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MONENSIN INHIBITS THE CONVERSION OF PROALBUMIN TO SERUM ALBUMIN IN CULTURED RAT HEPATOCYTES

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SUMMARY : Effects of monensin, a carboxylic ionophore, on intracellular transport of albumin were studied in primary cultured rat hepatocytes. The lag time after which newly synthesized albumin first appeared in medium was 10 min in the control cells, while it was prolonged to 40 min in the monensin-treated cells. In addition, this inhibition of secretion by monensin was accompanied by an intracellular accumulation of proalbumin. The results strongly suggest that monensin arrests the intracellular transport of proalbumin before the site where its conversion takes place.

INTRODUCTION

Monensin, carboxylic ionophore for K^+ and Na^+, inhibits the intracellular transport of secretory proteins (1-7) as well as membrane proteins (5-7). The Golgi complex is thought to be a target of monensin, which primarily causes a marked morphological alteration of the Golgi complex; numerous dilated vaculoles instead of typical stacked cisternae (1,2,8). Such morphological changes are shown closely related to an arrest of protein migration, as confirmed by electron microscopic autoradiography (1-3). In addition, these changes accompany an inhibition of oligosaccharide chain maturation of glycoproteins (3,7). From detailed analysis of glycosylation of IgM, Tartakoff and Vassalli concluded that monensin arrests the transport of IgM at the proximal (cis) Golgi elements and suggested that maturation of oligosaccharide chains may occur at the distal (trans) Golgi elements (3).

Serum albumin is synthesized on the membrane-bound ribosomes in hepatocytes, subsequently transported to smooth endoplasmic reticulum and the Golgi complex, segregated into secretory vesicles, and finally discharged into blood (9,10).

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The discovery of proalbumin (9-11), the precursor of serum albumin, has presented intriguing problems, including the conversion site(s) of the precursor, the specific protease involved in the processing and the physiological role of the propeptide. *In vivo* studies from several laboratories have demonstrated that the conversion takes place in smooth endoplasmic reticulum (12) and/or the Golgi complex (12-15). Recently Quinn and Judah have suggested that cathepsin B is a possible converting enzyme (16).

In the present report we describe the effects of monensin on the secretion of albumin in cultured rat hepatocytes, demonstrating that the drug arrests the intracellular transport of proalbumin before its conversion occurs.

MATERIALS AND METHODS

Preparation of rat serum albumin and antibody to serum albumin. These were prepared as described previously (17,18).

Cell isolation and culture. Hepatocytes were isolated from adult Wistar rats, weighing 200-250 g, according to the method of Seglen (19). 2×10^6 cells were suspended in 4 ml of Eagle's MEM** (20), supplemented with 5% fetal calf serum, 0.1 µM insulin, 1 µM dexamethasone, and 60 µg/ml kanamycin. Cells were cultured at 37°C in humidified incubator under air and CO_2 (95:5). The medium was changed after the first 24h and then once every two days.

Pulse-label experiments. 1 mM monensin in ethanol was added to each dish 30 min before the start of pulse-labeling (final concentration, 1 µM). After monolayer cells were washed twice with Dulbecco's phosphate buffered saline (PBS) (20), 1.5 ml of medium containing 1 µM monensin and 100 µCi [^{35}S]methionine but no cold methionine and serum, was added to each dish. The cells were incubated for 5 min at 37°C and the medium was aspirated. After washed twice with PBS, the cells were cultured in 2 ml of Eagle's MEM containing 100 µM methionine and 1 µM monensin. At various chase time, the cells were washed twice with PBS and lysed in 0.5 ml of distilled water containing 1% sodium deoxycholate and 1% Triton X-100. The lysates were centrifuged at 15,000 x *g* for 10 min, and used for immunoprecipitation of albumin or counting trichloroacetic acid insoluble radioactivity.

Immunoprecipitation. Two volumes of 1% Triton X-100-50 mM Tris-HCl(pH 7.4)-150 mM NaCl-5 mM EDTA were added to each cell lysate or medium. 0.2 mM phenylmethylsulfonylfluoride in *n*-propanol (finally 2 mM) and appropriate amounts of carrier serum albumin and anti-albumin antiserum were added, followed by incubating at 37°C for 1h and further at 4°C overnight. The resultant immunoprecipitates were washed either with 1% Triton X-100-2 mM EDTA-10 mM methionine-10 mM Tris-HCl(pH 7.4) (for radioactivity determination) or with 0.1% Triton X-100-0.1% sodium dodecyl sulfate-2 mM EDTA-10 mM Tris-HCl(pH 7.4), followed by an additional washing with 0.1% sodium dodecyl sulfate-2 mM EDTA-10 mM Tris-HCl(pH 7.4) (for gel electrofocusing). The washed immunoprecipitates were dissolved in 1% sodium dodecyl sufate-5 mM EDTA-1% β-mercaptoethanol-62.5 mM Tris-HCl(pH 6.8), boiled for 3 min and stored at -20°C until use.

Gel electrofocusing. Slab-gel electrofocusing was carried out according to Ames and Nikaido (21). Frozen samples were thawed, boiled again for 3 min

** Abbreviations: MEM, minimum essential medium; PBS, phosphate buffered saline.

and mixed with 2 volumes of sample dilution buffer containing 9.5 M urea, 2% ampholines (pH 5-7), 5% β-mercaptoethanol and 8% Nonidet P-40. Electrophoresis was initiated at 50 V and voltage was gradually stepped up to 300 V within 2h. After focused for 18h at room temperature, gel was fixed with 30% methanol-10% trichloroacetic acid-7% acetic acid for 1h, then treated with "Enhance" before drying and fluorography (22).

Determination of radioactivity. For trichloroacetic acid insoluble radioactivity, aliquots of the cell lysate and medium were precipitated with 10% trichloroacetic acid after the addition of 0.5 mg of bovine serum albumin as carrier, and washed three times with 5% trichloroacetic acid. Trichloroacetic acid insoluble materials and immunoprecipitates were solubilized in Protosol and subjected to radioactivity determination in toluene scintillator in a Packard liquid scintillation spectrometer.

Materials. L-[^{35}S]Methionine (1267.7 Ci/mmol), Omnifluor, Protosol, and Enhance were obtained from New England Nuclear, Boston, Mass.; monensin from Colbiochem-Behring Co., La Jolla, CA.; insulin and fetal calf serum from Sigma Chemicals, St. Louis, MO.; Eagle's MEM from Nissui Seiyaku Co., Tokyo.

RESULTS AND DISCUSSION

To study effect of monensin on the overall secretion of plasma proteins, cultured hepatocytes were pulse-labeled with [^{35}S]methionine and chased for various times. In the control cells, secretion of plasma proteins began at 10 min and the incorporated radioactivity increased linearly up to 60 min. In contrast, secretion in the monensin-treated cells was markedly inhibited. It was 60 min before we could detect substantial radioactivity in the medium. Dose effect studies of monensin on different plasma proteins such as serum albumin, α_1-antitrypsin, and α_{2u}-globulin yielded similar inhibition curves (data not shown).

The effect of monensin on the biosynthesis and secretion of albumin was examined by immunoprecipitation of [^{35}S]methionine-labeled albumin (Fig. 1). No significant difference was observed in biosynthesis of albumin between the control and monensin-treated cells. In the control cells, albumin appeared in medium after 10 min and accumulated progressively, and concomitantly the level of cellular albumin declined. However, a striking suppress-

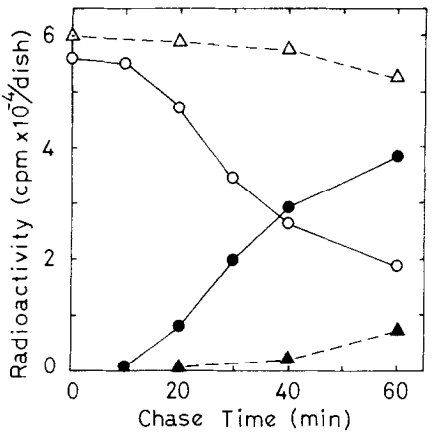

Fig. 1. Effect of monensin on synthesis and secretion of albumin in cultured hepatocytes. 2 x 10^6 cells were pulse-labeled with [^{35}S]-methionine and chased for the indicated times in the presence or absence of 1 uM monensin as described in Materials and Methods. Cellular and medium albumin were immunoprecipitated and counted for radioactivity. Control cells; cellular (○) and medium (●) albumin. Monensin-treated cells; cellular (△) and medium (▲) albumin.

serum albumin are 6.0 and 5.7, respectively (24). To analyze proalbumin and serum albumin, we carried out polyacrylamide gel electrofocusing. This is based on our previous finding that these two albumins were much better resolved by gel electrofocusing than by conventional ion exchange chromatography (13,15). When intracellular and medium albumins labeled with [^{35}S]-methionine were subjected to slab-gel electrofocusing followed by fluorography, five conponents (two major and three minor bands) were detected in the medium (Fig. 2A, lanes 5 & 6), while additional four basic components were found in the cell lysates (Fig. 2A, lane 2). The former five bands were found to correspond to multiple forms of serum albumin and the latter four to those of proalbumin (15).

The intracellular processing of proalbumin in the control cells and monensin-treated cells was compared as a function of chase time after pulse-label with [^{35}S]methionine. In the control cells, a distinct band of serum-type albumin was detected 20 min after the start of chase (Fig. 2A and 3A). As time after chase increased, the ratio of serum-type albumin to proalbumin increased, indicating that proalbumin was converted to serum-type albumin before it was discharged into medium. In the monensin-treated cells, however, the intensity of a major band in proalbumin did not significantly change

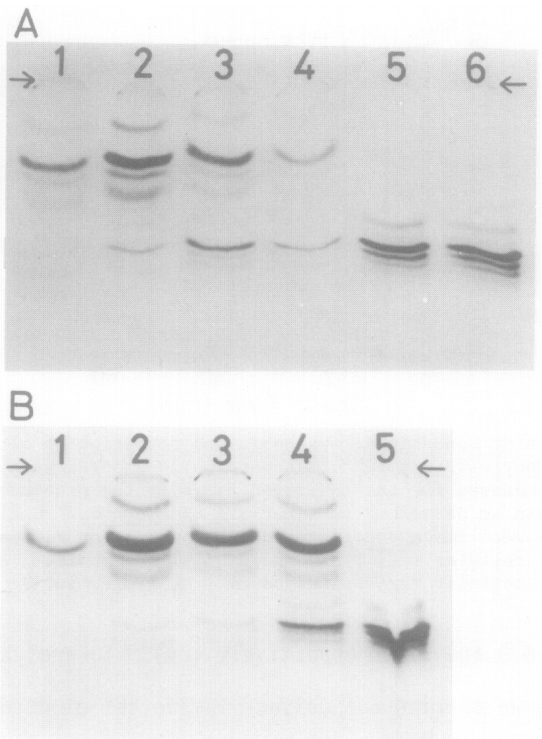

Fig. 2. Effect of monensin on the conversion of proalbumin to serum albumin. Cells were labeled and chased as described in the legend to Fig. 1. Cellular and medium albumins which were immunoprecipitated were subjected to gel electrofocusing as described in Materials and Methods. Fluorography was done using X-ray film (Kodak X-Omat XS-5). (A) Control cells; lanes 1-4, cellular albumin; lanes 5-6, medium albumin. Samples for each lane (1-6) were taken at 0, 20, 40, 60, 40, and 60 min after chase, respectively. (B) Monensin-treated cells; lanes 1-4, cellular albumin taken at 0, 20, 40, and 60 min after chase, respectively. Lane 5, medium albumin from the control experiment (60 min). In the case of monensin-treated cells, sufficient quantities of albumin for electrofocusing could not be obtained even at 60 min chase. Arrows indicate top of gels. Cathode at top; anode at bottom.

during the chase up to 60 min (Figs. 2B and 3B). No distinguishable band of serum-type albumin was detected in the cells even at 40 min. It was 60 min before we could find serum-type albumin within the cells (Figs. 2B and 3B, lane 4), and at this time a significant amount of albumin was released into medium (Fig. 1). These results strongly indicate that monensin blocks the intracellular transport of albumin before the conversion site of proalbumin. Taken together with the site specificity of monensin on the cis Golgi elements (3,25), these results suggest that the conversion of proalbumin to serum albumin takes place at the trans Golgi elements includ-

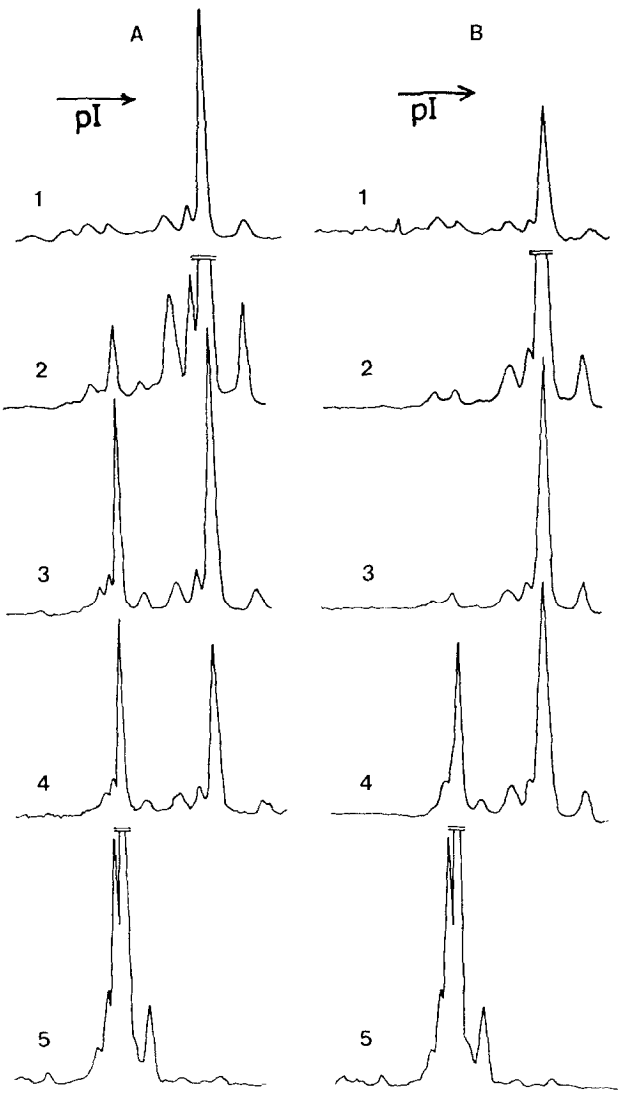

Fig. 3. Densitometric scanning of fluorograms. Fluorograms shown in Fig. 2 were scanned at 540 nm. (A) Control cells; (B) monensin-treated cells. Each number corresponds to the respective lane number shown in Fig. 2.

ing secretory vesicles. This is compatible with the previous results obtained from the in vivo experiments (13-15); most of the converted albumin was found in the secretory vesicle fraction, though some portion was found also in the Golgi cisternal fraction. Thus, our data do not support the possibility that endoplasmic reticulum is also involved in the conversion of proalbumin, as suggested by Edwards et al. (12).

The Golgi complex is now believed to be a major site for modifications (such as glycosylation, selective proteolysis, sulfation and phosphorylation)

and sorting of many different proteins for multiple destinations (extracellular, lysosome, and surface membrane), though most of the mechanisms still remain to be clarified (26). To analyze such a complexity of the Golgi function in detail, monensin, which possibly discriminates functions of the cis and trans Golgi elements, is a most useful and powerful agent, as demonstrated in the present and previous studies (3,7).

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