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Hepoxilin signaling in intact human neutrophils: biphasic elevation of intracellular calcium by unesterified hepoxilin A_3

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Abstract We have previously shown that the methyl ester of hepoxilin A_3 causes a receptor-induced rise in intracellular calcium through the release from intracellular stores in suspended human neutrophils. The corresponding free acid was devoid of activity. We now report that the action of the free acid form of hepoxilin A_3 is dependent on the type of vehicle used, i.e. it is active in releasing calcium when used in an ethanol vehicle but not in DMSO. The methyl ester is equally active in either vehicle. The pattern of calcium release between the free acid and the methyl ester is qualitatively different. Both compounds show a biphasic pattern, i.e. an initial rapid phase followed by a slow decline in calcium levels but never reaching pre-hepoxilin A_3 baseline levels. The methyl ester appears slightly more potent in the initial phase of calcium release than the free acid (methyl = 188 ± 14 S.D., free acid = 135 ± 11 S.D. nM, $P < 0.0005$). Both compounds appear to reach the same calcium levels at the plateau of the second prolonged phase (methyl = 88 ± 8 S.D., free acid = 107 ± 15 S.D. nM, not significant). Lanthanum chloride (an inhibitor of calcium influx) interfered with the second phase of the curve causing calcium levels to return to normal pre-hepoxilin levels for both compounds. Addition of lanthanum chloride prior to the hepoxilin addition or carrying out the experiments in calcium-free medium, eliminated the second phase completely, with the calcium peak returning rapidly to normal baseline levels, suggesting that the second phase is due to calcium influx. Again the methyl ester is more active than the free acid (methyl, 189 ± 12; free acid, 145 ± 6 S.D. nM, $P < 0.005$). Additional experiments with tritium-labelled methyl ester of hepoxilin A_3 demonstrated that the compound is hydrolyzed into the free acid intracellularly. These experiments demonstrate that DMSO interacts with hepoxilin free acid, interfering with its entry into the cell while ethanol does not. Once inside the cell, hepoxilin interacts with its own receptor to release calcium rapidly from stores, but it also causes a more prolonged influx of calcium from the extracellular milieu.

1. Introduction

We have recently shown that hepoxilin (Hx) A_3 interacts with a specific binding protein in the intact human neutrophil resulting in the release of intracellular calcium [1]. In those studies we used the methyl (Me) ester form in DMSO because we found that the free acid (FA) was without effect in intact cells both in terms of binding and calcium release [2]. We also found that both the FA and Me ester (in DMSO) were active in specific binding to disrupted neutrophil membranes [3]. This suggested that the FA did not penetrate into the intact cell and that the binding protein was intracellular and not at the outside surface of the plasma membrane. We now describe new findings which support a binding/calcium release coupling where both the FA and the Me ester are found active (in ethanol vehicle) in the intact neutrophil and demonstrate a biphasic action of HxA_3, one in releasing calcium from intracellular stores, a second in increasing calcium influx.

2. Materials and methods

2.1. Materials

Pure 8(R)-HxA_3 Me ester was prepared in our laboratory as previously reported [4]. The FA was prepared by hydrolysis of the Me ester with 1 N KOH/ethanol followed by mild acidification, extraction and further purification by chromatography [5]. [$^3H_6$](8R)-HxA_3 Me ester was prepared as previously reported [5]. Lanthanum chloride was purchased from Sigma (St. Louis, MO, USA).

2.2. Preparation of human neutrophils

Human neutrophils were prepared essentially as described previously [3]. Typically, neutrophils were obtained from 40 ml venous blood from volunteers that had not taken any medication. The blood was anti-coagulated with heparin sodium (Organon Tecknica, Durham, NC, USA). Erythrocytes were removed by 4.5% dextran sedimentation. Neutrophils were collected and pelleted after Ficoll-Paque gradient centrifugation. Further removal of contaminating erythrocytes was carried out by ammonium chloride lysis. After washing twice with centrifugation, neutrophils were resuspended in RPMI 1640 medium and were counted in a Coulter counter (Model 901). The cells were adjusted to a concentration of 10^7 cells/ml in RPMI 1640 medium.

2.3. Intracellular calcium release

Freshly prepared neutrophil suspensions (1 ml: 1×10^7 cells) were loaded with 3 mM (final concentration 3 μM) of the acetoxymethyl ester precursor of the calcium indicator (Indo-1-AM, Calbiochem, La Jolla, CA, USA) during 30 min at 37°C. Excess dye was removed by centrifugation and the cells were suspended in fresh RPMI 1640 medium. Dye-loaded cells were kept at room temperature on a rotator. Each measurement consisted of the following: 2×10^6 cells were suspended in 1 ml of assay medium of the following composition in mM: NaCl 140, KCl 5, $MgCl_2$ 1, $CaCl_2$ 1, HEPES sodium-free 10 and glucose 10, pH 7.3 and placed in a plastic cuvette (Diamed Lab., Toronto, Canada) whose temperature was controlled at 37°C. Calcium-free media contained no calcium and 1 mM EGTA was added. The cell suspension was continuously stirred magnetically. Fluorescence was continuously monitored with a Perkin-Elmer fluorescence spectrophotometer (model 650-40) and recorded on a chart recorder (LKB model 2210) set at 1 cm/min. The excitation wavelength was set at 331 nm, the emission wavelength at 410 nm, with slits of excitation

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Abbreviations: HxA_3, hepoxilin A_3 8(R)-hydroxy-11,12-epoxyeicosa-5Z, 9E, 14Z-trienoic acid; Me, methyl ester; FA, free acid; DMSO, dimethyl sulfoxide; ωH, omega-hydroxy; $TrXA_3$, trioxilin A_3, 8(R/S), 11(R), 12(S)-trihydroxyeicosa-5Z, 9E, 14Z-trienoic acid

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and emission set at 3 and 15 nm respectively. At the end of each test, a calibration was carried out to determine the maximal fluorescence by adding ionomycin (Sigma) at 1 mM (final concentration 1 μM) and minimal fluorescence by adding $MnCl_2$ (final concentration 3 mM) as previously described [6]. Test compounds were prepared in DMSO (Caledon, Georgetown, OR, USA) or glass-distilled ethanol (100%) at a stock solution of 1 μg/μl or 5 μg/μl and 1 μl of this solution was added to 2×10^6 cells in 1 ml.

2.4. Metabolism of [$^3H_6$]HxA_3 Me ester

Freshly prepared human neutrophils were incubated in the above assay medium in a siliconized glass tube to which had been added [^{3}H](8R)-HxA_3 Me ester (5×10^6 cpm) diluted with 3 μg of the unlabelled compound. The mixture was incubated for 1 or 5 minutes and the incubation mixture was extracted with ethyl acetate without prior acidification to extract the intact Hx structure and its products (which are acid sensitive). The solvent was evaporated to dryness with N_2 gas and the residue was dissolved in a small amount of ethyl acetate and spotted on TLC (silica gel G, ethyl acetate/acetic acid 99.5/0.5 (v/v)). The plate was developed for 60 min, then the solvent was dried with cold air and scanned for radioactivity on a TLC radiochromatogram scanner (Berthold). Authentic standards of HxA_3 Me ester and FA as well as the ω-hydroxy (ωH) metabolite FA (product of ω-oxidation [7]) and the trihydroxy metabolite FA (product of epoxide hydrolase) were spotted as reference compounds.

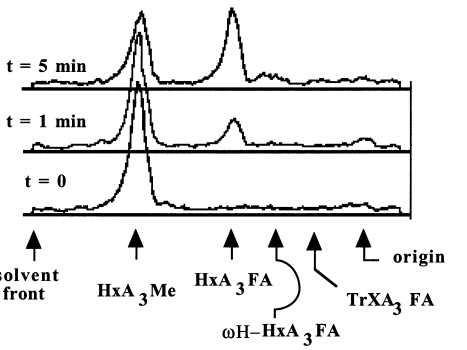

Fig. 2. TLC radioactivity profiles of ethyl acetate extracts of neutrophil 'short' incubations with [$^3H_6$]HxA_3 Me ester for various times indicated. Conditions are essentially similar to the biological assays shown in Fig. 1. Migration of authentic standards is shown. Note that within the time frame of biological action (1 min, see Fig. 1), HxA_3 Me is largely unchanged. The FA begins to appear around this time. Compare the calcium actions between the Me and the FA (Fig. 1B) with the metabolism profiles (Fig. 2). Also note that the ωH metabolite is not formed within this time frame, it is formed during longer incubations [7].

3. Results and discussion

We show herein that HxA_3 FA but not the Me ester is vehicle sensitive in evoking a rise in intracellular calcium in suspensions of human neutrophils. Fig. 1A shows a typical pattern of intracellular calcium resulting from the addition of HxA_3 Me ester (upper trace) and FA (lower trace) in DMSO as vehicle. This is compared with the same two compounds added in ethanol as vehicle (Fig. 1B). The following three points are apparent. First, the pattern for the Me ester is similar and independent of the vehicle used (compare upper traces in Fig. 1A and B). Second, the FA is inactive when made up in DMSO but is active when added in ethanol vehicle (compare lower traces in Fig. 1A and B). Third, both compounds added in ethanol show a rapid action (rise in intracellular calcium) and a slower more prolonged decrease in calcium levels but never decreasing to pre-HxA_3 levels (see Fig. 1B). The Me ester appears more potent in the rapid initial phase of calcium release than the FA (Me, 188 ± 14 S.D.; FA, 135 ± 11 S.D. nM, $P < 0.0005$, $n = 3$) and decreases to a similar plateau level as with the FA (Me, 88 ± 8 S.D.; FA, 107 ± 15 S.D. nM, not significant, $n = 3$). Hence, the decrease to the second phase (prolonged calcium rise) is greater with the Me ester (from 188 to 88 nM = 53%) than with the FA (from 135 to 107 nM = 20%). In order to address this second phase of calcium 'retention', we administered lanthanum chloride (1 μM final concentration) at the plateau of the profile (about 5 min after the addition of HxA_3). Lanthanum chloride blocks the entry of calcium by inhibiting calcium channels [8,9]. Lanthanum caused an immediate decrease in intracellular calcium levels towards the baseline for both the Me ester as well as the FA (Fig. 1B, upper and lower traces). In separate experiments (Fig. 1C) carried out in medium devoid of extracellular calcium (containing EGTA), the Me ester (tested in DMSO or ethanol) effected the same rapid initial phase of intracellular calcium rise but the peak response quickly returned to baseline (Fig. 1C, upper trace), suggesting that the second (longer) phase of the normal HxA_3 response was due to the entry of extracellular calcium. Similar results were observed with the FA of HxA_3 (Fig. 1C, lower trace). Again the Me ester is slightly more potent than the FA (Me, 189 ± 12 S.D.; FA, 145 ± 6 S.D. nM, $P < 0.005$, $n = 3$). Similar results to the extracellular calcium-free experiments were observed when lanthanum chloride was added to the cells prior to HxA_3 (data not shown).

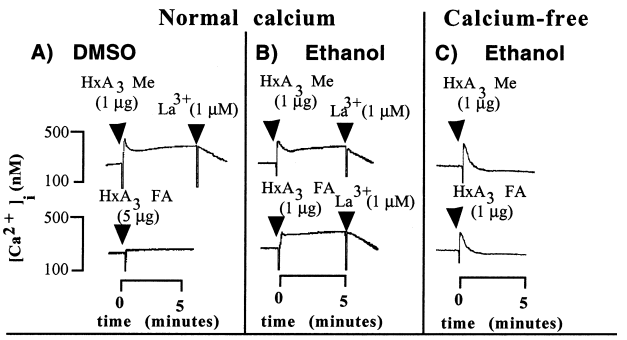

Fig. 1. Fluorescence profiles of intracellular calcium changes in suspended human neutrophils after administration of HxA_3 as the Me ester or FA prepared and administered in 1 μl of DMSO (Fig. 1A) or ethanol (Fig. 1B, C) as vehicles. Note that the FA is inactive in DMSO even at 5× the dose of the Me ester (Fig. 1A, bottom). The effect of lanthanum chloride (1 μM) is shown (Fig. 1B). Fig. 1C shows the patterns observed in calcium-free medium. Note the absence of the prolonged second phase (Fig. 1C): this is due to the influx of calcium (in Fig. 1A, B). When lanthanum chloride was added prior to HxA_3 in normal calcium, profiles similar to those observed in Fig. 1C were obtained, confirming that the second phase is due to calcium influx.

In order to investigate further whether the calcium releasing action of HxA_3 on neutrophils was mediated via its conversion into other compounds, we incubated tritiated HxA_3 Me ester with human neutrophils and analyzed the products after 1 and 5 min incubation, the periods of maximal change in intracellular calcium. The ethyl acetate extract of the incubation was analyzed by TLC. Fig. 2 shows that within a 1 min incubation (the period of sharp initial calcium rise) the Me ester is only partially hydrolyzed (about 10%) into the FA by intracellular esterases, demonstrating that the Me ester penetrates into the cell where it acts to release calcium from calcium stores (rapid initial phase). About 50% of the Me ester is

converted into the FA as the sole metabolite within 5 min of incubation. Hence, the rapid initial phase of the calcium profile of the Me ester of HxA_3 is due to a large part to the Me ester form itself while the prolonged phase occurs during and subsequent to the formation of the FA. The important experiment in Fig. 1C (bottom trace) demonstrates that the FA is also active on intracellular stores to cause the release of intracellular calcium, i.e. it has dual actions.

These experiments demonstrate that HxA_3, both as the FA as well as the Me ester, are taken up into the cell and act to release calcium from intracellular stores. Within the time frame of initial action (rapid phase, 1 min), studies with tritiated HxA_3 show that the Me ester is not substantially hydrolyzed into the FA, although this takes place subsequently and is likely involved in calcium influx. Supporting this is the finding that the second phase of calcium response is equally pronounced when the FA is used (Fig. 1B) and is absent in calcium-free medium (Fig. 1C). Finally, caution should be exerted in the choice of vehicle as we show that DMSO interacts with the FA form of HxA_3 (likely through formation of a polar hydrogen-bonded DMSO-HxA_3 adduct [10]) making the adduct incapable of entering the cell and rendering HxA_3 inactive. In ethanol as vehicle, the FA enters the cell where it is capable both of releasing calcium from intracellular stores (see Fig. 1C) and causing influx of extracellular calcium (compare Fig. 1B and C). Similar findings were observed with Hx binding studies. The FA dissolved in DMSO did not bind to intact neutrophils but did bind to membranes from disrupted cells, while the Me ester was equally well bound to the intact cell as well as disrupted cells [3].

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