Biochemical Pharmacology, Vol. 29, pp. 3103–3105.

0006-2952/80/1115-3103 $02.00/0

SHORT COMMUNICATIONS

Effect of cadmium acetate on the uptake and degradation of formaldehyde-treated ^{125}I-labelled human serum albumin in rat liver non-parenchymal cells *in vitro*

(*Received 19 November* 1979; *accepted* 4 *July* 1980)

Denatured ^{125}I-labelled human serum albumin (^{125}I-HSA) is widely used in the study of endocytosis in mammalian cells. Mego [1] demonstrated that in mouse liver ^{125}I-HSA is endocytosed and then degraded by hepatic secondary lysosomes.

In the rat liver, ^{125}I-HSA is preferentially taken up and degraded by non-parenchymal cells (NPC), as demonstrated by Nilsson and Berg [2]. This work suggested a system for *in vitro* studies of the uptake and degradation of ^{125}I-HSA in rat liver NPC.

Earlier investigations indicated that the cadmium ion stabilizes lysosomal membranes [3]. Following the fate of ^{125}I-HSA in mouse liver particles, Mego and Cain [4] proposed that the metal ion interferes with the formation of the primary lysosomes, and hence inhibits proteolysis.

Using a minor modification of the method developed by Nilsson and Berg [2], one can assess for the total amount of ^{125}I-HSA endocytosed but not degraded, as well as the total amount of ^{125}I-HSA degraded by suspended rat liver NPC. This procedure is used here to study the effect of cadmium acetate (Cd^{2+}) on the uptake and degradation of ^{125}I-HSA *in vitro*.

Materials and methods

Animals. Male Wistar rats, weighing about 200 g, were used. The animals were fed standard food pellets (Møllesentralen I/S, Oslo, Norway) and water *ad lib.*

Chemicals. Collagenase (type 1) and bovine serum albumin (fraction V) were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). ^{125}I-labelled human serum albumin (sp. act. 0.4 mCi/ml) was prepared by the Instutt for Atomenergi (Kjeller, Norway). The native ^{125}I-labelled human serum albumin was denaturated by treatment with 20% formaldehyde in the presence of 0.2 M carbonate buffer [5]. The denaturated ^{125}I-HSA was dialysed against 0.9% NaCl overnight before use. Phthalic acid-bis-3, 5, 5-trimethylhexylester ('dinonyl'-phthalate) and phthalic acid-dibutylester (dibutyl-phthalate) were from Fluka (Switzerland). All other reagents were analytical grade.

Preparation of liver cells. Liver cells were prepared by collagenase-perfusion of the liver [6, 7]. The liver cells were suspended in an ice-cold HEPES-buffered incubation medium containing 2% (w/v) bovine serum albumin to prevent aggregation of the liver cells, as described elsewhere [2].

Purification of NPC. The hepatocytes were sedimented by slow speed centrifugation in a Sorvall centrifuge, according to the procedure of Nilsson and Berg [2].

^{125}I-HSA as a tracer for uptake and degradation. Non-degraded ^{125}I-HSA was precipitated in ice cold trichloroacetic acid (TCA) at a final concentration of 5% (w/v).

Radioactivity measurements were carried out on a Packard auto gamma spectrometer (Packard Instruments Co., Downers Grove, IL, U.S.A.).

Uptake and degradation of ^{125}I-HSA in vitro. Suspensions containing, respectively, 2, 20, 200 and 2000 μM Cd^{2+} and one control were incubated at 37° in a shaking incubator (Heto, Denmark). The concentration of NPC was adjusted to about 5×10^6 cells/ml of cell suspension.

To all suspensions ^{125}I-HSA (final concentration: 1.9 μg/ml) was added 10 min after the addition of Cd^{2+}. The concentration of ^{125}I-HSA was determined by the method of Lowry *et al.* [8].

Samples of 500 μl were taken from the suspensions at chosen time intervals. The samples were placed on top of a mixture of 'dinonyl'-phthalate and dibutyl-phthalate (1 : 3) in a 1.5 ml centrifugation tube, and centrifuged at 7000 rpm for 1 min in a Beckman microfuge B. As demonstrated earlier, the NPC sediment through this oil mixture, while the medium remains at the surface [2]. After centrifugation, the bottom of the centrifugation tube, containing the sedimented NPC, was cut off (P), and 250 μl of the medium above the oil mixture was carefully pipetted off (S). To both samples ice-cold TCA was added to a final concentration of 5% (w/v). This procedure yielded information on the following parameters:

^{125}I-HSA in the medium which is not degraded and not bound to the NPC (i.e. acid precipitable radioactivity in the sample above the oil mixture: S ↓).

^{125}I-HSA which is bound to the NPC, but not degraded (i.e. acid precipitable radioactivity in the sample below the oil mixture: P ↓).

^{125}I-HSA which is bound to the NPC, and degraded (i.e. acid soluble radioactivity in the sample below the oil mixture: P ↑).

^{125}I-HSA which is degraded by the NPC and released into the medium (i.e. acid soluble radioactivity in the sample above the oil mixture: S ↑).

The following parameters were then calculated:

Total amount of ^{125}I-HSA degraded *in vitro* = S ↑ + P ↑.

Total amount of ^{125}I-HSA taken up *in vitro* = S ↑ + P ↑ + P ↓.

The quantity of ^{125}I-HSA taken up and degraded in the suspensions containing Cd^{2+} was expressed as per cent of the quantity of ^{125}I-HSA taken up and degraded in the control.

Effect of Cd^{2+} on the degradation of ^{125}I-HSA in vitro. A suspension of about 5×10^6 NPC/ml was preincubated for 30 min with 3.8 μg ^{125}I-HSA/ml of cell suspension. Thereafter, the ^{125}I-HSA was washed out at 0° by three centrifugations for 3 min at 5000 *g* in a Sorvall centrifuge. The NPC, now suspended in a medium without ^{125}I-HSA, were divided into two fractions. To the one 20 μM Cd^{2+} was added, the other served as control. The degradation pattern was followed by observing the increase in acid soluble radioactivity with time during incubation at 37°. The acid soluble radioactivity liberated was expressed as per cent of the acid precipitable radioactivity contained in the cell suspension at the start of incubation with Cd^{2+}.

Results and discussion

When the effect of Cd^{2+} on the uptake and degradation of ^{125}I-HSA *in vitro* was studied, the recovery of ^{125}I-HSA in the samples from the cell suspension yielded an average slightly above 100 per cent (104 ± 1.7 S.D.). This is expected, because no correction was made for the volume occupied by the cells in the samples placed on top of the oil mixture (see Materials and methods).

Cd^{2+} (20 and 200 μM) inhibited the uptake of ^{125}I-HSA. This inhibition increased with increasing concentrations of Cd^{2+} (Fig. 1). However, a biphasic effect of Cd^{2+} on the uptake was observed, and at an extremely high concentra-

tion (2000 μM), Cd^{2+} no longer seemed to have an inhibitory effect (Fig. 1).

At concentrations from 20 to 2000 μM, Cd^{2+} inhibited the degradation at an increasing rate with increasing concentrations of the metal ion (Fig. 2).

Preincubation with ^{125}I-HSA followed by addition of 20 μM Cd^{2+} to the cell suspension decreased the liberation of acid soluble radioactivity (Fig. 3). Thus, the depression in degradation following addition of Cd^{2+}, appears to be more than just a consequence of a reduced endocytosis of ^{125}I-HSA.

When Cd^{2+} added was dissolved in 0.02 ml rat serum/ml of cell suspension to give a final concentration of 20 μM, no significant alteration in the effects of Cd^{2+} was seen (results not shown). The formation of a complex between Cd^{2+} and α-globulins observed in rat plasma [9] thus does not seem to protect against the toxic effects of the metal ion.

No decrease in cell-bound acid-precipitable radioactivity was observed when the NPC were incubated in the presence of 20 μM Cd^{2+} at 0° (results not shown), suggesting that the inhibition of the uptake was not due to an inhibited binding of ^{125}I-HSA to cell surface receptors.

Endocytic activity seems to demand energy [10]. Prior investigations have shown that Cd^{2+} uncouples the oxidative phosphorylation *in vivo* [11] and *in vitro* [12]. The inhibition of the ^{125}I-HSA uptake by Cd^{2+} may thus be caused by an uncoupling of the oxidative phosphorylation.

An earlier investigation [4] had demonstrated that Cd^{2+} inhibits the degradation of ^{125}I-HSA in mouse liver *in vivo*. As ^{125}I-HSA is preferentially endocytosed by the NPC [2], it seems likely that the cadmium-induced inhibition of the ^{125}I-HSA degradation observed by Mego and Cain [4] is due to an effect of Cd^{2+} on NPC.

Mego and Cain proposed that Cd^{2+} inhibited the formation of primary lysosomes [4]. However, such an inhibition could not explain the depressed degradation of ^{125}I-HSA reported here. As seen from Fig. 3, degradation is inhibited almost immediately after the addition of 20 μM Cd^{2+}. If Cd^{2+} only interfered with formation of primary lysosomes, a depressed degradation would be expected to occur after a time lag following the addition of Cd^{2+}. A

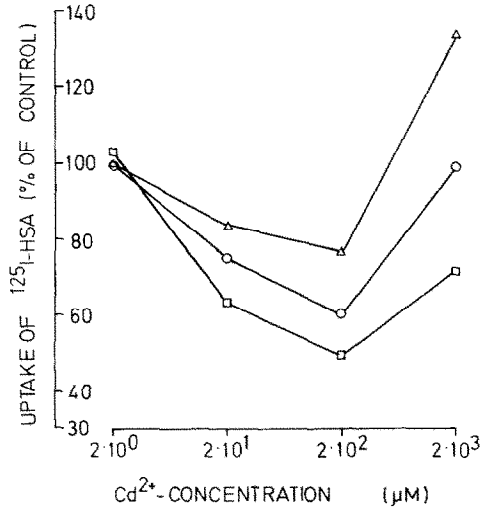

Fig. 1. Effect of Cd^{2+} on the uptake of ^{125}I-HSA *in vitro*. The quantity of ^{125}I-HSA taken up in the suspensions with Cd^{2+} added is calculated as per cent of the quantity taken up in the control. (△) 15 min incubation, (○) 30 min incubation, (□) 60 min incubation. The figure represents one typical of three identical experiments, and each value represents the average of three samples. The errors in these experiments did not exceed ± 10% S.D.

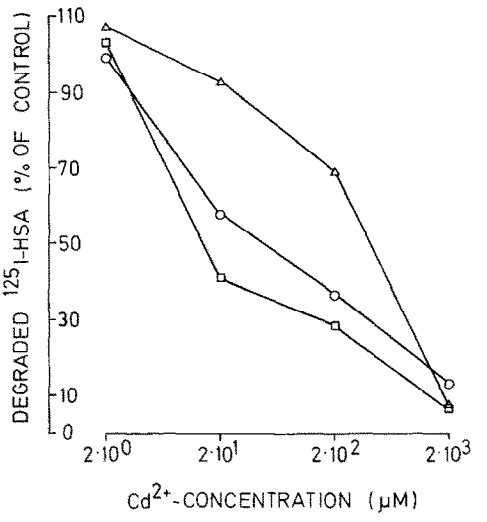

Fig. 2. Effect of Cd^{2+} on the degradation of ^{125}I-HSA *in vitro*. The quantity of ^{125}I-HSA degraded in suspensions with Cd^{2+} added is calculated as per cent of the quantity degraded in the control. (△) 15 min incubation, (○) 30 min incubation, (□) 60 min incubation. The figure represents one typical of three identical experiments, and each value represents the average of three samples. The errors in these experiments did not exceed ± 10% S.D.

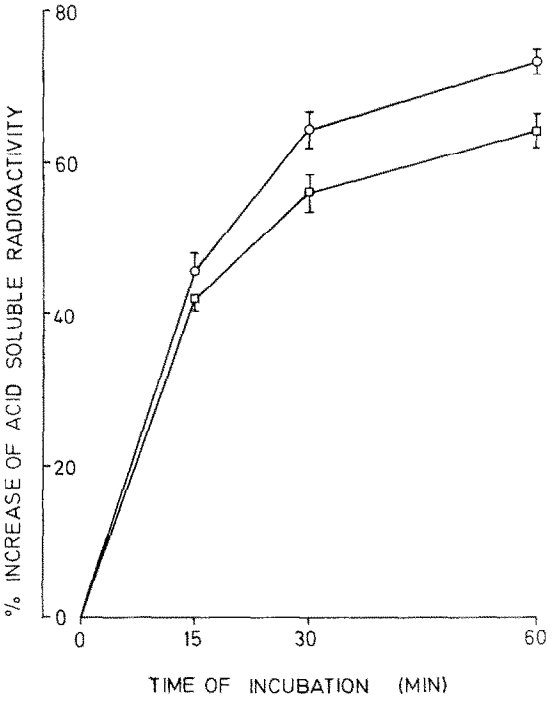

Fig. 3. Degradation of ^{125}I-HSA *in vitro*. 30 min of preincubation with ^{125}I-HSA was followed by addition of 20 μM Cd^{2+}, and the liberation of acid-soluble radioactivity with time was measured. The acid-soluble radioactivity thus liberated is calculated as per cent of the acid precipitable radioactivity contained in the cell suspension at the start of incubation with 20 μM Cd^{2+}. The uncertainty is presented as one standard deviation, calculated from three samples. The experiment was carried out in duplicate. (□) Suspension with 20 μM Cd^{2+}, (○) control.

more likely explaination might be that Cd^{2+} at concentrations of 20 and 200 μM stabilized the lysosomal membrane, inhibiting the fusion between lysosomes and phagosomes [13], and hence proteolysis. Indeed, a stabilizing effect of Cd^{2+} on lysosomal membranes has been reported [3].

Degradation by lysosomes requires energy either for an ATP-driven proton pump necessary for the maintainance of the intralysosomal acidity [14-16], and/or, for other processes involved in the catabolic pathways [17]. Hence, uncoupling of the oxidative phosphorylation by Cd^{2+} [11, 12] may also explain the observed inhibitory effect of Cd^{2+} on the degradation of ^{125}I-HSA.

Cd^{2+} might also inhibit lysosmal proteases, but probably only at extremely high concentrations [4].

In conclusion, the immediate inhibition of ^{125}I-HSA degradation by Cd^{2+} is most likely caused by a combination of membrane stabilization and inhibition of the oxidative phosphorylation.

Drugs usually classified as membrane stabilizing agents can also labilize membranes *in vitro* at extremely high concentrations [18–21]. Accordingly, the observed stabilization of lysosomal membranes by Cd^{2+} [3] could at extremely high concentrations (2000 μM) be replaced by labilization.

The experimental system described here should be a useful tool in the study of the endocytic and degradative activity of NPC. The number of drugs that may be used is large, and the conditions for *in vitro* incubation are readily controlled.

Acknowledgements—I am indebted to Dr. Trond Berg for his advice and careful reading of the manuscript. Mrs. Turid Berdal Gangnæs gave skilful technical assistance.

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Biochemical Pharmacology, Vol. 29, pp. 3105–3109.

0006-2952/80/1115-3105 $02.00/0

Effects of salicylate–copper complex on the metabolic activation in phagocytizing granulocytes

(*Received* 6 *December* 1979; *accepted* 1 *July* 1980)

The uptake of particles by human polymorphonuclear leucocytes (PMN) is associated with a strong increase in oxygen consumption [1–3] and with a concomitant generation of toxic oxygen metabolites such as superoxide anion (O_2^-) [4], hydrogen peroxide (H_2O_2) [5] and hydroxyl free radical (OH^-) [6]. Under normal conditions these toxic metabolites appear to promote the killing of bacteria in PMN [7, 8]. Moreover, immune complexes and aggregated immunoglobulin G also induce PMN to elaborate O_2^- and H_2O_2 [9]. The question arises as to whether this phenomenon causes a major part of PMN-mediated tissue injury that occurs with inflammation. Accordingly, a number of compounds endowed with anti-inflammatory activity interfere with the altered oxygen metabolism that accompanies the phagocytic process [12]. Recently, Sorenson [13] reported that salicylate–copper complex (Cu(II)–Sal_2) has stronger anti-inflammatory activity than salicylate alone, which suggests that chelate might be the active form of the drug. In view of this it is of interest to obtain information about the effects of Cu(II)–Sal_2 on oxygen-dependent PMN metabolism as compared with those induced by salicylate alone. We have therefore investigated the effects of salicylate and Cu(II)–Sal_2 on phagocytosis-induced PMN metabolic activation (measured by zymosan-stimulated oxygen consumption, NBT reduction and iodination) compared to the effects of these compounds on the extent of phagocytosis (measured by the ingestion rate of *Klebsiella pneumonie*).

Materials and methods

Chemicals. Zymosan A from *Saccharomyces cerevisae*, nitroblue tetrazolium (NBT) and superoxide dismutase were obtained from Sigma Chemical Co., St. Louis, MO,