papers on methodology

Simultaneous determination of hydroxyeicosanoid (HETE) binding to cells and its cellular metabolism

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Abstract In order to study the mechanism(s) through which certain biologically active lipids, such as the hydroxyeicosanoids (HETEs), exert their effects, it is necessary to distinguish between binding of these lipids to cells and their cellular metabolism. A novel and simple method is described for the simultaneous determination of [^{3}H]15-hydroxyeicosanoid (15-HETE) binding to cells and cellular [^{3}H]15-HETE metabolism. The method involves initial separation of radiolabeled cells by filtration, filter extraction of cellular lipids by methanol, and thin-layer chromatography (or high performance liquid chromatography) determination of both nonesterified 15-HETE bound to cells and 15-HETE incorporation into cellular phospholipids. The method was applied to both PT-18 mast/basophil cells and rat basophilic leukemia (RBL-1) cells and should be applicable to other cells as well as other metabolizable hydroxy fatty acids or lipids.—**Vonakis, B. M., and J. Y. Vanderhoek.** Simultaneous determination of hydroxyeicosanoid (HETE) binding to cells and its cellular metabolism. *J. Lipid Res.* 1993. **34:** 853–858.

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Leukotrienes, prostanoids, and thromboxanes are members of the biologically active eicosanoid lipid family that act as signalling molecules and mediate their responses by binding to specific receptors (1). Numerous binding studies using specifically radiolabeled ligands have delineated the properties of these different classes of receptors (2, 3).

One of the major problems in studying the receptor-binding kinetics of eicosanoids is metabolic conversions of the radioligand. A variety of ways have been reported to eliminate this problem. For example, the presence of cysteine or serine-borate buffer blocked LTD_4 metabolism and greatly facilitated the assay for the LTD_4 receptor (4). However, it is not always possible to block ligand metabolism and other approaches must be developed to differentiate ligand binding from metabolism.

Hydroxyeicosanoids or HETEs are also members of the biologically active eicosanoid family and are involved in the modulation of secretory responses and enzymatic pathways (5). We have previously reported that 15-HETE was a selective stimulator of the inactive 5-lipoxygenase in mast-basophil PT-18 cells (6). In investigating the mechanism of this stimulatory action of 15-HETE, it was necessary to differentiate between 15-HETE binding to cells and 15-HETE metabolism because the latter pathway could not be blocked. A novel yet simple procedure was developed that quantitatively assessed 15-HETE binding and metabolism and which can be utilized in studying other HETE receptors.

MATERIALS AND METHODS

15-HETE and [^{3}H]15-HETE were prepared from arachidonic acid (Nu-Chek Prep, Elysian, MN) and [^{3}H]arachidonic acid (2.2–3.7 TBq/mmol, NEN-DuPont, Boston, MA), respectively, and soybean lipoxygenase (Sigma Chemical Co., St. Louis, MO) as previously described (7). This procedure was also used to synthesize [^{3}H]5,15-diHETE from [^{3}H]5-HETE (7.8 TBq/mmol, Amersham, Arlington Heights, IL). All synthesized radioactive compounds were purified by RP-HPLC. Silica Gel 60 TLC plates were obtained from EM Science (Cherry Hill, NJ).

PT-18 and RBL-1 cells were grown, harvested, and washed as previously described (6, 8), and resuspended at either 14×10^6 (PT-18) or 10^7 (RBL-1) cells/ml of Dulbecco's phosphate-buffered saline (PBS), pH 7.0, containing 11 mM glucose (assay buffer). Cell-association studies were carried out at 4°C using 1.5-ml polypropylene microcentrifuge tubes. Cells (250 μl) were added to 250 μl cold assay buffer containing [^{3}H]15-HETE with or without unlabeled 15-HETE and the incubations (in quadruplicate) were maintained on ice for 3 h. The viability of PT-18 cells prior to assay was ≥95% [lactate dehydrogenase (LDH) release (9) or trypan blue exclusion)

Abbreviations: HETE, hydroxyeicosatetraenoic acid; LDH, lactate dehydrogenase; TLC, thin-layer chromatography; HPLC, high performance liquid chromatography.

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whereas the viability decrease of HETE-treated cells was <10% of untreated controls. The mixtures were then filtered through Whatman (Hillsboro, OR) GF/C glass microfiber filters (2.4 cm diam., #1822024) on a Millipore (Bedford, MA) 12-place sampling manifold to separate free ligand from cell-associated ligand. The filters were then washed with cold (4°C) 3 × 4 ml Dulbecco's PBS, pH 7.0 (wash buffer). Two of the quadruplicate filters were then air-dried, placed in separate scintillation vials containing 4 ml Safety-Solve (Research Products International, Mt. Pleasant, IL), and the filter-retained radioactivity on the unextracted controls was determined by liquid scintillation counting. In all cell-association studies described, the exact concentration of ligand used was determined in each experiment. As experiments were performed several times, the range of ligand concentration used for all experiments within a study is indicated in the respective figure or table. In determining the optimum extraction solvent to remove cell-associated radioactivity, filters were treated with either 3 × 5 ml of methanol or ethyl acetate or once with 10 ml 0.5% Triton X-100 in wash buffer. The Triton X solution was then extracted with 3 × 5 ml ethyl acetate. The amounts of radioactivity recovered in the organic extracts and retained on the filters were determined by liquid scintillation counting. In most other experiments, two of the quadruplicate filters were immediately placed in separate tubes and extracted with 4 ml methanol. The methanolic solutions were evaporated under nitrogen and the residues were resuspended in 50 μl chloroform. The chloroform extracts were then analyzed by TLC or HPLC.

The distribution of radioactivity on TLC plates was determined by dividing the plate into 1-cm bands, scraping them, and counting the radioactivity using Safety-Solve as scintillation cocktail. TLC separation of cell-bound 15-HETE from 15-HETE incorporated into phospholipids was accomplished using a solvent system consisting of petroleum ether–diethyl ether–acetic acid 50:50:1 where the R_f of 15-HETE was 0.3 and the phospholipids remained at the origin. Phospholipid values in the text represent the amount of saponifiable lipid at the origin on the TLC plate (10). Radioactivity measurements were corrected for silicic acid quenching. RP-HPLC analyses of [^{3}H]15-HETE-containing cell extracts as well as cell supernatants were carried out as previously described (11). identification of radioactive bands was based on comparative retention times (or R_f values) of authentic standards.

RESULTS

Incubation of 3.5 × 10^6 PT-18 cells (0.5 ml) with 100–350 pM [^{3}H]15-HETE for 3 h at 4°C resulted in the formation of radiolabeled cells. These radiolabeling conditions were found to be optimum and the cells were isolated by filtration rather than by centrifugation because 20–30% more radioactivity was recovered with the former method (data not shown). Although cell filtration is a commonly accepted method for determining ligand binding (12, 13), we first examined whether the filtration procedure caused greater cell lysis compared to radiolabeled cell isolation by centrifugation, which could lead to HETE binding to intracellular receptors or organelles as a result of the filtration procedure. Using LDH release as a measure of cell damage (9), the amount of LDH release (relative to maximum LDH release in the presence of 0.5% Triton X-100) by cells treated with [^{3}H]15-HETE for 3 h at 4°C was determined. It was found that 13.7 ± 1.1% (n = 6 experiments) of LDH was released and present in the filtrate when the cells were filtered as described in Materials and Methods. Alternatively, when the cells were pelleted by centrifugation, the supernatant contained 14.6 ± 0.3% LDH (n = 6). As these differences in LDH release are not statistically significant, the results indicate that cell filtration does not lead to increased cell lysis and thus cannot account for the higher binding observed with the filtration procedure. As shown in **Table 1,** 10.3 ± 1.3 (mean ± SEM) fmol of cell-associated radioactivity was recovered using the filtration procedure, which represented 10% of the ligand added. In the presence of 10 μM unlabeled 15-HETE, the cell-associated radioactivity decreased to 3.2 ± 0.40 fmol. As the addition of 30,000- to 100,000-fold excess unlabeled ligand only displaced about 70% of the radioactivity, it was decided to investigate the nature of this cell-associated radioactivity.

Extraction of the radioactive filters with several different solvents indicated that methanol was the optimum extraction solvent as 98% of the radioactivity could be removed from the filter and recovered (**Table 2**). When the cell extract was analyzed by TLC, it was found that there were two bands of radioactivity, the major one (70%) in which the [^{3}H]15-HETE was incorporated into phospholipids and a second band (30%) corresponding to non-

TABLE 1. Cell-associated radioactivity isolated from treatment of PT-18 cells with [^{3}H]15-HETE

	Radioactivity Recovered	
Treatment of Cells	fmol	% of Added Ligand
[^{3}H]15-HETE	10.3 ± 1.3	10.0 ± 0.89
[^{3}H]15-HETE + 10 μM 15-HETE	3.2 ± 0.40	

PT-18 cells (3.5 × 10^6 in 0.5 ml) were treated with the 100–350 pM [^{3}H]15-HETE in the absence or presence of unlabeled 15-HETE. After 3 h at 4°C the incubation mixture was filtered and the filter was washed, dried, and assayed for radioactivity as described under Materials and Methods. The results represent the mean ± SEM from 19 separate experiments, each of which was done in duplicate, and have been corrected for controls containing ligand but no cells. In each experiment, the difference between the individual samples was <20% of the mean.

TABLE 2. Determination of the optimal extraction solvent to remove and recover radioactivity from filters containing [^{3}H]15-HETE-associated cells

Extraction Solvent	Radioactivity: Solvent Extractable	Radioactivity: Remaining on Filter	Combined Recovery
		% Control	
Methanol	97 ± 1.9	1.5 ± 0.1	99
Triton X-100–ethyl acetate	61 ± 4.2	3.4 ± 0.5	64
Ethyl acetate	38 ± 3.3	65 ± 1.6	103

PT-18 cells (7 × 10^6/ml, 5 ml) were incubated with 193 pM [^{3}H]15-HETE for 3 h at 4°C. Aliquots (0.5 ml) were then removed and the cells were filtered. In three experiments, 9712 ± 1053 dpm remained on unextracted (control) filters after washing with buffer. Duplicate washed filters were extracted three times with either methanol, ethyl acetate, or 0.5% Triton X-100–ethyl acetate as described in Materials and Methods. The amounts of radioactivity in the extracts and retained on the filters were determined by liquid scintillation counting. The values represent the mean ± SEM from four separate experiments, each done in duplicate.

esterified [^{3}H]15-HETE that is bound to cells. **Fig. 1** shows the relative distribution of [^{3}H]HETE ligands into phospholipid and cell-bound ligand fractions. Confirmation that no additional 15-HETE metabolism occurred under these conditions was obtained by reverse phase HPLC analysis of the cell extract and the cell supernatant which did not reveal the presence of any other metabolites of [^{3}H]15-HETE (results not shown). In addition, controls with heat-inactivated cells showed that less than 0.5 fmol of radioactivity was incorporated into the phospholipid fraction. When the incubations were carried out at 25°C, there was a small shift to greater (78%) esterification into phospholipids. When [^{3}H]5,15-diHETE was used as the ligand at 25°C, the major band (about 90% of the radioactivity) was found to be cell-bound (nonesterified) [^{3}H]5,15-DiHETE. Further analysis showed that at 4°C, 50% of the [^{3}H]15-HETE was esterified into phosphatidylcholine, 36% into phosphatidylinositol, and the remainder into phosphatidylethanolamine.

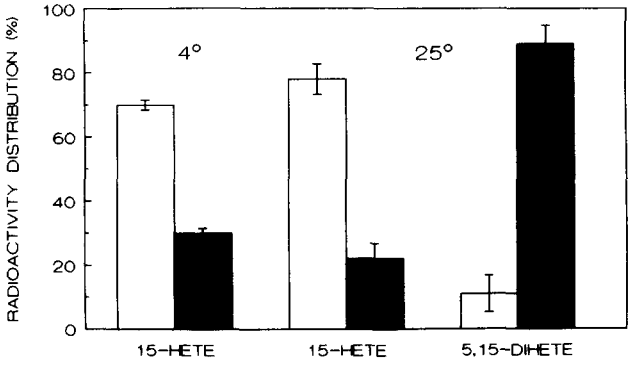

Fig. 1. Ligand distribution in PT-18 cells incubated with [^{3}H]15-HETE or [^{3}H]5,15-diHETE. Radiolabeled cells were isolated, extracted, and ligand distribution into cell-bound (total ^{3}H-labeled ligand binding, closed bars) and esterified (open bars) forms was determined as outlined in Materials and Methods. Corrections have been made for controls containing ligand but no cells. Values represent the mean ± SEM from [^{3}H]15-HETE cell-association determinations carried out at 4°C for 3 h (n = 42) and at 25°C for 20 min (n = 10) and from three separate experiments with [^{3}H]5,15-diHETE incubated for 30 min at 25°C.

Although HETE incorporation into phospholipids of various cells had been previously observed to occur at 37°C (5), the results with PT-18 cells indicated that the esterification process proceeded even at 4°C. In order to eliminate the possibility that this unexpected finding was due to a methodological artifact and to determine whether HETE incorporation at 4°C was observable with other cells, the distribution of [^{3}H]15-HETE (i.e., free 15-HETE and esterified 15-HETE) using RBL-1 cells at both 4°C and 37°C was examined. These cells were chosen because *a)* they were previously reported to incorporate HETEs at 37°C (14) and *b)* they were responsive to 15-HETE as 15-HETE was found to inhibit the 5-lipoxygenase in these cells (J. Y. Vanderhoek, personal communication). Treatment of RBL-1 cells (10^7 cells/ml) with 180 pM [^{3}H]15-HETE at 4°C for 3 h was followed by the isolation and analysis of radiolabeled cells by the procedure described above. The results indicated that 21 ± 5% (n = 2 separate experiments) of [^{3}H]15-HETE was esterified into phospholipids with the remainder being cell-associated but nonesterified 15-HETE. When RBL-1 cells were treated similarly at 37°C for 30 min, the relative amounts of these two pools were 49 ± 1 and 51 ± 1%, respectively (n = 2 experiments).

In view of these results, we examined the effects of increasing amounts of unlabeled 15-HETE on the PT-18 cell-associated radioactivity in terms of the levels of both cell-bound [^{3}H]15-HETE and [^{3}H]15-HETE esterified into phospholipids. As shown in **Fig. 2A,** unlabeled 15-HETE readily displaced [^{3}H]15-HETE bound to PT-18 cells in a concentration-dependent manner when the cells were treated simultaneously with both unlabeled and 160–200 pM ^{3}H-labeled 15-HETE. Thus 1 and

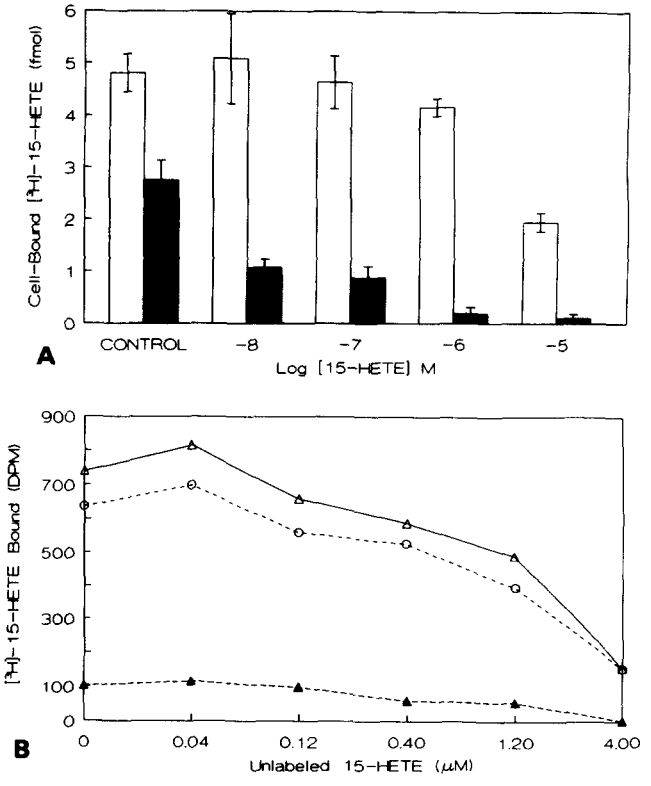

Fig. 2. Competition of unlabeled 15-HETE for [^{3}H]15-HETE associated with PT-18 cells. PT-18 cells (3.5×10^6, 0.5 ml) were incubated at 4°C for 3 h with 160–235 pM [^{3}H]15-HETE in the absence or presence of the indicated concentrations of unlabeled 15-HETE. A (top): The amounts of cell-associated [^{3}H]15-HETE incorporated either into phospholipids (open bars) or bound to cells (total [^{3}H]15-HETE binding, closed bars) were determined as indicated in Materials and Methods. Values represent the mean ± SEM from three separate experiments, each done in duplicate. B (bottom): Total [^{3}H]15-HETE binding (open triangles) and nonspecific binding (10 μM unlabeled 15-HETE, closed triangles) was assessed as described in Materials and Methods. The results shown are from one quadruplicate experiment representative of two such experiments. Corrections have been made in all experiments for controls containing ligand but no cells.

10 μM unlabeled 15-HETE displaced 93 and 97% (respectively) of cell-bound [^{3}H]15-HETE. As specific ligand binding is typically defined as the difference in the amount of ligand bound in the absence (total ligand binding) and presence (nonspecific binding) of a large excess (100- to 10,000-fold) of unlabeled ligand (12, 13), these findings indicate that treatment of PT-18 cells with [^{3}H]15-HETE for 3 h at 4°C resulted in 97% specific binding. Further confirmation was obtained from *1)* analysis of 42 separate binding determinations with [^{3}H]15-HETE which yielded the same amount (mean ± SEM = 96 ± 1.3%) of specific binding, and *2)* from competition experiments in which nonspecific binding (in the presence of 10 μM unlabeled 15-HETE) was assessed at each concentration of 15-HETE tested and shown to be <15% of total binding (Fig. 2B). On the other hand, displacement of [^{3}H]15-HETE incorporated into phospholipids only became significant in the presence of 10 μM unlabeled 15-HETE, which displaced about 60% of the total esterified [^{3}H]15-HETE or 72% of the original cell-associated [^{3}H]15-HETE of the control cells (Fig. 2A).

Finally, the possibility of whether the nonesterified, cell-associated [^{3}H]15-HETE entered an internal cellular pool was examined. PT-18 cells were first equilibrated with 111–350 pM [^{3}H]15-HETE for 3 h at 4°C, followed by addition of 10 μM unlabeled 15-HETE. The incubation was stopped 1 min later which resulted in a 85 ± 5% (n = 4 separate experiments) decrease in nonesterified [^{3}H]15-HETE but an insignificant decrease in [^{3}H]15-HETE incorporation into phospholipids. This finding makes it unlikely that nonesterified 15-HETE was internalized.

DISCUSSION

In order to investigate the mechanism(s) of action of biologically active lipids, it is important to distinguish between binding of these lipids to intact cells and lipid metabolism. Numerous studies have shown that HETEs, the predominant metabolites derived from arachidonic acid, exert a variety of biological actions and are readily metabolized (5). For example, it has been reported that 5-, 12-, and 15-HETE are metabolized via incorporation into cellular lipids, β- and ω-oxidation, and transformation into polyoxygenated fatty acids such as hepoxilins and lipoxins (5, 15–18). Various reports have suggested that there may be HETE receptors but all these studies have ignored the contribution of HETE metabolism (19–21). In studying the mechanism of the 15-HETE-induced stimulation of the inactive 5-lipoxygenase in PT-18 cells (6), it became apparent that a method was needed that could distinguish between 15-HETE metabolism and 15-HETE binding to cells.

Isolation of [^{3}H]15-HETE-labeled PT-18 cells in the presence and absence of 10 μM unlabeled 15-HETE (a 30,000- to 100,000-fold excess) indicated that only 70% of the label could be displaced. In examining this result and in view of the above reports, a three-step filtration-extraction-chromatography (TLC or HPLC) method was developed to simultaneously measure 15-HETE binding and metabolism. Using this method, it was found that there were two cell-associated radioactive bands. The major band (70% at 4°C, 78% at 25°C) represented [^{3}H]15-HETE esterification into phospholipids and the minor band, though not negligible, was due to [^{3}H]15-HETE binding to cells. However, with RBL-1 cells at 4°C, there was four times more 15-HETE binding to cells than HETE esterification. Similar results were obtained with PT-18 cells (at 25°C) and [^{3}H]5,15-diHETE as ligand as it was found that diHETE binding to PT-18 cells predominated (90%) over incorporation into phospho-

lipids. The latter finding that diHETEs were not readily esterified into cellular lipids confirmed previous reports (22, 23). Moreover, these results indicated that esterification of [^{3}H]15-HETE into phospholipids of PT-18 and RBL-1 cells was an important process even at 4°C as others have reported for different cells at 37°C (5, 14, 24). It appears that at 4°C this esterification is an extremely facile process that seems to require very little metabolic energy. Even at this low temperature, different cells appear to have different energy requirements as there was about three times more HETE incorporation into PT-18 cells than into RBL-1 cells. In addition, these findings would suggest that the reported properties of the other HETE receptors may be in error because the ubiquitous incorporation of HETE into lipids was ignored and hence should be reinvestigated (19–21).

The presence of 1 or 10 μM unlabeled 15-HETE displaced all but 7 and 3% (respectively) of the nonesterified, cell-bound [^{3}H]15-HETE which indicated that 93–97% of [^{3}H]15-HETE was specifically bound to cells. Since neither [^{3}H]15-HETE internalization nor other metabolism was observed, this strongly suggested that the displaceable, nonesterified [^{3}H]15-HETE corresponded to the binding of [^{3}H]15-HETE to specific sites on the cells. Thus the present results indicated that the contribution of specific cell-bound [^{3}H]15-HETE to the interaction of cells with HETEs should not be ignored (see for example, reference 24).

Although we routinely use TLC in our three-step method, it should pointed out that this was done because *1)* RP-HPLC analysis showed that no other HETE metabolism (besides incorporation into lipids) was observed in this PT-18 cell system, and *2)* many samples could be processed inexpensively and quickly. If, in other systems, additional routes of metabolism are operating, HPLC rather than TLC, should be the preferred analytical method to determine the levels of specific (nonesterified) bound HETE and metabolized HETE. Studies, now in progress, are using this method to delineate various properties of the 15-HETE receptor in PT-18 cells. In view of the simplicity of this method, it is expected that this procedure should be readily applicable to determining the binding and metabolism of other lipids.

In summary, a simple and novel procedure is described that simultaneously measures two pools of cellular HETE, one in which nonesterified HETE is bound to specific cell sites and a second where HETE is incorporated into cellular lipids. The method can easily be extended to other HETE isomers and lipids and should be very useful for investigating their mechanisms of action.

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