Volume 298, number 2,3, 249–252 FEBS 10756 February 1992

High-affinity binding sites for 12(*S*)-hydroxy-5,8,10,14-eicosatetraenoic acid (12(*S*)-HETE) in carcinoma cells

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Received 20 December 1991

12(*S*)-hydroxy-5,8,10,14-eicosatetraenoic acid (12(*S*)-HETE) enhances tumor cell adhesion to endothelial cells [Honn et al. (1988) Proc. Soc. Exp. Biol. Med. 189, 130–135]. The effect is correlated to surface expression of an integrin receptor, GpIIb/IIIa. Here, we describe evidence for high-affinity binding of 12(*S*)-HETE to Lewis lung carcinoma cells. Scatchard plot analyses indicated a single class of sites with apparent K_d and B_{max} values of 0.44 nM and 66,000 sites per cell, respectively. Competition experiments with unlabeled compounds shod d that the binding was reversible and saturable as well as stereo- and regiospecific. The 12(*S*)-HETE binding, demonstrated here, might be an important step in a series of events controlling surface expression of integrin receptors.

GpIIb/IIIa; 12-HETE; Integrin; Lewis lung carcinoma; Lipoxygenase; Scatchard plot

1. INTRODUCTION

Lipoxygenases catalyze reactions between O_2 and polyunsaturated fatty acids containing 1,4-*cis*,*cis*-pentadiene structures (recently reviewed in [1]). Structurally related enzymes occur both in plants and animals [2] suggesting that they catalyze reactions of fundamental biological importance. Mammalian arachidonate 5-lipoxygenase participates in leukotriene biosynthesis [3] and consequently plays an important role in allergy and inflammation. The functions of animal 12- and 15-lipoxygenases are less well understood. Reactions catalyzed by 15-lipoxygenase seem to be involved in the programmed disappearance of mitochondria during reticulocyte maturation [2,4] and in modifying LDL prior to lipoprotein uptake by macrophages during foam cell formation [2,5]. Recently, it has been suggested that 12-lipoxygenase products participate in the regulation of the surface expression of the integrin receptor GPIIb/IIIa on carcinoma and melanoma cells [6]. The reported stereo- and regio-specificity of this effect suggested to us that 12-HETE might interact with a specific binding site. The purpose of the present investigation was to determine if such binding sites could be demonstrated in Lewis lung carcinoma cells.

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Abbreviations: diHETE, dihydroxyeicosatetraenoic acid; Gp, glycoprotein; HETE, hydroxyeicosatetraenoic acid; HODE, hydroxyoctadecadienoic acid; LLC, Lewis lung carcinoma; LTB_4, leukotriene B_4.

2. MATERIALS AND METHODS

2.1. *Chemicals*

12(*S*)-Hydroxy-[5,6,8,9,11,12,14,15(*n*)-^{3}H]-eicosatetraenoic acid (specific activity 178 or 103 Ci/mmol) was purchased from New England Nuclear and Amersham, respectively. 5(*S*)-HETE, 12(*S*)-HETE, 12(*R*)-HETE, 15(*S*)-HETE, 5(*S*),12(*S*)-diHETE, 6-*trans*-LTB_4, 6-*trans*-12-epi-LTB_4 and 13(*S*)-HODE were purchased from Cayman Chemicals, Ann Arbor, MI. Leukotriene B_4 was kindly provided by Dr. A. Ford-Hutchinson, Merck Frosst, Pointe-Claire, Dorval, Quebec, Canada. Alternatively, 12(*S*)-hydroxy-5,8,10,14-eicosatetraenoic acid (12(*S*)-HETE) was prepared from arachidonic acid using bovine platelets as enzyme source [7]. Blood, collected at slaughter in acid citrate dextrose (ACD), was centrifuged for 4 min at 1,500 × *g*, 4°C. The supernatant was recentrifuged at 1,000 × *g* for 20 min. Arachidonic acid was incubated with the platelets as described [7]. The purification of 12(*S*)-HETE was carried out by chromatography on a column of 1 g SilicAR CC-4 (Mallinckrodt). The sample was applied to the column in diethylether/heptane (1:9, v/v) and eluted with 20 ml diethylether/heptane (1:1, v/v). It was further purified by reverse-phase HPLC on C_{18}-Nucleosil using methanol/H_2O/acetic acid (78:22:0.01, by volume; solvent A) as mobile phase at a flow rate of 1 ml/min.

2.2. *Cell culture*

Lewis lung carcinoma cells (LLC) were a generous gift from Dr. Gilbert Vaes (Laboratoire de Chimie Physiologique, Université de Louvain, Brussels, Belgium). The cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 100 IU/ml penicillin, 100 μg/ml streptomycin and 10% (v/v) heat-inactivated (30 min at 56°C) fetal calf serum (Gibco) at 37°C in a humified atmosphere containing 10% (v/v) CO_2. Cultures were passaged by use of 0.54 mM EDTA in an isotonically buffered saline. Cells were not used beyond 20 consecutive passages in vitro.

2.3. *Binding assay*

LLC cells were grown on 24-multiwell culture trays (Flow Laboratories) for 24–48 h before binding experiments were performed. The cultures were washed twice with phosphate-buffered saline (PBS) before incubation with 12(*S*)-[^{3}H]HETE (0.1 nM, except for Scatchard analyses) in Dulbecco's modified Eagle's medium (DMEM) buffered

with 10 mM HEPES and supplemented with 100 IU/ml penicillin and 100 μg/ml streptomycin. The incubation volume of each well was 400 μl and incubations were performed at 4°C with gentle shaking. The binding reactions were terminated by washing the cells with ice-cold incubation buffer 4 times. After solubilization of the cells with 0.1 M NaOH, the cellbound radioactivity was measured by liquid scintillation spectrometry. Non-specific binding was determined in the presence of 100 nM unlabeled 12(*S*)-HETE. Specific binding was defined as the difference between the total binding of 12(*S*)-[^{3}H]HETE and non-specific binding. All data are expressed as mean values from triplicate analyses. The time course of the binding reaction was analyzed by incubating 0.1 nM 12(*S*)-[^{3}H]HETE with LLC cells in the presence or absence of unlabeled 12(*S*)-HETE (100 nM) for various lengths of time. The reversibility was determined by adding a thousand fold excess of unlabeled 12(*S*)-HETE to all wells after 120 min incubation. To calculate binding constants at equilibrium, different concentrations of 12(*S*)-[^{3}H]HETE (0.1–2 nM) were incubated with LLC cells for 120 min. Non-specific binding was determined as described above. Cell numbers were obtained from parallel wells after detachment by 2.5 g/l trypsin, 2.7 mM EDTA in phosphate-buffered saline (PBS) using a haemocytometer. Linear curve fitting was performed on the result according to the least square method and gave $R^2 = 0.94$. Competition studies were performed by addition of different unlabeled compounds in the concentration range 0 to 10^{-6} M and non-specific binding was determined by adding with 100 nM unlabeled 12(*S*)-HETE.

2.4. *Stability of 12(S)-HETE in the binding assay*

LLC cells in a tissue culture flask were incubated with 0.1 nM 12(*S*)-[^{3}H]HETE for 120 min at 4°C in the same buffer as in the other experiments. The incubation buffer was removed and acidified to pH 3 by acetic acid before extraction with diethylether. The extract was analyzed on a C_{18}-Nucleosil HPLC column using solvent A at 1 ml/min.

3. RESULTS

3.1. *Metabolic stability of 12(S)-HETE in the binding assay*

When 12(*S*)-[^{3}H]HETE was incubated with LLC cells for 120 min at 4°C about 90% of the radioactivity recovered from the incubation buffer had the same retention time on HPLC as 12-HETE.

3.2. *Characteristics of the binding reaction*

Figure 1A shows a time course experiment of 12(*S*)-[^{3}H]HETE binding to LLC cells at 4°C. Equilibrium was reached within 90 min after the addition of ligand. A standard incubation time of 120 min was chosen. In Fig. 1B 12(*S*)-[^{3}H]HETE was first incubated with LLC cells for 120 min, then a thousand-fold excess of unlabeled compound was added. The cellbound radioactivity diminished by 80% within 90 min suggesting that 12(*S*)-HETE binding is reversible. The experiments in Fig. 1A and 1B were performed on different days and using separate batches of cells which explains the variation in total binding observed. Fig. 2 shows a Scatchard plot [8] obtained after incubating 12(*S*)-[^{3}H]HETE (0.1–2 nM) with LLC cells as described in Section 2. The results indicated a single population of binding sites with apparent K_d and B_{max} values of 0.44 nM and 66,000 sites per cell, respectively.

3.3. *Specificity of the binding reaction*

To examine the specificity of binding, competition experiments were performed. LLC cells were incubated with 12(*S*)-[^{3}H]HETE (0.1 nM) in the presence of different concentrations (0–10^{-6} M) of structurally related, unlabeled compounds. Table I shows the IC_{50} values obtained. Two positional isomers of 12(*S*)-HETE, 5(*S*)-HETE and 15(*S*)-HETE, formed from arachidonic acid by distinct lipoxygenases, and 13(*S*)-HODE, derived from linoleic acid by one type of 12- or 15-lipoxygenase [1], gave IC_{50} values of around 5×10^{-7} M. Unlabeled 12(*S*)-HETE, analyzed under the same conditions, had an IC_{50} value of ca. 5×10^{-8} M. 12(*R*)-HETE, the enantiomer of 12(*S*)-HETE, did not compete for binding even at a concentration of 10^{-6} M. Thus, the stereospecificity of binding was greater than the positional specificity. Two dihydroxy metabolites of arachidonic acid both having a 12(*S*)-hydroxyl group and in addition a 5(*S*)-hydroxyl group, 5(*S*),12(*S*)-diHETE and 6-

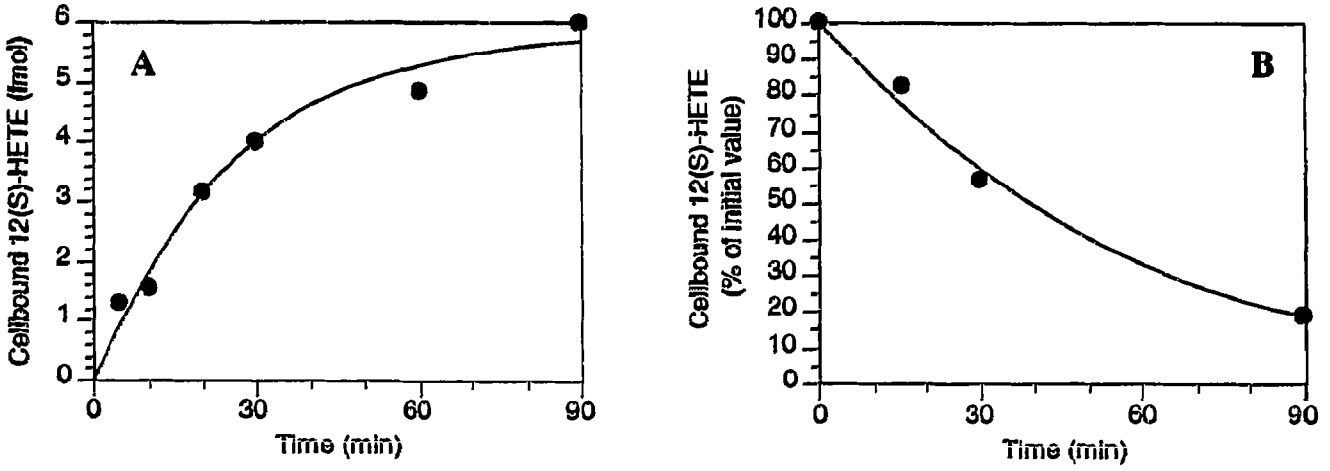

Fig. 1. (A) Time course of specific binding of 12(*S*)-[^{3}H]HETE to LLC-cells. (B) Reversibility of 12(*S*)-[^{3}H]HETE binding to LLC-cells. The cells were preincubated with 0.1 nM 12(*S*)-[^{3}H]HETE at 4°C for 120 min. A 1,000-fold excess of unlabeled 12(*S*)-HETE was then added. 100% corresponds to the total amount of bound ligand (1.2 fmol) after 120 min.

trans-12-epi-LTB_4, also did not compete for binding at concentrations up to 10^{-6} M. The effects of LTB_4 and 6-*trans*-LTB_4 were also determined. These two compounds have 5(*S*)- and 12(*R*)-hydroxyl groups. No competition was observed for 6-*trans*-LTB_4 ($IC_{50} \gg 10^{-6}$ M) whereas LTB_4 at 10^{-6} M reduced the binding to 64% of the initial value.

4. DISCUSSION

12-HETE is an important arachidonic acid metabolite in platelets [9], epidermis [10] and brain [11]. Its biological function in these cells and tissues is not well understood. It has been suggested that it has pathogenetic importance for psoriasis [10,12]. 12(*S*)-HETE is also synthesized in LLC cells [13]. A correlation has been reported between 12(*S*)-HETE synthesis and metastatic ability [14] which might be secondary to integrin GpIIb/ IIIa mediated tumor cell adhesion to endothelial cells, subendothelial matrix and fibronectin [6]. The high degree of stereospecificity reported for these effects suggested to us that binding to a binding site for 12(*S*)- HETE is an early step in a sequence of events regulating integrin expression. A binding assay was developed and specific binding was demonstrated. The characteristics of 12(*S*)-[^{3}H]HETE binding to LLC cells satisfies the criteria for a receptor; the binding is specific, reversible, saturable and has high affinity and low capacity for the ligand. The apparent dissociation constant (K_d) was 0.44 nM. This means that this binding site has a similar or higher affinity for its ligand compared to other eicosanoid receptors: 15(*S*)-HETE bound to a cloned strain of rat pituitary cells with a K_d of 0.75 nM [15] and 12(*S*)-HETE bound to squamous carcinoma cells with a K_d of 2.6 nM [12]. Two populations of receptor sites for LTB_4 were reported on polynuclear leukocytes with dissociation constants of 0.4 nM and 61 nM, respectively [16]. The dissociation constant for prostaglandin E_2 binding to a fraction from hypothalamus was 4.2 nM [17] and for prostaglandin D_2 binding to synaptic membranes of rat brain 28 nM [18]. Prostaglandin $F_{2\alpha}$ bound to a particular fraction of

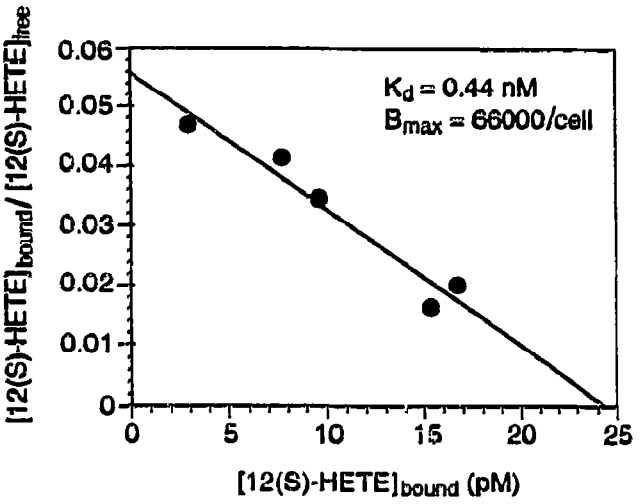

Fig. 2. Scatchard plot of binding data at equilibrium.

bovine and ovine corpora lutea with K_ds of 50 nM [19] and 100 nM [20], respectively. The binding site described here was highly specific for 12(*S*)-HETE. Table I lists the IC_{50} values for different competitors. These compounds were chosen because they occur naturally and are structurally related to 12(*S*)-HETE. The results indicate which parts of the 12 (*S*)-HETE molecule are most important for binding. 13(*S*)-HODE is also interesting because it has antagonistic effects to 12(*S*)-HETE [6]. The binding affinity of the various related compounds indicated that both the position and the stereochemistry of the hydroxyl group are important for binding. Furthermore, monohydroxy acids were preferred compared to dihydroxy compounds. Among the dihydroxy acids LTB_4 was a more potent competitor than 6-*trans*-LTB_4, 6-*trans*-12-epi-LTB_4 and 5(*S*),12(*S*)diHETE suggesting that the configuration at C12 is not the only determinant for binding affinity. The binding of 12(*S*)-HETE to Lewis lung carcinoma cells, reported here, might be an important step in regulating integrin surface expression. Further studies concerning the 12(*S*)-HETE binding site are in progress.

Table I

Competition by various lipoxygenase products on specific binding of 12(*S*)-HETE to LLC cells

Compound		IC_{50}
Abbreviated name	Systematic name	
5(*S*)-HETE	(6E,8Z,11Z,14Z)-(5(*S*))-5-hydroxyeicosa-6,8,11,14-tetraenoic acid	ca. 5×10^{-7} M
12(*S*)-HETE	(5Z,8Z,10E,14Z)-(12(*S*))-12-hydroxyeicosa-5,8,10,14-tetraenoic acid	ca. 5×10^{-8} M
12(*R*)-HETE	(5Z,8Z,10E,14Z)-(12(*R*))-12-hydroxyeicosa-5,8,10,14-tetraenoic acid	$\gg 10^{-6}$ M
15(*S*)-HETE	(5Z,8Z,11Z,13E)-(15(*S*))-15-hydroxyeicosa-5,8,11,13-tetraenoic acid	ca. 5×10^{-7} M
5(*S*),12(*S*)-diHETE	(6E,8Z,10E,14Z)-(5(*S*),12(*S*))-5,12-dihydroxyeicosa-6,8,10,14-tetraenoic acid	$\gg 10^{-6}$ M
6-*trans*-LTB_4	(6E,8E,10E,14Z)-(5(*S*),12(*R*))-5,12-dihydroxyeicosa-6,8,10,14-tetraenoic acid	$\gg 10^{-6}$ M
6-*trans*-12-epi-LTB_4	(6E,8E,10E,14Z)-(5(*S*),12(*S*))-5,12-dihydroxyeicosa-6,8,10,14-tetraenoic acid	$\gg 10^{-6}$ M
LTB_4	(6Z,8E,10E,14Z)-(5(*S*),12(*R*))-5,12-dihydroxyeicosa-6,8,10,14-tetraenoic acid	$> 10^{-6}$ M
13(*S*)-HODE	(9Z,11E)-(13(*S*))-13-hydroxyoctadeca-9,11-dienoic acid	ca. 5×10^{-7} M

Acknowledgements: We thank Mrs. Susanne Thunholm and Miss Åsa Schippert for their skilful technical assistance. This work was supported by the Swedish Medical Research Council (project 03X-5914).

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