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Influence of heavy metals on the *in vitro* interaction between human serum albumin and warfarin

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Heavy metals such as cadmium, mercury and lead can produce a variety of toxic lesions in human subjects and animals [1–3]. Most of these metals, when ingested, are carried in the circulating blood before distribution to the organs of the body. These metals are highly bound to serum protein [4], and albumin is thought to be the major agent in blood for the transport of metal ions [5]. It is also known that these metals are capable of reacting with a variety of protein binding sites including sulfhydryl groups, and (to a lesser extent) amino, phosphate, carboxylate, imidazole and hydroxyl groups of enzymes and other essential biological proteins [6–8]. It is possible, therefore, to foresee a potentiation or inhibition of a pharmacological or toxicological response to a given drug, caused by the interference of these metal ions with the drug–protein interactions. For example, Craig Snell *et al.* [9] observed a potentiation of the duration of hypnosis induced by hexobarbital in male rats that had been pretreated with cadmium. A significant increase in prothrombin time in the presence of mercury or cadmium was observed when male Sprague–Dawley rats were treated with warfarin [10]. One of the important mechanisms for such an anticoagulant response might involve the warfarin–albumin binding interaction, since *in vitro* protein binding studies with albumin and a highly bound drug such as warfarin are usually found to have clinical relevance [11–13]. Therefore, an *in vitro* study on the mechanism of potentiation of the anticoagulant response to warfarin, involving the effects of heavy metals on the interaction between human serum albumin and warfarin at the level of drug-protein binding, has been undertaken.

Human serum albumin (HSA) of a fatty-acid-free grade (< 0.005%) was obtained from Sigma Chemical Co., St. Louis, MO. The concentration of albumin was determined using the extinction coefficient of $E^{1\%}_{1\,cm} = 5.3$ at 280 nm [14] and the molecular weight of albumin as 69,000. Warfarin sodium of U.S.P. grade was used without further purification. Warfarin concentrations were measured with an absorption spectrophotometer (Unicam SP 8000) using an extinction coefficient of $1.39 \times 10^4\ M^{-1}\,cm^{-1}$ at 308 nm [15]. Zinc chloride, cadmium sulfate, lead acetate and mercuric chloride of reagent grades (A.C.S. certified) were used without further purification. All solutions were made up in sodium phosphate buffer (0.05 M, pH 7.4) except for the inorganic compounds which were made in distilled water.

Binding studies were carried out using the method of ultrafiltration with Centriflo membrane cones (CF-50 from Amico Corp., Lexington, MA). Each solution of HSA (200 μM) in the absence or presence of fixed amounts of the metal ions was first preincubated for 3 hr at 22°. These preincubated solutions were then incubated with various amounts of warfarin (60–1500 μM) for 35 min at 22°. Such a preincubation time was found to be necessary in order to allow the reaction between HSA and metal ions to attain equilibrium. After incubation, the solutions were ultrafiltered and the ultrafiltrates were analyzed for warfarin. About 10 per cent of the total sample volume was ultrafiltered. Corrections for the binding of warfarin to the cones, although found to be very small (5–10 per cent). were made whenever necessary, using a standard procedure [16]. The membrane cones were presoaked in demineralized water for 45 min before ultrafiltration.

Table 1. Effects of metal ions on the binding of warfarin with human serum albumin (200 μM) at 22°

Meta ion	Concn of metal ion (μM)	β*	K_1* ($M^{-1} \times 10^{-4}$)	K_2* ($M^{-1} \times 10^{-3}$)	n_1*	n_2*
None	0.00	0.96 ± 0.03	33.2 ± 4.3	15.1 ± 3.1	1.25 ± 0.1	2.7 ± 0.2
Zinc	4.2	0.95 ± 0.02	29.6 ± 2.9	14.2 ± 3.2	1.15 ± 0.1	2.5 ± 0.2
Zinc	30.2	0.95 ± 0.03	30.1 ± 3.4	13.1 ± 2.8	1.10 ± 0.1	2.9 ± 0.1
Zinc	110.6	0.95 ± 0.03	30.8 ± 3.7	12.5 ± 2.5	1.20 ± 0.1	3.0 ± 0.1
Mercury	2.5	0.92 ± 0.03	14.2 ± 2.1†	12.1 ± 3.0	1.26 ± 0.2	3.0 ± 0.2
Mercury	26.0	0.89 ± 0.02	11.4 ± 1.6‡	11.5 ± 2.8	1.28 ± 0.1	3.2 ± 0.1
Mercury	76.7	0.86 ± 0.02	7.8 ± 1.1§	10.3 ± 2.5	1.35 ± 0.1	3.2 ± 0.2
Cadmium	1.5	0.93 ± 0.02	16.8 ± 2.2‖	11.7 ± 2.9	1.25 ± 0.2	3.2 ± 0.2
Cadmium	38.5	0.90 ± 0.01	12.5 ± 1.5‡	12.8 ± 3.2	1.31 ± 0.1	3.5 ± 0.2
Cadmium	85.2	0.86 ± 0.01	7.9 ± 0.9§	10.5 ± 2.8	1.32 ± 0.1	2.9 ± 0.3
Lead	4.4	0.95 ± 0.03	29.5 ± 2.4	15.6 ± 3.5	1.30 ± 0.2	2.4 ± 0.3
Lead	39.8	0.94 ± 0.02	26.8 ± 2.1	13.9 ± 3.1	1.35 ± 0.1	2.8 ± 0.2
Lead	94.5	0.93 ± 0.01	18.5 ± 1.1‖	12.8 ± 3.2	1.29 ± 0.1	3.0 ± 0.2

*K_1 and K_2 are the binding constants of the warfarin–albumin complex for the primary (n_1) and secondary (n_2) drug-binding sites respectively. Results are expressed as mean ± S.E.M. of three sets of experiments. β = fraction of warfarin bound. Values of binding constants without statistical notations are taken as non-significant.

†$P < 0.02$.
‡$P < 0.01$.
§$P < 0.005$.
‖$P < 0.05$.

The pH of the solutions undergoing ultrafiltration was not affected by the addition of these metal ions, in the concentration ranges studied. The presence of the small concentrations of the metal ions used in this study did not affect the measurements of warfarin. However, the absorbance of warfarin was corrected for the small absorbance (about 10 per cent) due to the presence of higher concentrations of heavy metal ions.

The binding data were analyzed by plotting according to Scatchard [17] using the equation: $r/D_f = K_a(n - r)$, where r is the number of warfarin bound per mole of albumin, n is the number of warfarin-binding sites available per albumin molecule, K_a is the binding (or association) constant for the warfarin–albumin complex, and D_f is the concentration of free warfarin. The binding parameters were determined by an iterative process according to the method of Priore and Rosenthal [18], taking into account the two classes of binding sites. Student's t-test was employed for the statistical analysis. To study the conformational changes in the albumin structure, the fluorescamine-labeled albumins were prepared in the absence and presence of the metal ions according to the method described by Böhlen *et al.* [19]. Fluorescence of the fluorescamine-labeled albumins was measured in a Perkin–Elmer MPF-3A spectrofluorometer using the excitation wavelength at 390 nm and the emission wavelength at 475 nm. The polarization of fluorescence was measured as described previously [20].

The biphasic curves obtained from the Scatchard plot show the existence of heterogeneity of drug-binding sites (Fig. 1). It is evident from Table 1 that the binding charcteristics of warfarin to HSA were altered considerably in the presence of mercury, cadmium and higher concentrations of lead ions, whereas the affinity constant of the warfarin–HSA complex was found to be unaffected by the presence of zinc ions. Accordingly, the fraction of warfarin bound to the albumin (β) was also decreased proportionately in the presence of these heavy metal ions. Furthermore, it was observed that the inhibition of the warfarin–HSA interactions, as evident from these binding parameters in the presence of a given metal ion (except zinc), depends on the concentration of that metal ion. While the binding constants of warfarin for albumin were reduced, the number of warfarin binding sites in HSA remained very nearly the same in the presence of the heavy metal ions, cadmium, mercury and lead. When the inhibitions of the warfarin–HSA interaction in the presence of the lowest concentrations ($\sim \mu$M) of the heavy metal ions used in the present study were compared, mercury and cadmium seemed to have considerable inhibitory effect. The results from Table 2 indicate that the albumin has undergone conformational changes in its molecular structure as a result of its interaction with these heavy metal ions (except zinc), as reflected in the values of the degree of polarization of fluorescence of fluorescamine-labeled albumin. Since the fluorescence quantum yield of the fluorescamine–HSA complex was found to be unaffected by the presence of the heavy metal ions, changes in the fluorescence lifetime in the presence of the metal ions were not expected. Although direct displacement of warfarin by the heavy metal ion at the warfarin binding site is possible, conformational changes induced in the albumin molecule by heavy metals would be more important, according to the conditions of the binding experiments presently performed.

Table 2. Influence of heavy metal ions on the polarization of fluorescence of fluorescamine-labeled human serum albumin*

Metal ion	Concn of metal ion (μM)	Degree of polarization of fluorescence† (mean ± S.E.M.)
None	0	0.26 ± 0.01
Zinc	4.2	0.26 ± 0.01
Zinc	30.2	0.25 ± 0.01
Zinc	110.6	0.24 ± 0.01
Mercury	2.1	0.21 ± 0.01
Mercury	26.0	0.19 ± 0.005
Mercury	76.7	0.17 ± 0.005
Cadmium	1.5	0.21 ± 0.005
Cadmium	38.5	0.20 ± 0.01
Cadmium	85.2	0.18 ± 0.005
Lead	4.4	0.24 ± 0.01
Lead	39.8	0.24 ± 0.01
Lead	94.5	0.20 ± 0.01

†Results are the averages of each of three sets of experiments.

*The albumin solution (in phosphate buffer, pH 8.0) with metal ion was first incubated for a period of 3 hr. After incubation, 0.5 ml of 1 mM fluorescamine in dry acetone was added rapidly with shaking to the albumin solution. Details are in Ref. 19 and in the text.

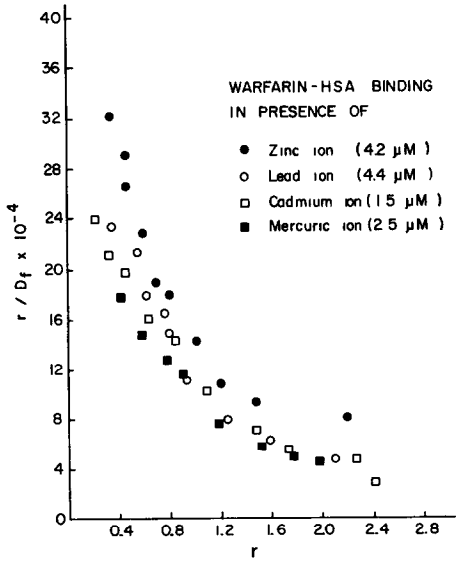

Fig. 1. The Scatchard plot showing the effects of different metals on the binding interactions between human serum albumin (200 μM) and warfarin at 22°. Details are in the text.

Because of the relative rapidity of the oxidation process of Hg^0 to Hg^{2+} (as well as of other heavy metals) in the blood and tissues, most of the mercury (or the other heavy metals) is in the form of the positive ion (+2). The works of Smith *et al.* [21] showed that typical blood mercury levels of occupationally exposed male workers exhibiting mercury toxicity were in the range of 2–5 μM. On the other hand, Lundgren *et al.* [22] found in human studies that the ratio of mercury in blood cells to that in plasma was 1 : 1. The minimum blood levels of camium and lead in humans displaying serious toxic symptoms are of the order of 0.5 and 3.5 μM respectively [23]. However, it is not certain whether these minimum concentrations of the metals causing typical toxic symptoms characteristic of each metal are, or are not, sufficiently high to produce an increasing pharmacological or toxicological response in humans. The ratio of heavy metal to protein is about three times greater in the present *in vitro* studies than would be found clinically. This ratio was used to study the *in vitro* mechanism of interaction with warfarin without making quantitative clinical extrapolations.

The present study suggests that the degree of potentiation of the anticoagulant response to warfarin as a result of displacement of bound warfarin by a heavy metal might be increased if persons were occupationally or environmentally exposed for a long time to mercury, cadmium and lead. Although experimental data are not available at the moment, preliminary results from our laboratory, using rats, have demonstrated such a phenomenon. For example, when male Sprague–Dawley rats were pretreated with mercuric chloride (2 mg/kg, i.p.) 24 hr before treatment with warfarin (0.75 mg/kg, i.p.), the prothrombin time was significantly increased from 22 sec in the control to about 49 sec in the treated animal. A significant increase in the anticoagulant response to warfarin was also noticed when both warfarin and mercury were injected simultaneously in rats [10]. At the same time, a diminution of warfarin binding and an initial significant increase in the amount of free warfarin in the plasma of mercury-treated rats were observed *in vivo* (M. Baril, S. Chakrabarti and J. Brodeur, unpublished results). Similarly, pretreatment with $HgCl_2$ (2 mg/kg, s.c.) 24 hr prior to the administration of thiopental (35 mg/kg, i.p.) significantly increased the thiopental sleeping time in rats without affecting hepatic microsomal drug metabolism (S. K. Chakrabarti and J. Brodeur, unpublished results).

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The cumulative effect of halothane and steroids on mitochondrial respiration

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General anaesthetic halothane has been shown to inhibit mitochondrial respiration [1, 2, 3]. The anaesthesia inducing concentration of halothane has been established as 1–1.5 mM *in vivo* and in whole brain tissue [4, 5]; however the effective mitochondrial concentrations of halothane in the course of narcosis remain still unknown. Some experiments performed indicate that mitochondria exposed to gaseous halothane *in vitro* contain about twice as much halothane as these isolated from anaesthetized animals [6]. This clearly indicates that the differences between halothane action in *in vivo* and *in vitro* conditions depend mainly on concentration of this compound. On the other hand, since general anaesthetic properties are attributed to some steroids [7, 8, 9] it was interesting to know whether *in vitro* the presence of such substances would modify effects of halothane. Among steroids investigated progesterone and pregnanolone were found to be a highly potent anaesthetics [8]. It has been also shown that steroids at high concentrations inhibit mitochondrial respiration and oxidative phosphorylation [10, 11].

Placental and cord plasma progesterone concentration has been estimated as 5×10^{-5} M [12]. The aim of the present investigation was to examine the effect of the comparable low steroid concentration on the mitochondrial respiration *in vitro* in the presence of halothane. Evidence is presented in this paper that the presence of progesterone and pregnanolone at low concentrations increases the inhibitory effect of halothane on mitochondrial state 3 respiration. A suggestion is presented that during the halothane induced narcosis, either endogenous or therapeutically administered steroids may potentiate halothane action.

Mitochondria were isolated from rat liver according to the procedure described by Loewenstein *et al.* [13] with omission of the last digitonin step, and resuspended in 0.33 M sucrose with 5 mM Tris–HCl pH 7.3. Protein was determined by biuret reaction in the presence of 1% deoxycholate. Solutions of Analar (Koch–Light) sucrose were passed through the Amberlite IRC–50 resin before use. Tris (Fluka AG) was used after recrystallization from water. Only mitochondria having respiratory control greater than or