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HEPOXILIN BINDING IN HUMAN NEUTROPHILS

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Hepoxilins have previously been shown to release intracellular calcium in human neutrophils. We show herein that tritium-labeled hepoxilin A_3 of high specific radioactivity binds to human neutrophils, and this binding is reversed by the addition of unlabeled compound, demonstrating that specific binding for these compounds exists in these cells. Specific binding of both the methyl ester derivative as well as the free acid form of the hepoxilin takes place in broken membrane fragments. In contrast *only* the methyl ester derivative binds specifically to the intact cells. We also show that intact neutrophils form hepoxilin A_3 when incubated in the presence of the hepoxilin precursor, 12(S)-HPETE. These data demonstrate that hepoxilin synthesis can occur in the neutrophil and that hepoxilin binding sites, which appear to be located intracellularly, exist in these cells.

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Hepoxilins are hydroxy epoxide metabolites of arachidonic acid formed via the 12-lipoxygenase pathway (1, 2). An enzyme system has been identified which selectively utilizes 12(S)-HPETE as substrate for hepoxilin A_3 synthesis; in contrast 12(R)-HPETE is not utilized by this enzyme (3). We have also shown that the methyl ester of hepoxilin A_3 releases intracellular calcium in human neutrophils in a dose dependent way and this release takes place in the absence of extracellular calcium (4, 5). In contrast, the free acid of hepoxilin A_3 is much less active in releasing intracellular calcium (6). The aim of this study was to determine if hepoxilin-specific binding sites exist in human neutrophils and to determine whether these sites exist at the cell surface on these cells or are located intracellularly.

MATERIALS AND METHODS

Materials: Pure 8(S) HxA_3 -methyl ester was kindly provided by Prof. E. J. Corey (Harvard University, Cambridge, MA). [$^3H_6$]-HxA_3 (8S) - Me (spec. act. 169 Ci/mmol)

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Abbreviations used: Me, methyl ester; FA, free acid; HPETE, hydroperoxyeicosatetraenoic acid; hepoxilin A_3, HxA_3 (8S), 8(S)-hydroxy-11(S),12(S)-*trans*-epoxyeicosa-5Z,9E,14Z-trienoic acid; ADAM, 9-anthryldiazomethane.

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was prepared in our laboratory by total chemical synthesis (7). It was hydrolyzed into the free acid (FA) through reaction with ethanol/1 N KOH (1/1, v/v) and extracted into diethyl ether after adjusting the solution to pH 5 as previously reported (1, 2).

Preparation of human neutrophils: Neutrophils were prepared according to Böyum (8) with slight modifications according to Dho et al (4). Typically, forty mL of venous blood was collected from normal drug-free human volunteers and anticoagulated with heparin sodium (Organon Tecknica Co., Durham, NC). Neutrophils were then obtained after removal of erythrocytes by 4.5% dextran sedimentation, and further removal of contaminating erythrocytes by lysis. Neutrophils were resuspended in RPMI 1640 medium and were counted in a Coulter counter (Model 901). The cells were finally adjusted to a concentration of 10^7 cells/mL in RPMI 1640 medium.

Binding studies: The binding assay was conducted in a clear medium (composition in mM: NaCl 140, KCl 5, $MgCl_2$ 1, $CaCl_2$ 1, HEPES sodium-free 10, and glucose 10, pH 7.3) containing the neutrophil suspension (2 x 10^6 cells/tube) as intact cells (or broken membranes resulting from cell sonication and centrifugation where specified) and [$^3H_6$]-HxA_3 (8S) - Me (or its free acid as specified)(50,000 cpm) in the presence or absence of 1 µg of unlabeled HxA_3 (8S) - Me (or its free acid as specified)(in 2 µL DMSO) to determine the total and non-specific binding. All binding experiments were carried out in 1 mL at 37°C for 60 min in siliconized glass tubes, and performed in duplicates. Preliminary experiments indicated binding saturation took place well within the 60 min period of incubation. These experiments also indicated that after 60 min, the radiolabel was displaced by the addition of 1 µg of unlabeld hepoxilin. In the present study, the reaction was started by the addition of the cells in the medium containing the radioactive ligand (and the unlabeled ligand where specified). The binding reactions were terminated by isolation of the ligand-receptor complexes through rapid vacuum filtration on Whatman GF/B glass fiber filters (Maidstone, England) prewashed with the clear medium. The tubes and the filters were washed with 3 x 3 mL of ice-cold medium. The radioactivity retained on the filters was counted in 10 mL of Ecolite scintillation fluid (ICN, St. Laurent, Quebéc) in a Beckman (Model LS 3800) liquid scintillation counter.

Metabolism of 12(S)-HPETE by human neutrophils: A suspension of 1 x 10^7 neutrophils in 1 ml cell medium used for calcium measurements (see above) was incubated at 37°C for 60 min in the absence of substrate, or in the presence of 1 µg of 12(S)-HPETE. The incubations were terminated by the addition of methanol (1 ml), followed by 2 ml of a 5% solution of ADAM reagent in diethyl ether. The mixture was magnetically stirred at 23°C for 60 min to extractively derivatize the products, followed by phase separation of the ether, washing of the aqueous phase twice more with ether, and evaporation of the combined ether phase to dryness. The residue was resuspended in ether, and the incubation mixture of ADAM esters of the hepoxilins was purified on TLC (silica gel G, Brinkmann, ethyl acetate and the appropriate zone was eluted with methanol/ethyl acetate (1:1, v/v)). The purified esters of the hepoxilins were analyzed on HPLC (C18 NovaPak, Waters/Millipore, Toronto, acetonitrile-water-triethylamine-acetic acid, 75/25/1/1, v/v, 1.5 ml/min) and the effluent was passed through an on line fluorescence detector (Model FS950, Kratos Analytical Instruments, Westwood, NJ) operated with a mercury lamp and filters set at exc. 254 nm, em. 400 nm.

Statistics: Comparisons were made between each sample using student's t-test for unpaired data (ANOVA) and found significant at the $p \leq 0.005$ level. All values represent the mean±SD, n=4 separate determinations.

RESULTS AND DISCUSSION

The crude membrane fraction of sonicates of human neutrophils bind hepoxilin A_3 in a specific way, in that the radiolabel is competed for by the presence of unlabeled compound. Specific binding represents about three- to four-fold greater than non-specific binding. The binding of the hepoxilin is not covalent to protein because it can be displaced by unlabeled compound (data not shown). Fig. 1A shows the binding of both the free acid

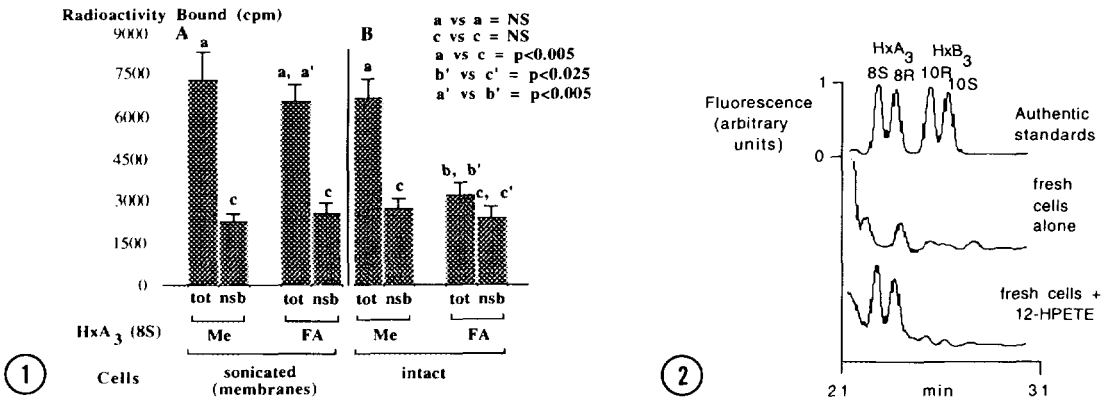

Figure 1. Experiment showing that hepoxilin A_3, as the methyl ester, binds to intact neutrophils (panel B) and broken membranes (panel A). In contrast the free acid form binds *only* to the broken membranes, indicating that the binding sites for the hepoxilin are internalized and that the methyl ester form permits the compound to permeate inside the intact cell. Details of the binding study are found in the Methods. Sonicated cells were centrifuged at 100 x g for 5 min and the sedimented membranes derived from 2 x 10^6 cells were used/tube. The same amount of intact cells were used. Data represent the Mean ± SD (n=2 separate experiments performed in duplicates). tot = total, nsb= nonspecific binding (obtained with 1 µg of unlabeled hepoxilin), NS = not significant, Me = methyl ester, FA = free acid.

Figure 2. Reverse phase HPLC profiles of incubates of intact neutrophils showing the metabolism of exogenous 12-HPETE (1 µg added to 1.10^7 cells) into mostly hepoxilin A_3 (bottom panel). Analysis was carried out by direct derivatization into ADAM esters of the hepoxilins in the incubates without prior acidification by a recently reported method (3). In this way acid-labile hepoxilins are fully recovered as they are stable to extraction as the ADAM esters.

form of hepoxilin A_3 as well as its methyl ester derivative to the crude membrane fraction, suggesting that binding of the compound does not require the presence of a free carboxylic acid group and that the hydroxy epoxide functionality is the required feature for binding. In contrast, specific binding in *intact* cells is observed *only* for the methyl ester form of the hepoxilin but not for the free acid (Fig. 1B). This data indicates that the free acid form of hepoxilin does not penetrate into the cell, while derivatization into the methyl ester is required for it to enter. In fact the methyl ester form is active in releasing calcium from inracellular stores, while the free acid is much less active (6). Support of this concept is derived from the well known studies with fluorescent indicator dyes used for intracellular free calcium measurements. An ester form (acetoxymethyl) of the dye is used to allow the compound to penetrate inside the cell. The ester is then hydrolysed by cytosolic esterases and the free acid form of the dye is retatined inside the cell where it binds to free intracellular calcium (9).

How does hepoxilin A_3 (free acid) act inside the cell in vivo? One possibility is that it is formed inside the cell. Fig. 2 shows results of experiments in which intact neutrophils were incubated with 12(S)-HPETE (free acid), the precursor of hepoxilin A_3, abundantly formed by platelets (10). As hepoxilins are unstable to acidic workup, the incubates were extractively derivatized without prior acidification of the incubate) into the ADAM esters.

The intact hepoxilins were analyzed by HPLC with fluorescent detection by a method recently described (3). Results show that hepoxilin A_3 (detected as the ADAM derivative) is indeed selectively formed by intact neutrophils from 12(S)-HPETE (Fig. 2, bottom panel). Thus transcellular delivery of 12(S)-HPETE could account for hepoxilin formation (as the free acid) within the neutrophil with subsequent action in releasing intracellular calcium.

Our experiments demonstrate that human neutrophils contain specific binding sites for hepoxilin A_3 and that these binding sites are intracellular in location. We have demonstrated this through use of the methyl ester derivative which allows the compound to penetrate the intact cell while coresponding experiments with the free acid show that the free acid form does not penetrate into the intact cell. Conversely, we have shown that both the free acid as well as the methyl ester bind to broken (sonicated) cells. Further experiments are in progress to characterize these binding sites.

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