Pergamon

Biochemical Pharmacology, Vol. 49, No. 1, pp. 91–96, 1995.

0006-2952/95 $9.50 + 0.00

0006-2952(94)00448-X

EFFECT OF NITRIC OXIDE ON ALBUMIN–PALMITATE BINDING

FRANK J. BURCZYNSKI,* GU-QI WANG and MARK HNATOWICH

Department of Pharmacology and Therapeutics, Liver Research Section, Faculty of Medicine, University of Manitoba, Winnipeg, Manitoba, Canada R3E 0W3

(*Received* 14 *May* 1994; *accepted* 15 *August* 1994)

Abstract—Bovine serum albumin (albumin) was modified by treatment with nitric oxide (NO) to form *S*-nitrosoalbumin. Analysis of the reduced sulfhydryl groups showed that more than 99% of the albumin was converted to *S*-nitrosoalbumin. Using a 1 : 1 molar ratio of protein : palmitate, the unbound palmitate fraction in the presence of *S*-nitrosoalbumin was determined to be greater (28%) than in the presence of albumin as determined by heptane : water partitioning. NO degradation products neither affected the palmitate heptane : water partition ratio in the absence of binding protein nor the hepatocyte uptake of [^{3}H]palmitic acid. The equilibrium association constants (K_a) for albumin–palmitate and *S*-nitrosoalbumin–palmitate complexes were determined using the stepwise equilibrium model. The K_a for the first and second palmitate binding sites were $(4.6 \pm 1.2) \times 10^8\,M^{-1}$ and $(3.3 \pm 0.5) \times 10^7\,M^{-1}$ and $(3.1 \pm 0.9) \times 10^8\,M^{-1}$ and $(1.3 \pm 0.8) \times 10^8\,M^{-1}$ for albumin and *S*-nitrosoalbumin, respectively. Thus, the increased unbound fraction of palmitate in the presence of *S*-nitrosoalbumin was apparently due to a decreased binding affinity at the first high-affinity binding site. Palmitate uptake by hepatocyte suspensions was 27% higher in the presence of *S*-nitrosoalbumin as compared with albumin. This increase paralleled the increased unbound palmitate fraction. When the albumin concentration was adjusted to account for the increased unbound fraction, there was no difference in the palmitate uptake rates between albumin and *S*-nitrosoalbumin. Our findings indicate that under conditions where NO concentrations are high (e.g. cirrhosis) and extensive *S*-nitrosylation of serum albumin occurs, the decreased ligand binding ability of *S*-nitrosoalbumin may be an important consideration when modeling drug uptake in pathological states.

Key words: nitric oxide; albumin; protein binding; palmitate; organic anions; uptake; hepatocytes; *S*-nitrosoalbumin

The discovery of NO† as a mediator in numerous cellular responses has opened new areas of biological research. Postulated roles for this low molecular weight substrate range from neurotransmission to the regulation of vascular tone [1–6]. As evidenced by the increasing number of articles devoted to NO in recent years, the more closely the effects of NO are investigated, the more firmly its role in cellular chemistry becomes established.

In the circulatory system, NO is rapidly inactivated ($T_{1/2} = 0.1$ to 6 sec) following its release from endothelial cells [2, 6]. The short half-life is due, in part, to the rapid formation of the nitrite anion (NO_2^-) upon reaction with oxygen. NO, and in special circumstances nitrite anions, may combine with reduced thiols of which plasma albumin constitutes an abundant source [7], to form the *S*-nitroso-substituted species. The formation of *S*-nitrosoalbumin complexes results when the single free cysteine of albumin reacts with NO. These complexes have been reported to be biologically active and more stable than NO [8, 9]. They also have been postulated to serve as NO donors. Thus, albumin may buffer the concentration of NO in plasma by serving as both an NO acceptor and donor [6].

Plasma albumin constitutes a major drug binding and delivery protein in the circulatory system. Covalent modification of albumin by NO may, as is often the case when ligands bind to macromolecules, cause conformational changes resulting in altered drug binding characteristics. Such conformational changes could, depending upon the ligand in question, modify protein binding and ultimately affect the cellular uptake rates [10–12]. The therapeutic and toxic consequences of alteration in the drug binding characteristics of albumin are obvious and prompted this investigation. In this report, we examined the binding of a long-chain fatty acid (palmitate) to albumin and *S*-nitrosoalbumin and the palmitate clearance rate by hepatocyte suspensions in the presence of albumin and *S*-nitrosoalbumin.

MATERIALS AND METHODS

Purification of palmitic acid. All reagents were purchased from the Sigma Chemical Co. (St. Louis, MO) and were of analytical grade. NO gas was purchased from Matheson (Edmonton, Alberta). Manufacturer-supplied [^{3}H]palmitic acid in ethanol (New England Nuclear, Mississauga, Ontario) was purified using the alkaline ethanol extraction

* Corresponding author. Tel. (204) 789-3457; FAX (204) 783-6915.

† Abbreviations: NO, nitric oxid; K_a, equilibrium association constant; $\bar{\nu}$, molar ratio of bound palmitate : protein; PR, partition ratio; and $T_{1/2}$, half-life.

procedure previously reported [13]. Briefly, manufacturer-supplied [^{3}H]palmitic acid was added to purified research grade water containing NaOH and thymol blue. Following the addition of heptane, the solution was vortexed and allowed to separate. The organic phase was discarded, fresh heptane added and the procedure repeated. After two such extractions, the ethanol phase was acidified and the procedure repeated. The heptane phase, now containing purified [^{3}H]palmitic acid, was harvested, fresh heptane added and the procedure repeated with the second heptane extract being combined with the first. The pooled heptane extracts were evaporated to dryness, dissolved in 95% ethanol, and stored at −20°.

Binding of NO to albumin. PBS (KCl 2.68 mM, KH_2PO_4 1.65 mM, NaCl 136.9 mM, and Na_2HPO_4 8.92 mM, pH 7.4) was degassed by vacuum (25 mm Hg) for ~20 min at room temperature. Bovine serum albumin (albumin; essentially fatty acid free) and palmitate were added to the PBS solution to a final concentration of 600 μM each (pH 7.4). Aliquots (20 mL) of the albumin–palmitate solution were dispensed into 25-mL Erlenmeyer flasks. Purified [^{3}H]palmitic acid was added to the flasks, which were then capped using rubber stoppers containing two 25-gauge needles. Nitrogen was bubbled into the flasks through one needle, while the other served as the outflow. After 30 min, the outflow needle was removed and NO gas (10 mL) was added to the test solution, which resulted in a calculated aqueous NO concentration of 1.9 mM [14]. The flasks were uncapped, and pH of the test solution was adjusted to 7.4.

Formation of *S*-nitrosoalbumin was confirmed by measuring the sulfhydryl levels spectrophotometrically with DTNB [5,5′-dithiobis(2-nitrobenzoic acid)] as reported by Ellman [15].

Determination of unbound palmitate. All binding studies were performed at pH 7.4 and 37°. The unbound palmitate fraction was determined by the heptane:water partition method as previously described [13, 16]. Following an 18-hr incubation, both phases were sampled, radioactivity was determined, and the heptane:water partition ratio was calculated. Previous work has shown that this time period is sufficient for equilibrium to be established [13].

An excess of nitrite anion (relative to NO) may result when reacting 1.9 mM NO with 600 μM albumin to form the *S*-nitrosoalbumin species. To determine the effect of nitrite anion on the binding properties of albumin, we added NO gas to the PBS solution such that a saturated NO solution was attained (1.9 mM NO). The mixture was allowed to stand open to the atmosphere for 2 hr at room temperature after which time we added albumin and palmitate each to a final concentration of 600 μM. The pH of the resulting solution was adjusted to 7.4. Heptane:water partitioning also was performed on a solution consisting of 1.9 mM $NaNO_2$ and 600 μM albumin–palmitate (molar ratio 1:1).

The time course of *S*-nitrosoalbumin–palmitate binding was investigated by comparing the palmitate heptane:water partition ratio values at 12 and 24 hr. The PBS solutions for these experiments were degassed in order to minimize any effects of dissolved oxygen. The effect of oxygenated solutions on *S*-nitrosoalbumin–palmitate binding was investigated by determining the palmitate heptane:water partition ratio using PBS solutions that were oxygenated after the formation of *S*-nitrosoalbumin.

Determination of the equilibrium association constant. Purified [^{3}H]palmitate was added to solutions of 5 μM albumin and 5 μM *S*-nitrosoalbumin containing various concentrations of unlabeled palmitate (Sigma). The palmitate-protein molar range investigated was 10^{-6} to 5. The unbound palmitate fraction was determined by heptane:water partitioning. In the presence of binding protein the unbound palmitate concentration in the aqueous phase cannot be determined directly. Therefore, it was necessary to calculate this value by dividing the concentration of palmitic acid in the heptane phase by 1445. This value has been shown to reflect the increased heptane:water partition ratio of palmitic acid in the absence of binding protein [13]. The equilibrium association constant for each protein was obtained by nonlinear regression analysis using the stepwise equilibrium model [17, 18]. Analysis consisted of fitting the experimental values of $\bar{v}$ (protein concentration was fixed at 5 μM) for each value of A (unbound palmitate) using the equation

$$\bar{v} = \frac{K_1[A] + 2K_1K_2[A]^2 + \ldots + nK_n[A]^n}{1 + K_1[A] + K_1K_2[A]^2 + \ldots + K_n[A]^n}$$

where K_n represents the equilibrium association constant for the n^{th} binding site of palmitate to albumin or *S*-nitrosoalbumin.

Uptake of palmitate by hepatocyte suspensions. This study was approved by the University of Manitoba Animal Care Committee. Female Sprague–Dawley rats (weight range 225–250 g; University of Manitoba breeding stock) were housed in a temperature–light controlled room. Room temperature was set at 22°, with lights set on a 12-hr on and 12-hr off cycle starting at 6:00 a.m. Rats were allowed Agway Prolab Animal Diet (Agway County Foods, Inc., Syracuse, NY) and water *ad lib.* Hepatocytes were prepared using the collagenase perfusion technique described previously [19]. Cells were stored at room temperature and used within 2 hr of isolation. Immediately prior to experiments, cells were equilibrated to 37°. Trypan blue exclusion was greater than 90% and not different before and after the uptake experiments.

Hepatocytes were added to solutions of PBS containing [^{3}H]palmitate and 5.0 μM *S*-nitrosoalbumin and 3.8 or 5.0 μM albumin. Because NO degrades rapidly to nitrite, we investigated the effect of nitrite anions on palmitate uptake. Uptake experiments were performed in the presence and absence of 1.9 mM $NaNO_2$ and 5.0 μM albumin. Final cell count in the uptake medium was ~290 × 10^3 hepatocytes/mL. Cells were prevented from sedimenting by gentle agitation. After specified intervals, 1.0-mL aliquots of cell suspensions were immediately filtered through GF/C glass microfiber filters (Fisher Sci.) by vacuum and washed with 7 mL of ice-cold PBS. Cell-associated radioactivity was determined by liquid scintillation counting using Ready Safe

Table 1. Heptane: water partitioning of [^{3}H]palmitate

Group	Protein	Palmitate (μM)	Heptane: PBS partition ratio	%*	P
1	600 μM ALB	600	0.0250 ± 0.0004 (N = 24)	28	†
	600 μM ALB-NO	600	0.0320 ± 0.0010 (N = 21)		
2	600 μM ALB	600	0.0250 ± 0.0003 (N = 6)	8	†
	600 μM ALB-NO + 600 μM ALB (1:9)‡	600	0.0270 ± 0.0004 (N = 6)		
3	600 μM ALB	600	0.0260 ± 0.0010 (N = 6)	4	NS§
	600 μM ALB + 3 mM NaN_3	600	0.0250 ± 0.0004 (N = 6)		
4	600 μM ALB-NO	600	0.033 ± 0.003 (N = 6)	3	NS
	600 μM ALB-NO + 3 mM NaN_3	600	0.032 ± 0.001 (N = 6)		
5	600 μM ALB	600	0.017 ± 0.001 (N = 6)	6	NS
	600 μM ALB + 1.9 mM $NaNO_2$	600	0.018 ± 0.001 (N = 6)		
6	5 μM ALB	0.01	0.90 ± 0.015 (N = 7)	24	†
	5 μM ALB-NO	0.01	1.12 ± 0.04 (N = 6)		
7	No protein	0.01	69.9 ± 1.9 (N = 6)	3	NS
	No protein + 1.9 mM $NaNO_2$	0.01	68.0 ± 3.1 (N = 6)		

Values are means ± SEM. ALB = albumin, ALB-NO = *S*-nitrosoalbumin, NaN_3 = sodium azide, and $NaNO_2$ = sodium nitrite.

* Percent difference within groups.

† $P < 0.01$.

‡ Molar ratio of ALB-NO to ALB.

§ NS = not statistically different.

liquid scintillation fluid (Beckman). Palmitate space was calculated as the ratio of recovered cell-associated radioactivity to the concentration of radioactivity in the bathing medium. Total ligand clearance rate was calculated from the least-squares linear regression coefficient for the slope of the plot of palmitate space versus time.

Statistics. Data are expressed as means ± SEM with the number of independent experiments shown in parentheses. Student's *t*-test was used for statistical comparisons.

RESULTS

S-Nitrosoalbumin formation. A decrease in pH resulted when an excess of NO gas was allowed to react with 600 μM albumin. The pH of the albumin solution decreased from 7.1 to 6.9 in deoxygenated PBS solutions. In the presence of oxygen, however, the pH decreased to 5.1. Once the pH was adjusted to 7.4, it remained stable throughout all the studies.

The extent of *S*-nitrosoalbumin formation was determined spectrophotometrically as described by Ellman [15]. This analysis revealed that less than 1% of the reduced sulfhydryl groups were intact following NO treatment. Thus, more than 99% of the albumin had been converted to *S*-nitrosoalbumin.

Palmitate binding. Table 1 shows the results from the palmitate heptane: water partition ratio experiments. In all cases, the palmitate PR was significantly greater in the presence of *S*-nitroso-albumin than in the presence of albumin. When a 600 μM *S*-nitrosoalbumin concentration was decreased 10-fold by dilution with 600 μM albumin, the difference between control and test was reduced from 28 to 8%. In the absence of binding protein, NO did not affect significantly the heptane: water partitioning of palmitate (Table 1). The bacteriostatic agent sodium azide (NaN_3; 3 mM) had no effect on the heptane: water partitioning of palmitic acid in the presence of albumin or *S*-nitrosoalbumin.

Addition of 1.9 mM $NaNO_2$ to 600 μM albumin: palmitate (albumin: palmitate molar ratio 1:1) did not affect the albumin binding properties for palmitate. Similar results were obtained when 600 μM albumin and 600 μM palmitate were added to a solution of NO (aqueous NO concentration 1.9 mM), which was allowed to stand at room temperature for 2 hr. The heptane: water partition ratio in the presence and absence of NO treatment was 0.018 ± 0.001 (N = 6) and 0.017 ± 0.001 (N = 12), respectively.

The palmitate heptane: water partition ratio using *S*-nitrosoalbumin (600 μM) dissolved in degassed PBS solutions was not statistically different between the 12-hr (PR = 0.029 ± 0.003, N = 6) and the 24-hr (PR = 0.028 ± 0.003, N = 6) samples. When the PBS solutions were gassed with O_2 following the formation of *S*-nitrosoalbumin, the 12-hr palmitate heptane: water partition ratio in the presence of *S*-nitrosoalbumin (PR = 0.052 ± 0.005, N = 6) was greater ($P < 0.05$) than the corresponding 12-hr albumin controls (PR = 0.045 ± 0.002, N = 6). Also, the 24-hr palmitate heptane: water partition ratio values were lower in the presence of *S*-nitroso-albumin (PR = 0.035 ± 0.002) than the corresponding 12-hr *S*-nitrosoalbumin–palmitate samples but were not statistically different from the 24-hr albumin–palmitate control samples (control PR = 0.039 ± 0.003; N = 6).

Figure 1 shows results from the palmitate binding isotherms with albumin and *S*-nitrosoalbumin. Nonlinear regression analysis using the stepwise equilibrium model fitted to these data showed that the equilibrium association constants for the first binding site of the albumin–palmitate and *S*-

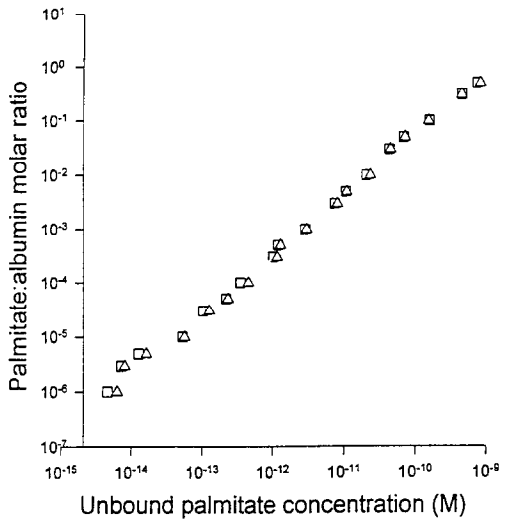

Fig. 1. Palmitate binding to 5 μM albumin (□) and 5 μM *S*-nitrosoalbumin (△). The ordinate and abcissa (log scales) show the total palmitate : albumin molar ratio and the unbound palmitate concentration, respectively.

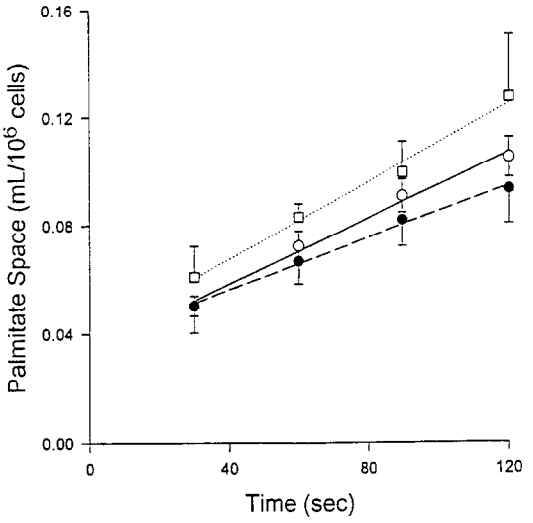

Fig. 2. Palmitate uptake curves by hepatocyte suspensions in the presence of 3.8 and 5 μM albumin (□ and ●, respectively) and 5 μM *S*-nitrosoalbumin (○). Palmitate space was calculated as the ratio of recovered cell-associated radioactivity to the concentration of radioactivity in the bathing medium. Values are means ± SEM, N = 6.

nitrosoalbumin–palmitate complexes were similar [albumin–palmitate = $(4.6 \pm 1.2) \times 10^8\ M^{-1}$ and *S*-nitrosoalbumin–palmitate = $(3.1 \pm 0.9) \times 10^8\ M^{-1}$]. The equilibrium association constant for the second binding site of the albumin–palmitate and *S*-nitrosoalbumin–palmitate complexes, however, differed considerably [albumin–palmitate = $(3.3 \pm 0.5) \times 10^7\ M^{-1}$ and *S*-nitrosoalbumin–palmitate = $(1.3 \pm 0.8) \times 10^8\ M^{-1}$; $p < 0.05$].

Palmitate uptake. Figure 2 shows the [^{3}H]palmitate uptake curves obtained with hepatocyte suspensions in the presence of albumin (3.8 and 5.0 μM) and *S*-nitrosoalbumin (5.0 μM). The total palmitate clearance rate was greater ($P < 0.01$) in the presence of 5.0 μM *S*-nitrosoalbumin than 5.0 μM albumin (0.61 ± 0.05 μL/sec/10^6 cells vs 0.48 ± 0.07 μL/sec/10^6 cells, respectively; N = 6) but was not statistically different when 3.8 μM albumin was used (0.72 ± 0.14 μL/sec/10^6 cells; N = 6). The presence of nitrite anion (supplied as 1.9 mM $NaNO_2$) did not affect the hepatocyte [^{3}H]palmitate clearance rate in the presence of 5.0 μM albumin (0.52 ± 0.12 μL/sec/10^6 cells vs 0.54 ± 0.15 μL/sec/10^6 cells in the presence and absence of $NaNO_2$, respectively; N = 5; $P > 0.05$).

DISCUSSION

The half-life of NO in oxygenated physiological solutions has been reported to range from 0.1 to 6 sec [3, 6]. The short half-life is due to the rapid conversion of NO to the nitrite anion (NO_2^-) in the presence of oxygen. Hydrogen ions are released as a result of this reaction. The reaction mechanism for the degradation of NO is as follows [7]:

$$NO + \tfrac{1}{2}O_2 \rightarrow NO_2^-$$
$$2NO_2^- \rightleftarrows N_2O_4$$
$$NO_2^- + NO \rightleftarrows N_2O_3$$
$$N_2O_3 + H_2O \rightarrow 2\,NO_2^- + 2H^+$$

Solutions in this study, therefore, were deoxygenated by degassing using vacuum for 20 min and subsequently equilibrating the solutions with N_2 for an additional 30 min. Using this procedure, the formation of NO_2^- was limited as noted by only a slight drop in pH (from 7.1 to 6.9). Most of the NO, therefore, was available for *S*-nitrosylation of albumin. Analysis of the reduced sulfhydryl groups showed that more than 99% of the albumin had been converted to *S*-nitrosoalbumin.

Many reports describe the binding properties of albumin for long-chain fatty acids [10, 11, 17, 18, 20]. Much of this work relates the ligand binding properties of albumin to its equilibrium association constant. This value originally has been described using the heptane : water partitioning technique. Spector *et al.* [17, 18, 20, 21] used this method together with the multiple stepwise equilibrium model to propose that fatty acids are distributed evenly over six to nine sites. Carter and Ho [10] have reviewed the literature and reported that a consensus showed that there are two high-affinity long-chain fatty acid binding sites and several lower affinity sites. If we focus on the two high-affinity albumin binding sites, the stepwise equilibrium model shows that the equilibrium association constants for these sites were 3.0×10^7 and $7.3 \times 10^6\ M^{-1}$ for the first and second sites, respectively [17, 18]. Although these results were much lower than those reported in this study

($4.6 \times 10^8\ M^{-1}$ vs $3.0 \times 10^7\ M^{-1}$), our K_a for the first high-affinity binding site was much closer to the other published reports, which used much different methodologies. Studies using equilibrium dialysis with polyethylene sheeting report the K_a to be $0.97 \times 10^8\ M^{-1}$ [22] and $1.4 \times 10^8\ M^{-1}$ [23], while fluorescence techniques set the K_a at $1.2 \times 10^8\ M^{-1}$ [24].

The difference between our results and the earlier work using heptane:water partitioning may be due to the palmitic acid partition ratio results obtained in the absence of binding protein. This value is of extreme importance for calculating the apparent K_a. We have shown previously that unless precautions are taken to eliminate the contribution of lipophilic, and especially hydrophilic, radiolabeled impurities, the overall apparent [^{3}H]palmitic acid partition ratio may vary by 80-fold [13]. We found that a limiting value for the [^{3}H]palmitic acid partition ratio was reached when the hydrophilic impurities were diluted by using a large aqueous:organic phase volume ratio (500:1). The hydrophilic impurities were not an important consideration when binding proteins were present because the radioactivity associated with [^{3}H]palmitate binding to albumin was much greater than the radioactivity due to the hydrophilic impurities. In this study, we took our previously reported value of 1445, which was very close to the expected partition ratio value of 1376 [23], to calculate the unbound palmitate concentration and the molar ratio of albumin-bound palmitate:total albumin concentration. This resulted in a calculated K_a value that was an order of magnitude greater than previous reports. Thus, the higher estimate of K_a obtained in this study suggests that the presence of radiolabeled impurities must be considered when calculating an apparent K_a. Failure to account for these impurities will lead to a lower palmitate heptane:water partition ratio value in the absence of binding proteins, which will result in a lower apparent K_a.

There is currently no information available regarding the ligand binding properties of *S*-nitrosoalbumin. Our study showed that the equilibrium association constant for the first high-affinity palmitate binding site of *S*-nitrosoalbumin was reduced by 33%, whereas the association constant for the second high-affinity binding site increased by 394%. Thus, using a 1:1 molar ratio of albumin:palmitate and *S*-nitrosoalbumin:palmitate, the unbound palmitate fraction was increased by 28% in the presence of *S*-nitrosoalbumin. At higher molar ratios the unbound palmitate fraction was lower (Fig. 1) owing to the increased affinity of the secondary sites.

Since the unbound palmitate fraction was higher in the presence of *S*-nitrosoalbumin than albumin, it was not unexpected that the palmitate clearance rate by hepatocyte suspensions was greater in the presence of 5 μM *S*-nitrosoalbumin than 5 μM albumin (27%). When the albumin concentration was decreased (3.8 μM) to reflect the increased unbound palmitate concentration in the presence of *S*-nitrosoalbumin, there was no significant difference in the palmitate clearance rate. Thus, the increased clearance rate paralleled the increase in the unbound ligand fraction. The concentration of albumin required to yield a free palmitate fraction similar to *S*-nitrosoalbumin was calculated using the binding data and verified experimentally using equilibrium dialysis [22].

Binding studies were performed after 18 hr of incubation, while the uptake studies were performed within 2 hr of preparing the *S*-nitrosoalbumin solutions. Although this time period was sufficient for equilibrium to be established [13], it was possible that a substantial portion of the *S*-nitrosoalbumin had degraded since the *in vitro* half-life of *S*-nitrosoalbumin has been reported to be 24 hr [9]. Thus, during the uptake experiments the unbound fraction may have been much higher than expected. Binding studies, however, showed that the unbound palmitate fraction did not change over a 12- to 24-hr incubation time. This indicated either that the *in vitro* half-life of *S*-nitrosoalbumin was much longer than previously reported, that nitrite anions affected the protein binding properties, or that the hypoxic experimental conditions used in the binding studies resulted in an NO half-life much longer than in normoxic conditions.

It is unlikely that nitrite anions or some other NO degradation products were responsible for the increased half-life because results of the palmitate heptane:water partition ratio experiments, performed in the presence of 600 μM albumin and 1.9 mM sodium nitrite or NO gas, which was added to the PBS and allowed to stand open to the atmosphere for 2 hr prior to the addition of albumin, were similar to the control albumin partition ratio values (no sodium nitrite or NO).

It is possible that the degradation of NO is dependent upon the amount of O_2 available in solution. The half-life of NO could be much longer in deoxygenated solutions than in oxygenated solutions. Numerical solutions to the third-order rate reaction $d[NO]/dt = K[NO]^2[O_2]$ [25, 26] show that in conditions where the oxygen content has been greatly reduced, the NO half-life may be increased from seconds (normoxic conditions) to hours (extreme deoxygenation). Thus, it is possible that the half-life of *S*-nitrosoalbumin in our experimental conditions (heptane:water partition ratio experiments) was much longer than the 24 hr reported *in vitro*. Repeating the palmitate heptane:water partition ratio studies using 600 μM *S*-nitrosoalbumin in oxygenated PBS solutions showed that oxygen affected the degradation of NO as noted by a decreased palmitate heptane:water partition ratio. Thus, the degradation of *S*-nitrosoalbumin is dependent upon the concentration of oxygen. Based on the half-life of 24 hr for *S*-nitrosoalbumin, it is estimated that in our uptake experiments approximately 4% of the *S*-nitrosoalbumin was degraded. A 4% reduction in the binding of palmitate by *S*-nitrosoalbumin does not affect our conclusion for the uptake of palmitate in the presence of *S*-nitrosoalbumin. Our data lead us to postulate that following the degradation of *S*-nitrosoalbumin the protein binding affinity returns to its former albumin value.

Since the degradation of NO results in the formation of nitrite ion, the concentration of nitrite

ion in our uptake solution was ~1.9 mM. We were interested to determine if nitrite ions influence the hepatocyte palmitate clearance rate. To examine this possibility, uptake experiments were performed in the presence of 1.9 mM sodium nitrite. These experiments showed that nitrite anions have no significant effect on the hepatocyte uptake of palmitic acid. Thus, the increased clearance rate of palmitate in the presence of *S*-nitrosoalbumin was due to the increased unbound palmitate fraction.

It is estimated that approximately 2% of the circulating plasma albumin is present as the *S*-nitrosothiol derivative in normal individuals [8]. If one assumes that the decreased binding affinity of bovine serum albumin upon nitrosylation is shared by other proteins (e.g. human serum albumin), then the present results suggest that the lowered binding affinity may not be of clinical importance. However, in pathological conditions such as cirrhosis, where the local tissue NO concentrations are much higher, the increased formation of *S*-nitrosoalbumin [27–29] may result in significant increases in unbound drug tissue concentrations. In this case increased toxicity may be of clinical importance especially for drugs that are highly protein-bound and possess a narrow therapeutic index [30]. Clearly, further studies are required to determine if our results apply to clinically used drugs and to proteins such as human serum albumin.

Acknowledgements—We gratefully acknowledge the Canadian Liver Foundation, the Manitoba Health Research Council and a Manitoba Health Research Council Scholarship award to F. J. Burczynski.

REFERENCES

1. Brady AJB, Poole-Wilson PA, Harding SE and Warren JB, Nitric oxide production within cardiac myocytes reduces their contractility in endotoxemia. *Am J Physiol* **263**: H1963–H1966, 1992.
2. Kelm M and Schrader J, Control of coronary vascular tone by nitric oxide. *Circ Res* **66**: 1561–1575, 1990.
3. Moncada S, Palmer RMJ and Higgs EA, Nitric oxide: Physiology, pathophysiology, and pharmacology. *Pharmacol Rev* **43**: 109–142, 1991.
4. Nathan C, Nitric oxide as a secretory product of mammalian cells. *FASEB J* **6**: 3051–3064, 1992.
5. Snyder SH and Bredt DS, Nitric oxide as a neuronal messenger. *Trends Pharmacol Sci* **12**: 125–128, 1991.
6. Stamler JS, Singel DJ and Loscalzo J, Biochemistry of nitric oxide and its redox-activated forms. *Science* **258**: 1898–1902, 1992.
7. Feelisch M, The biochemical pathways of nitric oxide formation from nitrovasodilators: Appropriate choice of exogenous NO donors and aspects of preparation and handling of aqueous NO solutions. *J Cardiovasc Pharmacol* **17** (Suppl 3): S25–S33, 1991.
8. Stamler JS, Jaraki O, Osborne J, Simon DI, Keaney J, Vita J, Singel D, Valeri CR and Loscalzo J, Nitric oxide circulates in mammalian plasma primarily as an *S*-nitroso adduct of serum albumin. *Proc Natl Acad Sci USA* **89**: 7674–7677, 1992.
9. Stamler JS, Simon DI, Osborne JA, Mullins ME, Jaraki O, Michel T, Singel DJ and Loscalzo J, S-Nitrosylation of proteins with nitric oxide: Synthesis and characterization of biologically active compounds. *Proc Natl Acad Sci USA* **89**: 444–448, 1992.
10. Carter DC and Ho JX, Structure of serum albumin. *Adv Protein Chem* **45**: 153–203, 1994.
11. Kragh-Hansen U, Molecular aspects of ligand binding to serum albumin. *Pharmacol Rev* **33**: 17–53, 1981.
12. Shaklai N, Garlick RL and Bunn HF, Nonenzymatic glycosylation of human serum albumin alters its conformation and function. *J Biol Chem* **259**: 3812–3817, 1984.
13. Burczynski FJ, Pond SM, Davis CK and Weisiger RA, Validity of albumin-fatty acid binding constants determined by heptane:water partition. *Am J Physiol* **265**: G555–G563, 1993.
14. Windholz M, (Ed.), *The Merck Index*, 10th Edn. Merck & Co., Rahway, NJ, 1983.
15. Ellman GL, Tissue sulfhydryl groups. *Arch Biochem Biophys* **82**: 70–77, 1959.
16. Goodman DS, The distribution of fatty acids between *n*-heptane and aqueous phosphate buffer. *J Am Chem Soc* **80**: 3887–3891, 1958.
17. Spector AA, Fletcher JE and Ashbrook JD, Analysis of long-chain free fatty acid binding to bovine serum albumin by determination of stepwise equilibrium constants. *Biochemistry* **10**: 3229–3232, 1971.
18. Spector AA, John K and Fletcher JE, Binding of long-chain fatty acids to bovine serum albumin. *J Lipid Res* **10**: 56–67, 1969.
19. Burczynski FJ, Cai Z-S, Moran JB and Forker EL, Palmitate uptake by cultured hepatocytes: Albumin binding and stagnant layer phenomena. *Am J Physiol* **257**: G584–G593, 1989.
20. Spector AA, Plasma albumin as a lipoprotein. In: *Biochemistry and Biology of Plasma Lipoproteins* (Eds. Scanu AM and Spector AA), pp. 247–279. Marcel Dekker, New York, 1993.
21. Spector AA and Fletcher JE, Transport of free fatty acids in the circulation. In: *Disturbances in Lipid and Lipoprotein Metabolism* (Eds. Dietschy JM, Gotto AM and Ontko JA), pp. 229–249. Williams & Wilkins, Baltimore, MD, 1978.
22. Moran JB, Burczynski FJ, Cheek RF, Bopp T and Forker EL, Protein binding of palmitate measured by transmembrane diffusion through polyethylene. *Anal Biochem* **167**: 394–399, 1987.
23. Burczynski FJ and Cai Z-S, Palmitate uptake by hepatocyte suspension: Effect of albumin. *Am J Physiol* **267**: 6371–6379, 1994.
24. Richieri GV, Anel A and Kleinfeld AM, Interactions of long-chain fatty acids and albumin: Determination of free fatty acid levels using the fluorescent probe ADIFAB. *Biochemistry* **32**: 7574–7580, 1993.
25. Kharitonov VG, Sundquist AR and Sharma VS, Kinetics of nitric oxide autoxidation in aqueous solution. *J Biol Chem* **269**: 5881–5883, 1994.
26. Moore WJ, *Physical Chemistry*, p. 536. Prentice-Hall, Englewood Cliffs, NJ, 1955.
27. Guarner C, Soriano G, Tomas A, Bulbena O, Novella MT, Balanzo J, Vilardell F, Mourelle M and Moncada S, Increased serum nitrite and nitrate levels in patients with cirrhosis: Relationship to endotoxemia. *Hepatology* **18**: 1139–1143, 1993.
28. Pizcueta P, Piqué JM, Fernández M, Bosch J, Rodés J, Whittle BJR and Moncada S, Modulation of the hyperdynamic circulation of cirrhotic rats by nitric oxide inhibition. *Gastroenterology* **103**: 1909–1915, 1992.
29. Whittle BJR and Moncada S, Nitric oxide: The elusive mediator of the hyperdynamic circulation of cirrhosis. *Hepatology* **16**: 1089–1092, 1992.
30. Rolan PE, Plasma protein binding displacement interactions—Why are they still regarded as clinically important. *Br J Clin Pharmacol* **37**: 125–128, 1994.