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ARTICLES

Potent Interaction of Histamine and Polyamines at Microsomal Cytochrome P450, Nuclei, and Chromatin From Rat Hepatocytes

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Abstract Histamine and polyamines have been implicated in the mediation of cell proliferation. Our previous work linked the growth-modulatory effects of histamine with its binding to intracellular sites in microsomes and nuclei of various tissues. In this study, we identify cytochrome P450 enzymes as a major component of microsomal intracellular sites in hepatocytes and demonstrate that polyamines compete with high affinity for histamine binding to them. Spectral measurement of histamine binding to P450 in liver microsomes resolved high and intermediate affinity binding sites (K_{s1} = 2.4 ± 1.6 μM; K_{s2} = 90 ± 17 μM) that corresponded to microsomal binding sites (K_{d1} = 1.0 ± 0.9 μM; K_{d2} = 57 ± 13 μM) resolved by ^{3}H-histamine binding; additional low affinity (K_{d3} ~ 3 mM), and probably physiologically irrelevant, sites were resolved only by ^{3}H-histamine radioligand studies. As determined spectrally, treatment of microsomes with NADPH/carbon monoxide decreased histamine binding to P450 by about 90% and, as determined by ^{3}H-histamine binding, abolished the high affinity sites and reduced by 85% the number of intermediate sites. Spermine competed potently for ^{3}H-histamine binding: in microsomes, K_i = 9.8 ± 5.8 μM; in nuclei, K_i = 13.7 ± 3.1 μM; in chromatin, K_i = 46 ± 33 nM. Polyamines inhibited the P450/histamine absorbance complex with the rank order of potency: spermine > spermidine >> putrescine. In contrast, histamine did not compete for ^{3}H- spermidine binding in nuclei or microsomes, suggesting that polyamines modulate histamine binding allosterically. We propose that certain P450 isozymes that modulate gene function by controlling the level of oxygenated lipids, represent at least one common intracellular target of growth-regulatory endogenous bioamines and, as shown previously, of exogenous growth-modulatory drugs including antiestrogens, antiandrogens, and certain antidepressants and antihistamines. J. Cell. Biochem. 69:233–243, 1998. © 1998 Wiley-Liss, Inc.

Key words: histamine; polyamines; cytochrome P450; cell growth; cell proliferation

There is increasing evidence that histamine (HA) and polyamines (PA) play essential, and often complementary, roles in mediating cell proliferation [Morgan, 1991; Kahlson and Rosengren, 1970]. For example, increased levels of histidine decarboxylase (HDC) and/or ornithine decarboxylase (ODC), the enzymes that generate HA and PA, respectively, are associated with lymphocyte mitogenesis [Oh et al., 1988], myelopoiesis [Dy et al., 1993], certain hormone responses [Sjoholm, 1993], and the growth of both normal and malignant cells [Luk and Casero, 1987; Bartholeyns and Bouclier, 1984]. Inhibitors of HDC and ODC suppress the proliferation of many cell types in a time- and dose-dependent manner [Luk and Casero, 1987; Bartholeyns and Bouclier, 1984; Brandes and LaBella, 1993]. The tumor promoter phorbol-12-myristate-13-acetate (PMA) induces both HDC and ODC in the epidermis of the mouse [Watanabe et al., 1981]. HA and PA each suppress ODC induction in Ehrlich ascites cells [Sanchez-Jimenez et al., 1993].

We have presented evidence that HA is an intracellular messenger, mediating human platelet aggregation induced by PMA [Saxena et al., 1989] or collagen [Saxena et al., 1990], and murine lymphocyte mitogenesis induced by concanavalin A [Brandes and LaBella, 1992].

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We showed that platelet aggregation [Saxena et al., 1989] and lymphocyte mitogenesis [Brandes et al., 1991] are accompanied by formation of new HA; both processes are inhibited by the specific irreversible HDC inhibitor, α-fluoromethylhistidine (α-FMH). These actions of HA are mediated at intracellular sites designated "H_{IC}" to distinguish them from plasma membrane-associated H_1, H_2, and H_3 receptors [Brandes et al., 1990]; H_{IC} sites are associated with antiestrogen (tamoxifen) binding sites in microsomes and nuclei [Brandes et al., 1991] and appear to be most abundant in the liver [Brandes et al., 1991, 1987].

Evidence that a proportion of microsomal H_{IC} sites represents HA binding to cytochrome P450 enzymes derives from the following: (1) in liver and adrenal microsomes, HA generates a P450 absorbance spectrum typical of ligands that bind directly to the heme iron atom [LaBella et al., 1992]; (2) thioperamide, an imidazole antagonist of H_3 receptors [Arrang et al., 1983], yields a P450 absorbance difference spectrum and inhibits HA binding to adrenal P450 (K_s = 0.3 μM), competes for ^{3}H-HA binding in the same preparation (K_i = 0.3 μM), and inhibits cortisol secretion from isolated adrenal cortical cells (IC_{50} = 0.2 μM)[LaBella et al., 1992], suggesting both a physiological role for intracellular HA to modulate, and for thioperamide to antagonize, P450 mono-oxygenases that generate steroids; (3) the potencies of certain H_1 antihistamines, antidepressants, antiestrogens, and other drugs to inhibit lymphocyte mitogenesis in vitro and to stimulate experimental tumor growth in vitro and in vivo, correlate with potencies to inhibit ^{3}H-HA binding in microsomes, the HA/P450 absorbance complex, and P450 catalytic activity [Brandes et al., 1994; LaBella and Brandes, 1996].

Definitive receptors that mediate growth and other cell functions of PA have yet to be delineated, although PA form complexes with, for example, N-methyl-D-aspartate (NMDA) receptors [Williams, 1994], ion channels [Ficker et al., 1994], nucleic acids [Tabor and Tabor, 1989], various cellular membranes [Shuber, 1989], and phospholipids [Tabor and Tabor, 1989]. A second messenger role for PA in response to hormonal stimulation has been demonstrated in renal cortical cells, where testosterone induces rapid induction (1–4 min) of ODC and Ca^{2+} flux; endocytosis, hexose and amino acid transport are blocked by difluoromethylornithine (DFMO), a specific irreversible inhibitor of ODC [Koenig et al., 1983]. In accordance with their parallel effects on cell function and growth [Watanabe et al., 1981; Sanchez-Jimenez et al., 1993], HA and PA assume similar spatial conformations in their minimal potential energy states [Sanchez-Jimenez et al., 1993]. These findings prompted us to determine whether the PA (putrescine [PUT], spermine [SP], and spermidine [SPD]) compete with HA at nuclear and microsomal sites.

MATERIALS AND METHODS

Materials

Buffer constituents were obtained from Sigma Chemical Co., St. Louis, MO, and from Mallinckrodt (Anachemia Science, Montreal, Quebec). HA, SP, SPD, and PUT were purchased from Sigma. ^{3}H-HA (28 Ci/mmol) and ^{3}H-SPD (15 Ci/mmol) were obtained from Dupont New England Nuclear Research Products (Boston, MA). Male Sprague-Dawley rats were obtained from the Central Animal Care Facility, University of Manitoba.

Preparation of Microsomes

2-amino-2-(hydroxymethyl)1,3,-propanediol (TRIS) buffer (pH 8.5) was found to be optimal for the spectral measurement of HA binding to P450. Thus, for comparison of binding and spectral data on HA and PA, we employed microsomes prepared in sucrose and washed in TRIS. Fresh or frozen (-80°C) livers from (200–250*g*) male Spague Dawley rats 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). The resulting pellet was resuspended in 5 mM TRIS (pH 8.5) at a final concentration of 4 mg protein per ml, centrifuged (144,000*g*, 35 min, 4°C), resuspended in TRIS buffer (10–20 mg protein per ml) and stored at -80°C. Ethylene diamine tetraacetic acid (EDTA) (1 mM) was added to the suspension prior to radioligand binding assays.

We assessed competition of HA, SP, SPD, and PUT for ^{3}H-HA binding in microsomes prepared in HCO_3 buffer, as originally described [Brandes et al., 1987]. Fresh livers were homogenized in 0.32 M sucrose and 10 mM TRIS (pH 6.9). After centrifugation (5,000*g*, 10 min, 4°C)

the supernatant was decanted, centrifuged (150,000*g*, 30 min, 4°C), and the resulting pellet suspended in 10 mM $NaHCO_3$, centrifuged (28,000*g*, 30 min, 4°C), resuspended, and stored (-80°C) in 10 mM $NaHCO_3$ (pH 7.4) containing 0.1 μM $CuCl_2$.

Effect of Copper Ion on Ligand Binding

The addition of copper ($CuCl_2$) markedly enhances the specific binding of ^{3}H-histamine to liver cell microsomes and nuclei. Since concentration-effect studies indicate that binding parameters obtained in the presence of 0.1–10 mM $CuCl_2$ are not significantly different from those obtained with copper-free buffers, all microsomal studies have been done in buffers containing 0.1 mM copper ion, whereas, in order to increase specific binding from very low basal levels, nuclear studies have been done in buffer containing 10 mM copper.

Preparation of Hepatocyte Nuclei and Chromatin

Nuclei and nuclear chromatin were prepared as described previously [Brandes et al., 1991]. Fresh rat livers were minced and added (1 g/ml) to Buffer I (10 mM TRIS, pH 6.9; 3 mM $CaCl_2$; 2 mM Mg acetate; 0.32 M sucrose; nonylphenoxypolyethoxy ethanol (NP-40), 5 μl/ml; 1 mM phenylmethylsulfonyl fluoride (PMSF)), homogenized as described for microsomes, filtered through cheese cloth, and the filtrate rehomogenized. The homogenate was diluted (5 ml/g of original liver tissue) by addition of Buffer II (10 mM TRIS, pH 6.9; 5 mM Mg acetate; 2.2 M sucrose). Aliquots of 17 ml were layered over 5 ml buffered 2.04 M sucrose (3 ml Buffer I plus 32 ml Buffer II) in 25 x 89 mm polyallomar tubes and centrifuged (40,000*g*, 60 min, 4°C) in a Beckman (Palo Alto, CA) SW-28 rotor to yield a pellet comprising cell nuclei.

To prepare chromatin, the nuclear pellet was homogenized in 2–3 ml Buffer I using 5–7 strokes of a hand-driven teflon pestle-glass vessel homogenizer, centrifuged (2,000*g*, 10 min, 4°C), the pellet resuspended in 0.7 ml per g original liver of Buffer III (10 mM piperazine-N,N'-bis-[ethanesulfonic acid] (PIPES), pH 7.0; 1 M hexylene glycol; 30 mM Na butyrate; 2 mM $MgCl_2$; 1% v/v, thiodiglycol), and treated with 1 mM $CaCl_2$ and micrococcal nuclease (25 U/ml). After incubation (20 min, 37°C) in a shaking water bath, the reaction was terminated by addition of EDTA (10 mM final concentration). Following centrifugation (2,000*g*, 10 min, 4°C) the pellet was suspended in 10 mM TRIS buffer (pH 8.0 at 22°C), stored at 4°C overnight, and centrifuged (7,800*g*, 10 min, 4°C) to yield the chromatin-containing supernatant.

Spectral Analysis of Ligand/P450 Complexes

The P450 absorbance difference spectra of P450-bound HA were measured [Jefcoate, 1978] with a Milton Roy (Rochester, NY) Spectronic 3000 Array Spectrophotometer; a Rapidscan computer software program controlled and operated the unit, and collected and plotted the spectral data [Estabrook and Werringloer, 1978]. Binding parameters from radioligand binding data (K_d, B_{max}) were analyzed with the McPherson modification of the LIGAND program [Munson and Rodbard, 1980]; spectral data were analyzed with ENZYME, a curve-fitting program employing weighted nonlinear least squares analysis, as described by Lutz et al. [1986], or with the weighted nonlinear regression option of PRISM (Graphpad Software, Inc., San Diego, CA). To obtain spectra, microsomes were suspended in 5 mM TRIS buffer (pH 8.5; 1.0 mg protein/ml) containing HA (3–2,000 μM) in the absence or presence of increasing concentrations (10^{-5}–10^{-2} M) of SP, SPD, or PUT (all stock solutions adjusted to pH 8.5).

Radioligand Binding Assays

Data were analyzed using the LIGAND program [Munson and Rodbard, 1980]. Each assay included 20–25 concentrations of HA or 15–20 concentrations of PA (all 3 replicates each) for microsomes and nuclei. Chromatin assays included 10–15 concentrations (3 replicates) for HA and SP.

Microsomes. Aliquots (0.8 ml) of microsomal suspension (1.25 mg protein/ml) in HCO_3 (pH 7.4) or TRIS buffer (pH 8.5) were added to 1.5 ml Eppendorf tubes containing 5 nM ^{3}H-HA or 5 nM ^{3}H-SPD in the presence or absence of increasing concentrations (10^{-9}–10^{-2} M) of unlabelled HA, SP, SPD, or PUT (1 ml total volume). After incubation (40 min, 22°C with TRIS or 4°C with HCO_3), the tubes incubated with TRIS were placed in an ice bath for 10 min with 20 μl of 0.5 M $MgCl_2$ to promote protein aggregation. The tubes were then centrifuged (10,000*g*, 15 min, 4°C). Each pellet was dissolved in NaOH and radioactivity determined in a Beckman liquid scintillation counter. Data were analyzed using the LIGAND program [Munson and Rodbard, 1980].

TABLE I. Effect of Polyamines on Binding of ^{3}H-Histamine in Liver Microsomes

Buffer	Site	Histamine [K_d (μM)]	Spermine [K_i (μM)]	Spermidine [K_i (μM)]	Putrescine [K_i (μM)]
HCO_3	1	0.143 ± 0.13	0.10 ± 0.06	0.62 ± 0.06	4.61 ± 1.2
	2	18.2 ± 5.9	5.6 ± 1.4	12.8 ± 1.6	242 ± 26
	3	1,647 ± 228	—	—	—
		n = 8	n = 7	n = 6	n = 7
TRIS	1	0.33 ± 0.22	9.8 ± 5.8		
	2	49 ± 14	286 ± 42		
	3	2,300 ± 1,200	17,400 ± 4,170		
		n = 4	n = 2		

Nuclei. Aliquots of intact nuclei (0.2–0.4 mg protein/ml) in HCO_3 or Krebs buffer, containing 10 μM $CuCl_2$, were incubated with 5 nM ^{3}H-HA in the presence of increasing concentrations of cold HA or SP (10^{-9}–10^{-2} M) at 22°C for 60 min (Krebs buffer) or at 4°C for 30 min (HCO_3 buffer). The reaction was teminated by centrifugation (12,000*g*, 15 min, 4°C) and radioactivity of the pellets measured. Data were analyzed using the LIGAND program [Munson and Rodbard, 1980].

Chromatin. Aliquots of chromatin solution (1–1.5 mg protein/ml) were incubated (60 min, 22°C) in 10 mM TRIS buffer (pH 8.0) containing 10 μM $CuCl_2$, to which was added 5 nM ^{3}H-HA in the presence or absence of cold HA or SP. The soluble chromatin-HA complex was precipitated with 10 μM $MgCl_2$ and centrifuged (12,000*g*, 15 min, 4°C). The pellet was dissolved in NaOH, and radioactivity counted.

RESULTS

Histamine and Polyamine Competition for ^{3}H-Histamine Binding in Microsomes

Three binding sites for HA were resolved in the microsomal fraction (Table I). The PA competed for ^{3}H-HA binding (Table I) with a rank order of potency: SP > SPD > PUT. Under conditions optimal for HA binding to P450 (TRIS buffer, pH 8.5), HA was more potent than SP at all three microsomal sites labelled by ^{3}H-HA (Table I), whereas in HCO_3 buffer SP was more potent than HA (Table I). In HCO_3, the PA competed only at the two higher HA sites; in TRIS buffer, SP competed for all 3 HA sites (Table I). The difference in K_i values for SP in the two assay systems may be due, at least in part, to the presence of Mg^{2+} in the TRIS assay. HA binding affinities were similar in both systems.

TABLE II. Effect of Spermine on the Histamine-Cytochrome P450 Absorbance-Difference Spectrum*

Site	Regression analysis	
	K_s (μM) HA	B_{max} HA
1	2.4 ± 1.6	0.0054 ± 0.0019 A.U./mg
2	90 ± 17	.044 ± 0.02 A.U./mg
	n = 2	
	Lineweaver-Burk analysis	
	K_s (μM) HA	K_i (μM) SP
1	4.9 ± .09	22.3 ± 5.3
	n = 3	
2	86.4 ± 9.0	161 ± 33
	n = 2	

*A.U. = absorbance units.

Absorbance Difference Spectra of Histamine or Polyamines with Microsomes

Addition of HA to microsomal suspensions generated a difference spectrum with a trough at 390–410 nm and a peak at 425–435 nm (Fig. 1). Application of Lineweaver-Burk analysis to the spectral data for HA yielded dissociation constants (K_{s1} = 4.9 ± 0.9 μM; K_{s2} = 86.4 ± 9.0 μM; Fig. 2A and B; Table II), similar to those obtained by nonlinear regression analysis using the PRISM program (K_{s1} = 2.4 ± 1.6 μM; K_{s2} = 90 ± 17 μM; Table II and Fig. 1). Jefcoate reported that the concentration of cytochrome P450-bound substrates can be calculated from the difference spectrum by assigning a molar extinction value of 40–50 mM^{-1} cm^{-1} [Jefcoate, 1978]. Based on our spectral estimations for ΔA_{max} (.0054 and .044 AU/mg protein; Table II), the calculated B_{max} values were approximately 0.12 and 0.98 nm/mg protein, respectively; the

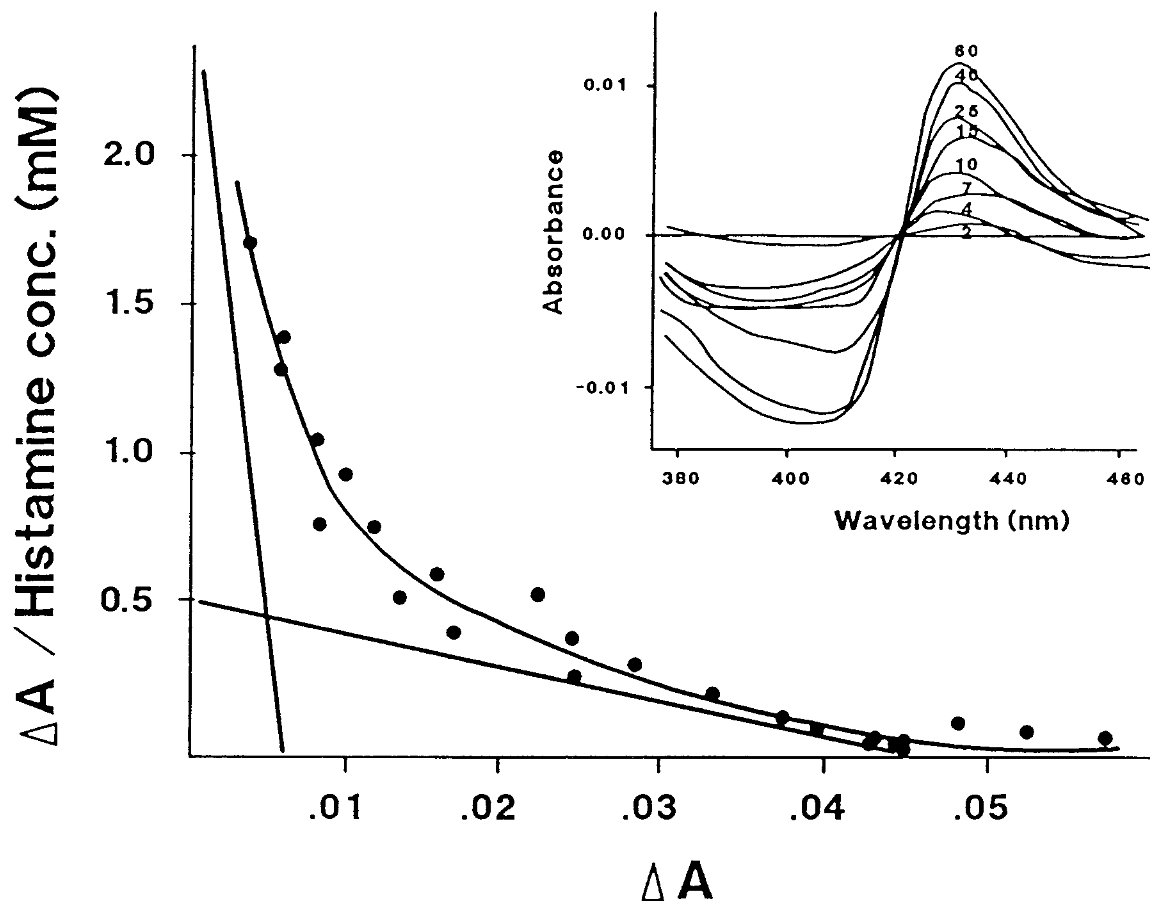

Fig. 1. Scatchard plot for HA (2–4,000 μM) binding to cytochrome P450. Actual K_s values, determined by employing the LIGAND program [Munson and Rodbard, 1980] to analyze absorbance difference spectra (K_s, A_{max}) from 2 experiments, are given in Table II. **Inset:** Absorbance-difference spectrum for HA (2–60 μM).

latter value compared favourably to the B_{max} value of the intermediate-affinity site determined by radioligand studies (0.85 nm/mg protein; Table IV). The lowest affinity (mM) radioligand site was not detected by P450 difference spectra. Although estimates of the two high affinity sites determined by both methods (Tables II, IV) were similar (1.0 and 2.4 μM), the B_{max} values were quite different between the two methods (0.12 and 0.0034 nm/mg). The concentration of P450, as measured by the method of Omura and Sato [1964], was about 1.0 nm/mg protein, corresponding to the combined density of the high and intermediate affinity sites resolved by ^{3}H-HA binding studies (Table IV).

SP inhibited HA binding to P450 (K_{i1} = 22.3 ± 5.3 μM; K_{i2} = 161 ± 33 μM; Figs. 2A,B, 3; Table II). These values are similar to K_i values for SP to inhibit ^{3}H-HA binding in microsomes (TRIS buffer; Table I). The inhibition was competitive at lower concentrations of HA and SP and noncompetitive at the higher amine concentrations. The addition of 100 μM SP (to both reference and sample cuvettes) caused a wavelength shift in the 10 μM HA difference spectrum curve of about 5 nm (Fig. 3). The interaction of amines with P450 can produce difference spectra with absorption maxima of 425–432 nm, between type IIa and IIb extremes, depending on experimental conditions [Walker et al., 1976].

The effects of the PA on the HA/P450 absorbing complex were determined in the presence of 500 μM HA; the IC_{50} value for SP was 150 ± 18 μM (Fig. 4), similar to its K_{i2} value calculated by Lineweaver-Burk analysis (161 ± 33 μM; Table II). SPD and PUT were weaker with IC_{50} values of 400 ± 55 μM and 4.3 ± .15 μM, respectively (Fig. 4). Despite inhibiting HA binding to P450, the PA did not themselves generate an absorbance difference spectrum over a wide range of concentrations.

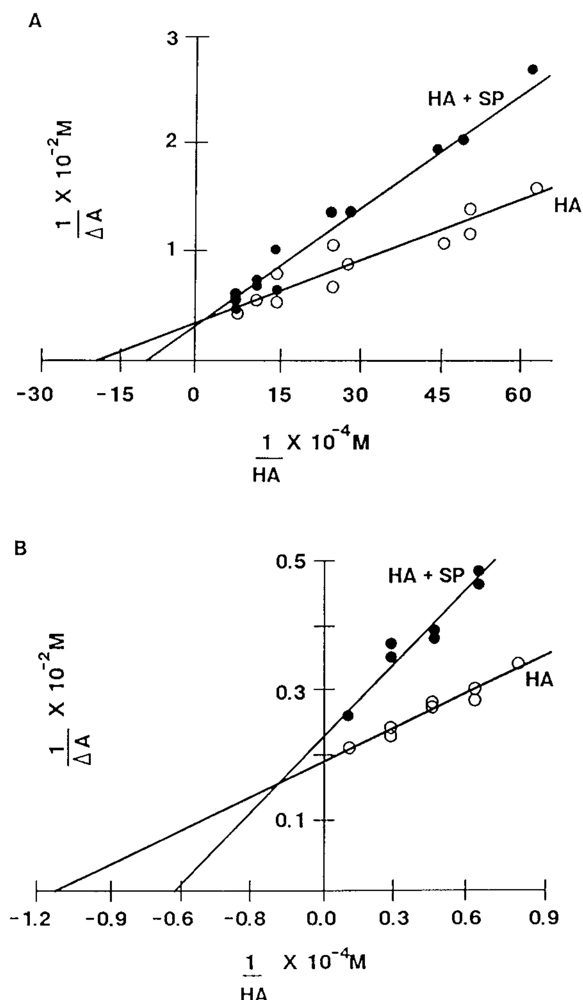

Fig. 2. Lineweaver-Burk plot of SP inhibition of the HA/P450 complex. **A:** Concentration range HA = 2–15 μM; concentration SP = 20 μM. **B:** Concentration range HA = 125–1,000 μM; concentration SP = 200 μM. K_s values for HA derived from these 2 experiments are similar to those obtained by Scatchard analysis (Fig. 2, Table II). The derived K_i values for SP (Table II) are similar to K_i values derived from radioligand binding studies (Table I).

Effect of NADPH/Carbon Monoxide on the Histamine/P450 Absorbance Difference Spectrum and on ^{3}H-Histamine Binding

(Note: high and low affinity sites determined by spectral analysis correspond to high and intermediate sites determined by ^{3}H-HA binding assays.) As measured spectrally, NADPH alone caused only increases, occasionally, in HA/P450 absorbance. With CO alone, complex formation was inhibited, but only at low (10 μM) concentrations of HA (data not shown). The combination of CO/NADPH inhibited complex formation in a concentration-dependent manner (Table III). At 10 μM HA, where high ($K_s \sim 2.5$ μM) and low ($K_s \sim 90$ μM) affinity sites bind approximately equal amounts of HA, 300 μM NADPH plus CO inhibited complex formation by 75% (Fig. 5). At HA concentrations of 1,000 μM, where low (90 μM) affinity sites represent 90% of the total bound HA, 100 μM NADPH plus CO caused greater inhibition (85%) than seen with 10 μM HA (60%); this difference was not observed with 3 mM NADPH plus CO (Table III). NADPH (5 mM)/CO treatment also abolished the high ($K_{d1} \sim 1$ μM) affinity sites (Table IV) and reduced (from $B_{max} = 0.85 \pm 0.19$ to 0.25 ± 0.06 nmol/mg protein) the intermediate ($K_{d2} \sim 60$ μM) affinity sites labelled by ^{3}H-HA; in contrast, the low ($K_{d3} \sim 3$ mM) sites were unaffected (Table IV).

Competition by Polyamines for ^{3}H-Histamine Binding to Nuclei and Chromatin

Two classes of binding sites for ^{3}H-HA were resolved in nuclei (Table V). A higher affinity ($K_d = 20$ nM) site that we had previously identified in nuclei prepared by a different method [Brandes et al., 1992b] was either absent, or unresolved, in this preparation. The k_d values for ^{3}H-HA binding to liver nuclei in HCO_3 (Table V) were similar to those for its binding to the intermediate and low affinity sites in microsomes in the same buffer (Table I). The rank order of potency of the PA to inhibit ^{3}H-HA binding was SP > HA > SPD > PUT (Table V). Although the affinities for ^{3}H-HA binding in microsomes were similar in HCO_3 and TRIS (Table I), a difference in ^{3}H-HA nuclear binding affinities were observed in HCO_3 and Krebs buffers. This may be due to differences in specific ions or to increased ionic strength of Krebs. The K_i values for SP were sensitive to changes in buffers (and/or pH) for both microsomes and nuclei (Tables I and V). ^{3}H-HA binding to purified chromatin revealed the highest affinity sites yet determined by us for both HA and SP (K_{d1} HA = 7.5 ± 6.9 nM; K_{i1} SP = 46 ± 33 nM; Table VI). HA was also more potent than SP at a lower affinity site on chromatin (K_{d2} HA = 1.9 ± 2.7 μM; K_{i2} SP = 53 ± 90 μM; Table II).

^{3}H-Spermidine Binding to Microsomes and Nuclei

Specific binding of ^{3}H-SPD was identified in microsomes ($IC_{50} \sim 30$ μM; Fig. 6) and nuclei ($IC_{50} \sim 0.5$ μM: data not shown). In contrast to the inhibition by SPD of ^{3}H-HA binding in mi-

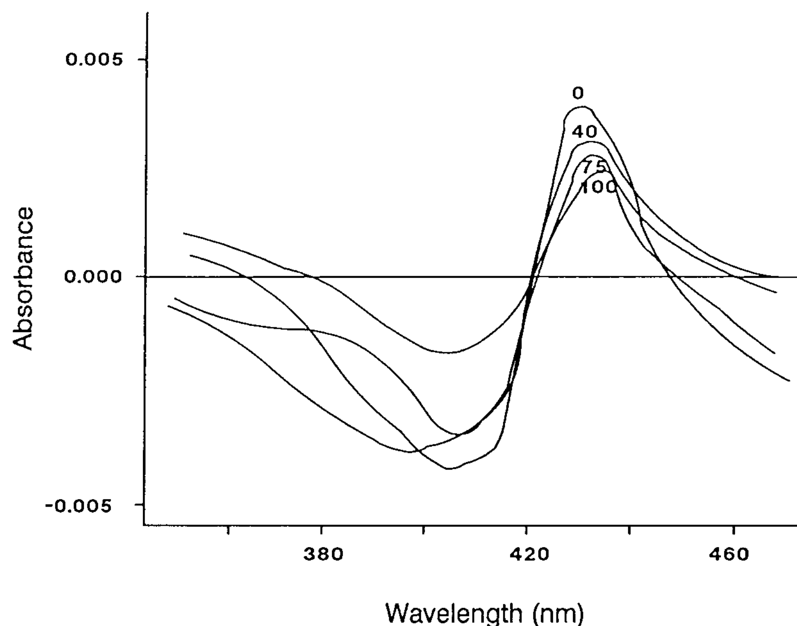

Fig. 3. Inhibition by SP (0–100 µM) of HA (10 µM) binding to cytochrome P450 in rat liver microsomes, as measured spectrally. HA yielded an absorbance difference spectrum with a peak at 425–435 nm and a trough at 390–410 nm. Despite its dose-dependent inhibition of HA binding to P450, SP and the other PA did not themselves generate an absorbance difference spectrum when tested over a wide range of concentrations, indicating that PA bind externally to the heme-containing pocket of the enzyme.

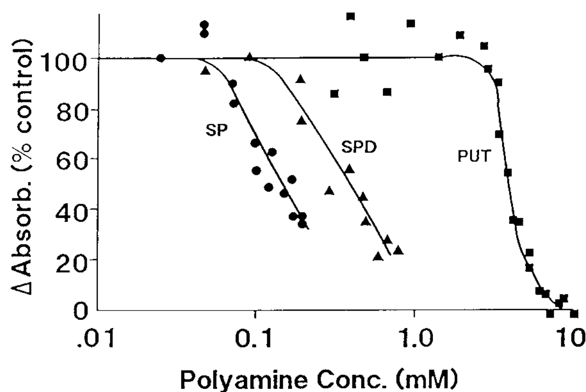

Fig. 4. Inhibition by SP, SPD, and PUT of HA (500 µM) binding to cytochrome P450 in rat liver microsomes, as measured spectrally. The IC_{50} values, derived from 2 experiments each, are typical of rank order of potency for PA in virtually all biological systems (SP > SPD > PUT).

TABLE III. Inhibition of the Histamine-Cytochrome P450 Absorbance-Difference Spectrum by NADPH ± Carbon Monoxide*

HA concentration (µM)	% Decrease in HA binding NADPH (100 µM) + CO	NADPH (300 µM) + CO	NADPH (3 mM) + CO
10	60	75	95
100	55	70	85
1,000	85	82	95

*NADPH alone stimulated binding at some HA concentrations.

crosomes and nuclei, HA did not compete for ^{3}H-SPD binding in either preparation.

DISCUSSION

In the present study, NADPH/CO treatment of microsomes reduced (by about 90%) both of the HA/P450 absorbance difference spectrum binding sites (K_s = 2.4 ± 1.6 µM and 90 ± 17 µM) as well as the intermediate affinity (~60 µM) sites labelled by ^{3}H-HA. It also abolished the high affinity (~1 µM) sites labelled by ^{3}H-HA. Thus, at least a proportion of microsomal H_{IC} sites previously identified by radioligand binding studies [Brandes and LaBella, 1993; Brandes et al., 1991] represents cytochrome P450 mono-oxygenases. The HA binding sites resolved from the spectral data may represent the direct binding of the imidazole moiety of HA to the Fe (III) atom of the heme [LaBella et al., 1992; Ribeiro and Walker, 1994], giving rise to the characteristic "type II" spectrum, and an additional well-recognized interaction between the non-imidazole moiety and the hydrophobic substrate-binding site [Lavrijsen et al., 1987;

TABLE IV. Effect of NADPH/CO on ^{3}H-Histamine Binding in Liver Microsomes

HA site	Control K_d (μM)	Control B_{max}	NADPH/CO K_d (μM)	NADPH/CO B_{max}
1	1.0 ± 0.9	3.4 ± 3.1 pm/mg	—	—
2	57 ± 13	0.85 ± 0.19 nm/mg	40 ± 8	0.25 ± 0.06 nm/mg
3	3,400 ± 1,800	21 ± 11 nm/mg	6,200 ± 6,000	28 ± 22 nm/mg
	n = 4		n = 1	

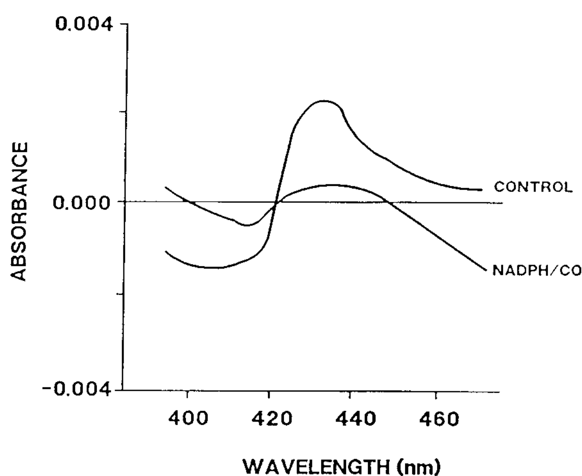

Fig. 5. Inhibition by NADPH/CO of the HA/P450 absorbance difference spectrum generated by 10 μM HA. NADPH reduces the heme iron, allowing the tight binding of CO, which then prevents the binding of HA.

TABLE V. Effect of Polyamines on Binding of ^{3}H-Histamine to Liver Nuclei

Buffer	Site	Histamine Kd (μM)	Histamine B_{max}
HCO_3	1	22.9 ± 4.4	3.4 ± .8 pm/mg
	2	2,747 ± 1,254	221 ± 106 pm/mg
Krebs	1	1.81 ± 1.16	2.17 ± 2.9 pm/mg
	2	36.4 ± 7.7	0.18 ± 0.023 nm/mg
			n = 9

	Ki (μM)	Spermine
Krebs	1	51 ± 12
	2	3,731 ± 1,044
		n = 4

	Ki (μM)	Polyamines Spermine	Spermidine	Putrescine
HCO_3	1	13.7 ± 3.1	50.7 ± 10.6	278 ± 62
	2	149 ± 26.5	523 ± 121	8,547 ± 1,972
		n = 5	n = 5	n = 4

Palmer and Cawthorne, 1974]. It has been demonstrated that at least three forms of cytochrome P450 in rat liver microsomes bind cyanide with different affinities and different A_{max}

TABLE VI. Effect of Spermine on the Binding of ^{3}H-Histamine to Chromatin

Site	Histamine K_d (μM)	Histamine B_{max}	Spermine [K_i (μM)]
1	.0075 ± .0069	15 ± 9 fm/mg	.046 ± .033
2	1.93 ± 2.74	0.7 ± 0.9 pm/mg	53.0 ± 90.2
	n = 7		n = 5

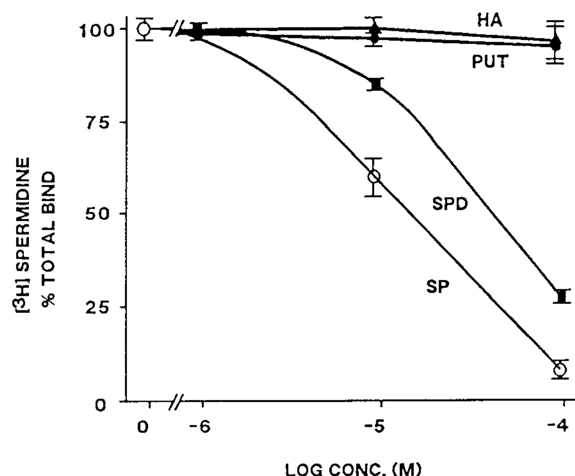

Fig. 6. Inhibition by HA and PA of ^{3}H-SPD binding to rat liver microsomes (bars, SD). Similar results were obtained in nuclei (not shown). PA inhibit HA binding but not vice versa, indicating that PA act allosterically.

values estimated from their "type II" spectra [Comai and Gaynor, 1973]. The H_2-antagonist cimetidine binds to at least two forms of P450 with affinities of 2.6 and 104 μM, [Reilly et al., 1983] similar to the HA spectral affinities of 2.4 and 90 μM reported in our study. Other NADPH-sensitive heme enzymes also may bind HA, possibly accounting for certain of the observed spectral affinities we have observed. For example, nitric oxide synthase has been shown to interact with imidazole to generate a "type II" spectrum [Chabin et al., 1996; McMillan and Masters, 1993]. The nature of the NADPH/CO-resistant low affinity (K_{d3} ~3 mM) sites labelled by ^{3}H-HA remains to be delineated.

Omura and Sato [Omura and Sato, 1964] reported that NADPH fully reduces P450 under anaerobic conditions, but that the heme iron is easily reoxidized in the presence of oxygen. Also, the reducing power of NADPH (reduction potential of -320 mV) is significantly less than that of dithionite (-500 mV) [Peterson and Boddupalli, 1992]. Dithionite-CO pretreatment abolished the difference spectra of 10, 100, and 1,000 μM HA (data not shown), whereas inhibition of the HA-P450 complex by CO in the presence of 3 mM NADPH was about 90% of that observed in the presence of dithionite. Like NADPH-CO, dithionite-CO pretreatment almost completely abolished both the 1 and 60 μM HA radioligand binding sites, but had little or no effect on the low (mM) affinity sites (data not shown). Since dithionite treatment is more drastic, and might have the potential to damage the integrity of the enzyme protein, we have presented only that data obtained with NADPH as the reducing agent.

PA antagonized binding of HA to P450 and of ^{3}H-HA to microsomes with the rank order: SP $>$ SPD $>$ PUT, consistent with that reported for virtually all biological systems [Tabor and Tabor, 1989; Shuber, 1989; Koenig et al., 1983]. The difference in K_i values in HCO_3 and TRIS for SP to compete for ^{3}H-HA binding at the cationic site may be due to the differences in buffer, pH, or the addition of Mg^{2+} to the TRIS. HA binding affinities were similar in both buffers. The potencies of PA at H_{IC} sites in nuclei and chromatin (K_i for SP = 46 ± 33 nM) appear to be among the highest reported thus far [Shuber, 1989]. Although the molecular nature of H_{IC} sites in chromatin is currently unknown, PA form complexes with nucleic acids [Morgan, 1991; Shuber, 1989] and cause DNA aggregation and condensation, responses associated with the promotion of cell division [Basu et al., 1990]. Correspondingly, intracellular depletion of PA is associated with alteration of nuclear chromatin structure and inhibition of cell proliferation [Morgan, 1991; Snyder, 1985]. Thus, nuclear/chromatin sites, like P450 microsomal sites [Brandes et al., 1994], may mediate growth-modulating effects of both HA and PA.

That PA appear to inhibit HA binding allosterically rather than competitively, derives from observations that (1) SP competed potently for ^{3}H-HA binding, but not vice versa, and (2) whereas HA generated a P450 absorbance difference spectrum, typical of ligands that bind within the heme cavity [LaBella et al., 1992; Ribeiro and Walker, 1994], the PA did not, suggesting their exclusion from the heme cavity. Others, similarly, have noted absence of a PA/P450 absorbance spectrum; an external cationic site on the enzyme with affinity for PA was identified on the basis that SP promoted an increase in the high spin content of camphor-bound P450 without exerting direct redox effects, indicating that the amine acts to alter enzyme configuration, not within the catalytic heme cavity, but on the peptide backbone [Hui Bon Hoa et al., 1990].

Modulation by HA and PA of catalytic activity of P450 and other heme proteins has been recognized. Whereas HA reportedly is not metabolized by P450 [Morris et al., 1989], it inhibits the P450-catalyzed 9-hydroxylation and O-demethylation of metaprolol (IC_{50} = 160 and 240 μM, respectively) and other reactions [Morris et al., 1989]. HA binds potently ($K_s \sim 0.1$ μM) to the nitric oxide-containing heme protein, nitrophorin [Ribeiro and Walker, 1994], but is a weak inhibitor ($IC_{50} > 500$ μM) of the P450 enzyme, thromboxane synthetase [Tai and Yuan, 1978]. PA stimulate or inhibit P450 catalysis, depending on the concentration. For example, SP (50 μM) stimulates by 3–4-fold, hydroxylation of benzphetamine and 7-ethoxycoumarin by liver microsomes [Andersson et al., 1981], and, at 1 mM, stimulates the oxidation of certain xenobiotics [Osimitz and Kulkarni, 1985]. Low concentrations (100 μM) of PA stimulate, and high concentrations (1 mM) inhibit, ribosomal peptidyltransferase [Drainas and Kalpaxis, 1994]. The $K_d/K_s/K_i$ values for HA and PA determined in the spectral and radioligand binding assays reported in the present study may represent a composite of interactions with multiple P450 isozymes.

Several decades ago, it was proposed that intracellular HA mediates growth and development of normal and malignant proliferating tissues [Kahlson and Rosengren, 1970]. Our identification of intracellular sites through which HA appears to mediate proliferation [Brandes and LaBella, 1993; Brandes et al., 1990] is in accordance with their hypothesis. More recently, Nebert proposed that P450 enzymes control the levels of endogenous lipid mediators that, in turn, modulate gene function, including expression of P450 isozymes themselves [Nebert, 1991]. On the basis of our findings, we suggest that intracellular HA regulates the catalytic activity of P450 enzymes, and that intracellular PA and various arylalkyl-

amine drugs that modulate normal and neoplastic growth, including certain antidepressants [Brandes et al., 1992a], H_1-antihistamines [Brandes et al., 1994], the antiestrogen tamoxifen [LaBella and Brandes, 1996], and the antiandrogen, flutamide [Brandes et al., 1997], perturb the HA/P450 interaction and reset the steady state levels of eicosanoids and other lipids involved in growth regulation.

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