membrane. Using this technique, the nonspecific adsorption of compounds to sampling tubes is not fully taken into account. This may explain the discrepancy between data obtained from ultracentrifugation with those obtained from ED and ultrafiltration.[12] Nakai *et al.* describe a microscale ultracentrifugation procedure for protein binding analysis using a table-top ultracentrifuge. Human plasma protein binding ratios for 10 drugs estimated by this method correlate well with reported values determined by other methods, such as ultrafiltration and equilibrium dialysis.[16]

High Performance Frontal Analysis Shibukawa *et al.* developed a novel analytical method, High-performance frontal analysis (HPFA), which enables the simultaneous determination of total and unbound drug concentrations under drug-plasma protein binding conditions. An ultramicro injection volume may be used, which is especially ideal for binding studies on proteins in scarce supply or difficult to obtain. In addition, HPFA does not suffer from undesirable drug adsorption to the membrane nor leakage of the bound drug through the membrane, since it can be achieved using separation systems such as HPLC and CE. By coupling HPFA with a chiral HPLC column, the concentration of an unbound racemic drug can be determined enantioselectively.[17]

High-performance affinity chromatography is based on examining the retention and competition of solutes as they pass through an immobilised HSA column. Qualitative and quantitative information that can be obtained include comparison of relative binding affinities, competitive displacement by other agents or measurement of equilibrium and rate constants based on immobilized albumin columns.[18] Both frontal and zonal, or a combination of zonal elution and frontal affinity chromatographies can be used to examine association constants of various compounds. The use of a combination of a zonal elution and frontal affinity chromatography method for determining association constants showed several advantages compared to traditional methods. Apart from rapidity, other advantages include ease of automation and ability to distinguish association constants of chiral compounds at the same time.[19]

Solid-phase microextraction (SPME) is a rapid, solventless alternative to conventional sample extraction techniques. It can be used in protein-drug interaction studies, provided that no analyte depletion occurs. In SPME, analytes establish equilibria among the sample matrix headspace above the sample, and polymer-coated fused fiber. They are then desorbed from the fiber to a chromatography column. Because analytes are concentrated on the fiber, and are rapidly delivered to the column, minimum detection limits are improved and resolution is maintained. Quantification of the amount of ligand extracted by SPME is accomplished by liquid chromatography coupled with tandem mass spectrometry.[20,21] An automated solid-phase microextraction (SPME) coupled to liquid chromatography-tandem mass spectrometry has been reported by Vuckovic et al. A new multifibre SPME system, which relies on multi-well plate technology and permits parallel preparation of up to 96 samples was used to obtain all the data points of the binding curve in a single experiment. The proposed method can be further extended to study plasma-protein binding or drug binding to whole blood.[22]

Spectroscopic methods

Circular dichroism has been used extensively to investigate the binding of ligands to albumin.[23] This technique is capable of estimating binding constants as well as conformational change of the protein upon ligand binding.[4] Using this technique, Yamasaki *et al.* reported phenomenon of site-to-site displacement on human albumin.[24]

Flourescence spectroscopy is a commonly used method for investigating the protein binding of albumin. Binding parameters for a binding site can be estimated by a modified Bjerrum method and the Scatchard method if there is enhancement in fluorescence on the binding of a ligand to albumin. The stereospecificity of binding and microenvironmental studies can be investigated with this method.[25-27]

Other methods

NMR has been used to study the structure of small proteins in solution. It has become a powerful tool for examining interactions between macromolecules and various ligands from qualitative screening, completing three-dimensional protein-ligand complex structure determination, to obtaining dissociation constants.[28,29] However, the binding affinities of ligands determined by NMR methods are universally weaker because NMR methods are susceptible to interference from additional non-specific binding.[30]

Surface plasmon resonance (SPR) is a new biosensor technology that precludes the need for label ligands and can be used in conjunction with a small sample volume. The analysis is rapid and automated. It has been used to classify ligands of albumin into high, medium and low affinity.[31] Rich *et al.* develop a high resolution and high throughput protocol using this technique to characterize drug-albumin interactions (**Fig. 2**).[32] The good correlation shown in **Figure 2C** indicates the utility of SPR to test compounds for HSA. This technique allows protein-protein interactions to be examined and detects differences in the binding of enantiomeric drug compounds with immobilized albumin.[33]

Differential Scanning Calorimety is primarily used to characterize stability and folding. And may be used to study binding interactions between proteins and small molecules, drugs and other proteins.[34] DSC measures binding constants from T_m shifts due to the binding of a drug (or other small molecule) to a protein. The binding

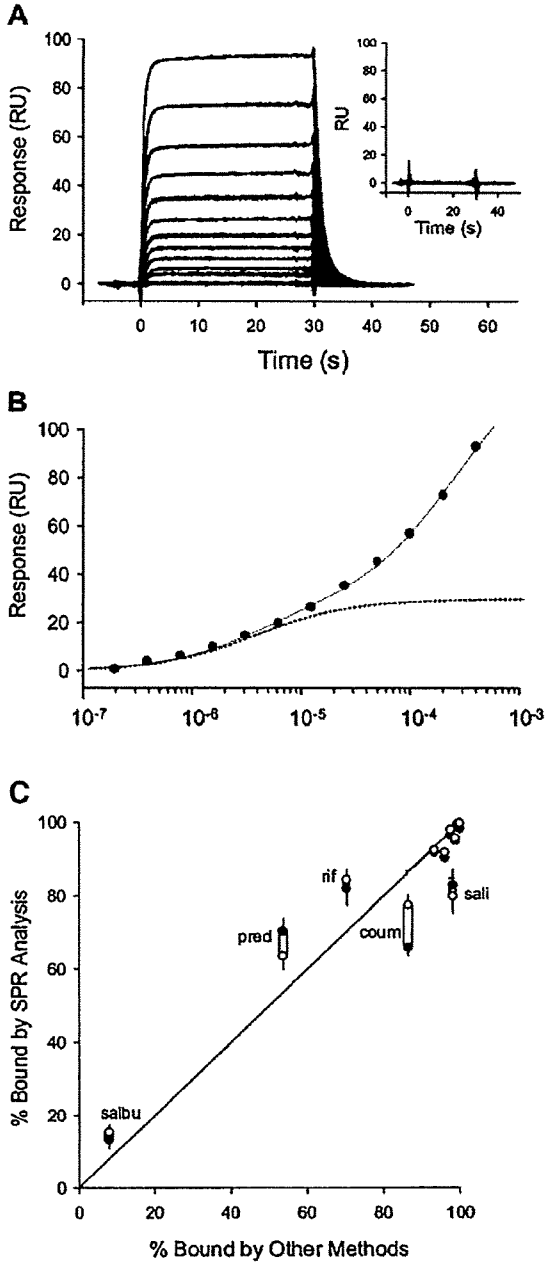

Fig. 2. Drug-HSA binding experiment using Biacore (from ref. 32)
(A) Sensorgrams; Responses collected from a non derivatized surface. (B) Response data; the solid line depicts the fit of the data treated by Eq. 1 $R = \Sigma(R_{max}(C/Kd)/1 + C/Kd)$ where R_{max} is the maximal response, C is the drug concentration, and Kd is the equilibrium dissociation constant. The dashed line represents the saturation point of the high-sffinity site. (C) Comparison with other methods

constant of the ligand can be estimated from T_ms in the presence and absence of a ligand, provided the concentration of the ligand in the DSC cell is known.[35] This method, together with isothermal titration calorimetry (ITC), provides a more comprehensive description of the thermodynamics of an associating system.[36,37]

Identification of Binding Sites

Identification of drug binding sites on a protein ensures that drug-drug interactions can be predicted accurately. Topology analysis for the binding sites on HSA has been carried out using several methods including X-ray crystal analysis and site directed mutagenesis (**Fig. 3**). This information is also invaluable for the molecular modification of drugs.

Photoaffinity labeling Although photolabeling has been used in identifying the binding of a ligand to a protein, only a few studies have been reported for HSA. Muramoto *et al.* investigated the interaction of the pituitary hormone corticotropin (ACTH) with bovine serum albumin by photoaffinity labeling with 2-nitro-4-azidophenylsulfenyl (2,4-NAPS) derivatives of aCTH and [Trp-(SH)9]ACTH.[38] Eckenhoff *et al.* attempted the first application of direct photolabeling of bovine albumin with [^{14}C]halothane.[39] Chuang *et al.* used [^{14}C]ketoprofen, a site II ligand, as a photolabeling agent without further modification, to identify the binding site of arylpropionic acid drugs on HSA[40] (**Fig. 4**). A similar attempt has been made by Kaneko *et al.* using dog albumin.[41] Photolabeling of HSA with [^{14}C]ketoprofen has been carried out in the presence of fatty acids of different chain lengths. The results shed light on how fatty acids influence the binding of site II drugs.[42]

Site directed mutagenesis Advancement in biotechnology has made recombinant technology an important tool for investigating protein properties. Watanabe et al. confirmed the role of arginine 410 and 411 tyrosine in the binding of site II ligands, by performing binding studies on 410 mutants of human albumin.[43] They further studied the binding of warfarin to site I mutants.[44] Thirteen recombinant isoforms of human albumin mutated in four different subdomains were produced by Kragh-Hansen *et al.* to investigate the high-affinity binding sites of fatty acids using a rate-of-dialysis technique.[45]

X-ray crystallography Ho *et al.* reported the crystal structure of equine serum albumin in 1993.[46] It was not until 1998 that Curry *et al.* reported and submitted the first crystal structure coordinates of recombinant human albumin complexed with myristate.[47] Subsequent publication on drug-albumin complex crystal structures revealed massive information on key amino acids at the binding sites involved in ligand binding.[48–50]

Conclusion

All Techniques have their own shortcomings. For example, ED generates errors due to protein leakage, a shift in concentration and binding equilibrium during separa-

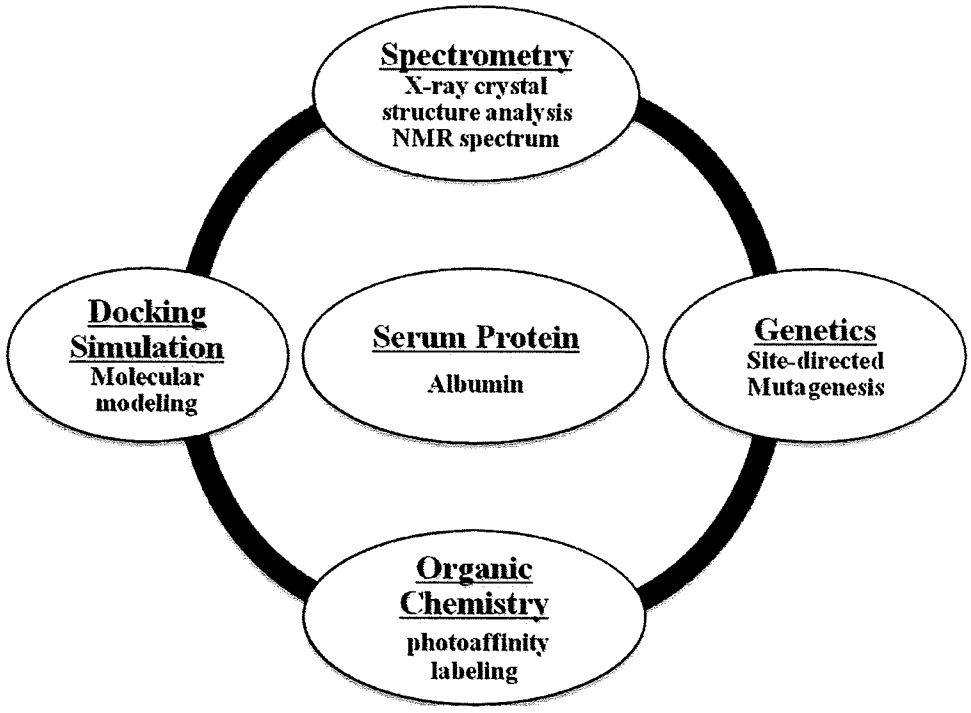

Fig. 3. Approach for topology analysis for drug-HSA interaction

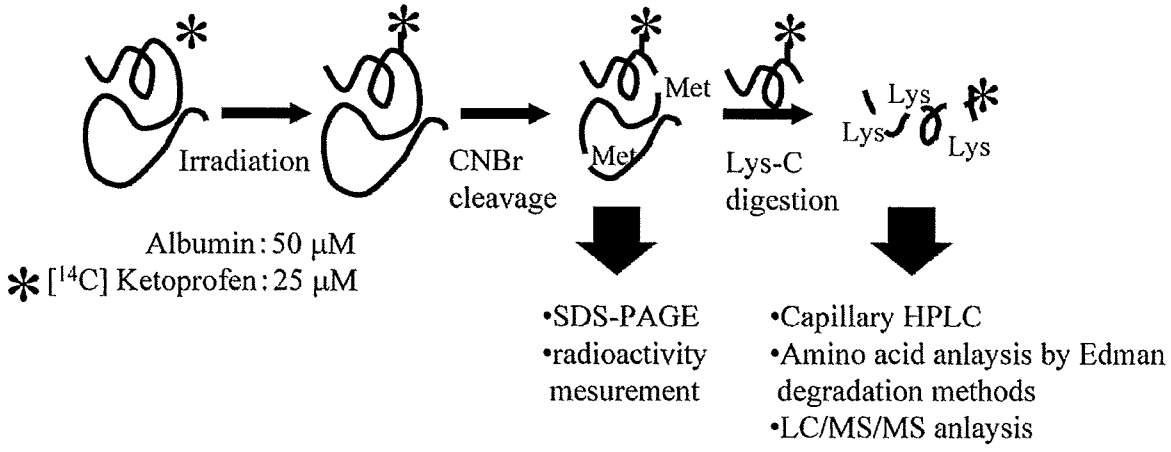

Fig. 4. Outline of photoaffinity labeling experiments

tion and is time consuming. Chromatographic methods based on immobilized human albumin columns only assay the fraction of drugs bound to albumin and not to the whole plasma. CE methods are restricted to certain buffer solutions, a single protein at a time and do not allow precise control of temperature. Ultrafiltration is attended with an intrinsic non-specific binding problem.

Using a combination of techniques to produce complementary data is the best solution. Otagiri *et al.* studied the binding of pirprofen to human serum albumin using dialysis and spectroscopy techniques.[2] Eckenhoff *et al.* evaluated the importance of the binding of a large domain III cavity and compared it to the binding characteristics of the 214 interdomain cleft for inhalation anesthetics using a combination of site-directed mutagenesis, photoaffinity labeling, amide hydrogen exchange, and tryptophan fluorescence spectroscopy.[51] Petersen et al combined crystallographic analysis with site directed mutagenesis to investigate the binding site of warfarin.[52] Simard *et al.* attempted to locate high-affinity fatty acid-

binding sites on albumin using a combination of x-ray crystallography and NMR spectroscopy.[53]

Finding a feasible method to evaluate the binding of compounds with low water solubility to albumin continues to be the most challenging issue in protein binding studies. A new high-throughput assay based on the distribution of drugs among plasma water, plasma proteins, and solid-supported lipid membranes (Transil) has been reported to produce valid results, even for drugs strongly bound to plasma proteins. The results obtained by this new method are identical with those by the erythrocyte partitioning technique or more conventional methods (ultrafiltration and equilibrium dialysis).[54,55] This new method may be suited for highly lipophilic drugs that adsorb onto surfaces due to their low aqueous solubility. Such a method would be invaluable during drug discovery and drug development for the high-throughput determination of protein binding.

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Drug Metab. Pharmacokinet. 24 (4): 308–317 (2009).

Review

Albumin as a Nitric Oxide-Traffic Protein: Characterization, Biochemistry and Possible Future Therapeutic Applications

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Full text of this paper is available at http://www.jstage.jst.go.jp/browse/dmpk

Summary: Nitric oxide (NO) is a ubiquitous molecule involved in multiple cellular functions. Inappropriate production of NO may lead to disease states. To date, pharmacologically active compounds that release NO within the body, such as organic nitrates, have been used as therapeutic agents, but their efficacy is significantly limited by rapid NO release, toxicity and induction of tolerance. Therefore, novel NO donors with better pharmacological and phamacokinetic properties are highly desirable. The *S*-nitrosothiol fraction in plasma is largely composed of *S*-nitrosylated human serum albumin (SNO-HSA) and that is why we are testing whether this albumin form can be used as a NO traffic protein. We have found that oleate and other endogenous ligands increase SNO-HSA formation *in vitro*. The cytoprotective effect of SNO-HSA in a ischemia/reperfusion model and its antiapoptotic effect on HepG2 cells treated with anti-Fas antibody were pronounced and could be enhanced by binding of oleate. The enhancement of *S*-transnitrosation to the HepG2 cells could be completely blocked by filipin III, a caveolae inhibitor. These findings indicate that a clinical application of SNO-HSA is expected as potent NO supplementary therapy and that fatty acids may serve as novel types of mediators for *S*-transnitrosation.

Keywords: human serum albumin; *S*-nitrosylation; *S*-transnitrosation; oleate; bilirubin; copper ion; ischemia/reperfusion liver injury model; HepG2 cells; cytoprotection; filipin III

Introduction

Depending on the location of its release, nitric oxide (NO) serves a wide range of biological functions.[1–27] For example, low concentrations of NO produced by constitutive NO synthase (cNOS) isoforms have several cytoprotective effects, such as regulation of local blood flow as an endothelium-derived relaxing factor,[12] inhibition of platelet aggregation,[28] attenuation of neutrophil adherence,[29] removal of superoxides,[30] and inhibition of superoxide anion production by neutrophils.[31] Defects of NO production can lead to many cardiovascular abnormalities such as essential hypertension, stroke, atherosclerosis and ischemia/reperfusion injury.[32] Therefore, replacement of or supplementing endogenous NO production by exogenously administered NO is an important and effective treatment for cardiovascular diseases. However, NO therapy still has some problems that should be overcome, such as the following.

First, administration of NO gas has limited utility, partly because of its short half-life *in vivo* ($\sim$0.1 s).[33] Therefore, pharmacologically active compounds that can release NO or lead to its formation in the body have been synthesized. Organic nitrates and nitrite esters have been used for many years to treat patients with ischemic heart disease. However, there are well-known side-effects and limitations to these NO donors, including potentially adverse hemodynamic effects, drug tolerance, lack of selectivity and limited bioavailability.[34,35] Thus, it is essential to develop reliable NO donors with better pharmacologi-

Received; April 7, 2009, Accepted; June 12, 2009
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This research was supported in part by grants-in-aid from the Japan Society for the Promotion of Science (JSPS), a grant-in-aid from the Ministry of Education, Culture, Sports, Science, and Technology (18390051), Japan, and by Fonden af 1870.

cal and pharmacokinetic parameters.

Secondly, it is important to be able to control reaction selectivity and dose of the NO donor against reactive oxygen species such as in NO therapy in inflammatory diseases. A high concentration of NO produced by inducible NOS (iNOS) is protective against bacterial infection in inflammatory processes, but too much NO will induce apoptosis and cellular damage.[1,8,36,37] The latter effect is due to the formation of peroxynitrite ($ONOO^-$), the reaction product of the interaction between superoxide (O_2^-) and NO, a potent proinflammatory nitroxide implicated in acute and chronic inflammatory conditions of many etiologies.[38-41] In addition, tissue injury and inflammation often accompany rapid development of hypersensitivity to noxious and nonnoxious stimuli (hyperalgesia and allodynia, respectively). In fact, sodium nitroprusside (SNP), which has been parenterally administered for the treatment of hypertension and heart failure, also induces an increase in the vascular production of superoxide leading to the formation of $ONOO^-$, which is associated with cytotoxic effects of SNP.[42,43]

Thirdly, local application of NO may be a very effective and safe form of NO therapy. To develop a method for the targeted delivery of NO, several groups of researchers have synthesized NO donors that hopefully can release NO selectively at a target site. For example, V-PYRRO/NO and 2-(acetyloxy) benzoic acid 3-(nitrooxymethyl) phenyl ester (NCX-1000) can selectively release NO in the liver. NO release from V-PYRRO/NO is mediated by cytochrome P450 which removes the vinyl group of the drug to generate free PYRRO/NO ion.[44-46] NCX-1000 is a prototype of a family of NO releasing derivatives of ursodeoxycholic acid. The two compounds are selectively metabolized in the liver and biologically active NO enters the liver microcirculation with no detectable effect on systemic circulation.[47] However, these NO donors have not yet been applied in clinical situations because the reaction mechanisms are not yet fully clarified. Thus, although NO release from V-PYRRO/NO is mediated by cytochrome P450, the isoform of cytochrome P450 catalyzing the process has not been identified. The enzyme that mediates NO release from NCX-1000 is still unknown. The enzymes that mediate NO release from V-PYRRO/NO and NCX-1000 must be identified in order to optimize their therapeutic efficacy.[48]

In our search for a reliable and safe NO donor, we have followed a different approach, namely to examine the possibility of using a NO-traffic protein. By a NO-traffic protein is meant a protein with i) high efficiency of *S*-nitrosylation, ii) high stability of the *S*-nitroso form in the circulation and iii) high efficiency of *S*-transnitrosation into cells which need NO. As a candidate in this respect we focus on human serum albumin (HSA), because HSA is the most abundant plasma protein (35–50 g/L) and because endogenous *S*-nitrosothiols in human plasma is largely associated with HSA.[49] *S*-nitrosylated HSA (SNO-HSA) is significantly more stable than low molecular weight *S*-nitrosothiols.[48] Also others have attempted to produce NO delivery systems using a NO-albumin conjugate. Marks *et al.*[50] and Ewing *et al.*[51] have synthesized a macromolecular *S*-nitrosothiol, poly SNO-BSA, in which several *S*-nitrosothiols are formed in bovine serum albumin (BSA) after reduction of the proteins disulfide bonds. Independently, Beak *et al.* have developed a macromolecular NONOate, diazeniumdiolated BSA, in which several NONOate moieties are conjugated to native BSA.[52] In a porcine coronary angioplasty model, the two BSA-forms, poly SNO-BSA and diazeniumdiolated BSA, were applied locally to a site of vascular injury and showed high retention at the administration site and reduced platelet attachment and activation. These effects were due to high binding of the modified albumins to the injured vessel.

In the development of targeted NO delivery systems for intravenous use, tissue distribution characteristics of the NO-carrier conjugate should be evaluated in vivo in order to identify the various obstacles to targeted delivery, such as extensive uptake by mononuclear phagocyte systems and rapid loss by glomerular filtration. Katsumi et al. have examined the pharmacokinetic properties of SNO-BSA. The results showed that serum albumin is a promising carrier to control pharmacokinetic properties of NO after intravenous injection, because *S*-nitrosylated albumin shows a relatively high retention in the blood circulation after intravenous injection into mice. However, targeted NO delivery after intravenous injection using a macromolecular carrier has not been successfully achieved so far.[53] To achieve targeted NO delivery from SNO-HSA after intravenous injection, we need to understand the different functions and structure of HSA and its biological fate in detail. Therefore, we have recently examined the relationship between *S*-nitrosylation and reversible binding of ligands to HSA. This review summarizes the effects of endogenous ligands on *S*-nitrosylation and *S*-transnitrosation of HSA both *in vitro* and in biological systems.

Structure and *S*-Ritrosylation of HSA

HSA is a single-chain, non-glycosylated protein with a molecular weight of 66.5 kDa. It is synthesized in and secreted from liver cells. The polypeptide organizes to form a heart-shaped protein with approximately 67% α-helix but no β-sheet.[54] It is composed of three homologous domains (I-III) each of which can be subdivided into subdomains (A and B) with distinct helical folding patterns connected by flexible loops (**Fig. 1**). All but one (Cys-34) of the 35 cysteine residues are involved in the formation of stabilizing disulfide bonds. In the circulation, the protein has an average half-life of 19 days, and

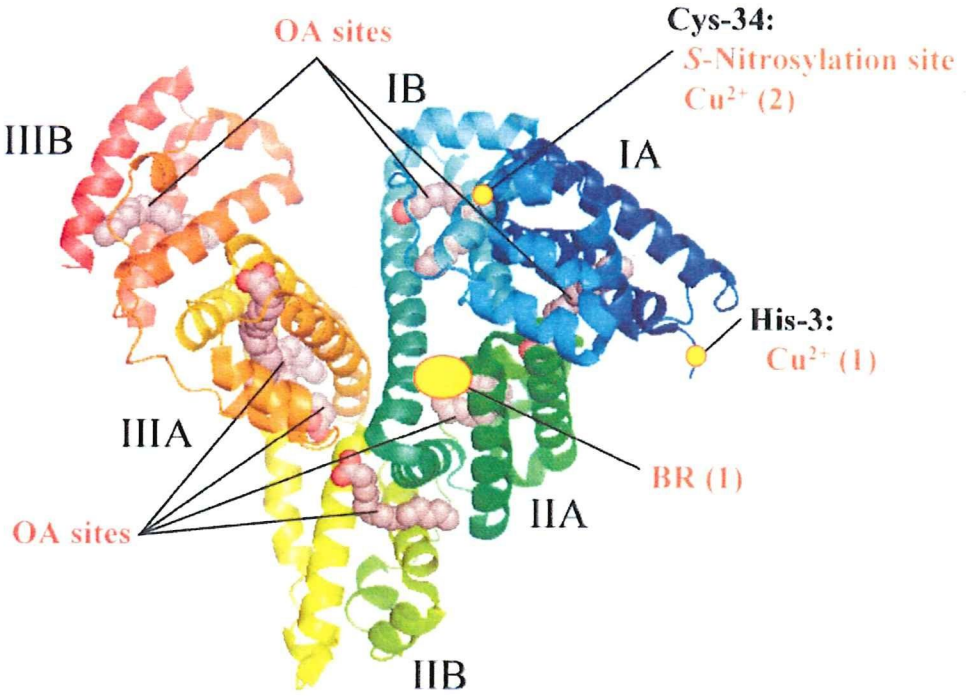

Fig. 1. Crystal structure of HSA showing locations of Cys-34 and OA, BR and Cu^{2+} binding sites
In addition to the seven OA binding sites, the locations of the high-affinity binding site for BR (BR(1)) and the high-affinity binding site (Cu^{2+}(1)) and a secondary binding site for Cu^{2+} (Cu^{2+}(2)) are indicated. Very recently, crystallographic analysis of HSA complexed with BR has shown, that the ligand is bound with high affinity in subdomain IB rather than in subdomain IIA.[70] The subdivision of HSA into domains (I-III) and subdomains (A and B) is given. The structure was simulated on the basis of X-ray crystallographic data for HSA-AO (PDB ID code 1gni) and modified with the use of Rasmol (downloaded from http://www.openrasmol.org).

normally about half of the Cys-34 residues are freely accessible; *i.e.*, they are not oxidized or involved in ligand binding and represent the largest fraction of free thiols in the blood.[54]

In the blood, HSA serves, among other things, as a transport and depot protein for numerous endogenous and exogenous compounds.[54,55] Therefore, we have studied, as a first step, the effects of the strongly bound ligands, oleate (OA) (C18:1), bilirubin (BR) and Cu^{2+} and the weakly bound ligands, L-tryptophan, progesterone, ascorbate, Zn^{2+} and Fe^{2+} on *in vitro* *S*-nitrosylation of HSA by *S*-nitrosoglutathione (GS-NO) and 1-hydroxy-2-oxo-3-(N-3-methyl-aminopropyl)-3-methyl-3′-triazene (NOC-7). We also studied the effects of the strongly bound ligands on *S*-nitrosylation of HSA in a biological system using stimulated RAW264.7 cells.

S-Nitrosylation of Mercaptalbumin with Bound Ligands

HSA purified from serum sossesses bound endogenous ligands, in particular fatty acids and perhaps also exogenous ligands. Any effect of these ligands on the *S*-nitrosylation of HSA was examined by incubating non-defatted and charcoal-treated albumin, with GS-NO. The *S*-nitroso moiety of the former preparation was significantly higher ($P<0.01$) than that of the latter.[56] Thus, the presence of ligands greatly enhance the efficiency of *S*-nitrosylation. In order to identify ligands of importance for *S*-nitrosylation, individual ligands were added to HSA previously delipidated by charcoal and dialyzed extensively against deionized water. In these experiments, two kinds of *S*-nitrosylating agents were used, namely GS-NO which *S*-transnitrosates via NO^+, and NOC-7 which *S*-

Reaction of NOC-7 with R-SH

$$\bullet NO + O_2 \longrightarrow \bullet OONO$$

$$\bullet OONO + \bullet NO \longrightarrow 2\ \bullet NO_2$$

$$\bullet NO_2 + \bullet NO \longrightarrow N_2O_3$$

$$N_2O_3 + R\text{-}SH \longrightarrow R\text{-}SNO + NO_2^- + H^+$$

$$\bullet NO + R\text{-}SH \xrightarrow{+\ metal} R\text{-}SNO + NO_2^- + H^+$$

Reaction of GS-NO with R-SH

$$GS\text{-}NO + R\text{-}SH \longrightarrow R\text{-}SNO + GSH$$

Fig. 2. Chemical reactions of NOC-7 and GS-NO with R-SH

Table 1. Effect of ligands on *S*-nitrosylation of HSA by GS-NO and NOC-7

	Oleic acid	Bilirubin	$CuSO_4 \cdot 5H_2O$	$(CH_3COO)_2Zn$	$FeCl_2$	L-Tryptophan	Ascorbate	Progesterone
GS-NO	↑	↑↑	↗	→	↗	→	→	→
NOC-7	→	→	↑↑	→	→	→	→	→

↑: $P<0.05$, ↑↑: $P<0.01$ as compared with HSA alone

nitrosylates mainly via NO and N_2O_3 (**Fig. 2**). The results obtained with equimolar amounts of protein and ligand are indicated in **Table 1**. It can be seen that OA and BR enhance the efficiency of GS-NO, but not that of NOC-7, whereas Cu^{2+} increases *S*-nitrosylation by NOC-7 but not that caused by GS-NO. In contrast, no significant effect was observed when adding L-tryptophan, progesterone, ascorbate, $(CH_3COO)_2Zn$ or $FeCl_2$. We studied in more detail the positive effects of OA, BR and Cu^{2+}, which bind to different high-affinity sites of HSA (**Fig. 1**).

Effect of AO binding: We investigated the effect of increasing OA on *S*-nitrosylation of HSA by GS-NO in more detail. Increment was found to be dose-dependent up to a OA:HSA molar ratio of 3; increasing the molar ratio further to 4 or 5 did not result in additional *S*-nitrosylation. Because OA does not bind to Cys-34 (**Fig. 1**), the effect observed most probably is due to binding-induced conformational changes of HSA making Cys-34 more accessible to GS-NO.[56] This proposal is supported by the finding that OA binding results in an almost linear increment in binding of the test-compound 5,5′-dithiobis -2-nitrobenzoic acid (DTNB) to Cys-34.[56] The proposal is also supported by crystal structure analysis.[57-59] According to analysis, the reactive SH group of Cys-34 is located in a crevice on the surface of HSA. Binding of OA induces conformational changes in the protein, leading to a slight opening of the interface between the two halves of the albumin molecule (subdomains IA-IIA and IIB-IIIB, respectively) and a rotation of domain I. These structural changes result in greater opening of the crevice that contains Cys-34 rendering the SH-group of Cys-34.[58]

Effect of BR binding: The effect of BR binding on *S*-nitrosylation by GS-NO was also studied at different molar ratios of ligand to protein. Binding of 1 mol of BR resulted in significantly higher *S*-nitrosylation ($P<0.01$); increasing the molar ratio to 3 or 5 did not cause additional *S*-nitrosylation. Thus, only high-affinity BR binding increases *S*-nitrosylation. Because this kind of binding takes place in another region of HSA to a greater extent than that possessing Cys-34 (**Fig. 1**), the improving effect must be due to conformation change in the protein related to accommodation of the large BR molecule. In contrast to GS-NO, high-affinity binding of BR does not influence *S*-nitrosylation by NOC-7 (**Table 1**). To test whether this lack of effect could be caused by interaction between NO and HSA-bound BR, we performed spectrophotometric experiments. The experiments showed that exposure of HSA-BR to NOC-7, but not to GS-NO, resulted in fast decrease of absorbancy at 470 nm (representing λ_{max} for HSA-BR) and concomitant and pronounced increase at 650 nm (representing λ_{max} for HSA-biliverdin). Therefore, the following reaction seems to take place: (^{34}Cys-SH)-HSA-BR + $\cdot$NO → (^{34}Cys-SH)-HSA-BV + NO_2^-. Thus, lack of effect of BR is due to its conversion to biliverdin (BV), and neither ligand nor NO_2^- formed improves *S*-nitrosylation.

Effect of Cu^{2+} binding: In contrast to the *S*-nitrosylating effect of GS-NO, the effect of NOC-7 was significantly increased by Cu^{2+} (**Table 1**). The increasing effect was the same, whether the molar ratio of Cu^{2+} to protein was 1:1, 3:1 or 5:1. Cu^{2+} binds with a very high affinity to a specific site in the N-terminal region of HSA, and His-3 is an essential element at that site[54] (**Fig. 1**). To test whether high-affinity binding of Cu^{2+}, which takes place at a distance from Cys-34, is responsible for the improving effect of NOC-7, or whether the effect is caused by other means, *e.g.* secondary binding, we mutated His-3 for an alanine. Positive effect of Cu^{2+} disappeared when mutating His-3. This finding strongly proposes high-affinity binding as the reason for improved effect of Cu^{2+} on *S*-nitrosylation by NOC-7. The positive effect of high-affinity Cu^{2+} binding is most probably caused by conformational change induced in the HSA molecule, which renders the SH-group of Cys-34 more reactive. Such a mechanism seems supported by the results of Zhang and Wilcox.[60] These authors, using isothermal titration calorimetry and different spectroscopic techniques, found evidence for interaction between the first Cu^{2+} binding site and Cys-34 in BSA. However, the conformational changes are different from those caused by OA, because in contrast to the binding of OA, that of Cu^{2+} does not affect the accessibility of Cys-34.[56] In contrast to the above studies, Stubauer *et al.*[61] found no effect of high-affinity bound Cu^{2+} on RS-NO formation. RS-NO formation was only initiated, when that binding site was saturated and the authors proposed *S*-nitrosylation of Cys-34 when Cu^{2+} binds with a low affinity to the same residue. However, they used BSA and NO gas in their studies.

Experiments with a cell line: To study *S*-nitrosylation of HSA in a biological system, we investigated the process caused by the murine macrophage cell line RAW264.7. The cell line had been activated by interferon-γ and lipopolysaccharide for expressing inducible

NO synthase. Binding of OA or BR to HSA did not affect *S*-nitrosylation of the protein by the cells. In contrast, Cu^{2+} binding facilitated significantly *S*-nitrosylation ($P<0.01$). We also found that binding of Cu^{2+}, but not binding of OA or BR, decreased significantly ($P<0.01$) the production of NO_2^-. Taken together, these results show that the formation of SNO-HSA by the cell line takes place via NO. This is supported by findings showing that the effects of GS-NO in a similar experiment differ from those of NOC-7 and RAW 264.7 cells.

Concluding remarks: Thus, high-affinity binding of ligands such as OA, BR and Cu^{2+} facilitate *S*-nitrosylation of Cys-34 in HSA. A common aspect to the binding of the three ligands is that they all bind with high affinity to the same part of HSA as that housing Cys-34, namely domain I (see legends to **Fig. 1**). The improving effect of ligand binding was observed *in vitro* and in a biological system, but depended on the NO donor.

Effect of Fatty Acids on *S*-Transnitrosation of HSA

In the following, the effect of endogenous fatty acids on *S*-transnitrosation from SNO-HSA is described. The effect was studied using an animal model and cultured cells. Again, OA was used as a representative for the fatty acids, because quantitatively it is the most important fatty acid in human depot fat and because it is a major contributor to the albumin-bound fatty acids. As in the studies on *S*-nitrosylation, we used OA:HSA molar ratios up to 5:1. This was done for investigating the potential effect of physiological and pathological fatty acid concentrations. Thus, HSA usually carries a total amount of 1–2 molar equivalents of fatty acids. However, this value can rise to 4 or more after maximal exercise or other adrenergic stimulation.[54]

Cytoprotection against ischemia/reperfusion liver injury in rats: To determine the effect of OA binding on *S*-transnitrosation from SNO-HSA *in vivo*, we used an ischemia/reperfusion liver injury model.[62] To evaluate liver injury, the extracellular release of the liver enzymes asparatate aminotransferase (AST) and alanine aminotransferase (ALT) was measured via plasma enzyme values. Injecting HSA, OA or HSA-OA into the portal vein immediately after reperfusion had no effect on plasma concentrations of AST and ALT. But administration of SNO-HSA diminished, to a significant extent ($P<0.01$), the enzyme concentrations measured at 60 min and 120 min. The protection of the liver cells by SNO-HSA was more pronounced, if the protein also carried OA. The effect of OA on SNO-HSA-mediated cytoprotection appeared to depend on OA content; *e.g.*, the binding of 5 mol OA had more pronounced effect than binding of 3 mol. We also found that caprylate (C8:0), a short-chain and saturated fatty acid, potentiated the cytoprotective effect of SNO-HSA. However, improved effect of caprylate was slightly less than that of OA.

Table 2. Uptake clearance and plasma concentrations of SNO-HSA, with and without 5 mol of OA, 120 min after injection into mice

		Uptake clearance (ml/h)			Plasma concentration after 120 min (% of injected dose)
		Liver	Kidney	Spleen	
SNO-HSA	OA (−)	186±20	48±18	12±8	52.6±4.4
	OA (+)	232±14	66±14	15±7	53.4±7.2

Results are expressed as means ± SEM ($n=3$).

The pharmacokinetic characteristics of SNO-HSA have been studied in mice. The study showed that binding of as much as 5 mol of OA per mol of SNO-HSA does not effect neither the protein forms plasma half-life nor its uptake by liver, kidney or spleen (**Table 2**).[62]

The advantageous effects of fatty acids on cytoprotection found in the ischemia/reperfusion model may involve multiple mechanisms, including maintenance of tissue blood flow, induction of heme oxygenase-1 (a cytoprotective enzyme), suppression of neutrophil infiltration and reduction of apoptosis.[63] Therefore, we investigated the effect of fatty acid binding in a simpler system, a cell line.

Cytoprotection of HepG2 cells exposed to anti-Fas antibody: NO and related species have been reported able to induce both antiapoptotic and proapoptotic response in cells, the type of response depending on the concentration of NO donor and type of cell and apoptosis-inducing reagent.[64] We have examined the influence of OA binding on the antiapoptotic effect of SNO-HSA on HepG2 cells treated with anti-Fas antibody. The presence of HSA, with or without bound OA, or OA alone had no effect on induced apoptosis. In contrast, addition of SNO-HSA resulted in concentration-dependent protection of the cells. This protection was greatly increased by binding 5 mol of OA per mol of SNO-HSA. Thus, fatty acid binding also improves the cytoprotective effect of SNO-HSA in an *in vitro* system.

The above findings clearly show that HepG2 cells *S*-transnitrosate to SNO-HSA. This aspect we have studied in a direct way, namely by measuring the concentration of SNO-HSA. We found that the presence of the cells caused decrease in SNO-HSA, and that decrease was faster and quantitatively more pronounced in the presence of OA. The effect increased with OA concentration: from 1:1 to 3:1 to 5:1 molar ratios. The OA-mediated promotion of SNO-HSA decay can be explained by increased accessibility to the *S*-nitroso moiety of HSA and/or by an intensified interaction between SNO-HSA and cell surface thiols.

We examined whether the improving effect of OA binding on *S*-transnitrosation is unique for that fatty acid, or whether the effect can also be exerted by a mixture of

Table 3. Plasma concentrations of HSA and fatty acids in 7 patients before and after hemodialysis

	Sex (M/F)	Albumin (g/dl)	Fatty acids (mM)	
			Before dialysis	After dialysis
No. 1	F	2.9±0.2	0.070±0.03	0.159±0.03
No. 2	F	3.9±0.4	0.114±0.04	0.614±0.06
No. 3	F	4.0±0.3	0.174±0.05	0.261±0.03
No. 4	F	3.6±0.3	0.182±0.06	0.934±0.07
No. 5	M	4.1±0.2	0.322±0.05	0.718±0.05
No. 6	M	3.9±0.4	0.230±0.05	0.380±0.03
No. 7	F	4.1±0.3	0.130±0.05	0.651±0.03

Results are expressed as means±SEM (n=3–4).

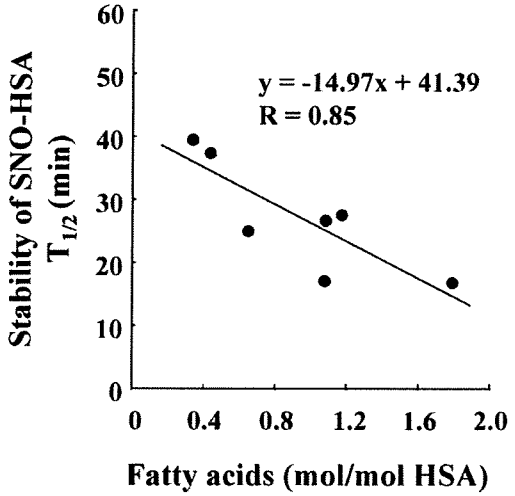

Fig. 3. *S*-transnitrosation of SNO-HSA made from HSA isolated from hemodialytic patients after dialysis by HepG2 cells
HSA samples were isolated by polyethylene glycol fractionation of blood plasma followed by chromatography on a Blue Sepharose column.[62] The HSA samples were *S*-nitrosylated using isoamyl nitrite. SNO-HSA and HepG2 cells were incubated for different periods of time and $T_{1/2}$ for *S*-transnitrosation of the different SNO-HSA samples were determined. Data represent means of four experiments.

endogenous fatty acids. We used HSA preparations isolated from hemodialysis patients, because such treatment increases fatty acid concentrations in the blood (**Table 3**). *S*-transnitrosation was quantified by determining the time necessary for HepG2 cells to cause halving of the SNO-HSA concentration ($T_{1/2}$ of SNO-HSA). As seen in **Figure 3**, there is good linear correlation between $T_{1/2}$ and the amount of fatty acid bound to SNO-HSA. Thus, in addition to OA, a mixture of endogenous fatty acids facilitates the decay of SNO-HSA by HepG2 cells. This finding has biological and clinical implications, because the plasma concentrations of non-esterified fatty acids can be increased in a number of situations. In addition to hemodialysis, increase in fatty acids is seen in connection with exercise and other adrenergic stimulation and in pathological conditions such as the metabolic syndrome and diabetes mellitus.

S-Transnitrosation of SNO-HSA by GSH: Kinetic aspects of *S*-transnitrosation of SNO-HSA have also been studied *in vitro* by using glutathione (GSH) as a NO-acceptor. The amount of GS-NO formed after 30 min incubation with SNO-HSA and decay in SNO-HSA, was more pronounced when OA was bound to SNO-HSA. Surprisingly, the effect was the same, whether SNO-HSA bound 1, 3 or 5 mol of OA per mol of protein. Thus possibly, only protein conformational changes caused by binding of the first OA molecule are essential for *S*-transnitrosation to GSH.

NO Uptake of HepG2 Cells

As mentioned above, fatty acid binding accelerates SNO-HSA decomposition by HepG2 cells. We investigated whether this decomposition is accompanied by NO uptake by the cells using intracellular DAF-FM DA fluorescence (fluorescence of diaminofluorescein-FM diacetate). Intracellular NO concentration increased with incubation time and with increasing OA/SNO-HSA molar ratios. To clarify the *S*-transnitrosation properties of a saturated fatty acid, we tested the effect of stearate (C18:0) on NO uptake by HepG2 cells. Stearate had an effect on the *S*-transnitrosation of SNO-HSA, which is very similar to that of OA.

Fatty acid-induced increment in the transfer of NO from SNO-HSA into the hepatocytes is completely blocked by the addition of filipin III. However, a basal mechanism, not affected by filipin III addition, slows the transfer of small amounts of NO or modifications thereof (*e.g.* NO^+). Both systems may involve a membrane protein and operate by transferring NO^+ from one thiol to another.

We examined whether NO uptake involves contact between albumin and the cell membrane or components thereof using FITC-fluorescence (fluorescence of fluorescein isothiocyanate). SNO-HSA was labeled with FITC and interaction with the HepG2 cells was analyzed by fluorescence microscopy.[62] FITC-SNO-HSA was found to bind to the cells, and that binding increased in a dose-dependent manner by cobinding of OA. Similar results were obtained with HSA that was not *S*-transnitrosylated. HSA binding to HepG2 cells is thus not affected by *S*-transnitrosation. Adding filipin III had no effect on the protein-cell interaction. Thus, OA is proposed to enhance the interaction between SNO-HSA and HepG2 cells.

Conclusion

Figure 4 proposes a model for *S*-transnitrosation of HepG2 cells by SNO-HSA. Binding of fatty acids in-

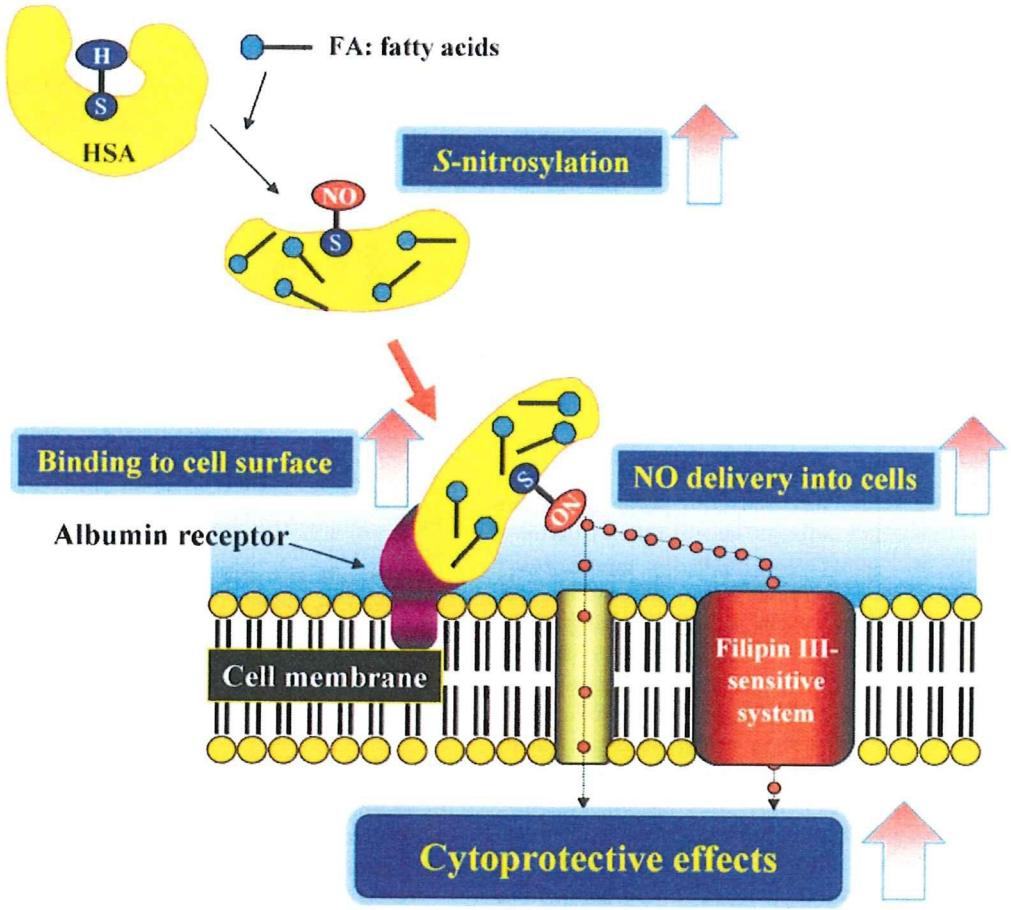

Fig. 4. Proposed model for fatty acid-mediated increase in *S*-transnitrosation of HepG2 cells by SNO-HSA
The model operates with two types of *S*-transnitrosation. A basal one and a much more pronounced process, caused by albumin-binding of fatty acid, and which can be blocked by filipin III. The model also proposes that *S*-transnitrosation takes place without involving low molecular weight thiols.

troduces conformational change in HSA that renders the SH-group, without or with *S*-nitrosylation, more accessible. Fatty acid binding also facilitates the binding of albumin to a receptor on hepatocytes, perhaps the albumin binding adaptor protein gp60.[65] When bound to the receptor, SNO-HSA-OA *S*-transnitrosates to HepG2 cells via two (or more) systems. Because OA-induced transnitrosation is completely blocked by filipin III, an inhibitor of caveolae,[66] it is proposed that caveolae are important for this type of *S*-transnitrosation. These findings strongly suggest that OA and NO of SNO-HSA-OA are transported by caveolae-associated proteins. Further studies are needed to identify and clarify the mechanism for the caveolae-associated proteins.

It is widely assumed that *S*-transnitrosation from SNO-HSA to cells takes place solely or mainly via low molecular weight thiols.[67–69] However, it should be noted that all the experiments with HepG2 cells were performed in the absence of GSH and other low molecular weight thiols.

Thus, fatty acid binding improves the cytoprotective effect of SNO-HSA *in vivo*, and reinforcement of an antiapoptotic effect by fatty acid binding contributes to this. Fatty acid bound to SNO-HSA enhances the interaction between SNO-HSA and HepG2 cells and the *S*-transnitrosation of SNO-HSA. This enhances NO transfer from SNO-HSA into the hepatocytes and the antiapoptotic effect. We found a novel filipin III-sensitive mechanism for the transfer of NO from SNO-HSA into hepatocytes. Taken together, further study is now warranted to explore the roles of fatty acid binding in the pharmacological benefits of SNO-HSA, for which clinical application is expected as a potent NO supplementary therapy as summarized in **Figure 5**.

Acknowledgments: We wish to thank Drs. T. Akaike and T. Sawa, Graduate School of Medical Sciences, Kumamoto University for their valuable advice and kind consideration. We also thank Dr. H. Katsumi of Kyoto

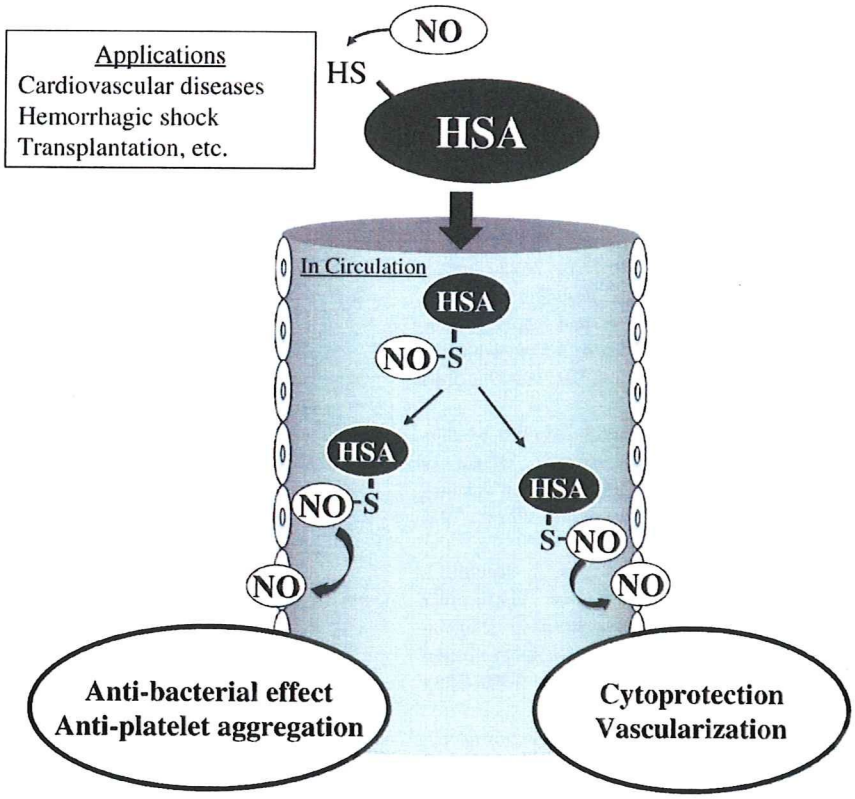

Fig. 5. Biological effects and potential therapeutic applications of SNO-HSA
The figure indicates some beneficial effects of circulating SNO-HSA, which most probably can be clinically useful. Our work shows that *S*-nitrosylation of and *S*-transnitrosation from albumin is greatly improved by binding of oleate and perhaps also by other high-affinity bound ligands.

Pharmaceutical University for his valuable advice.

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YAKUGAKU ZASSHI 129(4) 413—425 (2009) © 2009 The Pharmaceutical Society of Japan

—Reviews—

薬物の血清タンパク結合に関する研究

小田切優樹

Study on Binding of Drug to Serum Protein

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(Received December 12, 2008)

After being distributed in the circulating blood, drugs bind to serum proteins varying degrees. In general, such binding is reversible, and a dynamic equilibrium exists between the bound and unbound molecular species. It is believed that unless there is a specific transport system (*e.g.* receptor-mediated endocytosis, protein-mediated transport), only unbound drugs are able to penetrate through biomembranes, are distributed to tissues, and undergo metabolism and glomerular filtration. It is also believed that only unbound molecules present in target tissues can exert their pharmacological effects, and that the concentration of unbound molecules in tissues is in proportion to the drug serum concentration. Therefore, drug-serum protein binding is critically involved in the manifestation of the pharmacological effects of a drug as well as its pharmacokinetics. Among serum proteins, human serum albumin (HSA) and α_1-acid glycoprotein (AGP) play important roles in protein binding for many drugs, which is of key importance to drug distribution in the body. In addition, they are widely used in clinical settings as blood preparations and drug delivery system carriers. It is thus of great importance from the viewpoint of pharmaceutical science to clarify the structure, function, and pharmaceutical properties of HSA and AGP. Accordingly, since starting my laboratory, the focus of my research has involved molecular pharmaceutical studies on the interactions of drugs and HSA and AGP for the purpose of applying these findings to clinical fields, such as drug treatment, diagnosis and drug discovery. In this review, the molecular properties of HSA and AGP will be briefly outlined. The static and dynamic topology of drug binding sites on these proteins, investigated by various spectroscopic techniques, X-ray crystallography, quantitative structure-activity relationships, molecular modeling, photo affinity labeling, site-directed mutagenesis *etc.*, changes in the serum protein binding of drugs in pathological conditions, such as liver and kidney failure and various inflammation diseases and factors contributing to the changes will then be summarized. Finally, cases in which protein binding displacement can be applied to medical fields will also be introduced.

Key words——protein binding; drug; albumin; α_1-acid glycoprotein; clinical application

1. はじめに

薬物は循環血液中に移行したのち，薬物物性の差異を反映して程度の違いはあるものの，血清タンパク質と結合する．この現象を薬物の血清タンパク結合と言う．一般に，薬物の血清タンパク結合は可逆的であり，結合型と非結合型分子種の間に動的平衡が保たれている．薬物の輸送は特殊な輸送系（例えば受容体介在性エンドサイトーシスやタンパク介在性輸送）が存在しない限り，非結合型薬物分子のみが生体膜を透過して，組織に移行し，代謝や糸球体ろ過を受けると考えられている．また，薬理効果を発揮できるのは薬効発現組織における非結合型の分子とされているが，この濃度は血清中の非結合型濃度と比例関係にある．それゆえ，薬物の血清タンパク結合はその体内動態，さらには薬効発現に大きな係わり合いを有している．[1-7]

近年，疾病治療の標的となる受容体や酵素に対して高い親和性を有する薬物の開発が進んでいるが，通常，薬物は脂溶性が高く，かさ高い構造を有している．一般に，薬物の血清タンパク結合の推進力は疎水性相互作用であるため，血清タンパク質に対し

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本総説は，平成 20 年度日本薬学会賞を記念して記述したものである．

て高い親和性を有する薬物が多い．事実，上市される薬物に占める高タンパク結合型薬物の割合は以前に比べて大きく増加しており，薬物の中には，血清タンパク質に対する親和性が著しく強いことが原因で開発が中止となったものもある．そのため，従前以上に血清タンパク結合を適確に評価する必要がある．

ところで，ヒトにおいて薬物との結合に関与する血清タンパク質としては，血清アルブミンとα_1-酸性糖タンパク質（AGP），リポタンパク質，免疫グロブリンなどが知られているが，中でも最も多くの薬物と親和性を有しているのが，血清アルブミンとAGPである．これらのタンパク質は，薬物の体内分布の鍵を握る血清タンパク結合に重要な役割を果たすのみならず，医療現場では血液製剤やドラッグデリバリーシステムキャリアーとして広く利用されている．したがって，これらの血清タンパク質の構造・機能や製剤学的特性を明らかにすることは，医療薬学上，極めて意義深い．このような背景の下，筆者は研究室を主宰した当初から一貫して，基礎から臨床への応用を念頭におき，薬物とアルブミンやAGPとの相互作用に関する分子薬剤学的研究を行い，これら血清タンパク質の構造と機能に関する基盤情報を構築するとともに，それらを活用して治療や診断あるいは創薬・創剤といった医薬への応用を試みてきた．

本稿では，薬物の血清タンパク結合に関与する血清タンパク質の特性について簡単に述べ，筆者がこれまで行ってきた研究成果のうち，結合サイトの動的・静的トポロジー解析結果を紹介する．ついで，病態時における血清タンパク結合の変動とその要因について紹介し，最後にタンパク結合置換現象を医療分野に応用した事例について紹介する．

2. 薬物の体内動態に関与する血清タンパク質の構造と機能に関する基盤情報の構築

2-1. ヒト血清アルブミンとAGPの性状　ヒト血清アルブミン（HSA）は585個のアミノ酸残基からなる分子量約66500の単純タンパク質である．HSAは血清中に約4 g/dlと多量に存在し，血清タンパク質の約半分を占めている．HSAの生体内半減期は約15–18日であり，血管内プールに約40%が，血管外プールに約60%が分布している．HSAの構造特性としてα-ヘリックス構造からなる3つのドメインの繰り返し構造を有する．[8–12] HSAは膠質浸透圧の調節能を有しており，正常血漿の膠質浸透圧の約80%を維持しているため，古くから出血時やネフローゼ症候群に膠質浸透圧維持を目的としてアルブミン製剤が汎用されてきた．HSAは，脂肪酸，ビリルビン，尿毒症物質，一酸化窒素（NO），カルシウム，ホルモンなどの内因性物質や薬物，毒劇物や農薬といった外因性物質の血中輸送担体として働き，また組織への拡散防止としての解毒作用やエステラーゼ様活性等を有し，これら結合リガンドの体内動態や生理活性に影響を及ぼしている．また最近では，HSAの抗酸化効果に注目が集まっており，抗酸化物質の乏しい細胞外液中における抗酸化物質としての役割が見い出されるようになってきた．[13–18] このようにHSAは生体内の恒常性維持に必要不可欠な機能を数多く有していることから，各種病態時に伴うHSAの量的・質的変動は，上述した機能低下ひいては恒常性維持機能の脆弱化を引き起こすと考えられている．

一方，AGPの場合は183個のアミノ酸残基と5本の糖鎖からなる分子量約44100の血清糖タンパク質である．AGPの糖鎖含量は分子量の約45%と他の糖タンパク質の中でも極めて高く，その糖鎖末端にシアル酸残基を有しているために，血清中で最も負に帯電している（pI値：2.7）．[19–21] 現在までのところ，AGPの生物学的意義は不明であるが，疎水性化合物の輸送担体として機能しているタンパク質群，いわゆるリポカリンファミリーの一員として分類されている．事実，AGPは多くの塩基性薬物やステロイドホルモン類の主要結合担体として機能しており，結合薬物の体内動態に影響を及ぼしている．[14,22,23] また，通常，AGPは血清中に50–100 mg/dlで存在しているが，ストレス，外傷，炎症，腫瘍等，生体反応の急性期に合成が亢進し，血中及び組織中で著しく増加する急性相反応物質の1つであるため，これらの疾病時には血中濃度が3–5倍増加する．[24,25] また，免疫グロブリンと相同性を有していることや多様な糖鎖構造を有していることから，生体内で抗炎症作用や免疫調節能を発揮しているのではないかと考えられている．[26] 最近では，動物実験レベルではあるが，AGP自身を抗炎症剤として投与する試みも行われている．[27] このようにAGPの生物活性については新たな作用が見い出されてい

る半面，相反する報告も見受けられるため，生理的な役割についてはいまだ十分に明らかにされていないのが現状である．

2-2. HSA及びAGP分子上の薬物結合サイトのトポロジー解析　HSAやAGPは，非常に多くの物質を結合するにも係わらず，その分子上における結合サイトは限定されていることから，分光学的手法や構造活性相関，光アフィニティラベル法，部位特異的変異法，X線結晶解析，分子モデリングなどを駆使して，薬剤学領域では他に先駆け，これら血清タンパク質分子上のリガンド結合部位のトポロジー解析に着手した．

筆者が血清アルブミンの研究に着手した当時，Sudlowらのグループが一連のダンシル誘導体を用いた蛍光プローブ置換実験により，HSA分子上に2つの薬物結合サイト，いわゆるサイトⅠ，Ⅱの存在を見い出した．[28-30] しかしながら，ジキトキシンやジゴキシンの結合が2つのサイトでは説明できないことから，これらの化合物を結合する第3のサイトが存在するのではないかと考え，クマリン系化合物をはじめ種々の蛍光プローブを用い，ジギトキシンやジゴキシンなどの薬物が特異的に結合する新たなサイトの存在を明らかにし，これをサイトⅢと名付けた．[31] また，従来，サイトⅠは単一な結合領域と考えられてきたが，この点に関して多角的な検討を行った結果，結合領域はサイトⅡより柔軟で幅広く，少なくとも3つのサブサイトから形成されていることを初めて明らかにした．[32,33] さらに，サイトⅠのうち，サイトⅡと境界を接しているサブサイトⅠbだけがサイトⅡと相互作用を引き起こし，サイトⅡ結合リガンドの親和性を低下させることや，このサイト間相互作用がpH依存的な構造転移により厳密に制御されていることを見い出した．[34]

一方，サイトⅡについては，カルボン酸のような負に強く帯電した化合物が強く結合することから，従来より結合サイトにおける正電荷の存在が示唆されてきた．そこで，サイトⅡに強く結合する薬物をピックアップし，これらの負電荷をマスクした誘導体を合成して結合性を検討した．その結果，負電荷の消失はサイトⅡへの親和性を著しく低下させるだけでなく，サイト指向性にも大きな影響を及ぼし，サイトⅡのリガンド結合の認識過程における静電荷の重要性が実証された．[35] この知見がきっかけとなり，結合過程に関与するアミノ酸残基の同定に関する研究がスタートし，後述する部位特異的変異法によるアミノ酸残基の同定につながっていった．また，アリルプロピオン酸系消炎鎮痛薬カルプロフェンのHSA結合性を詳細に検討した結果，本薬物の高親和性結合サイトがサイトⅡに相当すること，他方，低親和性結合サイトはサイトⅠであることを同定した．[36] 興味深いことに，カルプロフェンを高親和性サイトのみに結合している条件下でサイトⅡ結合薬物を共存させても，予想に反して結合率には大きな影響は生じず，一見，競合置換が起きていないように思われる現象を見い出した．そこで，種々の分光学的手法を駆使して，この機序を検討した結果，サイトⅡに結合しているカルプロフェン分子が併用薬によりいったん置換されるものの，その後，低親和性サイトであるサイトⅠに再結合するというHSA分子内におけるサイト-サイト移行機構を提唱した．[37,38] 薬物結合サイト以外にも，内因性リガンドである脂肪酸の結合部位に関する分子マップを作成し，[39] 薬物結合サイトとの位置関係を明らかにするとともに，種々の条件下で結合サイト間のダイナミクスを網羅的に解析して，HSA分子上におけるリガンド結合サイトの静的プラス動的トポロジーを構築した（Fig. 1）．

薬物結合に関与するアミノ酸残基を明らかにしようと試みたが，従来の化学修飾方法では特異性が低く，残念ながら残基と部位の同定には至らなかった．そこで部位特異的変異法を導入して，サイトⅠではArg-218が，他方，サイトⅡではArg-410とTyr-411が重要であること，[40,41] 特にArg-410のグアニジノ基がリガンドの負電荷と静電的相互作用をしていることを明らかにし，これが前述したサイトⅡにおける正電荷であることを実証した．[41] また，Arg-410とTyr-411をいずれもアラニンに二重置換した変異体（R410A/Y411A）ではリガンド結合性が著しく低下し，サイトⅡへの指向性が消失することから，この変異体をサイトⅡノックダウンHSAと称し，野生型HSAと組み合わせて結合性を調べることにより，結合サイトを簡便かつ迅速に同定する新たな評価法を開発した．[14]

ところで，HSAにはドメインⅠに局在するCys-34が唯一遊離状態で存在しているが，筆者らはこの残基とSH含有化合物との共有結合体の形成

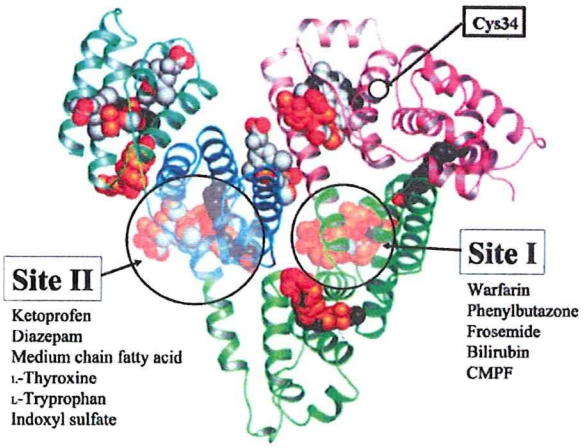

Fig. 1. Summary of the Ligand Binding Capacity of HSA as Defined by Crystallographic Studies to Date

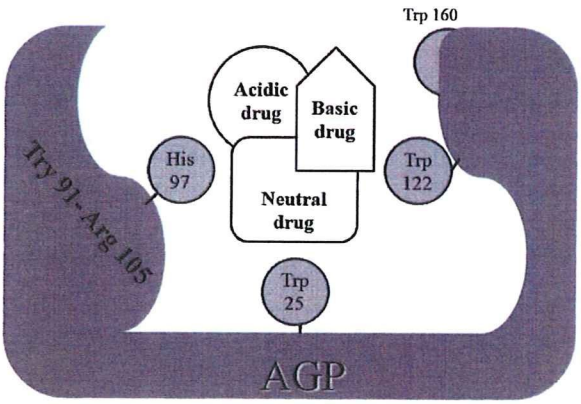

Fig. 2. Proposed Drug-binding Region on AGP

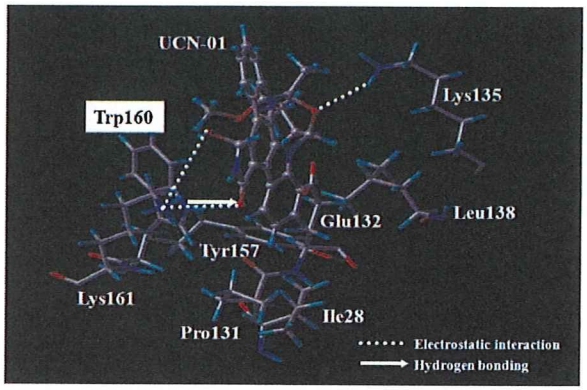

Fig. 3. Amino Acid Residues in a Surface Cleft around Trp-160 That Interacts with UCN-01 Exhibited in Type II Docking Model

を直接的に定量する方法を開発し，システインやグルタチオンなどの内因性物質やカプトプリル，*N*-アセチルシステインといったSH含有薬物が可逆的な共有結合体を形成することを見い出すとともに，この反応に影響を及ぼす要因を明らかにした.[42,43] また，この複合体形成の生理的な意義を明らかにするため，免疫学的検討を重ねた結果，反応に伴うハプテン形成が薬物の免疫学的な副作用の発現機序に関与していることを証明した.[44] さらに，Cys-34を蛍光プローブのアクリロダンでラベル化し，種々の条件下でその反応性を検討した結果，Cys-34の反応性がSH基の溶媒への露出度や周囲の静電的ポテンシャルといったミクロ環境に依存することを初めて実証した.[45,46]

シグナル伝達分子であるNOは多様な活性を有するものの，その生体内寿命は非常に短く，生体ではタンパク質などのチオール基と反応し，S-ニトロソチオールへと変換され，比較的安定な状態を保っている．そのため，タンパク質のS-ニトロソ化はNOリザーバーとして働き，生体内のNO濃度の調節に関与している.[47,48] 中でもHSAは血漿中において多量に存在する上に，優れた血中滞留性を有していることから，NOリザーバーとしての役割も大きいことが期待される.[49] 筆者らは，HSAにおけるS-ニトロソ化反応やS-ニトロソ転移反応が脂肪酸，銅イオン，ビリルビンなどの内因性結合物質によって絶妙にコントロールされていることを見い出した.[50,51] これまで数多くのS-ニトロソ化タンパク質が同定されてきたが，結合リガンドにより反応性が調節されている例は見当たらないことから，この機序はHSAに特異的なものであるかもしれない．これらの内因性結合リガンドは疾病時に大きく変動するため，リガンド結合によるS-ニトロソ化反応の制御は病態時におけるHSAの新たな役割を考える上で非常に興味深い．現在，種々の疾病モデル動物を用いて，この反応の生理的意義について追及している．

一方，AGPの場合，糖鎖含量が極めて高いため，X線結晶構造解析やNMR解析等による立体構造の解明はなされていない．これまでのところ，円二色性（CD）スペクトル解析や分子モデリングによる検討から，水溶液中ではβ-シート構造に富んでいることが報告されているだけであり，[19,20] 薬物結合サイトのトポロジーについては不明な点が多く残さ

れている.

研究開始当初，AGP 分子上のリガンド結合サイトについては，AGP が塩基性薬物の主要結合タンパク質であることから，これらリガンドに対する結合サイトの同定が試みられ，HSA とは対照的にリガンド結合サイトは 1 つであると信じられてきた.[21,22] しかしながら，筆者らは AGP には塩基性薬物だけでなく，ワルファリンをはじめとするクマリン系薬物などの一部の酸性薬物やベンゾジアゼピン系薬物やステロイドホルモンといった疎水性物質も HSA と同等かそれ以上に強く結合することを見い出した.[52,53] これらの知見に基づき，塩基性薬物以外の結合サイトも存在するのではないかと考え，種々の蛍光，CD プローブを用いて薬物結合サイトのトポロジー解析を行った結果，AGP 分子上の薬物結合サイトは，塩基性薬物，酸性薬物及びステロイドホルモンをはじめとする中性薬物の結合する 3 つのサブサイトから構成され，これらが一部オーバーラップした位置関係で互いに干渉しているモデルを提唱した（Fig. 2）.[54-56]

また，抗がん薬 UCN-01 は AGP に $10^8\ M^{-1}$ という非常に高い親和性を有しているが，光アフィニティラベル法，部位特異的変異法，構造活性相関，ドッキングシュミレーションなどのアプローチを駆使することにより，この強固な結合が UCN-01 のインドカルバゾール環と Trp-160 のスタッキングによることを明らかにした（Fig. 3）.[57,58] これまでも，化学修飾法や蛍光スペクトル法の検討結果から，AGP と薬物の結合における Trp 残基の重要性は示唆されてきたものの，特定の残基は明らかにされていなかったため，本研究で初めて Trp-160 の関与を同定できたことは，AGP の薬物結合サイトのマッピングを作成する上で有用な基礎資料になるものと考えられる.

AGP には 2 つのバリアント（F1*S 体と A 体）が存在し，薬物によってはいずれかのバリアントに選択的に結合する.[59-61] 例えば，F1*S 体にはワルファリン，ジピリダモールなどが，他方，A 体にはプロパフェノン，ジソピラミドなどが選択的に結合する．この興味深い現象は今から約 10 年前に見い出されたものの，その機序については明らかにされていない．そこで筆者らは，これまで構築した薬物結合サイトに関するトポロジーデータとリポカリンファミリーの立体構造モデルを精査し，薬物結合に関与するアミノ酸残基を絞り込み，さらに部位特異的変異法により両バリアント間でアミノ酸残基をスイッチさせ，結合選択性の変化を調べた．その結果，92 番目のアミノ酸残基（F1*S 体では Val, A 体では Glu）をバリアント間でスイッチすることにより，薬物選択性が逆転したことから，この残基が AGP バリアント間の薬物結合選択性に関与していることを実証した.[62] 本知見は AGP の薬物結合選択性に関与するアミノ酸残基の存在を初めて明らかにしたものであり，今後，AGP のリガンド結合及び結合サイトのトポロジーを構築する上での重要な基礎情報になるだろう.

ところで，医薬品開発過程では，種々の実験動物を用いて体内動態における種差が検討され，その結果に基づきヒトへの外挿が試みられている．従来より，血清タンパク結合においても種差の存在が明らかにされていたが，その機序については不明であった．そこで，これらの差異がアルブミン及び AGP 分子上の薬物結合サイトの違いに起因すると考え，上述の手法を用いて結合サイトのトポロジー解析を試みた．その結果，いずれのタンパク質においてもヒトと全く同等なトポロジーを有するものは存在せず，動物種間で若干異なっていた．例えば，アルブミンの場合，ラットはヒトのサイト I に酷似した部位を保持しており，ウサギもサブサイトの重なり度合いは多少異なるもののヒトに類似した構造を有していた．しかし，ウシではサブサイトが独立していた.[63] イヌの場合は，サイト I に相当する結合部位の存在を見い出すことができなかった．対照的に，サイト II ではヒトとイヌが 2 つの重なり合ったサブサイトから構成されており，非常に類似したトポロジーを有していることが判明した．一方，ウシ，ラット，ウサギではサイト II の存在はあるものの，ヒトに比べると不完全な形であった．最近，このモデルの妥当性が光アフィニティラベル実験により裏付けられた.[64] また，これらアルブミン種における構造の熱安定性を示差熱量計で検討した結果，ヒト，ウシ，ラットでは 1 成分ピークによる 2 状態転移を取るが，イヌやウサギは 2 成分ピークで多状態転移を示すことを明らかにした.[65] 一方，AGP の場合，ヒトと同様，ウシやイヌでは塩基性薬物及びステロイドホルモンの結合サイトが互いに重なり合っ