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Displacement of Histamine From Liver Cells and Cell Components by Ligands for Cytochromes P450

Lorne J. Brandes,[1,2,3]* Gary M. Queen,[2] and Frank S. LaBella[2]

[1]Department of Medicine, Faculty of Medicine, University of Manitoba, Canada
[2]Department of Pharmacology/Therapeutics, Faculty of Medicine, University of Manitoba, Canada
[3]The Manitoba Institute of Cell Biology, 675 McDermot Ave. Winnipeg, Manitoba R3E 0V9, Canada

Abstract Intracellular histamine (HA) and cytochrome P450 monooxygenases (P450) each have been proposed as mediators of cell function, growth, and proliferation. The P450 family of heme enzymes is found in virtually all cells and generates, transforms, or inactivates steroids and other lipids that participate in cell regulation. We previously demonstrated a second messenger role for HA in blood platelets and the formation of a HA-P450 heme complex when exogenous HA was added to microsomes isolated from rat liver cells or to purified human P450 isozymes. Employing a radioimmunoassay, we now demonstrate that rat liver slices, microsomes derived from the livers of adult male rats and mast cell-deficient mice, and hepatoma cells, all contain endogenous HA. HA release from microsomes into the incubation medium, as determined by radioimmunoassay, is enhanced in the presence of carbon monoxide, steroids, and certain drugs, all agents that unite either directly with the iron atom or bind elsewhere within the heme cavity. Rat liver slices preincubated with ^{3}H-HA release labeled amine into the medium in the presence of those same ligands. These findings provide evidence of an in situ HA-P450 complex and offer further support that the imidazole, HA, is a physiological, intracellular modulator of cytochromes P450 in liver cells, and perhaps of these and other heme proteins in tissues in general. J. Cell. Biochem. 85: 820–824, 2002.

Key words: histamine; in situ histamine-P450 complex; displacement of histamine

Our laboratory previously reported a second messenger role for intracellular histamine (HA) in the mediation of human platelet aggregation [Saxena et al., 1989; Brandes et al., 1990] and cell proliferation [Brandes and LaBella, 1993; Brandes et al., 1994]. These effects are linked with the intracellular binding of HA to cytochromes P450 [LaBella and Brandes, 1996, 2000]. Intracellular HA and P450 enzymes each have been proposed as regulators of cell function and proliferation [Kahlson et al., 1970; Nebert, 1991]. Polyamines, various sex hormones, their drug antagonists, and a variety of growth-modulatory arylalkylamine medicinals all displace exogenous HA from cytochromes P450 [LaBella and Brandes, 1996; Brandes et al., 1998; LaBella et al., 2000], suggesting that monooxygenases may be common intracellular targets for an array of diverse endogenous and exogenous growth-regulating substances.

We now report evidence of an in situ HA-P450 complex in liver microsomes obtained from normal male rats and from mast-cell deficient (WW^{v}) mice. Moreover, both endogenously-bound HA in microsomes and labeled exogenous HA added to liver slices are displaced by agents that unite either directly with the iron atom or bind elsewhere within the heme cavity of P450 enzymes.

MATERIALS AND METHODS

Materials

Buffer constituents and phenobarbital were obtained from Sigma Chemical Company (St. Louis, MO) or Mallinckrodt (Anachemia Science, Montreal, Quebec). ^{3}H-HA (28 Ci/mmol) was purchased from Mandel (Dupont-New England Nuclear Research Products, Guelph, ON) and androstenedione from Steraloids (Wilton, NH). Culture media and sera

*Correspondence to: Lorne J. Brandes, The Manitoba Institute of Cell Biology, 675 McDermot Ave., Winnipeg, Manitoba, MB R3E 0V9, Canada.
E-mail:brandes@cc.umanitoba.ca

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were obtained from Grand Island Biological Company (GIBCO-BRL, Burlington, ON). Rat hepatoma cell lines (H4-IIE-C3; McA-RH-7777) were purchased from American Type Culture Collection (Manassas, VA). Adult male Sprague–Dawley rats were bred at the Central Animal Care Facility, University of Manitoba. Mast cell-deficient 8-week-old male mice (WW^v) and control mice (+/+) were purchased from Jackson Laboratory (Bar Harbor, ME). HA was measured by radioimmunoassay (Immunotech, Beckman Coulter Company, Burlington, ON).

Preparation of Microsomes

Fresh or frozen (− 80°C) livers from (200–250 gm) male Sprague–Dawley rats or WW^V (mast cell deficient) and control (+/+) mice were minced, suspended (10% wt/vol) in 0.25 M sucrose, homogenized with 6–8 passes in a motor-driven teflon-pestle glass vessel and centrifuged (15 min, 13,000*g*, 4°C). The supernatant was decanted and centrifuged (95 min, 144,000*g*, 4°C). For rat liver microsomes, the pellet was resuspended in 5 mM Tris buffer pH 8.5, centrifuged (30 min, 144,000*g*, 4°C) and stored at − 80°C (10–20 mg protein/ml).

Preparation of Liver Slices

Livers were removed from male Sprague–Dawley rats (150–200 g) and placed in ice-cold Krebs-Ringer. Pieces of liver were cut into slices of 0.25 mm thickness and chopped with a Mickle tissue chopper.

Hepatoma Cell Cultures

Monolayers of H4-IIE-C3 or McA-RH-7777 rat hepatoma cells were incubated (37°C, 95% air, 5% CO_2) and grown to confluency in wells containing Dulbecco's modified Eagle's medium, 5% glucose, 20% horse serum, and 5% fetal calf serum (GIBCO-BRL).

Measurement of HA

Microsomes. Microsomes (14–20 mg protein) were suspended in 1 ml buffer containing 0.1% bovine serum albumin (BSA) and the suspension placed in dialysis tubing with a 3,500 mol. wt. exclusion. The dialysis tube was suspended in 7 ml Tris-BSA at 4°C with stirring. To determine displacement of HA, test drugs at various concentrations were added to buffer both inside and outside the tubing. Aliquots were removed from the bathing medium at 8 min, and the concentration of HA/ml dialysate determined by radioimmunoassay (Immunotech).

Hepatoma cells. Cell cultures were replenished with fresh medium, washed with phosphate-buffered saline (PBS), and incubated in PBS for 60 min at 37°C. Aliquots of cells in PBS were centrifuged at 100*g* for 10 min and HA in the supernatant determined by radioimmunoassay (Immunotech).

Liver slices. To assess release of ^{3}H-HA following incubation with various agents, liver slices were washed three times in 80-ml ice-cold Krebs-Ringer buffer without glucose, with repeated pipetting of slice suspensions. The liver slice suspensions were filtered through a 0.33-mm nylon screen and the tissue weighed and put into flasks containing 10 ml buffer (24 mM glucose and 100 μM aminoguanidine, a histaminase inhibitor)/g tissue. The suspension was flushed with 95%/5% O_2/CO_2, and the flasks stoppered and incubated for 20 min at 37°C with gentle shaking in a Dubinoff shaker, washed two times in 30 ml buffer, and adjusted to yield 2 ml/g original slice. Unlabeled HA (0.32 μM final concentration) and ^{3}H-HA (0.06 μM; 2.4 μCi/ml) were added, and the flasks containing the suspension flushed with 95%/5% CO_2, stoppered, and incubated with gentle shaking for 30 min at 37°C. The ^{3}H-HA-loaded tissue was washed four times at 1 min intervals and three times at 10 min intervals, each time with 20 ml buffer, and suspended in Krebs-Ringer buffer containing about 87 mg tissue/ml, or in Krebs-Tris buffer (25 mM, pH 7.4), both containing glucose and histaminase inhibitor. Two milliliter aliquots of tissue suspension were incubated in the presence or absence of test compounds for up to 60 min at 8°C. For studies with N_2 or CO, the flasks were flushed, bubbled for 20 s, and again flushed with the appropriate gas. After 60 min incubation, aliquots were centrifuged at 5,000*g* for 5 min and ^{3}H-HA measured in the supernatant.

Spectral studies. Microsomes were incubated with 100 μM HA in 5 mM Tris buffer (pH 8.5) for 30 min at room temperature, centrifuged at 150,000*g* for 20 min and pellets (approximately 1 mg/ml protein) resuspended with gentle homogenization in buffer. Similarly-prepared microsomes, not incubated with HA, served as controls. Buffer or test compound was added to both reference and sample cuvettes in 1–25 μl aliquots and mixed. The change

TABLE I. HA Content in Microsomes From Mast-Cell Deficient Mice and in Hepatoma Cell Culture Medium

Cultures	HA content
Mouse liver microsomes	
+/+ (fmol HA/mg protein)	147 ± 20[a] (n = 3)
WW^v (fmol HA/mg protein)	52 ± 35 (n = 3)
Hepatoma culture supernate	
H4-IIE-C3 (pmol HA/ml)	1.58 ± 0.16 (n = 5)
McA-RH-7777 (pmol HA/ml)	442 ± 84 (n = 12)

[a]Mean ± SE.

in the absorbance difference spectrum with time was measured [Jefcoate, 1978].

RESULTS

Microsomes prepared from rat liver homogenates reproducibly contained substantial amounts of HA (mean ± SD, 23.3 ± 5.5 pmol HA/875 ± 93 pmol P450/mg protein). Washing the microsomes in distilled H_2O an additional two times did not diminish the amount of measured HA, suggesting that the amine was tightly bound.

To exclude the possibility that mast cell disruption was the source of the HA content of these homogenates, liver microsomes were also prepared from mast cell-deficient (WW^V) mice (Table I), and shown to contain about one-third as much HA as microsomes from control (+/+) mice (52 ± 35 vs. 147 ± 20 fmol HA/mg protein).

Supernatants from the two hepatoma cell lines also contained measurable amounts of HA, although McA-RH-7777 cells secreted approximately 250-fold more HA into the medium than did H4-IIE-C3 cells (Table I), corresponding with a total cell HA content of 130 pmol/mg protein in the former and 0.35 pmol/mg protein in the latter. Marked variability among hepatoma and other tumor cell lines in intracellular HA content and/or in HA export from the cell was previously demonstrated by others [Bartholeyns and Bouclier, 1984; Falus et al., 1997].

In isolated microsomes, various hydrophobic and/or nonpolar compounds displaced endogenous HA (Table II), which presumably is highly specific for the heme iron atom. HA complexed to P450 enzymes forms a stable bond, which was disrupted immediately upon addition of a competing ligand (Fig. 1). Both "type II" ligands that bind to the heme iron atom (imidazole) and "type I" compounds that bind to sites within the catalytic cavity (fluoxetine, androstenedione) promptly displaced HA, a "type II" imidazole derivative.

Radiolabeled HA was taken up by liver slices and its release enhanced by addition of androstenedione, pentobarbital, and carbon monoxide, all ligands for P450 (Fig. 2).

DISCUSSION

Previous work from our laboratory showed that in microsomes, the complex formed between added HA and cytochromes P450 was disrupted by various ligands, including hydrophobic and/or nonpolar compounds that would not be expected to displace HA from sites other than the P450 catalytic cavity [LaBella and Brandes, 1996; LaBella et al., 2000].

The findings reported here now support the notion that intracellular (endogenous) HA naturally complexes to the heme iron atom within the catalytic pocket of at least certain P450 isozymes. Although the occupancy of P450 by tightly-bound endogenous HA in isolated rat liver microsomes is only about 3%, we previously demonstrated that in addition to higher affinity (K_{d1}, ~1 μM) microsomal sites, ^{3}H-HA also binds to a much larger number of lower affinity (K_{d2}, ~60 μM) microsomal sites in rat liver [Brandes et al., 1998]. The B_{max} value for these intermediate affinity sites (850 ± 190 pmol/mg protein) [Brandes et al., 1998],

TABLE II. Release of Endogenous HA From Rat Liver Microsomes by Various Ligands

Drug	Control (mean ± SE) (pmol HA/ml dialysate)	Drug (mean ± SE) (pmol HA/ml dialysate)	n[a]
Androstenedione (250 μM)	3.05 ± 0.72	5.54 ± 0.93[b]	6
Fluoxetine (250 μM)	2.06 ± 0.43	4.91 ± 1.11[c]	7
Cetirizine (250 μM)	2.33 ± 0.57	2.74 ± 0.74	5

[a]Replicates = 3–4 per experiment.
[b]$P < .001$.
[c]$P < .01$ (paired two-tailed t-test).
The difference observed for cetirizine was not significant ($P = 0.3$).

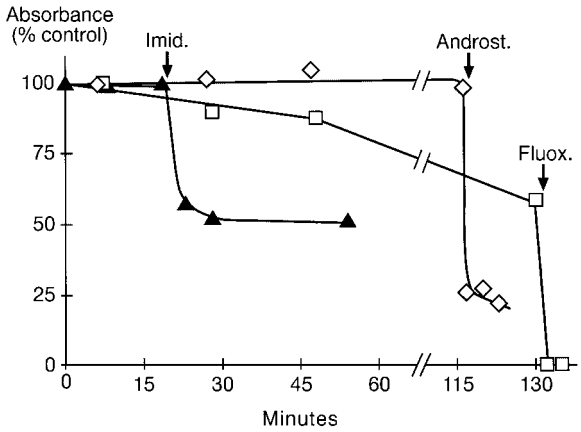

Fig. 1. Effect of various compounds on the HA-P450 complex as determined by the absorbance-difference spectrum. Microsomes were incubated with 100 μM HA in 5 mM Tris buffer, pH 8.5, for 30 min at room temperature, centrifuged at 150,000*g* for 20 min, and pellets resuspended at about 1 mg/ml protein with gentle homogenization in buffer. Microsomes in the absence of HA served as reference controls. X-axis: The measured difference in absorbance at 430 nm (peak) and 395 nm (trough) in the absence of ligand represents 100% of control HA binding. Buffer or test compound was added to both reference and sample cuvettes in 1–25 μl aliquots and mixed. The change in the absorbance difference spectrum was measured over time. Arrows indicate time of addition of the various ligands; a decrease in absorbance on the addition of the various ligands indicates displacement of HA. Imid., imidazole; Androst., androstenedione; Fluox., fluoxetine.

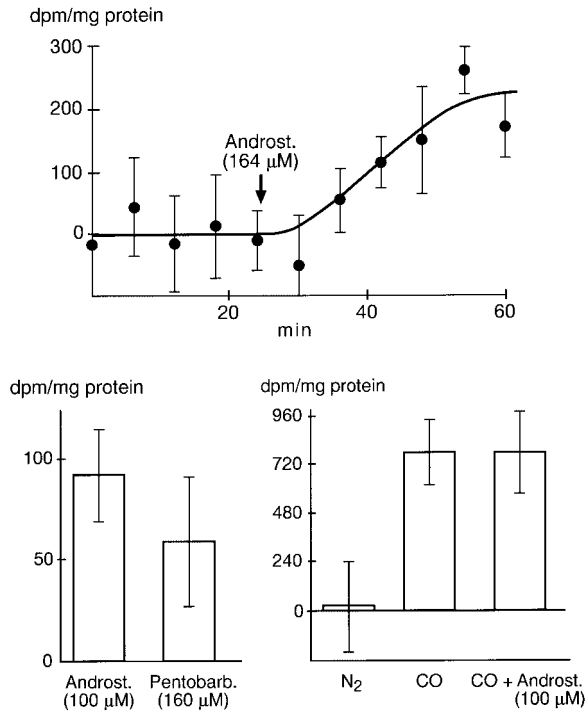

Fig. 2. Release of ^{3}H-HA from liver slices preincubated with various agents. At time indicated by arrow (top graph), or after 60 min (lower graphs), aliquots of incubation medium were centrifuged at 5,000*g* for 5 min and ^{3}H-HA measured in the supernatant. Top graph: As opposed to the rapid release of HA from microsomes (Fig. 1), the more gradual release of ^{3}H-HA from slices may reflect time-dependence of cell uptake of androstenedione and export of displaced ^{3}H-HA. Bottom graphs: Bars represent the difference between ^{3}H-HA release from slices in the presence of ligands and from control slices in the absence of ligands. Androst., androstenedione; Pentobarb., pentobarbital; CO, carbon monoxide; N_2, nitrogen.

approximates the total concentration of P450 in rat liver microsomes. Therefore, it is possible that a large amount of loosely bound, but physiologically relevant [Saxena et al., 1989], endogenous HA occupies most, or all, P450s, but is lost during the preparation of microsomes. The demonstration here that liver slices take up ^{3}H-HA, which can be displaced by ligands for P450 also suggests that in addition to endogenously-produced amine, mast cell HA may enter hepatocytes in vivo and bind to P450.

Of the compounds tested, only cetirizine failed to significantly enhance the release of endogenous HA from microsomes (Table II), correlating with our previous observation that this H_1-anti-HA is relatively weak to inhibit catalytic activity of P450 and to displace HA added to isolated microsomes [LaBella and Brandes, 1996]. In contrast to microsomes, we were unable, by immunoassay, to demonstrate chemically-induced release of endogenous HA from liver slices. Perhaps, in slices, the number of cells penetrated by the ligands was small, resulting in binding to P450 that although sufficient to measure release of added ^{3}H-HA, was too low to detect release of endogenous HA. Alternatively, background release of endogenous HA from other sites may have masked ligand-induced release.

That release of microsomal HA by CO, androstenedione, and certain drugs is through an interaction at P450, supported by their lack of effect on HA release from McA-RH-7777 hepatoma cells that contain high levels of intracellular HA, but contain levels of P450 insufficient for spectrophotometric quantitation of the complex with carbon monoxide; even when P450 was induced in these cells, levels increased to only about 5% of that of normal liver microsomes, and no enhanced release of HA by androstenedione could be detected using intact cells or microsomes (data not shown).

In summary, evidence is presented for (i) an in situ P450-HA complex in liver microsomes from

adult male rats and mast-cell-deficient mice and (ii) the rapid displacement of endogenous HA from this microsomal complex by both natural and synthetic agents that also bind to P450. The presumed consequence of this interaction is alteration of the metabolism of endogenous substrates, which in turn influences the level of cell-regulatory lipid and prostanoid mediators of cell growth and function [Nebert, 1991]. In support of this suggestion, we found, for a group of compounds representing several therapeutic classes of drugs, a strong correlation between the in vitro potencies to inhibit (i) formation of a HA-P450 complex, (ii) P450 catalytic activity, and (iii) lymphocyte mitogenesis and the in vivo potencies to enhance experimental tumor growth [Brandes et al., 1994; LaBella and Brandes, 1996]. Finally, our novel findings may be applicable to other heme proteins and to other bioamines [LaBella and Brandes, 2000].

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