Hepoxilin A₃-specific binding in human neutrophils

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Hepoxilins have been shown to release calcium from intracellular stores in human neutrophils [Dho, Grinstein, Corey, Su and Pace-Asciac (1990) *Biochem. J.* **266**, 63–68; Laneuville, Reynaud, Grinstein, Nigam and Pace-Asciac (1993) *Biochem. J.* **295**, 393–397]. In this paper we report that tritium-labelled hepoxilin A₃ (8S) binds to broken neutrophil membranes in a time-, substrate- and temperature-dependent fashion. Specific binding was displaced with unlabelled hepoxilin A₃. Specific binding was greatest at 37 °C. Competitive binding was best observed with unlabelled hepoxilin A₃ (8S); the glutathione conjugate, HxA₃-C (8S or 8R), or 12(S)-hydroxyeicosatetraenoic acid was less active. Similarly inactive in displacing the bound radiolabelled

hepoxilin A₃ was leukotriene B₄ as well as a variety of prosta-glandins and thromboxane B₂. Formylmethionyl-leucylphenyl-alanine was similarly inactive in competing for the hepoxilin binding sites. Specific binding was inhibited by pretreatment of the broken membranes during 30 min at 37 °C with proteinase K, while specific binding of the intact cells was unaffected. Scatchard analysis of binding data revealed a single population of binding sites with apparent K_D and B_{max} of 79.3 ± 9.1 nM and 8.86 ± 1.4 pmol/ml per 2×10^6 cells ($\pm$ S.E.M.) respectively reflecting approx. 2.67×10^6 sites/cell. These results demonstrate for the first time that neutrophils contain specific binding sites to hepoxilin A₃.

INTRODUCTION

The hepoxilins (HxA₃ and HxB₃) are 8(*S,R*)-hydroxy- and 10(*S,R*)-hydroxy-11,12-epoxide metabolites of arachidonic acid (20:4, *n*–6) formed through the 12-lipoxygenase pathway via a haemoglobin or other haemoprotein-assisted intramolecular rearrangement of 12(*S*)-hydroperoxyeicosatetraenoic acid (HPETE) [1–5]. Recently we have shown that the formation of hepoxilins is carried out by an enzymic process in intact slices of tissues/intact cells [6]. HxA₃ is metabolized via two independent pathways, one involving an epoxide hydrolase to form trioxilin A₃ (TrXA₃) [7–9], the other via a glutathione S-transferase to form a glutathione conjugate, hepoxilin A₃-C (HxA₃-C) [10–13]. The Y_{b2} subunit of liver glutathione S-transferases was shown to preferentially catalyse the formation of HxA₃-C [14]. Hepoxilins are formed by a variety of tissues and possess a diversity of actions based on their ability to modulate ion fluxes, including calcium and potassium ions in the cell [15]. Hence hepoxilins are capable of enhancing the secretion of insulin from pancreatic islets [2,16], to potentiate the vascular contraction in the aorta and trachea [17,18], to potentiate the bradykinin-induced vascular permeability in the skin [19,20], and to modulate synaptic release of neurotransmitters in the brain [11] and synaptic neurotransmission in both mammalian [21,22] and molluscan neurons [23–25].

We have previously shown that HxA₃ is capable of raising the free intracellular concentration of calcium in human neutrophils [26]. Recent experiments have shown that HxA₃ appears to deplete the calcium stores and prevent the uptake of calcium, since the response to a second addition of hepoxilin is inversely

related to the amplitude of the first response. In fact HxA₃ dose-dependently inhibits the response to a subsequent addition of formylmethionylleucylphenylalanine (fMLP), platelet-activating factor (PAF) as well as leukotriene B₄ (LTB₄) [27].

The present study was carried out to determine whether HxA₃-binding sites exist in human neutrophils.

MATERIALS AND METHODS

Materials

Pure HxA₃ (8S) methyl ester was kindly provided by Professor E. J. Corey (Harvard University, Cambridge, MA, U.S.A.). [³H]₆HxA₃ (8S) methyl ester (specific radioactivity 169 Ci/mmol) was prepared in our laboratory by total chemical synthesis [28]. The corresponding free acid was prepared by hydrolysis in ethanol/1 M KOH (1:1, v/v) for 30 min at 23 °C followed by careful neutralization and extraction with ethyl acetate. The integrity of the free acid was confirmed by TLC (silica gel; ethyl acetate/acetic acid, 99:1, v/v). The free acids of LTB₄ and racemic 12-hydroxyeicosatetraenoic acid (12-HETE) were from Cayman Chemical (Ann Arbor, MI, U.S.A.). Prostaglandins and thromboxane B₂ (TxB₂) were gifts from the Upjohn Co. Proteinase K, RPMI 1640 medium and all reagent-grade chemicals for buffers were purchased from Sigma, U.S.A. Dextran T-500 and Ficoll-Paque were purchased from Pharmacia, Sweden.

Preparation of human neutrophils

Neutrophils were prepared according to Böyum [29] with slight modifications according to Dho et al. [26]. Samples (40 ml) of

Abbreviations used: 12-HETE, 12-hydroxyeicosatetraenoic acid; HPETE, hydroperoxyeicosatetraenoic acid; HxA₃, hepoxilin A₃; HxA₃ (8S), 8S-hydroxy-11S,12S-*trans*-epoxyeicosa-5Z,9E,14Z-trienoic acid; HxA₃ (8R), 8R-hydroxy-11S,12S-*trans*-epoxyeicosa-5Z,9E,14Z-trienoic acid; HxA₃-C (8S), 8S-hydroxy-11R-glutathionyl-12S-hydroxyeicosa-5Z,9E,14Z-trienoic acid; HxA₃-C (8R), 8R-hydroxy-11R-glutathionyl-12S-hydroxyeicosa-5Z,9E,14Z-trienoic acid; TrXA₃, trioxilin A₃ (8S,11S/R,12S-trihydroxyeicosa-5Z,9E,14Z-trienoic acid); LTB₄, leukotriene B₄; PAF, platelet-activating factor; fMLP, formylmethionylleucylphenylalanine; TxB₂, thromboxane B₂.

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venous blood were collected from normal drug-free human volunteers and anticoagulated with heparin sodium (Organon Tecknica Co., Durham, NC, U.S.A.). Erythrocytes were removed by 4.5 % dextran sedimentation for 45 min. The neutrophil-rich plasma was centrifuged at 1500 rev./min (350 *g*) for 5 min. The resulting pellet was gently suspended in PBS whose composition was (in mM): NaCl, 140; NaH₂PO₄, 10; Na₂HPO₄, 10; pH 7.2. This suspension was subjected to Ficoll-Paque gradient centrifugation at 2600 rev./min (1100 *g*) for 20 min. The entire supernatant was aspirated and discarded. The pellet containing the neutrophils was resuspended in 0.5 ml of RPMI 1640 medium (free of sodium bicarbonate but supplemented with L-glutamine and 25 mM Hepes buffered to pH 7.2) and the contaminating erythrocytes were eliminated by lysis in 10 ml of 0.85 % ammonium chloride containing 17 mM Tris, pH 7.2, during 15 min at 37 °C. The suspension was centrifuged at 900 rev./min (140 *g*) for 5 min. The neutrophils were then resuspended in 5 ml of RPMI 1640 medium and allowed to re-equilibrate at 37 °C for 15 min. After an additional centrifugation at 900 rev./min (140 *g*) for 5 min, the cells were resuspended in RPMI 1640 medium and were counted in a Coulter counter (Model 901). The cells were finally adjusted to a concentration of 10⁷ cells/ml in RPMI 1640 medium.

Binding studies

Neutrophil membranes equivalent to 2 × 10⁶ cells/tube were prepared by sonication (Model XL 2020, Sonicator Ultrasonic Processor, Heat Systems Inc., Farmingdale, NY, U.S.A.) of the cells and centrifugation at 1000 *g* for 5 min. The binding assay was conducted in a clear medium (composition in mM: NaCl, 140; KCl, 5; MgCl₂, 1; CaCl₂, 1; Hepes sodium-free, 10; glucose, 10; at pH 7.3) containing the broken neutrophil membrane suspension and [³H]₆HxA₃ (8S) (50000 c.p.m.) in the presence or absence of 1 μg of unlabelled HxA₃ (8S) (in 2 μl of DMSO) to determine the total and non-specific binding. All binding experiments were carried out in 1 ml for different times at 37 °C unless otherwise specified, and performed in triplicate. The reaction was started by the addition of the membranes in the medium containing the radioactive ligand (and the unlabelled ligand where specified). The binding reactions were terminated after the appropriate incubation times by isolation of the ligand-receptor complexes using vacuum filtration through Whatman GF/B glass-fibre filters (Maidstone, U.K.) prewashed with the clear medium. The tubes and the filters were washed with 3 × 3 ml of ice-cold medium. The radioactivity retained on the filters was counted in 10 ml of Ecolite scintillation fluid (ICN, St. Laurent, Québec, Canada) in a Beckman (Model LS 3800) liquid scintillation counter. For competition binding experiments, drugs (500 ng in 2 μl of DMSO) were mixed with radioligand prior to the addition of the membranes in 1 ml total volume.

Treatment with proteinase K

Proteinase K treatment was adapted from a published procedure [30]. Intact neutrophils and broken membranes were separately treated in the clear binding medium with proteinase K (200 μg) for 30 min at 37 °C. PMSF (5 μl of a 0.1 M solution) was then added to terminate the proteolysis, and the cells or the membranes were analysed for total and specific binding as described above, but using the methyl ester of HxA₃, since this form of the compound binds to both the intact cell and the broken membranes [31].

Statistics

Binding parameters (*K_D* and *B_{max}*) were derived from saturation isotherms by computer analysis using the BioSoft EBDA and LIGAND programs [32] modified for an Apple Macintosh computer. Comparisons between samples were evaluated with Student's *t*-test for unpaired data and found significant at the *P* < 0.005 level.

RESULTS

When [³H]₆HxA₃ is exposed to broken neutrophil membranes, specific binding is observed (Figure 1). This binding is time-dependent, reaching maximal binding by 30 min incubation at 37 °C. Binding of the radioligand then remains stable for at least 90 min. The binding is reversible and can be displaced by 1 μg of unlabelled HxA₃ (Figure 1). Under our binding conditions, specific binding reached about 70 % of total binding. We found that specific binding was greatest at 37 °C (Table 1). The

Figure 1 Time course of total binding of [³H]₆HxA₃ and its reversibility with unlabelled HxA₃

The Figure shows the time course of total binding (○) of the free acid form of [³H]₆HxA₃ (8S) (50000 c.p.m.) to broken membranes of human neutrophils (2 × 10⁶ cells) in 1 ml of buffer at 37 °C, and its reversibility (●) after 60 min with unlabelled HxA₃ (8S) (1 μg) added in 1 μl of DMSO. Non-specific binding (□) is shown in the lower curve in which 1 μg of unlabelled compound was mixed at the start with the labelled compound.

Table 1 Effect of temperature on the binding of HxA₃ to neutrophil membranes

Neutrophil membranes equivalent to 2 × 10⁶ cells were incubated in 1 ml for 60 min at the temperatures indicated with [³H]₆HxA₃ (8S) free acid (50000 c.p.m.). Specific binding was obtained by subtracting the amount of label bound in the presence of 1 μg of unlabelled ligand (non-specific binding) from the total amount of label bound by membranes exposed to labelled ligand alone. Values represent the mean ± S.D. of two separate experiments performed in triplicate.

Condition	Total binding (c.p.m.)	Specific binding (c.p.m.)
4 °C	3834 ± 544	1669 ± 387
23 °C	3874 ± 278	1691 ± 266
37 °C	4046 ± 107	2702 ± 113

Figure 2 Relationship of number of cells (1 ml total volume) to total (○), specific (●) and non-specific (□) binding to [³H]₆HxA₃ (8S)

The binding was carried out with broken membranes derived from the number of cells indicated and incubated at 37 °C for 60 min under conditions detailed in Figure 1. Binding conditions were selected for future experiments to contain membranes derived from 2 × 10⁶ cells/ml. Data represent the mean ± S.D. of triplicates.

Figure 3 Displacement of [³H]₆HxA₃ (8S) binding to neutrophil membranes by increasing amounts of unlabelled HxA₃ (8S) (50–1 μg)

Unlabelled compound was added 60 min after incubation of the membranes with [³H]₆HxA₃ (8S) (50 000 c.p.m.). Note the similarity in displacement by 1 μg (■) versus 500 ng. Data represent the mean ± S.D. (triplicates).

extent of specific binding was proportional to the number of cells used, increasing as the number of cells was increased (Figure 2). Typical experiments were carried out with membranes from 2 × 10⁶ cells/ml at 37 °C containing 50 000 c.p.m. radioactive ligand. Binding of [³H]₆HxA₃ was reversed by unlabelled HxA₃ in a concentration-dependent fashion up to a maximum of 1 μg of HxA₃ (Figure 3). The specific binding of [³H]₆HxA₃ (8S) to human neutrophils at 37 °C was saturable with radioligand concentrations within the range 0.3–1400 nM. Binding parameters, i.e. the equilibrium dissociation constant (K_D) and the total number of binding sites (B_{max}), were obtained from three separate saturation isotherms (performed in triplicate) and

Figure 4 Saturation isotherm from a typical experiment (performed in triplicate) showing specific binding of [³H]₆HxA₃ (8S) to human neutrophil membranes derived from 2 × 10⁶ cells

Conditions were as described in Figure 1. Inset: Scatchard plot of the specific binding is illustrated with the best fit using the EBDA/LIGAND computer program. The following constants can be derived from this plot; K_D = 94 nM, B_{max} = 9.82 pmol/ml per 2 × 10⁶ cells. Data averaged from three separate experiments (each point performed in triplicates) showed the following parameters: K_D = 79.3 ± 9.1 nM (± S.E.M.), B_{max} = 8.86 ± 1.4 pmol/ml per 2 × 10⁶ cells (± S.E.M.) from which the number of binding sites/cell can be calculated to be 2.67 × 10⁶ sites/cell.

subsequent Scatchard analysis of specific binding. A representative saturation isotherm from one of three experiments and its Scatchard plot are shown in Figure 4. Scatchard analysis of the data from three separate experiments performed in triplicate determined by the LIGAND program for one population of sites indicated a K_D value of 79.3 ± 9.1 nM (± S.E.M.) and a binding capacity (B_{max}) of 8.86 ± 1.4 pmol/ml per 2 × 10⁶ cells (± S.E.M.), reflecting 2.67 × 10⁶ binding sites/cell. The fit of the data was not improved by a two-population model.

A set of experiments was carried out with related unlabelled compounds to determine the specificity of binding of [³H]₆HxA₃. Based on the data shown in Figure 3, where 500 ng of unlabelled ligand was almost as effective as 1 μg in displacing radioactive HxA₃ (indicating a region of the binding curve that may reveal high sensitivity), we decided to test all compounds at the 500 ng/ml concentration. All drugs were added in 2 μl of DMSO at the same time as the radiolabelled HxA₃. As shown in Table 2, maximum competition of [³H]₆HxA₃ (8S) was observed with unlabelled HxA₃ (8S), although the (8R) diastereomer also competed with the labelled ligand almost to the same extent. In contrast, the glutathione analogue, HxA₃-C (8S or 8R) was ineffective in displacing the radioligand. 12(S/R)-HETE showed marginal activity. Similarly, a variety of prostaglandins and TxB₂ also failed to compete for the hepoxilin-binding site. Since we previously showed that HxA₃ inhibited the calcium-mobilizing actions of LTB₄ and fMLP, we investigated whether these compounds (at concentrations in excess of those used in the calcium studies) would interfere with the hepoxilin binding. Indeed, as shown in Table 2, neither of these compounds competed for the hepoxilin-binding sites in broken neutrophil membranes, indicating that hepoxilin binding was uniquely related to this ligand and not to the other ligands.

Additional evidence that the binding was receptor related was derived from experiments in which intact neutrophils and broken membranes were treated with proteinase K. We used the methyl ester of HxA₃ in these studies since we have shown that the free acid does not bind to the intact cell, but only to the broken

Table 2 Specificity of binding of HxA₃ to neutrophil membranes: lack of competition of binding of some analogues

Neutrophil membranes equivalent to 2×10^6 cells were incubated in 1 ml for 60 min at 37 °C with [³H]HxA₃ (8S) free acid (50 000 c.p.m.) in the absence (total binding) and presence of unlabelled HxA₃ (8S) free acid (1 µg) (non-specific binding) or in the presence of each of the compounds indicated also in the free acid form (500 ng). Data are expressed as a percentage of the specific binding observed by competition of the radiolabelled ligand with the addition of 1 µg of HxA₃ (8S). Values represent the mean of triplicate determinations $\pm$ S.D. Abbreviations: 6-keto-PGF_{1 α} , 6-ketoprostaglandin F_{1 α} ; PGD₂, prostaglandin D₂; PGE₂, prostaglandin E₂; PGF_{1 α} , prostaglandin F_{1 α} .

Compound	Competition against HxA ₃ (8S) specific binding (% $\pm$ S.D.)
HxA ₃ (8S) (1 µg)	100
HxA ₃ (8S) (500 ng)	90.1 $\pm$ 2.0
HxA ₃ (8R) (500 ng)	90.0 $\pm$ 3.7
TrXA ₃ (8S) (500 ng)	15.0 $\pm$ 3.8
HxA ₃ -C (8S) (500 ng)	0
HxA ₃ -C (8R) (500 ng)	0
12-(S/R)-HETE (500 ng)	11.3 $\pm$ 0.3
TxB ₂ (500 ng)	8.8 $\pm$ 0.6
6-Keto-PGF _{1α} (500 ng)	1.5 $\pm$ 0.1
PGD ₂ (500 ng)	0
PGE ₂ (500 ng)	3.3 $\pm$ 0.2
PGF _{1α} (500 ng)	8.4 $\pm$ 0.3
LTB ₄ (500 ng)	13.1 $\pm$ 0.3
FMLP (2×10^{-6} M)	10.8 $\pm$ 0.9

Table 3 Effect of proteinase K on [³H]HxA₃ (8S) binding in intact or broken membranes of human neutrophils

Cells (intact or broken) at a concentration of 2×10^6 /ml were incubated with 200 µg of proteinase K at 37 °C for 30 min. The incubation was terminated with the addition of 5 µl of a 0.1 M solution of PMSF. Cells were then incubated with [³H]HxA₃ (8S) methyl ester (50 000 c.p.m.) in the presence or absence of 1 µg of HxA₃ (8S) methyl ester during 60 min at 37 °C. Control cells received only PMSF before incubation with the radiolabelled ligand. Values represent the mean $\pm$ S.D. of triplicate samples.

Conditions	Total binding (c.p.m.)	Non-specific binding (c.p.m.)
Intact cells	9707 $\pm$ 427 ^a	2793 $\pm$ 93
Intact cells + proteinase K	9996 $\pm$ 983 ^a	3399 $\pm$ 155
Broken cells	9111 $\pm$ 348 ^b	3296 $\pm$ 594
Broken cells + proteinase K	6810 $\pm$ 340 ^b	3301 $\pm$ 496

^a versus ^a = not significant.
^b versus ^b = $P < 0.005$.

membranes, and that the methyl ester binds to both cell preparations [31]. As shown in Table 3, proteinase K inhibited the specific binding of HxA₃ to broken membranes, but did not affect the binding when intact cells were used. This finding demonstrates that binding is effected by a membrane-bound protein (receptor) that is partially hydrolysed under our experimental conditions by proteinase K. Since proteinase K does not penetrate into the intact cell, these experiments also show that the binding of HxA₃ is intracellular.

DISCUSSION

In this paper we report on the presence of specific binding sites for HxA₃ in human neutrophils. We used mostly broken membranes in this study instead of intact cells to avoid any unknown

factors which may be involved in the uptake of these lipophilic compounds in intact cells, e.g. carrier-mediated transport, or differences in lipophilicities of the compounds tested. However, important information could be derived when we compared the effect of proteinase K on the binding of HxA₃ in intact cells and broken membranes. While the free acid form of HxA₃ binds to broken membranes only, the methyl ester form binds to both intact and broken cells [31]. These experiments provided additional important evidence demonstrating that the binding is intracellular since proteinase K affected the binding of HxA₃ only in the broken membrane preparation (Table 3).

Both C8-epimers of HxA₃ appear to be equally active in displacing the radioactive HxA₃ (8S). Both are also equally active in raising intracellular calcium [26]. The binding of HxA₃ to neutrophils appears to be specific to the hydroxy epoxide moiety as evidenced by the inability of analogues lacking the epoxide moiety to compete for [³H]HxA₃ binding, i.e. analogues which either have a glutathione moiety at carbon position 11, as in HxA₃-C, or two hydroxyl groups at C-11 and C-12, as in the trihydroxy metabolite TrXA₃, or compounds which have only a 12-hydroxyl group, as in 12-HETE. The binding sites for HxA₃ are not shared by LTB₄ as this compound, which greatly raises intracellular calcium in neutrophils, does not compete with HxA₃ binding.

Hepoxilins appear to deplete calcium stores in the neutrophil, as the response to a subsequent challenge with either heparin itself or with other agonists such as fMLP, PAF and LTB₄ is inhibited [27]. The extent of this inhibition is directly proportional to the extent of calcium depletion by HxA₃ [27]. HxA₃ may indeed have a dual effect, one in depleting the calcium stores on which these agonists act, and a second effect may be in inhibiting the refilling of the stores. The former is supported by the finding that HxA₃ greatly potentiates the rise in free intracellular calcium caused by thapsigargin, an inhibitor of the refilling of the calcium stores [27]. Evidence for an additional action of heparin inhibiting the refilling of the calcium stores was obtained from lack of recovery of agonist-evoked calcium release in neutrophils pretreated with a dose of heparin large enough to deplete the calcium stores [27].

The present experiments reveal a single population of binding sites for HxA₃ with a binding capacity of 2.67×10^6 sites per neutrophil [$B_{\max}$, 8.86 ± 1.4 pmol/ml per 2×10^6 cells ($\pm$ S.E.M.) or 14.7 pmol/mg of protein] and a K_D of 79.3 ± 9.1 nM ($\pm$ S.E.M.). This contrasts with data generated for LTB₄ in neutrophils showing a K_D of 0.46 nM and a binding capacity of 19 600 sites per cell [33]. Recently, 12(S)-HETE was shown to bind to carcinoma cells with a K_D of 0.44 nM and a binding capacity of 66 000 sites per cell [34]. We have previously shown that HxA₃ inhibits the calcium-mobilizing ability of LTB₄ in neutrophils [27]. It is clear from the present data that the heparin-binding sites are distinct from those that bind LTB₄ although the calcium signal transduced by LTB₄ appears to be inhibited by heparin, probably because HxA₃ depletes the calcium stores [27]. As neither LTB₄ nor 12-HETE displaces bound HxA₃, it is apparent that they operate through different binding proteins. LTB₄-binding sites as well as 12(S)-HETE-binding sites appear to be at the cell surface, while we feel that heparin binding appears to be intracellular, as only the methyl ester form binds to the intact cell [31]. Interestingly, HxA₃ binding parameters are similar to those reported for the intracellular receptor, InsP₃, i.e. K_D 85 nM and $B_{\max}$ 6 pmol/mg of protein [35]. Our data suggest that heparin are not intercellular messengers but appear to act intracellularly within the cell that forms them. This finding contrasts with the actions of other eicosanoids which appear to act as intercellular mediators.

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