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Effect of Free Fatty Acid Concentration on the Transport and Utilization of Other Albumin-Bound Compounds: Hydroxyphenylazobenzoic Acid

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SUMMARY

The purpose of this study was to determine whether changes in long-chain free fatty acid concentration would alter the transport and utilization of another organic ligand that was bound to human plasma albumin. Ehrlich ascites cells were incubated in medium containing human albumin, and the uptake of 2-(4′-hydroxyphenylazo)benzoic acid was measured relative to the free fatty acid concentration of the medium. As the molar ratio of free fatty acid to albumin was raised from 1 to 4, hydroxyphenylazobenzoic acid uptake by the cells increased. In contrast, cells "loaded" with large quantities of fatty acid took up no more hydroxyphenylazobenzoic acid from an albumin-free medium than did cells loaded with only small amounts of fatty acid. Equilibrium dialysis binding measurements indicated that the binding capacity of human albumin for hydroxyphenylazobenzoic acid decreased as the molar ratio of free fatty acid to albumin was raised. Therefore, it is likely that the free fatty acid-induced increase in hydroxyphenylazobenzoic acid uptake by the Ehrlich cells was due to displacement of this compound from strong to weaker albumin binding sites rather than to a direct effect of fatty acids on the cells. These results suggest that variations in the molar ratio of free fatty acid to albumin may influence the transport and utilization of other albumin-bound metabolites or drugs.

INTRODUCTION

Free fatty acid is the form in which fat is transported from adipose tissue storage depots to peripheral sites of utilization (1, 2). Albumin is the transport vehicle for FFA[2] in the plasma (3). The plasma FFA concentration can vary considerably over relatively short periods of time, and values ranging from 0.2 to 2.5 μEq/ml have been observed in humans (4, 5). In addition to FFA, albumin serves as the plasma transport protein for metabolites such as uric acid (6) and bilirubin (7), and many commonly used drugs. Examples of the latter include salicylates (8), the coumarin anticoagulants (9), digitalis (10), and chlorophenoxyisobutyrate (11). Since the phar-

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[2] The abbreviations used are: FFA, free fatty acid or acids; HABA, 2-(4′-hydroxyphenylazo)-benzoic acid.

macological effectiveness of these and many other drugs depends in part upon binding to albumin, it is important to investigate those factors that may influence the binding process. The effect of FFA concentration on the utilization of another albumin-bound organic ligand has not been examined thoroughly. However, there is evidence from binding studies that changes in FFA concentration within physiological limits may affect the transport of a second organic ligand by albumin (8, 12, 13).

The purpose of this study was to determine whether changes in FFA concentration would affect the utilization of another compound that was transported by human plasma albumin. A model compound, 2-(4′-hydroxyphenylazo)benzoic acid, was selected for this introductory investigation because it can be assayed rapidly by spectrophotometry (14). Ehrlich ascites cells were employed for the uptake studies. FFA metabolism has been investigated extensively in this model system (15), and we thought that this information might permit a more definitive interpretation of the present findings. Our data indicate that HABA uptake was altered when the FFA to albumin molar ratio was varied within the physiological range and that this probably resulted from competition for albumin binding between HABA and FFA.

MATERIALS AND METHODS

Crystalline human serum albumin was purchased from Research Products Division, Miles Laboratories. The protein was extracted with activated charcoal to remove inherent FFA (16), dialyzed, and adjusted to the required salt concentration and pH as described previously (17). Fatty acids of the highest purity available commerically were purchased from the Hormel Institute, Austin, Minn. FFA were added to albumin solutions by incubation with fatty acid-coated Celite (18). FFA concentration was measured by titration (19), and protein concentration was determined by the biuret method (20). HABA was purchased from Dajac Laboratories, Borden Chemical Company. The absorbance spectrum and extinction coefficient of these dye preparations were identical with those reported by Baxter (14). All the solutions contained 132 mM NaCl, 4.9 mM KCl, 1.2 mM $MgSO_4$, and 16 mM sodium phosphate, pH 7.4. Absorbance measurements were made with a Beckman DU spectrophotometer in quartz cuvettes having a 1-cm light path.

Ehrlich ascites cells were transplanted, harvested, and prepared as reported previously (21). The washed cells were suspended in the phosphate-buffered salt solution described above, and cells were counted with a clinical hemocytometer and microscope (21). Suspensions of Ehrlich cells were incubated in 5 ml of medium containing albumin and HABA in a 37° water bath with shaking at 80 oscillations/min. Air served as the gas phase. Appropriate control incubations containing no cells were present in each experiment. The incubation was terminated by transferring the contents of each flask to a chilled centrifuge tube and sedimenting the cells at 0° for 5 min at 4000 × g. The supernatant solution was removed and centrifuged again at 8000 × g, and aliquots of this supernatant solution were assayed spectrophotometrically for HABA content (14). HABA uptake was calculated by difference, using the appropriate medium incubated without cells as the control. Absorbance measurements were made at both 350 and 480 nm, and concentrations were calculated from standards containing the appropriate amount of albumin and various amounts of HABA. Identical results were obtained when either set of readings was employed. All the cell uptake data with albumin-containing media are based upon the 480 nm absorbance measurements. Incubation of HABA with palmitate did not alter the absorbance spectrum of the dye.

In additional experiments, aliquots of cells were incubated initially for 5 min at 23° with 0.1 mM albumin solutions containing from 0 to 0.55 mM palmitate. After washing three times with cold buffer solution (21), the cells were incubated for 10 or 20 min at 37° in protein-free medium containing either 0.057 or 0.107 mM HABA. Control media containing no cells also were incubated, and HABA uptake was calculated as above, using absorbance measurements made at 350 nm.

The equilibrium dialysis technique employed for the binding measurements was a modification of the one described by Baxter (22). Dialysis cells (model 250) and membranes were purchased from Bel-Art Products, Pequannock, N. Y. Before use, membranes were placed in 200 ml of boiling distilled water for 15 min and then soaked for 24–48 hr in an additional 200 ml of distilled water at 23°. Preliminary experiments demonstrated that neither human plasma albumin nor palmitate-1-^{14}C passed through these dialysis membranes at pH 7.4. Complete equilibration of HABA in this system occurred within 20 hr at 23°. Small quantities of HABA were adsorbed on the dialysis membrane, and corrections were made for this in the calculation of the binding results. In a typical experiment, 6–13 dialysis cells were loaded with 0.1 mM albumin in one compartment, and a solution containing 0.06–1.5 mM HABA was placed in the second compartment. Incubation was performed at 23° for 24 hr in a water bath incubator that was shaken at 40 oscillations/min. The HABA concentration in the albumin-free compartment was measured spectrophotometrically at 350 nm. The pH of the solutions on both sides of the dialysis membrane remained identical during incubation, indicating that no appreciable Donnan effect occurred. Therefore, corrections for the Donnan effect were not made in these binding experiments. The unbound HABA concentration and the molar ratio of bound HABA to albumin were calculated, and the data were analyzed by the method of Scatchard (23).

RESULTS

Effect of FFA on HABA uptake by Ehrlich cells. Ehrlich cells took up HABA from media containing albumin and from protein-free media. In both cases, uptake increased during the course of a 1-hr incubation. HABA uptake was 3 times greater at 37° than at 0°. The initial studies revealed that the amount of HABA taken up at each time point from media containing albumin was dependent upon the FFA to albumin molar ratio. In order to investigate this observation in greater detail, additional experiments were performed using a 1-hr incubation at 37°. These conditions were selected because the HABA incorporation was large enough to permit the measurement of uptake by

TABLE 1

Effect of palmitate to albumin molar ratio on HABA uptake by Ehrlich cells

Ehrlich cells were incubated in 5 ml of a medium containing 132 mM NaCl, 4.9 mM KCl, 1.2 mM $MgSO_4$, 16 mM sodium phosphate (pH 7.4), 0.064 mM albumin, and 0.107 mM HABA. The palmitate content of the medium was varied from 0 to 0.211 mM. Incubation was performed at 37° for 60 min with air as the gas phase. Each value is the mean ± standard error of eight separate determinations. The difference between "FFA-free" albumin and the palmitate to albumin molar ratio of 1.04 is not significant ($p > 0.1$); that between FFA-free albumin and the palmitate to albumin molar ratio of 2.04 or 3.24 is significant ($p < 0.01$).

Palmitate to albumin molar ratio	HABA uptake
	nmoles/10^6 cells
0	88 ± 8
1.04	76 ± 9
2.04	123 ± 10
3.24	178 ± 10

TABLE 2

Effect of different fatty acids and albumins on HABA uptake by Ehrlich cells

The conditions of incubation were the same as those described for Table 1, except that the FFA concentration was either 0.064 or 0.21 mM. Each value is the mean ± standard error of five determinations.

Fatty acid	Albumin	HABA uptake	
		$\bar{\nu} = 1.0$[a]	$\bar{\nu} = 3.3$
		nmoles/10^6 cells	
Palmitate	Human	134 ± 3.1	221 ± 1.7[b]
Oleate	Human	128 ± 2.2	158 ± 2.5[b]
Laurate	Human	117 ± 4.5	151 ± 9.2[c]
Octanoate	Human	122 ± 1.7	128 ± 1.1[c]
Palmitate	Bovine	83 ± 3.2	127 ± 1.4[b]

[a] Ratio in moles of FFA to albumin present in the incubation medium.

[b] $P < 0.01$.

[c] $0.01 < p < 0.05$.

differences in medium HABA content and because sufficient time was available to manipulate large numbers of incubation flasks. Table 1 illustrates the effect of the palmitate to albumin molar ratio on HABA uptake by the cells. The molar ratio of HABA to albumin was 1.67 in each medium. There was no significant difference in HABA uptake from the FFA-free medium as compared with the one in which the palmitate to albumin ratio was 1.04. However, HABA uptake increased markedly when the palmitate to albumin ratio was raised further. As compared with the FFA-free medium, HABA uptake was 39% greater when the palmitate to albumin ratio was 2.08 and 102% greater when the ratio was 3.24.

Similar effects on HABA uptake were produced by FFA other than palmitate and when bovine albumin was substituted for human albumin (Table 2). In this series of experiments, there was a 65% increase in HABA uptake from media containing human albumin when the palmitate to albumin molar ratio was 3.3 as compared with 1.0. Oleate and laurate at molar ratios of 3.3 produced increments in HABA uptake of 23% and 29%, respectively, as compared with a molar ratio of 1.0. In contrast, only a very small increase in HABA uptake occurred when the medium-chain fatty acid octanoate was tested under these conditions. In media containing bovine albumin, palmitate at a molar ratio of 3.3 produced a 53% increase in HABA uptake as compared with a molar ratio of 1.0.

Table 3 illustrates the effect of palmitate on HABA uptake at different HABA to albumin molar ratios. The palmitate to albumin molar ratio was either 0.8 or 3.3. Independently of the palmitate content in the medium, HABA uptake increased considerably as the HABA to albumin molar ratio was raised. However, at each HABA to albumin ratio, more HABA was taken up when the larger amount of palmitate was present.

A second factor regulating HABA uptake was the amount of HABA-albumin complex present in the incubation medium. As shown in Table 3, HABA uptake increased when the concentration of the HABA-albumin complex was raised. This also occurred independently of the amount of palmitate present in the medium. However, at each albumin concentration, more HABA was taken up when the palmitate to albumin ratio was 3.3 than when it was 0.8.

TABLE 3

Effect of HABA to albumin molar ratio and albumin concentration on FFA-induced increment in HABA uptake

The conditions of incubation were the same as those described in Table 1. In experiment 1, the albumin concentration remained constant and the HABA concentration was raised so that the HABA to albumin ratio varied from 0.83 to 3.33. The palmitate concentration was either 51 or 211 μM. In experiment 2, two different albumin concentrations were employed. The HABA concentration was varied concomitantly so that the HABA to albumin molar ratio was 1.67 in both cases. Likewise, the palmitate concentration was adjusted so that the palmitate to albumin molar ratio remained either 0.8 or 3.3. Each value is the mean ± standard error of six separate determinations. All the mean values obtained when the palmitate to albumin molar ratio was 3.3 are significantly larger than those obtained when the molar ratio was 0.8 ($p < 0.01$).

Expt	Incubation medium		HABA uptake	
	Albumin	HABA	$\bar{\nu}$ = 0.8[a]	$\bar{\nu}$ = 3.3
	μM		*nmoles/10⁶ cells*	
1	64	53	11 ± 0.7	47 ± 2.8
	64	107	50 ± 1.6	117 ± 6.4
	64	213	114 ± 2.8	196 ± 2.5
2	80	133	95 ± 1.9	149 ± 4.0
	240	400	139 ± 4.0	213 ± 5.3

[a] Palmitate to albumin molar ratio present in the incubation medium.

HABA uptake relative to cell fatty acid content. Did the FFA-induced increase in HABA uptake result from an effect of fatty acid on the ability of albumin to bind HABA, or on the cellular uptake process? Previous studies showed that FFA uptake by Ehrlich cells increased markedly as the FAA to albumin molar ratio was raised (15, 24). Therefore, the increase in HABA uptake could be secondary to the presence of larger

amounts of fatty acid associated with the cells. In order to examine the possibility of a direct effect of FFA on the cells, the following experiments were performed. Cells were incubated briefly in albumin medium containing either high or low palmitate concentrations. The cells were isolated, washed thoroughly, and resuspended in a protein-free buffered salt solution (24). Cells prepared in this way after incubation with medium having a palmitate to albumin molar ratio of 1 contained only about 8 nEq of newly incorporated fatty acid per 10^8 cells; those incubated with medium in which the molar ratio was 5.5 contained about 175 nEq (24). Aliquots of these "loaded" cells were incubated briefly in a second medium containing HABA but no albumin. It was necessary to use a protein-free medium for the second incubation in order to prevent release of much of the newly incorporated fatty acid from the cells (15, 21, 24). In addition, the second incubation had to be brief because newly incorporated fatty acids are metabolized rapidly by Ehrlich cells "loaded" in this way (24, 25). As shown in Table 4, there was essentially no change in HABA uptake when cells initially containing large amounts of newly incorporated fatty acid were compared with those containing little or no newly incorporated fatty acid. These observations suggest that the FFA-induced increase in HABA uptake probably did *not* result primarily from an effect of fatty acid on the cells.

Effect of FFA on HABA binding to human serum albumin. Another possible explanation for the FFA-induced increase in HABA uptake was that FFA altered the affinity of albumin for HABA, thereby making HABA more available for uptake by the cells. Binding studies were performed in order to test this possibility. Figure 1 illustrates the differences in HABA binding to human albumin relative to the amount of palmitate present in the medium. HABA binding was decreased when the palmitate to albumin molar ratio was 3 as compared with "FFA-free" albumin, and it was decreased even further when the palmitate to albumin molar ratio was raised to 5.9. The binding isotherm with FFA-free albumin was resolved by the Scatchard method (23) into a minimum of two components: $n_1k'_1 = 2.4 \times 10^4$ M^{-1} and $n_2k'_2 = 2.1 \times 10^4$ M^{-1}. This suggests the presence of at least two classes of HABA-binding sites (23). When the palmitate to albumin molar ratio was 3, the binding isotherm also was resolved into a minimum of two components: $n_1k'_1 = 1.6 \times 10^4$ M^{-1} and

TABLE 4

Comparison of HABA uptake from protein-free medium by Ehrlich cells loaded with different amounts of palmitate

The cells were divided into two batches in each experiment. One batch (low FFA) was incubated for 5 min at 23° in a medium containing 0.1 mM albumin and either no FFA or 0.1 mM palmitate. The other batch (high FFA) was incubated under the same conditions with either 0.4 mM or 0.55 mM palmitate. Each cell preparation was washed three times with the protein-free buffered salt solution. Aliquots of these cell suspensions then were incubated for either 10 or 20 min at 37° in protein-free buffered salt solution containing either 0.24 or 0.48 mM HABA. In each experiment, the cells incubated initially in medium containing little or no FFA were compared with those incubated initially in medium containing a high FFA concentration. Media containing no cells also were incubated as controls, and HABA uptake was calculated from the difference in absorbance of the media at 350 nm. Each value is the mean ± standard error of three determinations. The differences in the mean values are not significant.

Expt	HABA[a]	Incubation time[b]	HABA uptake	
			Low FFA[c]	High FFA
	mM	*min*	*nmoles/10⁸ cells*	
1	0.24	10	12 ± 0.9	13 ± 0.2
2	0.48	10	36 ± 1.0	35 ± 1.0
3	0.48	10	45 ± 1.1	41 ± 1.0
4	0.48	20	61 ± 0.7	63 ± 0.7

[a] HABA concentration in the second incubation medium. No protein was present in this medium.

[b] Period of incubation in the medium containing HABA.

[c] FFA content of the medium used for the preliminary incubation. In experiments 1 and 2, the low FFA medium contained 0.1 mM palmitate and the high FFA medium contained 0.4 mM palmitate. In experiments 3 and 4, the low FFA medium contained no palmitate and the high FFA medium contained 0.55 mM palmitate.

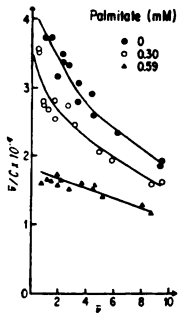

FIG. 1. *Effect of palmitate concentration on HABA binding to human serum albumin*

Binding was measured by equilibrium dialysis at 23° for 24 hr. The albumin concentration was 0.1 mM, and the incubation media contained 132 mM NaCl, 4.9 mM KCl, 1.2 mM $MgSO_4$, 16 mM sodium phosphate (pH 7.4), and various quantities of HABA. A single preparation of defatted albumin was used for these experiments. The top most curve (●) represents data obtained with "FFA-free" albumin; the center curve (○), data obtained when 0.3 mM palmitate was present; and the lowest curve (▲), data obtained when 0.59 mM palmitate was present. The symbol $\bar{\nu}$ refers to the HABA to albumin molar ratio; C refers to the molar concentration of unbound HABA.

$n_2k'_2 = 2.0 \times 10^4$ M^{-1}. Hence, the major change produced by the presence of palmitate was a reduction in the contribution of the stronger component (primary class of sites) to HABA binding. When the palmitate to albumin molar ratio was 5.9, the binding data could be fitted adequately by the method of Scatchard to a straight line with $n_1k_1' = 1.8 \times 10^4$ M^{-1}. This suggests that the stronger class of HABA-binding sites was not functional when this relatively large quantity of palmitate was present. In contrast, HABA binding to the weaker secondary sites still was not much affected.

Because of the poor resolution between the data obtained with "FFA-free" albumin and those with 3 moles of palmitate, we performed additional binding studies in order to determine the significance of the observed differences. Results taken from a series of experiments with palmitate and oleate are listed in Table 5. Different preparations of albumin were used for the palmitate and oleate experiments. In general, the data suggest that the affinity of albumin for HABA decreased progressively as the FFA to

TABLE 5

Effect of FFA to albumin molar ratio on HABA binding to human serum albumin

The procedure employed for the binding measurements was identical with that described for Fig. 1. Different "FFA-free" albumin preparations were employed for the palmitate and oleate experiments. Each value for unbound HABA concentration is the mean ± standard error of six separate determinations.

Fatty acid	FFA to albumin molar ratio	Unbound HABA		
		$\bar{\nu} = 0.9$[a]	$\bar{\nu} = 2.5$	$\bar{\nu} = 5.0$
			$M \times 10^4$	
Palmitate	0	0.33 ± 0.03	0.96 ± 0.07	2.52 ± 0.17
	1.5	0.36 ± 0.04	1.17 ± 0.12	3.13 ± 0.12[b]
	3.0	0.39 ± 0.04	1.26 ± 0.10[b]	3.22 ± 0.19[b]
	4.4	0.58 ± 0.04[c]	1.79 ± 0.08[c]	3.99 ± 0.18[c]
Oleate	0	0.42 ± 0.02	1.19 ± 0.06	2.45 ± 0.10
	1.5	0.43 ± 0.08	1.20 ± 0.04	2.94 ± 0.09[c]
	3.0	0.50 ± 0.04	1.28 ± 0.04	2.90 ± 0.08[c]
	4.4	0.57 ± 0.01[c]	1.43 ± 0.04[c]	3.38 ± 0.17[c]

[a] Molar ratio of bound HABA to albumin.

[b] Significance of the difference from the corresponding value obtained with "FFA-free" albumin is $0.01 < p < 0.05$.

[c] $p < 0.01$.

albumin molar ratio was raised. However, only certain of the observed differences between FFA-free albumin and albumin containing FFA were statistically significant. When FFA-free albumin was compared with albumin containing 1.5 moles of FFA, only the difference in unbound HABA concentration obtained at the highest HABA to albumin molar ratio was significant. In contrast, all the differences between FFA-free albumin and albumin containing 4.4 moles of FFA were significant. With albumin containing 3 moles of FFA, only three of the six values were significantly different from those obtained with FFA-free albumin at the level of $p < 0.05$ or less.

DISCUSSION

The amount of HABA taken up by Ehrlich ascites cells from media containing albumin was dependent upon the FFA to albumin molar ratio. No significant change in HABA uptake occurred when the palmitate to albumin molar ratio was raised from 0 to 1. However, a progressive increase in HABA uptake occurred when the molar ratio was increased from 1 to 4. Similar effects were produced by oleate and laurate, but not by octanoate. Based upon indirect experiments, it appears that the FFA-induced alterations in HABA uptake did not result primarily from an effect of fatty acid on the cells. On the other hand, equilibrium dialysis studies indicated that the binding capacity of albumin for HABA decreased as the FFA to albumin molar ratio was raised. Similar competitive effects for binding to albumin have been observed between FFA and methyl orange (12), azorubin (13), thyroxine (26), triiodothyronine (27), and salicylates (8). Therefore, it seems reasonable to suggest that the FFA-induced increase in HABA uptake was secondary to competition between HABA and FFA for albumin binding sites. Albumin binds FFA much more tightly than HABA (3, 17). Hence, it appears that when more than 1 mole of FFA is bound, HABA is displaced from strong to weaker albumin binding sites, thereby increasing the availability of HABA for uptake.

The present studies were performed *in vitro* with an experimental model system and a model ligand. Therefore, one must be extremely cautious in extrapolating these results to other situations in which FFA and a drug compete for albumin binding sites. On the other hand, the possibility is suggested that the effectiveness or metabolism of certain albumin-bound drugs in systems *in vitro* may be influenced by FFA concentration. Commerically available albumin preparations contain quite variable amounts of FFA (16). Hence, careful attention should be paid to the FFA content of albumin that is added to incubation media *in vitro*. Moreover, the possibility that FFA added to or accumulating in these media may influence the results obtained with an albumin-bound drug must be considered.

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