MOLECULAR PHARMACOLOGY, 13, 987-992

Effects of Fatty Acids on Two Specific Drug Binding Sites on Human Serum Albumin

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(Received February 8, 1977)
(Accepted May 27, 1977)

SUMMARY

BIRKETT, DONALD J., MYERS, SHIRLEY P. & SUDLOW, GILLIAN (1977) Effects of fatty acids on two specific drug binding sites on human serum albumin. *Mol. Pharmacol.*, 13, 987-992.

Two distinct binding sites, I and II, for anionic drugs on human serum albumin have previously been demonstrated using fluorescent probe techniques. 5-Dimethylamino-naphthalene-1-sulfonamide (DNSA) and dansylsarcosine are specific fluorescent probes for sites I and II, respectively. The addition of fatty acids results in differing effects at the two binding sites, and the specificity of site II is lost. The order of potency of various fatty acids in causing these changes is oleic > stearic > linoleic ≧ palmitic, and this is the same as the order of association constants for these fatty acids. It is concluded that chain length and degree of unsaturation determine both binding affinity and the extent to which the fatty acids induce configurational adaptations in the albumin molecule. Furthermore, studies with varying ratios of oleic acid to albumin suggest that the conformational changes induced in the protein are different for each molecule of oleic acid added. Addition of oleic acid at a 3:1 molar ratio with albumin significantly increased the binding of warfarin and DNSA to site I.

INTRODUCTION

Previous reports in this series have presented evidence for the existence of two specific binding sites for acidic drugs on human serum albumin (1, 2). The two sites have been identified by the use of fluorescent probe molecules (mainly dansylamino acids) which bind specifically to one or the other site, and the structural requirements for binding to the two sites have been investigated. It was also shown that stearic acid caused different effects at the two sites (2). A number of authors have reported effects of fatty acids both on drug binding to albumin and on the conformation of the albumin molecule (3-8).

In this report the effects of fatty acids on binding sites I and II are presented. It is shown that the structures of both sites are altered when fatty acids bind, and that this can result in changes in drug binding at these sites.

This work was supported by a grant from the National Health and Medical Research Council of Australia.

METHODS

Human serum albumin was obtained from Hoechst, Australia, Ltd. (electrophoretically pure human serum albumin, batch 4791) and from Sigma Chemical Company (essentially fatty acid-free human serum albumin, lot 34C-7010). The molar ratios of fatty acid to albumin were 1.1:1 for Hoechst albumin and 0.03:1 for

ISSN 0026-895X

Sigma FAF[1] albumin. The drugs were obtained as pure substances from the various manufacturers. Iophenoxic acid was a gift from Dr. Gilbert H. Mudge. Dansyl-L-proline, 5-dimethylaminonaphthalene-1-sulfonamide, dansylsarcosine, and dansyl-L-norvaline were obtained from Sigma. Stearic acid, linoleic acid, palmitic acid, and oleic acid as the sodium salts were also obtained from Sigma. [^{14}C]Warfarin (specific activity, 23 mCi/mmole) was obtained from the Radiochemical Centre, Amersham.

All experiments were carried out using sodium phosphate buffer (0.1 M, pH 7.4) containing 0.9% NaCl and at a temperature of 22° unless otherwise stated. When necessary, the drugs were dissolved initially in a small volume of 0.1 M NaOH or 0.1 M HCl. The final pH of the stock solutions of drugs was 7.2–7.6. The sodium salts of the fatty acids were evenly dispersed in buffer by sonication and then incubated with HSA at 37° until a clear solution was obtained. Control solutions of HSA were treated in an identical fashion. Nitrogen was bubbled through the solutions of oleic and linoleic acids to prevent oxidation.

The fatty acid content of the albumin preparations was measured by the method of Duncombe (9). The binding of [^{14}C]-warfarin in HSA was measured by dialysis as described by Sudlow *et al.* (10), and the binding of iophenoxic acid was measured by quenching of protein fluorescence (11). The displacement of dansylamino acids by drugs was measured fluorometrically or by dialysis as previously described (1). Titrations to measure the limiting fluorescence of probe molecules when completely bound to albumin were also carried out as previously described (11). Fluorescence measurements were made at 22° using a Perkin-Elmer MPF-3 spectrofluorometer.

RESULTS

The effects of various fatty acids on the two drug binding sites are shown in Tables 1 and 2. The fluorescence intensity of DNSA bound to site I was increased by addition of each of the four fatty acids, whereas the fluorescence intensity of dansylsarcosine (site II) was decreased (Table

[1] The abbreviations used are: FAF, essentially fatty acid-free; HSA, human serum albumin; DNSA, 5 - dimethylaminonaphthalene - 1 - sulfonamide.

TABLE 1

Effect of fatty acids on fluorescence intensity of probes bound to sites I and II

Palmitic, stearic, oleic, and linoleic acids were added at 2:1 molar ratios to Hoechst or Sigma FAF albumin. The limiting fluorescence intensities of DNSA (site I) and dansylsarcosine (site II) were determined by titrating the probes (2 μM) with albumin or albumin-fatty acid complex until the fluorescence intensity reached a limiting value. The results are expressed relative to those obtained for the albumin with no added fatty acid.

Fatty acid	DNSA	Dansylsarcosine
	%	%
Hoechst albumin		
None	100	100
Palmitic acid	108	96.5
Linoleic acid	122	89.3
Stearic acid	133	81.5
Oleic acid	128	79.6
Sigma FAF albumin		
None	100	100
Palmitic acid	120	97.2
Linoleic acid	125	93.8
Stearic acid	160	87
Oleic acid	188	86.5

TABLE 2

Effect of fatty acids on specificity of albumin binding sites I and II

Hoechst albumin was prepared with or without added palmitic, stearic, oleic, or linoleic acids. Fatty acids were added at 2:1 molar ratios with albumin. The effects of phenylbutazone and ibuprofen on the fluorescence of DNSA (site I) or dansylsarcosine (site II) are expressed as a percentage of the initial probe fluorescence before addition of drugs. The final concentrations were 2 μM for DNSA and dansylsarcosine and 20 μM for albumin, and the two drugs were added to final concentrations of 20 μM.

Fatty acid	Phenylbutazone		Ibuprofen	
	DNSA	Dansyl-sarcosine	DNSA	Dansyl-sarcosine
	% initial fluorescence			
None	36	94	121	62
Palmitic acid	39.8	91	113.5	60
Linoleic acid	37.5	79.2	111.4	60
Stearic acid	50	79.5	103	76
Oleic acid	56.8	68	101	79.7

1). The fluorescence values in Table 1 are limiting values obtained when the probes were completely bound and therefore reflect the fluorescence quantum yield of the probes at the two specific binding sites.

The effects of varying ratios of oleic acid to albumin are shown in Fig. 1. DNSA fluorescence was increased in a linear fashion by addition of up to 2 Eq of oleic acid. Addition of 3 Eq more of oleic acid produced no further change in probe fluorescence. Dansylsarcosine fluorescence was not altered by addition of 1 mole of oleic acid per mole of albumin, but decreased progressively as the ratio of oleic acid to albumin was increased from 1:1 to 5:1.

The effects of oleic acid on the binding of DNSA and dansylsarcosine were studied by equilibrium dialysis at concentrations similar to those used in the fluorescence experiments. The proportion of free DNSA was reduced from 69.4% ± 3.83% to 25.2% ± 2.64% ($p < 0.001$) by addition of oleic acid at a 3:1 ratio with albumin. In contrast, there was a slight increase in the proportion of free dansylsarcosine, from 20.8% ± 1.81% to 29.7% ± 4.23% ($p < 0.005$).

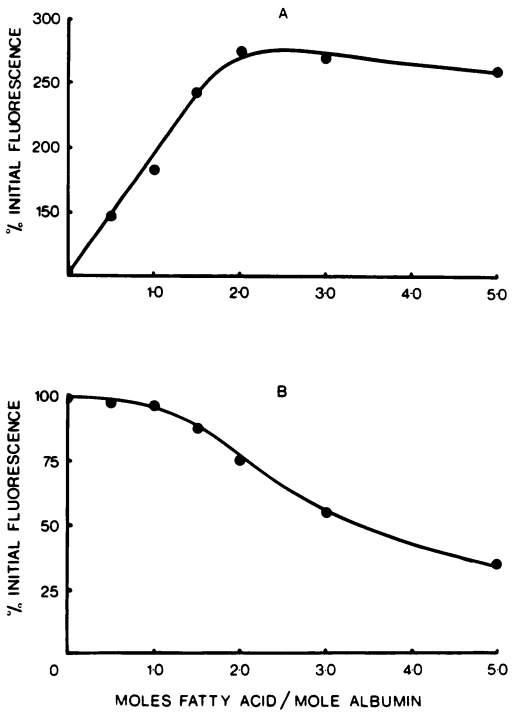

FIG. 1. *Effect of varying amounts of oleic acid on fluorescence of albumin-bound DNSA and dansylsarcosine*

Sigma FAF albumin, 20 μM, was prepared with oleic acid at concentrations from 0 to 100 μM. DNSA (A) or dansylsarcosine (B) was added at a final concentration of 2.5 μM, and the fluorescence was measured. Results are expressed relative to the fluorescence in the absence of oleic acid.

The displacement of the two probes by site I and site II drugs was also altered by addition of some fatty acids (Table 2). The specificity of site I remained unchanged, so that DNSA was still displaced by phenylbutazone but not by ibuprofen. However, the displacement by phenylbutazone was less in the presence of oleic and stearic acids, and the ibuprofen-induced enhancement of DNSA fluorescence was abolished. By contrast, the specificity of site II was lost. The displacement of dansylsarcosine by phenylbutazone was increased markedly in the presence of some fatty acids, whereas the displacement by ibuprofen was decreased. With oleic acid, dansylsarcosine was displaced more by the Site I drug, phenylbutazone, than by the site II drug, ibuprofen.

The change in the pattern of displacement of dansylsarcosine could be due to occupation of site II by fatty acids. Alternatively, the binding of fatty acids to other areas of the protein could alter the structure and, therefore, the specificity of the two drug binding sites. The binding of iophenoxic acid was studied by quenching of protein fluorescence, and the drug was found to bind tightly to one site in the presence and absence of oleic acid (Fig. 2). Previous studies have shown that iophenoxic acid binds to site I (1, 2, 11). Dansylsarcosine bound to Sigma FAF albumin was not displaced by iophenoxic acid. However, a 39% displacement by iophenoxic acid was observed when oleic acid was added at a 3:1 molar ratio with albumin, and there was a slight increase in the displacement of DNSA by iophenoxic acid.

Table 3 shows the effect of iophenoxic acid on the displacement of dansylsarcosine by phenylbutazone or ibuprofen in the

presence and absence of oleic acid. Iophenoxic acid alone had little effect on the displacement of dansylsarcosine. Oleic acid alone resulted in displacement of dansylsarcosine by both site I and site II drugs. In the presence of both iophenoxic acid and oleic acid, the site II pattern was restored, with only slight displacement of dansylsarcosine by phenylbutazone and extensive displacement by ibuprofen. Similar results were obtained using warfarin instead of phenylbutazone, and flufenamic or flurbiprofen instead of ibuprofen. Also, two other site II probes, dansyl-L-proline and dansyl-L-norvaline (2), behaved in a fashion similar to dansylsarcosine.

The fluorescence results were confirmed by equilibrium dialysis studies of dansylsarcosine displacement. The conditions used in the fluorescence experiments could not be exactly reproduced, as the ligands distribute to varying extents between the protein and buffer sides of the dialysis cells during dialysis. Despite this, similar patterns of dansylsarcosine displacement were found with the two methods (Table 3).

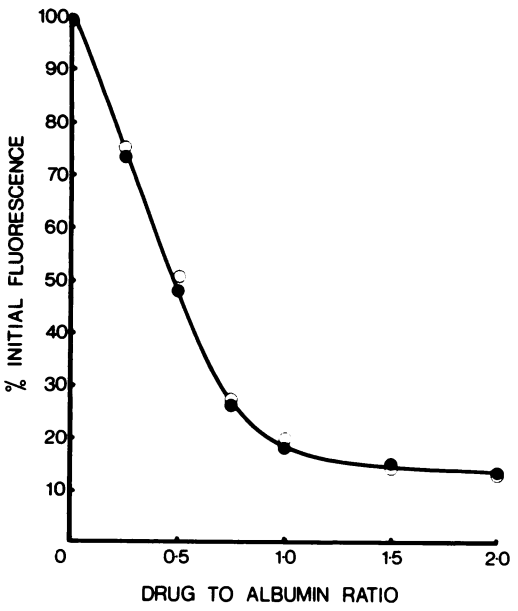

FIG. 2. *Binding of iophenoxic acid to HSA*

Sigma FAF albumin, 2 μM, with (○——○) or without (●——●) oleic acid, 8 μM, was titrated with iophenoxic acid, and the protein fluorescence was measured at 335 nm with excitation at 285 nm. Oleic acid at concentrations of 2 and 4 μM (not shown) gave similar results.

The effect of oleic acid on the binding of a site I drug, warfarin, was determined by equilibrium dialysis. The binding of warfarin was enhanced by the addition of oleic acid, resulting in a 60% decrease in the proportion of free drug, from 11.63% ± 0.55% to 4.47% ± 0.47% (mean ± 1 SD).

DISCUSSION

A number of previous reports have suggested that the binding of fatty acids causes change in the structure of human serum albumin. Woolley and Hunter (12) found that the binding of fatty acids induced changes in the absorption spectrum and circular dichroism of albumin-bound bilirubin without altering bilirubin binding. They suggested that this was due to a fatty acid-induced change in the structure of the bilirubin binding site. Spector *et al.* (4) suggested that displacement of chlorophenoxyisobutyrate by fatty acids involved an allosteric mechanism. Support for this interpretation was provided by the effects of fatty acids on the fluorescence of albumin-bound 1-anilino-8-naphthalenesulfonate and by the effect of fatty acids on the quenching of protein fluorescence by KI. Rippie (13) concluded that the data of Solomon *et al.* (3) on the displacement of warfarin and phenylbutazone by fatty acids were consistent with an allosteric mechanism as a dominant mode of fatty acid displacement of drugs from albumin.

The findings in the present study leave no doubt that some fatty acids cause widespread changes in the structure of human albumin. Oleic acid caused changes in the limiting fluorescence of probes, DNSA and dansylsarcosine, bound to two distinct drug binding sites (I and II). The limiting fluorescence (when the probe is completely bound) is a measure of the fluorescence quantum yield, and this is a sensitive index of changes in protein structure around the binding sites of the probes. The oleic acid-induced changes in the binding sites caused a marked increase in DNSA binding to site I, but a slight decrease in dansylsarcosine binding to site II.

TABLE 3

Effects of oleic acid and iophenoxic acid on dansylsarcosine binding to albumin

Sigma FAF albumin was prepared with or without oleic acid added at a 3:1 molar ratio. The displacement of dansylsarcosine by phenylbutazone or ibuprofen was measured in the absence and presence of iophenoxic acid, 20 μM. The conditions for the fluorescence experiments were 2 μM dansylsarcosine and 20 μM albumin, and phenylbutazone or ibuprofen was added to a final concentration of 20 μM. For the dialysis experiments, the initial concentrations on the albumin side of the dialysis cell (1 ml on either side) were 3 μM for dansylsarcosine, 20 μM for albumin, and 40 μM for phenylbutazone or ibuprofen. The percentage displacement is $(B - B_d)/B \times 100$, where B is the concentration of dansylsarcosine bound initially, and B_d the concentration bound after addition of phenylbutazone or ibuprofen.

Addition	Decrease in fluorescence		Displacement by dialysis	
	Phenylbutazone	Ibuprofen	Phenylbutazone	Ibuprofen
	%	%	%	%
Albumin, 20 μM	3.9	32	0	35.8
With iophenoxic acid, 20 μM	7.1	37.7	1.2	51.5
With oleic acid, 60 μM	26.5	27	12.9	26.4
With iophenoxic acid, 20 μM, and oleic acid, 60 μM	12.4	64.5	10.9	56.8

Effects of fatty acids on the structure of the primary binding site for bilirubin have previously been reported (12). As this site is likely to be distinct from drug binding sites I and II, it is clear that fatty acids induce changes in albumin conformation which affect at least three binding areas on the protein.

Detailed fatty acid binding studies by Ashbrook *et al.* (14) showed that the binding energy did not depend simply on the fatty acid chain length. They suggested that this was due to varying degrees of configurational adaptation of the albumin molecule as the fatty acid increased in length. This interpretation is confirmed by the data presented in Tables 1 and 2. Ashbrook *et al.* (14) reported association constants (K_1) for fatty acids in the order oleate > stearate > linoleate ≧ palmitate, and this corresponds to the order of potency of these four fatty acids in causing changes in the structures of sites I and II. The association constants found by Ashbrook *et al.* (14) suggest that, at the concentrations used in the present study, palmitate, linoleate, stearate, and oleate would all be almost totally bound. The differences in potency therefore are not likely to be due to varying degrees of binding of the fatty acids to albumin. The differences between stearate and palmitate and between stearate, oleate, and linoleate suggest that both steric factors and chain length are important determinants of fatty acid binding affinity and of the ability to induce configurational adaptations in the protein.

The effects of the oleic acid on the two drug binding sites were not a simple function of the fatty acid to albumin ratio (Fig. 1). The binding of 1 oleic acid molecule altered the structure of site I but had almost no effect on site II. Also, the effect of oleic acid on site I reached a maximum when 2 Eq had been added, whereas the maximum effect on site II required addition of at least 5 Eq of oleic acid. These results suggest that the binding of some fatty acids causes the albumin molecule to assume conformations which are different for each successive fatty acid molecule bound.

We have shown previously that the binding of some drugs to site II causes changes in the structure of site I (2) and that binding of the radiopaque dye iophenoxic acid also induces a conformational change in albumin (11). It is clear, therefore, that human albumin shows a high degree of conformational mobility and that binding sites for both endogenous and exogenous compounds are unlikely to be independent.

Oleic and stearic acids caused a change in the specificity of the site II probe, dansylsarcosine, so that it was displaced by both site I and site II drugs. At the concen-

trations used, iophenoxic acid binds stoichiometrically to site I (1, 2, 11), so that this site is not available for the binding of other compounds. In the presence of both oleic acid and iophenoxic acid, dansylsarcosine was displaced by only site II drugs. The effect of iophenoxic acid in restoring the specificity of dansylsarcosine suggests that in the presence of fatty acids this probe binds to both site I and site II and is therefore displaced by drugs specific for each site. This may be due to an increased affinity of this probe for site I and/or a decreased affinity for site II.

The effects of fatty acids on drug binding have been the subject of a number of studies, but the results have been conflicting. There is, however, general agreement that bilirubin binding is not affected by addition of fatty acids at molar ratios with albumin of up to 4:1 (12, 15, 16). Thyroxine was displaced from defatted albumin by the addition of fatty acids at a 3:1 molar ratio with albumin (17). The order of effectiveness was oleate > linoleate > palmitate, which is similar to that reported here. Solomon *et al.* (3) reported the displacement of phenylbutazone and warfarin from albumin by lauric, myristic, and stearic acids, but the fatty acids were added at 35:1 molar ratios with albumin. Rudman *et al.* (8) found that palmitate and oleate, at 7:1 molar ratios with albumin, displaced salicylate, octanoate, and diphenylhydantoin. However, there was little or no displacement when the molar ratio of fatty acid was reduced to 3.5:1. Spector *et al.* (4) found that the binding of chlorophenoxyisobutyrate was reduced by palmitate at ratios with albumin of 1:1, 2:1, and 4:1. In contrast, halofenate binding was not altered. The binding of digitoxin was not altered by fatty acids (5). In contrast to these reports, Mukherjee (18) found that penicillin binding to human albumin was increased by addition of palmitic acid.

The present results indicate that, in general, fatty acids do not compete for drug binding sites on albumin but may, at concentrations commonly found *in vivo*, change the binding of some drugs by allosteric mechanisms. This effect may well be of clinical significance for drugs such as warfarin, whose metabolic clearance is restricted to the free drug (19).

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