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Biological Actions of the Free Acid of Hepoxilin A_3 on Human Neutrophils

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ABSTRACT. In earlier reports and reviews, it was suggested that unlike its methyl ester, the free acid form of the 12-lipoxygenase-derived eicosanoid hepoxilin A_3 (HXA_3) does not enter neutrophils and other cells. Therefore, in the past, most studies on the biological activities of HXA_3 on human neutrophils were conducted with its methyl ester. Here, we present evidence that free HXA_3 is biologically active towards human neutrophils at submicromolar concentrations, which may occur under certain circumstances *in vivo*. Thus, HXA_3 caused chemotaxis at concentrations as low as 30–40 nM, an effect which was attenuated at higher concentrations of this eicosanoid. Its chemotactic potency proved to be comparable to that of leukotriene B_4, but higher than that of the chemotactic peptide formyl-methionyl-leucyl-phenylalanine (fMLP), and greatly exceeded that of the other 12-lipoxygenase metabolite, 12(*S*)-hydroxy-5,8,10,14-eicosatetraenoic acid, which was inactive at comparable concentrations. The chemotactic activity of HXA_3 was not abolished by serum albumin, but it was suppressed by pertussis toxin. Unlike fMLP, at this concentration range HXA_3 did not cause respiratory burst or aggregation of the neutrophils or activation of protein kinase C. These observations suggest a remarkably selective and specific receptor-mediated process. At concentrations higher than 1 μM, HXA_3 gives rise to an instantaneous release of calcium from intracellular stores which causes, however, only a slight, if any, liberation of arachidonic acid. On the other hand, pretreatment of the neutrophils with submicromolar concentrations of HXA_3 significantly blunts the liberation of arachidonic acid caused by fMLP. BIOCHEM PHARMACOL **59**;4: 435–440, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. arachidonic acid metabolism; chemotaxis; cell signalling; hepoxilins; neutrophils; 12-lipoxygenase-derived eicosanoids

Hepoxilins are hydroxyepoxy eicosatrienoic acids (HEpETrE) that are formed by a number of mammalian cells via the 12-lipoxygenase pathway of the arachidonic acid metabolism [1–4]. The first hepoxilin was isolated from human platelets and identified as 10-hydroxy-11,12-epoxyeicosa-5,8,14-trienoic acid [5], subsequently named hepoxilin B_3 [6]. Platelets have also been shown to convert arachidonic acid under special metabolic conditions to a mixture of several trihydroxyeicosatrienoic acid isomers (later also collectively named "trioxilins") that were tentatively identified as hydrolytic secondary products from the corresponding hydroxyepoxy eicosanoids [7–9]. In 1983, Pace-Asciak and co-workers demonstrated the conversion of 12-HpETE† to a mixture of HXA_3 and hepoxilin B_3 by a protein fraction from rat lungs [10]. One year later, Pace-Asciak and Martin observed the first biological activity of hepoxilins, a stimulation of the glucose-induced release of insulin from the pancreatic islets of Langerhans [6]. A number of other biological activities have been found since, most of them selectively exerted by HXA_3, which therefore appears to be the most important bioactive hepoxilin [1, 2, 4]. Formation of hepoxilins and their hydrolysis products has been observed in a number of cells and tissues (for a review, see [3]). The capability of synthesising hepoxilins appears to coincide with the presence of 12-lipoxygenase. The mechanism of conversion of 12-HpETE to hepoxilins is not yet fully understood. Apparently, several routes may exist in mammalian cells. Both non-enzymatic catalysis by haem compounds [11] and a putative hepoxilin A_3 synthase in rat pineal glands [12] have been reported. We recently demonstrated that with 12-HpETE as substrate even 12-lipoxygenases *per se* possess intrinsic hepoxilin synthase activity by virtue of their hydroperoxidase activities.‡ In earlier work, it was presumed that the free acid of HXA_3 cannot enter the cell, in contrast to its methyl ester [13]. Therefore, the effects of this eicosanoid on human neutrophils were studied by several investigators via the use of HXA_3 methyl ester [14–16]. It was found that the methyl ester causes a release of calcium from intracellular

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† *Abbreviations:* HXA_3, hepoxilin A_3 (8-hydroxy-11,12-epoxyeicosa-5,9,14-trienoic acid); 12-HpETE, 12-hydroperoxy-5,8,10,14-eicosatetraenoic acid; 12-HETE, 12-hydroxy-5,8,10,14-eicosatetraenoic acid; fMLP, formyl-methionyl-leucyl-phenylalanine; Fura-2/AM, 1-[2-(5-carboxyoxazol-2-yl)-6-aminobenzofuran-5-oxyl]-2-(2′-amino-5′-methylphenoxy) ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid pentaacetoxymethyl ester; and Glc, glucose.

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stores and a liberation of arachidonic acid. Since the methyl ester is intracellularly hydrolysed into the free acid and both forms seem to be active [17], it is difficult in such experiments to discriminate by what form of HXA_3 any biological action is exerted; only the effects of the free acid may be considered as biologically relevant.

Because of these difficulties, we investigated the action of the free acid form of HXA_3 on the release of intracellular calcium in human neutrophils and found it to be active,* which was in sharp contrast to earlier reports. Following on our work, Reynaud *et al.* restudied the actions of HXA_3 and its methyl ester and found that the failure to demonstrate biological activity of the free acid was due to an artifact caused by the use of an unsuitable solvent [17]. In this study, we describe further biological activities of HXA_3 on human neutrophils not recognised up to now and confirm thereby that the free acid of HXA_3 is bioactive towards human neutrophils.

MATERIALS AND METHODS

Chemicals

Unesterified hepoxilin A_3 was purchased from both Biomol and Calbiochem and gave identical results; for the experiments, the compound was generally used as a stock solution in methanol or ethanol and added to the set-ups in such a way that the final concentration of solvent was less than 0.5% by volume. The identical volume of solvent was added to the control samples. fMLP and Fura-2/AM were products from Sigma, [1-^{14}C]arachidonic acid from Amersham.

Cell Preparation and Pretreatment

Peripheral blood was withdrawn from healthy volunteers by veinpuncture into citrated tubes. Neutrophils were isolated as described earlier [18], with some modifications. Briefly, Ficoll-Hypaque gradient centrifugation followed by dextran sedimentation was employed. Contaminating erythrocytes were eliminated by hypotonic lysis in water for 20–30 seconds. The isolated cells were suspended in PBS/BSA/Glc medium without $CaCl_2$ in a final concentration of 20×10^6 cells/mL. For labelling of membrane lipids, the neutrophils were incubated at 37° for 20 min with 1.4 μM [1-^{14}C]arachidonic acid (specific activity: 50 mCi/mmol). Thereafter, the cells were washed twice with PBS/BSA/Glc to remove unesterified fatty acids and resuspended in the same medium at a final concentration of 5×10^6 cells/mL. For kinetic measurements of intracellular calcium concentration, the neutrophils were incubated with 5 μM Fura-2/AM at 37° for 40 min. Excess Fura-2/AM was removed by centrifugation and washing of the cells, which were then resuspended in PBS/BSA/Glc (5×10^6 cells/mL) and kept on ice until measurement.

Measurement of Chemotaxis and Aggregation of Neutrophils and Release of Intracellular Calcium

Chemotaxis was performed using a Neuro Probe standard 48-well chemotaxis chamber (Neuro Probe Inc.) and polycarbonate filters (Costar) of 3-μm pore size. Briefly, the neutrophils were suspended in PBS/BSA/$CaCl_2$ (2×10^5 cells/mL). Stock solutions of HXA_3 and fMLP were diluted in the same buffer and added to the bottom wells (27 μL). Neutrophil suspensions (50 μL) were placed on the top wells and incubated at 37° (5% CO_2, 100% humidity) for 2 hr. The cells were visualised by staining with either acridine orange or the Dade® Diff-Quik® staining set and counted. Each estimate was performed in duplicate and six squares of equal size per well were randomly counted. The aggregation of human neutrophils and the release of intracellular calcium were measured as described earlier [19], with minor modifications. For the intracellular calcium measurement, the cells were preincubated at 37° for 5 min in the presence of 1.2 mM $CaCl_2$ and 1 mM $MgCl_2$ before the addition of HXA_3 or fMLP. The fluorescence changes of Fura-2/AM were measured by the use of the F-4500 fluorescence spectrophotometer and the F-4500IC software package (Hitachi). The excitation wavelengths were 340 and 380 nm. The fluorescence signals were calibrated by the addition of 10% Triton X-100 to the cells to permit equilibration of intracellular and extracellular Ca^{2+} (maximum fluorescence), followed by addition of 250 mM $MnCl_2$ to completely quench the Ca^{2+} signal (minimum fluorescence).

Measurements of Respiratory Burst and Protein Kinase C Activity

Two independent methods were applied. Agonist-induced antimycin A-insensitive oxygen uptake of 10^6 neutrophils was recorded in PBS/BSA/Glc at 37° with an Oxygen Meter Model 781 (Strathkelvin Instruments) equipped with a micro Clark electrode 1302 in a closed chamber of 500 μL. Under these conditions, 100 nM fMLP caused a strong transient oxygen uptake ("respiratory burst") for 1–2 min (initial rate: 3.5 ± 0.3 nmol/min) that was completely prevented by pretreatment of the cells with 1 μM staurosporin, an inhibitor of protein kinases C. A second method was the spectrophotometric measurement of the superoxide anion production with 5×10^5 cells in PBS/BSA/Glc due to the superoxide dismutase-inhibited reduction of ferricytochrome *c*. A UVmax Kinetic Microtitre Reader (Molecular Devices) was used. Similar results were obtained with the microoxygraphic and spectrophotometric methods. Protein kinase C activity was measured in permeabilised cells as described by Heasley and Johnson [20].

Liberation of Arachidonic Acid

The [1-^{14}C]arachidonic acid-labelled cells were incubated at 37° for 5 min in the presence of 1.2 mM $CaCl_2$ and 1

* Sutherland M and Nigam S, unpublished results.

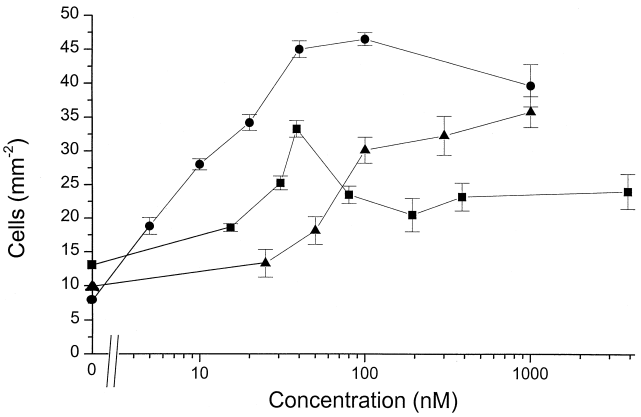

FIG. 1. HXA_3 evoked chemotaxis of human neutrophils. Chemotaxis by HXA_3 (squares), leukotriene B_4 (circles), and fMLP (triangles) was measured as described in Materials and Methods. Mean values ±SE of six estimations using three different preparations of neutrophils are given.

mM $MgCl_2$ before the addition of fMLP or HXA_3. Unless stated otherwise, the cells were incubated for another 5 min in the presence of the agonists. The effect of HXA_3 on fMLP-induced arachidonic acid release was determined by incubating the cells with HXA_3 for 5 min before the addition of fMLP. Incubations were performed in duplicate and terminated at the denoted intervals by the addition of 3.5 mL chloroform/methanol (2:5 v/v) and extracted as described earlier [15]. Samples were applied on heat-activated silica gel 60 TLC plates and separated in the solvent *n*-hexane/diethyl ether/acetic acid (50:50:1; v/v/v). Following staining with iodine vapour, the spots containing the free fatty acid fraction were scraped off and added to 10 mL scintillation fluid (Ultima Gold, Packard), and the radioactivity was quantified by scintillation counting. Phospholipid remodelling analysis was performed using [1-^{14}C]arachidonic acid-labelled cells (see above) and by applying two-dimensional TLC as previously described by Serhan *et al.* [21].

RESULTS

Chemotactic Activity of Hepoxilin A_3 on Neutrophils

The free acid of HXA_3 caused strong chemotaxis of human neutrophils at concentrations as low as 30–40 nM (Fig. 1). The dose dependence of the chemotactic activity of HXA_3 revealed a biphasic behaviour in that it was attenuated at higher concentrations of this eicosanoid. The latter effect, not observed with leukotriene B_4 or fMLP, might be due to an overlap with other effects of HXA_3 on cell signalling in neutrophils (see below) which could hinder the cell movement. Damage to cells at higher concentrations of HXA_3 seemed to be unlikely, since we failed to observe a staining of the cells by trypan blue at concentrations up to 10 μM HXA_3 (data not shown). From the comparison of the dose–response curve of HXA_3 with those of the known chemotactic agents leukotriene B_4 and fMLP (Fig. 1), it can be seen that the concentrations needed to achieve the maximal response are comparable in the case of HXA_3 and leukotriene B_4, whereas fMLP requires higher concentrations under our experimental conditions. The maximal intensity of the chemotactic effect was, however, markedly higher for leukotriene B_4 than for HXA_3 and fMLP. It should be noted that 12-HETE, another eicosanoid of the 12-lipoxygenase pathway of arachidonic acid metabolism, did not exhibit chemotactic activity at all at the concentration range investigated (data not shown), which is in line with data from the literature [22]. HXA_3-induced chemotaxis was completely abolished by pretreatment of the cells with pertussis toxin (2 μg/mL) for 90 min, suggesting the involvement of a G-protein-dependent receptor protein in this activity. It was, however, not affected by BSA (1 mg/mL), indicating that the affinity of the putative HXA_3 receptor toward this eicosanoid in neutrophils is higher than that of the fatty acid-binding domain of BSA (data not shown). HXA_3 did not elicit aggregation of neutrophils within the concentration range at which strong chemotaxis occurred (data not shown). Therefore, chemotactic activity by HXA_3 appears to be mechanistically different from that exerted by fMLP and, moreover, more specific.

Action of Hepoxilin A_3 on Cell Signalling in Neutrophils

Contrary to earlier reports, addition of the free acid of HXA_3 (7 μM) to Fura-2/AM-loaded neutrophils caused an immediate transient release of calcium, the extent of which was about one-half of that produced by 100 nM fMLP (Fig. 2). The extent of this calcium signal was dose-dependent in the concentration range between 1 and 7 μM; it was, however, not detectable at concentrations less than 1 μM. Presence of BSA in the reaction medium shifted the

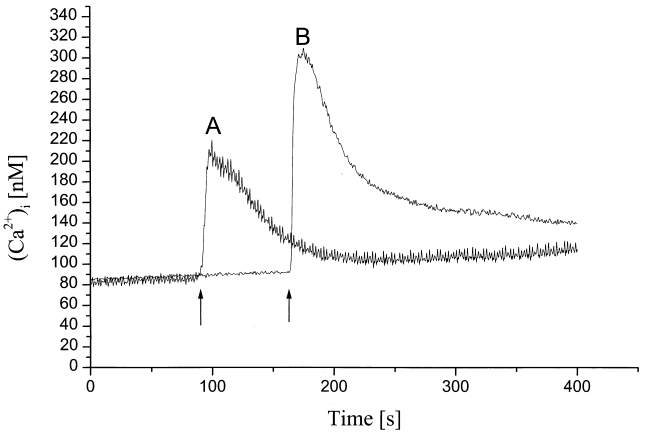

FIG. 2. Calcium release in human neutrophils by the free acid of HXA_3 (7 μM; trace A) and fMLP (0.1 μM; trace B). The cells were loaded with Fura-2/AM as described in Materials and Methods and then preincubated at 37° for 5 min. The agonists were added at the time indicated by the vertical arrow as solutions in ethanol. The vehicle did not produce a measurable effect at the final concentration applied. Fluorescence measurement was carried out as described in Materials and Methods. $[Ca^{2+}]_i$, intracellular free calcium.

threshold concentration to achieve the Ca^{2+} signal to higher concentrations of HXA_3. A lesser intensity of the eicosanoid-mediated calcium signal as compared with that of fMLP was also observed earlier for lipoxins [15]. The calcium signals produced by HXA_3 and by fMLP resembled each other with respect to their time profile; the instantaneous rise in calcium was followed by a rapid return to the basal level within one minute. Addition of the non-permeable calcium chelator EGTA reduced, but did not abolish the calcium signal evoked by the HXA_3, excluding a primary role of an influx of extracellular Ca^{2+} for this signal. The signal was abolished, however, by pretreatment of the cells for 5 min with 0.25 μM thapsigargin, a blocker of the intracellular membrane calcium pump [23], in the presence of 0.5 mM EGTA (data not shown), which indicated that the calcium was released from intracellular stores that become devoid of calcium upon pretreatment with thapsigargin. Unlike fMLP, 100 nM μM HXA_3, as well as its methyl ester and its glutathione conjugate, did not cause NADPH oxidase-mediated respiratory burst in neutrophils, as judged from both measurement of oxygen uptake and determination of superoxide anion production. Only at a concentration of HXA_3 as high as 5 μM did we observe a very small oxygen uptake, the extent of which amounted to about one-tenth of that produced by 100 nM fMLP under identical conditions (data not shown). Moreover, HXA_3 failed to cause activation of protein kinase C in human neutrophils at varying concentrations up to 5 μM (data not shown).

Action of Hepoxilin A_3 on Arachidonic Acid Metabolism in Neutrophils

The release of calcium from intracellular stores observed at higher concentrations of HXA_3 was expected to activate phospholipases and a concomitant liberation of arachidonic acid, as observed earlier for the methyl ester of HXA_3 [15]. Contrary to this expectation, however, HXA_3 in a concentration range between 1 and 5 μM caused only a very slight, if any, enhancement of the liberation of arachidonic acid as compared with the control without agonist, whereas under identical conditions a strong effect by 100 nM fMLP was observed (data not shown). This difference in the responses toward HXA_3 and fMLP may be in part due to the different intensities of the calcium signals; it could, however, also suggest that the appearance of the calcium signal is not a sufficient precondition for a sizeable activation of phospholipases.

When the neutrophils were pretreated with varying concentrations of HXA_3 for 5 min, we observed a dose-dependent modulation of the fMLP-induced liberation of arachidonic acid as measured 5 min following addition of fMLP. At a concentration of 100 nM, HXA_3 blunted the effect of 100 nM fMLP significantly (Table 1). This blunting action disappeared, however, at higher concentrations of HXA_3 in a similar way as for the chemotactic action of this eicosanoid. At concentrations higher than 1 μM, HXA_3 rather caused a slight increase in the fMLP-evoked liberation of arachidonic acid (data not shown). The latter effect might be due to a modulation of the kinetics of the action of fMLP rather than to a true stimulation. As shown in Fig. 3, in cells not treated with HXA_3 there was a lag period in the liberation of arachidonic acid of about 15 sec, which was abolished upon pretreatment with 1 μM HXA_3. The fMLP-induced arachidonic acid liberation in HXA_3-primed cells was partially inhibited by propranolol, which is known to inhibit phosphatidic acid phosphohydrolase activity [24] (Table 2), suggesting not only the involvement of phospholipases A_2, but also that of the phospholipase D pathway in this process, which is in agreement with earlier observations using the methyl ester of HXA_3 [25]. The arachidonic acid liberation following stimulation of the cells by 1 μM HXA_3 plus 0.1 μM fMLP originated exclusively from the phospholipids and mainly from the phosphatidylethanolamine

TABLE 1. Submicromolar concentrations of HXA_3 blunt fMLP-evoked arachidonic acid release in human neutrophils

HXA_3 (nM)	AA release (% of vehicle control)
0	339 ± 13 (6)
40	307 ± 15 (5)
100	278 ± 16 (6)*
1000	318 ± 7 (5)

The cells were prelabelled with [1-^{14}C]arachidonic acid (AA), pretreated with $CaCl_2/MgCl_2$ as described in Materials and Methods, preincubated in the absence or presence of varying concentrations of HXA_3 for 5 min at 37°, and incubated for another 5 min at 37° in the presence of 100 nM fMLP. Then the lipids were extracted, separated by TLC, and the radioactivity of the AA fraction was determined (see Materials and Methods). The values are expressed as percentage of the vehicle control ± SE. The number of experiments using different preparations of neutrophils is indicated in parentheses. The asterisk denotes significance against the control according to the Student's *t*-test ($P < 0.02$).

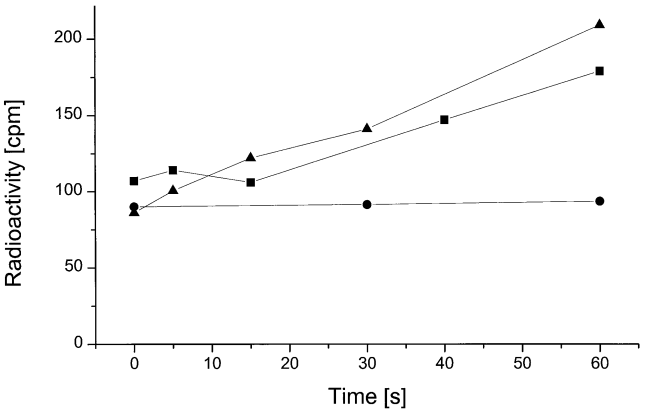

FIG. 3. Effect of HXA_3 (free acid) on the initial phase of fMLP-induced liberation of arachidonic acid. The neutrophils were prelabelled with [1-^{14}C]arachidonic acid and treated as described in Materials and Methods. The cells alone (circles) were stimulated with 0.1 μM fMLP in the absence (squares) or presence of 1 μM HXA_3 (triangles). A representative example from 5 independent experiments that revealed consistent time profiles is shown.

TABLE 2. Effect of priming by HXA_3 on fMLP-mediated liberation of arachidonic acid (AA) and the effect of propranolol

Agonist, addition	Priming by 1 μM HXA_3	AA release (cpm)
None	−	92 ± 10
None	+	104 ± 9
fMLP (100 nM)	−	212 ± 25
fMLP (100 nM)	+	246 ± 24
fMLP (100 nM) propranolol (100 μM)	+	164 ± 21

The cells were preincubated in the absence or presence of 1 μM HXA_3 for 5 min at 37°, and incubated for another 5 min at 37° in the presence of the compounds indicated. Other conditions as in the experiments in Table 1 (see also Materials and Methods). Mean values ± SE of 5 experiments are given. The effects of priming by HXA_3 were not statistically significant.

pool, as judged by phospholipid remodelling experiments, whereas the triacylglycerol pool was not affected (data not shown). Similar observations were also made for the formation of leukotriene B_4 following stimulation by HXA_3 or fMLP (data not shown). Again, HXA_3 caused only a very slight, if any, formation of leukotriene B_4, which was significantly higher following stimulation by fMLP. The two agonists share the property of being poor releasers of leukotriene B_4 in human neutrophils. Therefore, their chemotactic activities cannot be accounted for by the formation of this 5-lipoxygenase-derived eicosanoid.

DISCUSSION

Here we provide ample evidence that HXA_3 is biologically active towards human neutrophils in its unesterified form. In particular, we demonstrate for the first time that HXA_3 is a potent chemotactic mediator in the 10 to 100 nM range. HXA_3 (100 nM) also blunted the fMLP-induced liberation of arachidonic acid significantly, suggesting modulation of certain cell signalling processes. At higher concentrations (1 to 10 μM), free HXA_3 caused a rise in cytosolic Ca^{2+} by releasing it from intracellular stores, as also reported earlier for the methyl ester of HXA_3 [14]. Taken together, our data argue against the presence of a membrane barrier for unesterified HXA_3 at both submicromolar and micromolar concentrations of this compound, in opposition to earlier suggestions [2]. The present observations also imply that HXA_3, like other eicosanoids, can exert its biological activities elsewhere than in the cell in which it is formed. Human neutrophils do not contain a 12-lipoxygenase and are thus unable to synthesise HXA_3 from arachidonic acid. A *de novo* synthesis of HXA_3 in these cells is only possible if external 12-HpETE is supplied [26]. However, neutrophils have been reported to contain a hepoxilin-binding protein [13] that may function as an HXA_3 receptor and mediate the actions observed by us. The putative role of a hepoxilin receptor is supported by the suppression of the chemotactic activity of HXA_3 by pertussis toxin. Pertussis toxin was also shown earlier to block the liberation of arachidonic acid and of diacylglycerols by HXA_3 methyl ester [15].

The chemotaxis of neutrophils caused by HXA_3 appears to be more specific than that caused by formyl peptides or leukotriene B_4, since we failed to detect concomitant aggregation–stimulatory activity and induction of the respiratory burst. The lack of activation of the NADPH oxidase system leading to the oxidative burst is also in line with the observed absence of activation of protein kinase C. Therefore, we conclude that cell signalling by HXA_3 does not involve protein kinase C-dependent processes, in contrast to that evoked by formyl peptides. Moreover, the HXA_3-evoked chemotaxis appears to be independent of a cytosolic Ca^{2+} signal, since we failed to observe such a signal at concentrations of HXA_3 that exhibit chemotactic activity. Rather, it is tempting to speculate that a rise in cytosolic Ca^{2+}, which was observed at higher concentrations of HXA_3, counteracts the chemotactic activity of this eicosanoid. Such an assumption would explain the unique bell-shaped dose–response curve for the chemotactic action (Fig. 1). The detection of chemotactic activity towards neutrophils identifies HXA_3 as a proinflammatory mediator. In the same concentration range, however, we also observed a significant attenuation of the fMLP-induced arachidonic acid release by this eicosanoid, which may be regarded as an anti-inflammatory action. The latter phenomenon could be due to a thapsigargin-like effect of HXA_3 as proposed earlier [16], i.e. HXA_3 may block the endoplasmic Ca^{2+}-ATPase pump leading to a depletion of the intracellular Ca^{2+} stores, so that the biological effects of subsequently added fMLP are blunted.

Our data raise new aspects as to the possible biological role of HXA_3. Hepoxilins are formed via the 12-lipoxygenase pathway of the arachidonic acid metabolism. This pathway is bifurcated at the level of 12-HpETE into two alternative routes, the reduction route yielding 12-HETE and an isomerisation route leading to hepoxilins. The share of the two routes is determined by the competition for 12-HpETE by the corresponding catalysts involved. In many cells possessing 12-lipoxygenase, formation of 12-HETE predominates because of the high enzymatic capacity of glutathione peroxidases, whereas the share of hepoxilin formation in the total 12-lipoxygenase pathway of the arachidonic acid metabolism is usually small. For this reason, a sizeable hepoxilin formation in intact cells can only occur if the enzymatic capacity of glutathione peroxidases or related enzymes is either basally low or exceeded by special metabolic conditions such as severe oxidative stress or selenium deficiency. Thus, in cells having a high capacity of glutathione peroxidases, HXA_3 may be produced in response to oxidative stress. The specific type of the chemotactic action of HXA_3, which differs from that of leukotriene B_4 and formyl peptides, may enable these cells to challenge neutrophils to the site of oxidative stress without causing sizeable release of arachidonic acid and other signs of strong cell activation, which would lead to uncontrolled release of inflammatory mediators and finally

to cell death of the neutrophils. In this way, HXA_3 may function as a stress-induced protective eicosanoid.

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