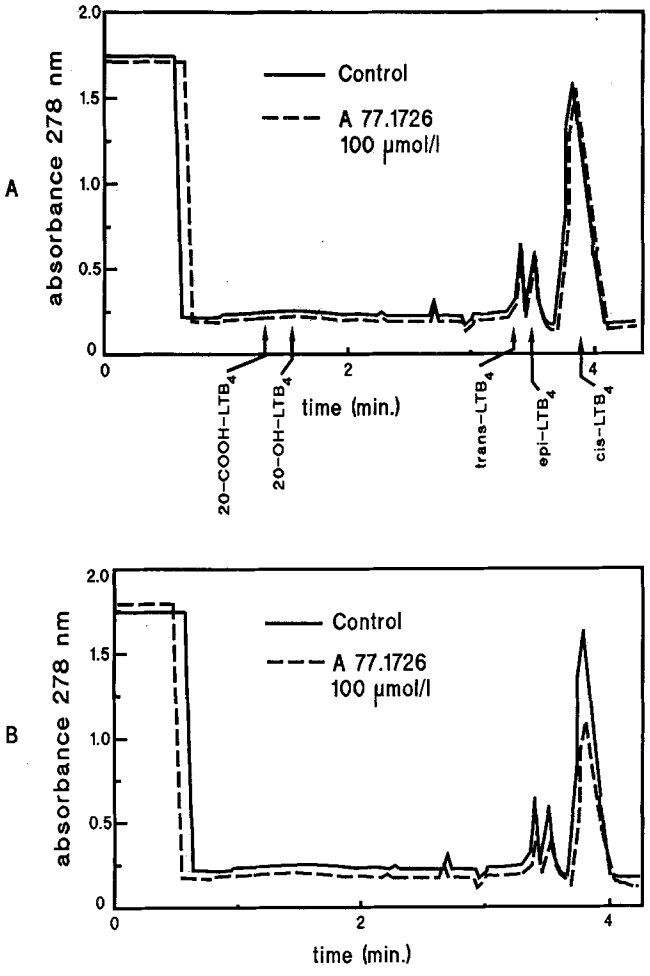

Fig. 1A. Effect of 100 µmol/l A 77 1726 on Ca-ionophore stimulated metabolism of endogenous arachidonic acid to the isomers of LTB_4 in human WBC *in vitro*. LTB_4 was separated by HPLC/UV-detection, and identified by comparison with authentic standard metabolites, as indicated. Control experiment was performed without drug.

Fig. 1B. Effect of 100 µmol/l A 77 1726 on Ca-ionophore stimulated metabolism of exogenously added arachidonic acid to the isomers of LTB_4 in human WBC. Experiment was performed as described under Fig. 1A, with the exception that 100 µmol/l arachidonic acid was incubated along with the drug.

In purified human PMNL, the metabolism of 1-C-14-arachidonic acid, induced by the Ca-ionophore A 23187 could be analysed by HPLC with radiodetection. As demonstrated in Fig. 2 no cyclooxygenase products are detectable in the pure human PMNL system. 100 µmol/l A 77 1726 inhibited the metabolism of arachidonic acid to LTB_4 and 5-HETE by about 50% whereas the metabolites of other lipoxygenase pathways, 12- and 15-HETE, remained unaffected. The effects of several concentrations of A 77 1726 on generation of LTB_4 and 5-HETE is summarized in Fig. 3.

In Fig. 4 it is shown that the murine bone marrow cells employed here, were able to produce cyclooxygenase as well as lipoxygenase metabolites upon stimulation with A 23187. 100 µmol/l leflunomide suppressed the

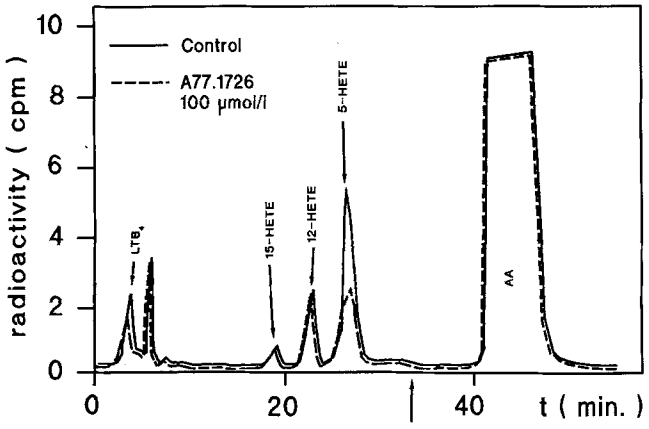

Fig. 2. Effect of 100 µmol/l A 77 1726 on Ca-ionophore stimulated metabolism of C-14-arachidonic acid in human PMNL *in vitro*. The formed eicosanoids were separated by HPLC/radiodetection, and identified by comparison with authentic metabolites. The unidentified peak is presumably HHT. Arrow indicates change of solvent system. Control experiment was performed without the addition of drug.

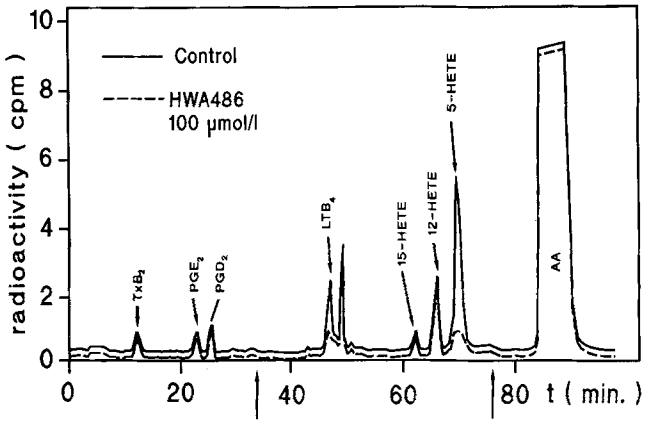

Fig. 4. Effect of 100 µmol/l leflunomide (HWA 486) on the Ca-ionophore stimulated metabolism of C-14-arachidonic acid in murine bone marrow cell culture. The formed eicosanoids were separated by HPLC/radiodetection, and identified by comparison with authentic metabolites. The unidentified peak is presumably HHT. Arrows indicate changes of solvent systems. Control experiment was performed without the addition of drug.

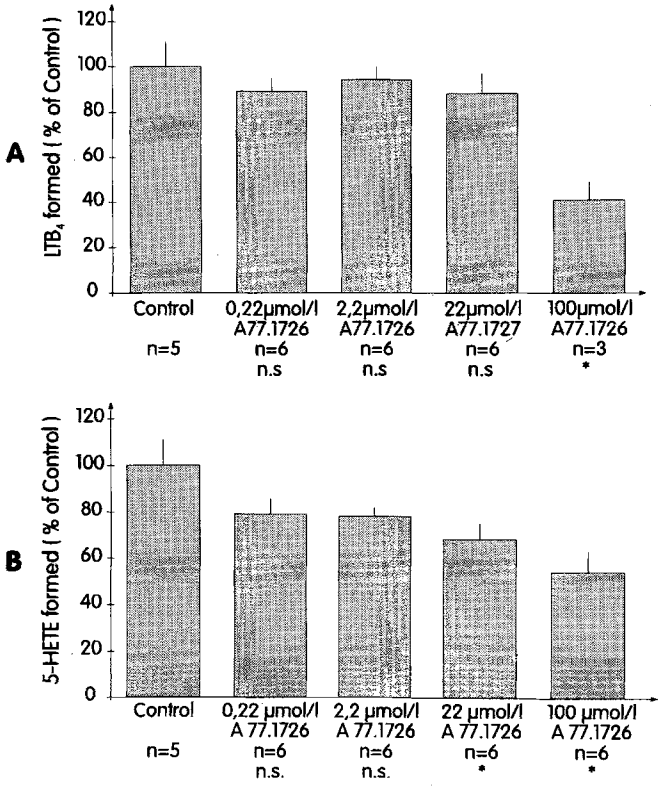

Fig. 3A. Effect of indicated concentrations of A 77 1726 on Ca-ionophore stimulated metabolism of C-14-arachidonic acid to C-14-LTB_4. Experiment was performed as described under Fig. 2.

Fig. 3B. Effect of indicated concentrations of A 77 1726 on Ca-ionophore stimulated metabolism of C-14-arachidonic acid to C-14-5-HETE. Experiment was performed as described under Fig. 2.

formation of 5-HETE and LTB_4 almost completely, whereas there were no such effects towards 12- and 15-HETE and the prostaglandins.

As summarized in Table 1, neither leflunomide nor its metabolite A 77 1726 exerted a marked inhibitory effect on systems dependent on constitutive cyclooxygenase (COX-1), such as sheep seminal vesicles or homogenized rabbit mucosa. Furthermore, these drugs showed no marked effects in the model of arachidonic acid induced aggregation of human platelets.

As shown in Fig. 2 there are practically no cyclooxygenase products to be found after incubation of 1-C-14-arachidonic acid with Ca-ionophore stimulated purified human PMNL. However, as first demonstrated by Herrmann et al. [11], incubation of the cells with LPS for several hours led to the appearance of the cyclooxygenase product PGE_2 in amounts high enough to be detected by the ELISA-technique. This inducible activity is in general referred to a newly generated cyclooxygenase (COX-2). As demonstrated in Fig. 5A, B the induction of this new enzymatic activity could be demonstrated after incubation of the cells with 0.1 mg/ml LPS for 18 h. After this incubation period the addition of A 23187 led to the generation of PGE_2, whereas after a similar treatment of the cells with LPS for 30 min only negligible amounts of this prostaglandin could be generated by the Ca-ionophore. The addition of 0.1 mmol/l acetylsalicylic acid at this stage led to an insignificantly stronger suppression of prostaglandin synthesis. After removal of the acetylsalicylic acid the LPS induced long term induction of this enzymatic activity could be suppressed, when 10 µmol/l of the protein biosynthesis inhibitor cycloheximid (CHX) where incubated along with LPS, as shown in Fig. 5A.

The PGE_2 generating activity, once induced after 18 h incubation with LPS, could not be inhibited by A 77 1726 or by the classical NSAID indomethacin even by incubating times up to 30 min, and in rather high drug concentrations up to 100 µmol/l for each drug (Fig. 5B).

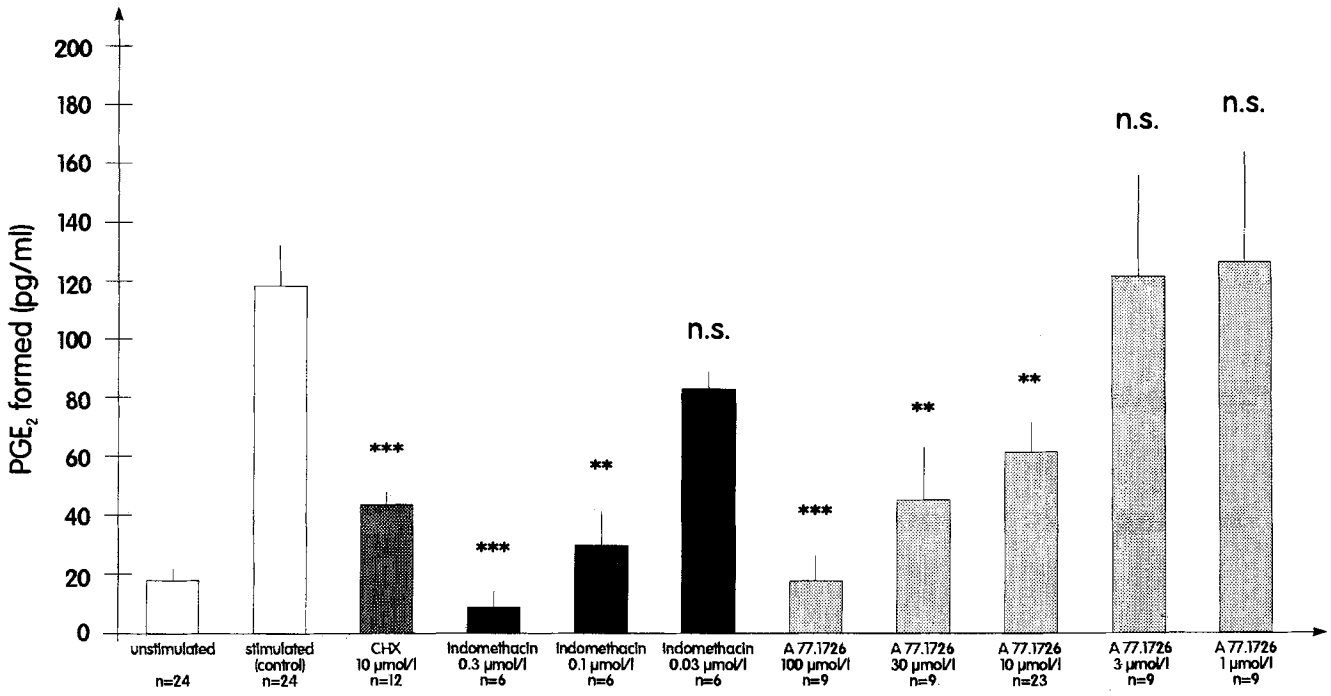

Fig. 5A. Effect of cycloheximide (CHX), indomethacin and A 77 1726 on activation of PGE_2-pathway in human PMNL by LPS. Column marked with *unstimulated* represents the Ca-ionophore induced PGE_2-level in PMNL supernatant immediately after the addition of 10 µmol/l LPS. *Stimulated (control)* means the Ca-ionophore induced PGE_2-level after the activation of PMNL with LPS for 18 h. Simultaneous addition of LPS with CHX or indomethacin or A 77 1726 in the indicated amounts for 18 h led dose dependently to a significant suppression of the Ca-ionophore inducible PGE_2-formation, as indicated by the respective columns.

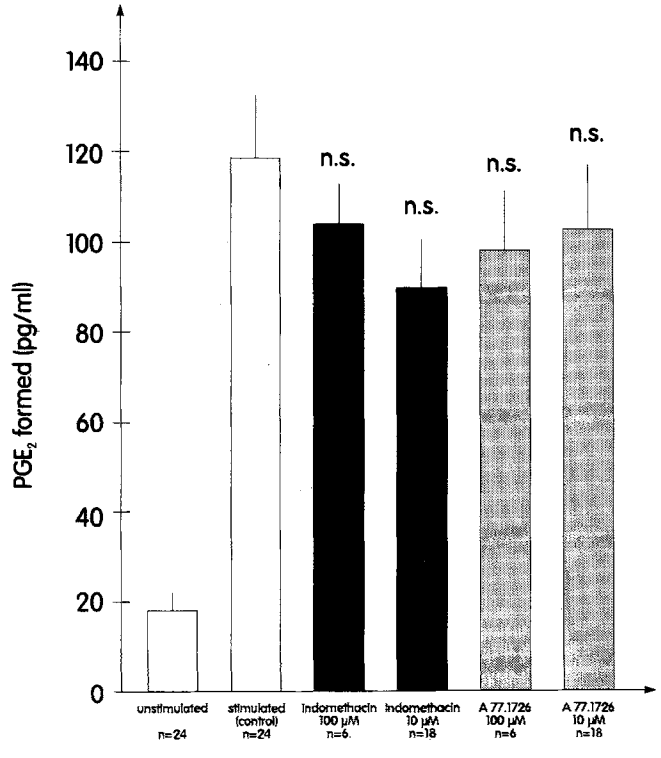

Fig. 5B. Effects of indomethacin and A 77 1726 on LPS-activated PGE_2-pathway in human PMNL. Indicated drugs were added after the 18 h activation period, and their effect on Ca-ionophore inducible PGE_2-formation after further 0.5 h of incubation was determined.

However, the LPS-induction of the PGE_2 generating enzymatic activity could significantly be suppressed by 10 µmol/l of A 77 1726 and 0.1 µmol/l indomethacin. This indicates that both drugs are able to inhibit the long term induction of cyclooxygenase activity, but not the enzymatic activity itself (Fig. 5A).

Discussion

In search for new antiinflammatory compounds numerous targets within the arachidonic acid cascade have been evaluated. Due to the significant inflammatory effects of the metabolites of 5-lipoxygenase, appropriate inhibitors of the enzyme or of the closely associated 5-lipoxygenase activating protein have attracted considerable attention [25]. An additional specific target represents the LTA_4-hydrolase, which stereospecifically hydrolyses LTA_4 to the biologically active cis-LTB_4, instead of the biologically inactive hydrolysis products trans- and epi-LTB_4.

In our experiments, we stimulated the liberation of endogenous arachidonic acid and the subsequent 5-lipoxygenase pathway by Ca-ionophore in a preparation of human white blood cells.

In this system we found that A 77 1726, as the active metabolite of leflunomide, did not exhibit any significant effect towards the 5-lipoxygenase pathway since the drug was not able to suppress the formation of the LTB_4-isomers. Furthermore, we did not detect any increase of

the nonenzymatic products trans- and epi-LTB_4 at the expense of decreased cis-LTB_4, which would be expected after inhibition of LTA_4-hydrolase.

Thus, it is unlikely that the activity of the drug is associated with any inhibitory effect towards the 5-lipoxygenase protein complex or the LTA_4-hydrolase in the white blood cell system. The preceding step of arachidonic liberation, which is catalysed by phospholipase A_2, can also be ruled out as target of the drug.

In contrast, the drug exerted inhibitory effects on 5-lipoxygenase metabolites as soon as exogenous arachidonic acid was added to the system. There was again no virtual alteration of the LTB_4-isomer ratio. Similar inhibitory effects of leflunomide or A 77 1726 towards the metabolism of C-14-arachidonic acid to 5-HETE and LTB_4 could be verified in human PMNL, rat peritoneal cells, and in a murine bone marrow cell line. The formation of other eicosanoids, especially the prostaglandins, which are formed by the two latter mentioned cell types, were not inhibited. An obvious explanation for these effects might be that the drug is able to interfere with the access of exogenous arachidonic towards the 5-lipoxygenase system. A more simple explanation could be that 5-lipoxygenase in certain cell types is inhibited by the drug, whereas in others it is not.

The prostaglandin pathway via cyclooxygenase is generally accepted as the major target of classical NSAIDs, such as acetylsalicylic acid or indomethacin. Their antiinflammatory effects are thought to be related to their ability to inhibit cyclooxygenase in synovium and inflammatory cells. However, prostaglandins are found ubiquitously in many different cell types. They are involved in maintaining physiological functions, for instance regulating gastric, renal, and vascular homeostasis. Thus, the general inhibitory effects on prostaglandin formation by the classical NSAID explain why they are often associated with adverse effects, such as prolongation of bleeding time, nephrotoxicity, and gastrointestinal erosion.

In contrast to the classical NSAIDs, leflunomide or its metabolite A 77 1726 did not exert any inhibition on various prostaglandin generating systems. In detail, we found no significant effects on the formation of PGE_2 in a rabbit gastric mucosa preparation and in sheep seminal vesicles. There was also no alteration in the pattern of the several cyclooxygenase metabolites formed in human white blood cells, rat peritoneal cells and the murine bone marrow cell line. The drug is also not an inhibitor of the cyclooxygenase dependent arachidonic acid induced aggregation of human platelets.

Resting PMNL are not able to produce significant amounts of cyclooxygenase products. However, as first shown by Herrmann et al. [11], it is possible to induce a new prostaglandin generating system by stimulation with inflammatory mediators, such as LPS. As already pointed out, this new cyclooxygenase, generally refered to as COX-2, differs from the constitutive COX-1, and can be induced in many cell types by appropriate activation.

Employing purified human PMNL, we were able to activate these cells by long term incubation with LPS. Instead of the subsequent addition of exogenous arachidonic acid [11], we stimulated the formation of PGE_2 with Ca-ionophore A 23187. In our experiments, this new enzyme activity, once formed, could not significantly be inhibited by indomethacin, even in the high amount of 100 µmol/l, evidencing again that this new induced cyclooxygenase activity is significantly different from COX-1.

The leflunomide-metabolite A 77 1726 showed a similar action in the activated cell system, and did not exert significant effects on COX-2.

In our experiments we could confirm, that the LPS induced activation of the new PGE_2-generating pathway could be suppressed by cycloheximide, indicating that protein biosynthesis is involved [11].

Similar effects in this system could be shown for indomethacin as well as for A 77 1726. Both compounds significantly and dose dependently suppressed the LPS induced activation of the newly formed cyclooxygenase system.

It is evident, that these specific inhibitory effects may contribute to the understanding of the mode of action of these drugs. Yet our experimental data do not allow to describe the molecular target of the drugs. Thus, it remains to be elucidated whether the drugs interfere directly with protein biosynthesis or, for instance, with preceding steps related to the action of LPS.

Evaluation of various antiinflammatory drugs as direct inhibitors of murine COX-1 and COX-2 revealed that in particular 6-methoxy-2-naphthylacetic acid preferentially inhibited COX-2 and thus might spare cytoprotective prostaglandin synthesis [26]. Although there is apparently no such direct enzyme inhibition by indomethacin and the leflunomide metabolite, our investigation allows the conclusion, that both of these drugs suppress the COX-2 catalysed formation of prostaglandins at the earliest stage, and that is the biosynthesis of the COX-2 protein. Moreover, the investigated drug metabolite A 77 1726, in contrast to indomethacin, does not affect the constitutive COX-1.

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