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Effects of Monensin on Vesicular Transport Pathways in the Perfused Rat Liver

Thomas M. Kloppel, William R. Brown, and Juerg Reichen

Department of Medicine, Veterans Administration Medical Center (T.M.K., W.R.B.), and the Departments of Medicine (W.R.B.) and Biochemistry, Biophysics, and Genetics (T.M.K.), University of Colorado School of Medicine, Denver, Colorado

In the rat hepatoctye, the internalization and degradation of asialoglycoproteins and the secretion of plasma and biliary proteins require specific intracellular sorting of vesicles. To aid in the biochemical characterization of these different vesicular pathways, we examined the effects of the ionophore monensin on the uptake and degradation of ^{125}I-asialoorosomucoid (ASOR) and on the secretion of plasma and biliary proteins by the in situ perfused rat liver. In control livers, 77% of injected ^{125}I-ASOR was extracted on first pass; 93% of the extracted radioactivity was released back into the circulation (totally degraded and some intact ASOR was found); and approximately 2% was recovered in the bile, some of which was intact. Monensin treatment decreased first pass uptake of ^{125}I-ASOR to 57% and abruptly blocked the release of radioactivity into the perfusate and the bile. When hepatic proteins were biosynthetically labeled with ^{3}H-leucine, monensin treatment dramatically reduced and delayed the secretion of newly synthesized proteins into both the perfusate and the bile. In contrast with control livers, in which secretion of protein into the perfusate preceded secretion of protein into the bile, TCA-precipitable ^{3}H-protein appeared in bile about 20 min before TCA-precipitable ^{3}H-protein appeared in the perfusate in monensin-treated livers. Thus, monensin treatment in the perfused liver blocked the degradation of asialoglycoproteins and inhibited the secretion of plasma proteins but had less effect on biliary protein secretion. These data document physiologic effects of monensin in an intact organ and suggest that biochemical distinctions between different vesicular pathways exist in the rat hepatocyte.

Key words: monesin, vesicular transport pathways, liver perfusion, asialoorosomucoid

The processes that regulate and direct the movement and sorting of intracellular vesicles are unexplained. Endocytic vesicles that internalize extracellular ligands via

Abbreviations used; ASOR, asialoorosomucoid; pIgA, polymeric immunoglobulin A; SC, secretory component; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis; TCA, trichloroacetic acid.

Dr. Reichen's present address is Department of Pharmacology, University of Berne, Berne, Switzerland.

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receptors on the cell surface migrate to various cellular locations, thereby allowing vesicle contents to receive differential treatment [1,2]. Often, internalized ligands are delivered to lysosomes for degradation, and the receptors are recycled to the plasma membrane for reutilization [3,4,5]; but in a few systems, the ligand avoids intracellular degradation and is transported across the cell to be released at an opposing cell surface [6,7,8].

Similar vesicle sorting problems exist for newly synthesized proteins that are destined to be inserted into or secreted from different domains of polarized cells, ie, those cells with morphologically and biochemically distinct plasma membrane domains. These proteins are synthesized in the rough endoplasmic reticulum, processed in the Golgi, and then packaged into vesicles that migrate to the appropriate plasma membrane domain either for insertion into the membrane or for exocytosis [9]. Navigational signals on individual proteins may provide the key for proper targeting of proteins to different intracellular destinations [10]; however, the mechanisms that allow for proper routing of transport vesicles are unknown.

The hepatocyte is a polarized cell that must sort a variety of biogenetic and endocytic vesicles. It synthesizes many proteins destined for release into the plasma and/or bile; it maintains polarized plasma membrane domains that contain unique protein constituents; it endocytoses many different ligands that are destined for degradation or transcellular transport.

To aid in characterizing and dissecting the mechanisms of vesicle movement, we have monitored the effects of the ionophore, monensin, on the degradation of asialoorosomucoid and on the secretion of newly synthesized proteins into blood or bile in the in situ perfused rat liver.

MATERIALS AND METHODS

Animals and Liver Perfusion

Male Sprague-Dawley rats (200–300 g; Charles River, Wilmington, MA) were housed in a climate-controlled environment with a 12-hr light-dark cycle and were fed rat chow and water ad libitum. The rats were anesthetized with sodium pentobarbital (60 mg/kg), and the common bile duct was cannulated with PE-10 tubing (Clay Adams, Parsippany, NJ). Perfusion of the liver was performed in situ as described [11]. ^{125}I-Asialoorosomucoid (ASOR), ^{3}H-leucine, and monensin (Calbiochem-Behring, San Diego, CA), when employed, were injected into the portal vein. Perfusate samples were obtained from the hepatic venous effluent; bile samples were collected at timed intervals on ice in the presence of 1 mM phenylmethylsulfonyl fluoride and 50 μg/ml soybean trypsin inhibitor [12].

Proteins

Human ASOR, provided by Dr. G. Ashwell, NIADDK, NIH, was radioiodinated by using a solid-phase lactoperoxidase technique [13]. Purity of ^{125}I-ASOR was confirmed by SDS-PAGE and autoradiography. Precipitability of the iodinated ASOR in trichloroacetic acid (TCA) at a final concentration of 10% was greater than 95%.

Uptake and Degradation of ^{125}I-ASOR

After injection of a known amount of ^{125}I-ASOR (300,000 cpm/0.5 μg), samples of perfusate were collected at 30-sec intervals for the first 5 min and then at

5-min intervals for 1 hr; 1-ml aliquots then were removed, and their radioactivity was counted. During this time, the perfusate exiting the liver was not recirculated through the liver. At the end of the experiment, the liver was removed and cut into several pieces, and the radioactivity was counted in a gamma counter (Beckman Instruments, Irvine, CA). The percent pass-through radioactivity of the ^{125}I-ASOR was calculated as the ratio of the radioactivity observed in the first 5 min of perfusate effluent over the radioactivity injected times 100. The percent first pass uptake was obtained by subtracting the percent pass through activity from 100. The percent of ^{125}I-ASOR remaining in the liver 1 hr after injection was calculated as the ratio of radioactivity present in the liver 1 hr after injection over the radioactivity extracted initially by the liver times 100. The TCA precipitability of non-extracted radioactivity (pass through radioactivity), radioactivity released at peak of release (radioactivity that was extracted and then released), and radioactivity remaining in the liver 1 hr after injection were examined. In some experiments, the radioactivity in the perfusate was characterized by gel chromatography (Sephacryl 200).

Biliary Secretion of ^{125}I-ASOR and Biliary Proteins

Radioactivity in bile samples was determined by gamma counting, and the proportion of radioactivity precipitated by TCA was determined. The molecular size of radiolabeled products was estimated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) [14] and autoradiography. Bile samples destined for SDS-PAGE analysis were collected directly into sample buffer [12,14] (5 min of bile plus 50 μl of sample buffer). Gel lanes that were to be Coomassie-stained were loaded with 50 μl of the bile mixture.

Biosynthetic Labeling and SDS-PAGE

Newly synthesized hepatic proteins were biosynthetically labeled continuously with 0.4 mCi of ^{3}H-leucine (130 Ci/mmole; Amersham, Arlington Heights, IL) injected into the portal vein 40 min after initiation of perfusion; perfusion medium was recycled through the liver during the labeling period. Perfusate and bile samples, collected at timed intervals after the administration of isotope, were analyzed by TCA-precipitability and SDS-PAGE and fluorography [15].

Monensin Treatment

The dose of monensin required to inhibit ASOR degradation was determined in preliminary experiments. Thus, in the experiments described below, 300 μl of monensin was injected into the portal vein (stock solution: 6.9 mg monensin/ml ethanol; final concentration in the perfusion medium about 10^{-6}M) 30 min after beginning liver perfusion. Tests of liver viability were measured before and 30 min after the administration of monensin. ^{125}I-ASOR was injected into the portal vein at intervals of 1 min, 5 min, or 10 min after the injection of monensin; ^{3}H-leucine was injected 10 min after monensin treatment.

RESULTS

Effects of Monensin on the Perfused Liver

Monensin treatment altered many of the physiologic properties of the perfused liver (Table I). Portal pressure was increased by about 50% while portal blood flow

TABLE I. The Effect of Monensin Treatment on Physiology of the Perfused Rat Liver*

	Control	Monensin
Portal pressure (cm H_2O)	10.1 ± 0.5	15.7 ± 0.7**
Portal flow (ml $min^{-1}g^{-1}$)	1.37 ± 0.08	0.98 ± 0.17**
Bile flow (μl $min^{-1}g^{-1}$)	1.78 ± 0.35	0.48 ± 0.22**
Glutamate-oxaloacetate transaminase release (IU/h)	5 ± 2	12 ± 5***
[K^+] perfusate (meq/1)	4.8 ± 0.7	5.1 ± 0.8
Oxygen consumption μmoles $min^{-1}g^{-1}$	2.2 ± 0.2	1.9 ± 0.2

*Values are the mean ± standard deviation (n=6). The livers were perfused for 30 min; control values were taken; monensin was then administered, and all measurements rechecked after 30 min.
**p < 0.001 monensin-treated liver vs control liver.
***p < 0.01 monensini-treated liver vs control liver.

TABLE II. Fate of ^{125}I-ASOR Injected Into Control or Monensin-Treated Perfused Rat Liver*

		Time after monensin injection		
	Control	1 min	5 min	10 min
First-pass extraction[a]	77 ± 6 (5)	55 ± 5 (3)**	57 ± 8 (6)**	20 (2)**
Extracted radioactivity remaining in liver at 60 min[b]	5 ± 5 (4)		26 ± 18 (6)***	
Injected radioactivity recovered in bile (60 min)	2 ± 0.5 (3)		0.3 ± 0.3 (5)****	

[a]Given as the percentage of the injected radioactivity not recovered in the first 5 min of collection of hepatic venous effluent after isotope injection; most of the pass-through radioactivity (approximately 23% in controls) was recovered in the first 2 min.
[b]Given as the percentage of radioactivity remaining in the liver at 60 min relative to the initially extracted radioactivity. About 90% of extracted radioactivity (controls) was released into the perfusate medium (see Fig. 1); the remainder was recovered in the liver or in the bile.
*Values are average percent ± standard deviation. Numbers in parentheses indicate number of samples analyzed. ^{125}I-ASOR was injected into the portal vein at indicated times after injection of monensin.
**p < .005
***p < .05
****p < .001

and bile flow decreased by 30% and 70%, respectively. The two most sensitive indicators of liver damage, oxygen consumption and release of K^+, were not significantly altered. These data indicate that, while liver physiology may be affected by monensin treatment, the monensin-treated liver should be considered viable. That the release of glutamate-oxalocetate transaminase was doubled indicates that only minimal liver damage had occurred or, more likely, that the elevation resulted from the modest red blood cell lysis observed.

Uptake and Release of ^{125}I-ASOR

In control livers, the first-pass uptake of ^{125}I-ASOR was 77 ± 6% of the injected sample (Table II; Fig. 1). The majority of pass-through radioactivity appeared in the first 2 min of perfusate collection. About 90% of the ^{125}I- ASOR extracted by the liver was released back into the perfusate during the 60 min of perfusion (Fig. 1); approximately 5% remained within the liver. The release of initially extracted radioactivity from the liver into the perfusate began about 10 min after the injection of ^{125}I-ASOR, and peak release occurred at 21–25 min (Fig. 1). Some of the ^{125}I-ASOR

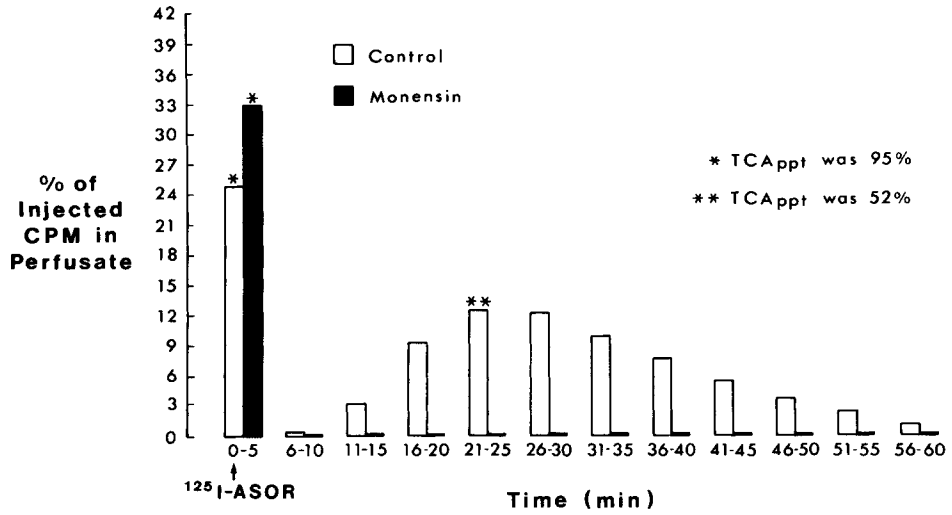

Fig. 1. Radioactivity recovered in perfusate from a control and monensin-treated liver after ^{125}I-ASOR injection. This figure shows a single representative experiment. The percent of injected radioactivity (counts per minute, CPM) recovered in the perfusate at the indicated time is shown on the ordinate. ^{125}I-ASOR was injected at time 0; samples of perfusate were collected as described in the text. The pair of bars on the left (0–5 min) show the cumulative percent of radioactivity in the pass-through (not extracted) fraction. TCA precipitability of recovered radioactivity is indicated.

released into the perfusate was degraded as indicated by the decrease in TCA precipitability, 39 ± 22% versus 95%. Further characterization of the released products by gel chromatography (Sephacryl 200; data not shown) indicated that both intact and totally degraded molecules were present; no partially degraded molecules were observed. The percent of radioactivity eluting the mobility of intact ASOR corresponded well with TCA-precipitation values. The TCA precipitability of radioactivity remaining in the liver was 67 ± 12%. A small portion (about 2%) of the injected ^{125}I-ASOR was recovered also in the bile (Table II). Release of radioactivity into the bile began at 10 min and peaked at 21–25 min (Fig. 2). TCA precipitability of radioactivity recovered in the bile at peak release was 54–69%; the presence of some intact ^{125}I-ASOR in the bile was confirmed by SDS-PAGE and autoradiography (data not shown).

In monensin-treated livers, the first-pass uptake of ^{125}I-ASOR was significantly less than in control livers (Table II). This difference was modest but significant when the ^{125} I-ASOR was injected 1 min or 5 min after monensin, but was marked when injected after 10 min of monensin treatment (77% vs 20%). When ^{125}I-ASOR was injected into monensin-treated livers, virtually no radioactivity was released back into the perfusate (Fig. 1) or into the bile (Fig. 2), and increased amounts of radioactivity remained within the liver (Table II). Thus, monensin treatment in the perfused rat liver caused a modest decrease the first-pass uptake of ^{125}I-ASOR and almost totally prevented the degradation and release of degraded ^{125}I-ASOR into the perusate or bile.

Biliary Protein Secretion

The major protein in the bile from a rat liver perfused with serum-free medium is secretory component (SC), the receptor for polymeric immunoglobulins (identified

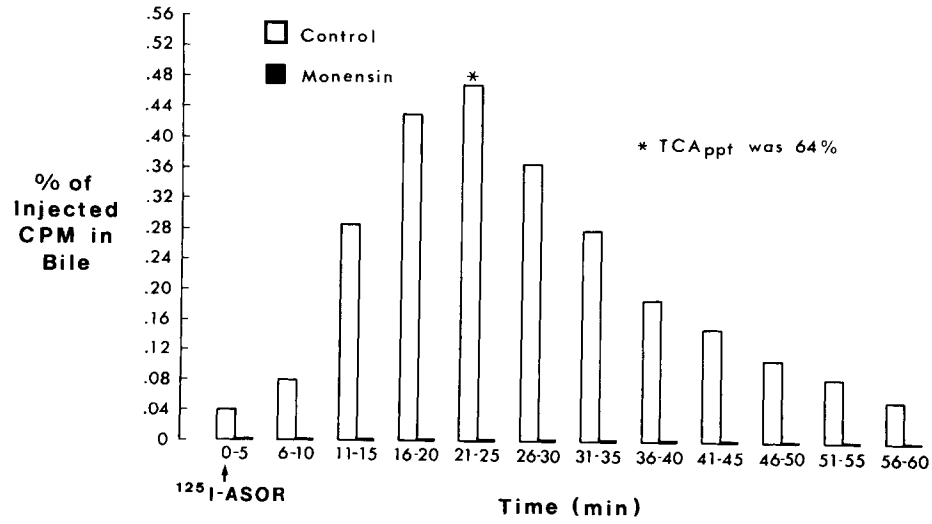

Fig. 2. Radioactivity recovered in bile from control and monensin-treated livers after ^{125}I-ASOR injection. This figure shows data from the same experiment as in Figure 1. The percent of injected radioactivity recovered in the bile at the indicated time is shown on the ordinate. TCA precipitability of radioactivity at peak release is indicated.

using immunoblot techniques, [12; Kloppel et al, Hepatology, in press]. In control livers, the amount of SC secreted into bile decreased slightly over the 60-min experimental period (Fig. 3A). In monensin-treated livers, there was a greater reduction in, but not cessation of, the secretion of SC (Fig. 3B). Based on calculations of bile flow (5-fold decrease and densitometric tracings of the SC bands (6-fold decrease), the amount of SC secreted into bile 60 min after ^{125}I-ASOR injection in monensin-treated livers was approximately 30-fold less than in control livers. However, during the time periods when ASOR degradation occurred in control livers (10–30 min) and was inhibited in monensin-treated livers, SC secretion was only inhibited by the monensin treatment about 4-fold.

It was also noted that the amount of bovine albumin transported into the bile from the perfusate medium increased in the monensin-treated livers (Fig. 3B). (In experiments not illustrated, monensin increased the biliary secretion of ^{125}I-BSA by two-fold). Transport of bovine albumin from the perfusate to bile and, similarly, endogenous rat serum albumin from the serum to bile is thought to occur through both intercellular and transcellular pathways [16, 17; Kloppel et al, Hepatology, in press]. Hence, monensin treatment might have disrupted the intercellular junctions, allowing more albumin to pass from the perfusate into bile. (Several of the other proteins present in the bile profiles from the monensin-treated liver (Fig. 3B) were contaminants of the bovine serum ablumin (BSA) that was added to the perfusate.)

Secretion of Newly Synthesized Proteins Into the Perfusate or Into the Bile

Newly synthesized, biosynthetically labeled proteins were released into the perfusion medium and into the bile from control livers. About 15 different ^{3}H-proteins were observed in the perfusion medium, becoming evident at the 30-min sampling period after injection of ^{3}H-leucine (Fig. 4). (In previous, unpublished experiments we had observed that secretion of ^{3}H-proteins by perfused livers begins

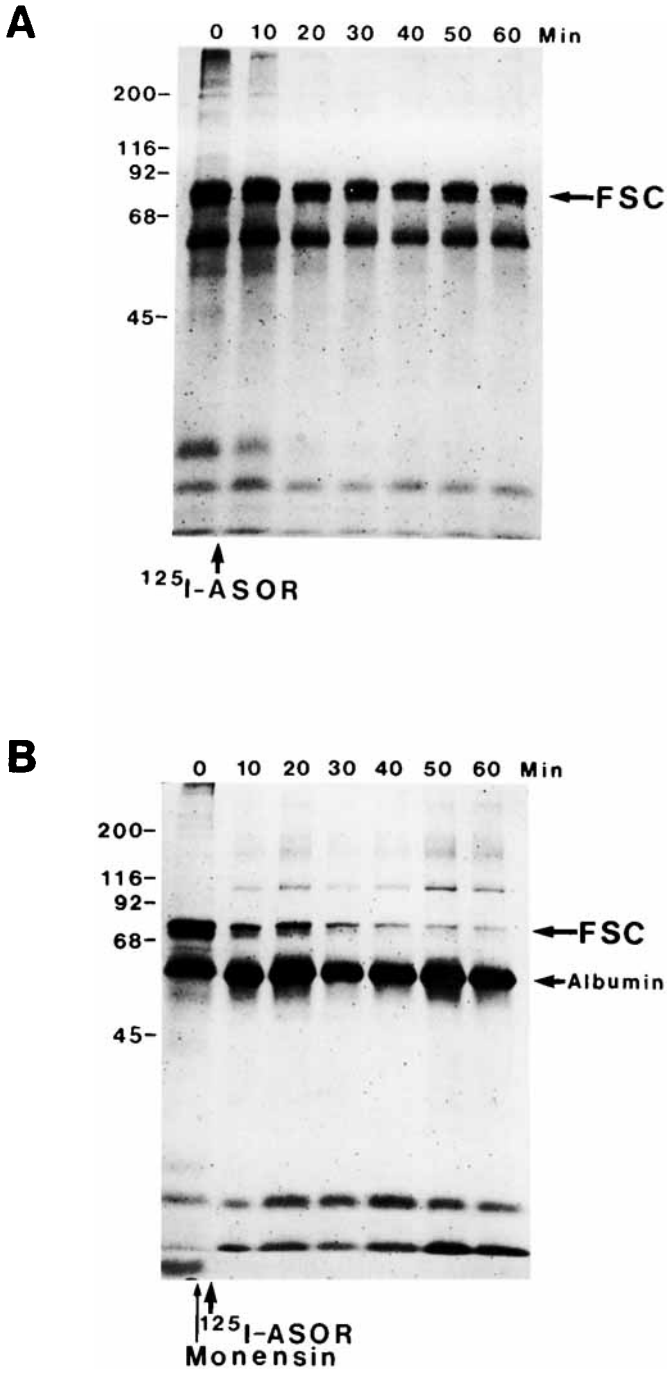

Fig. 3. SDS-PAGE of bile from a control (A) and a monensin-treated (B) liver injected with ^{125}I-ASOR. This figure shows data from the same experiment as described in Figure 1. Biliary proteins in bile samples collected at 10 min intervals for 0–60 min are stained with Coomassie blue. Migration of soluble SC (FSC; identified by immunoblot, 12) and bovine serum albumin is indicated. (The bovine serum albumin originated in the perfusion medium and was transported across the liver into bile; Kloppel et al, Hepatology, in press.) ^{125}I-ASOR was injected at time 0 or 5 min after monensin treatment. Migration of molecular weight markers is indicated (10^{-3}).

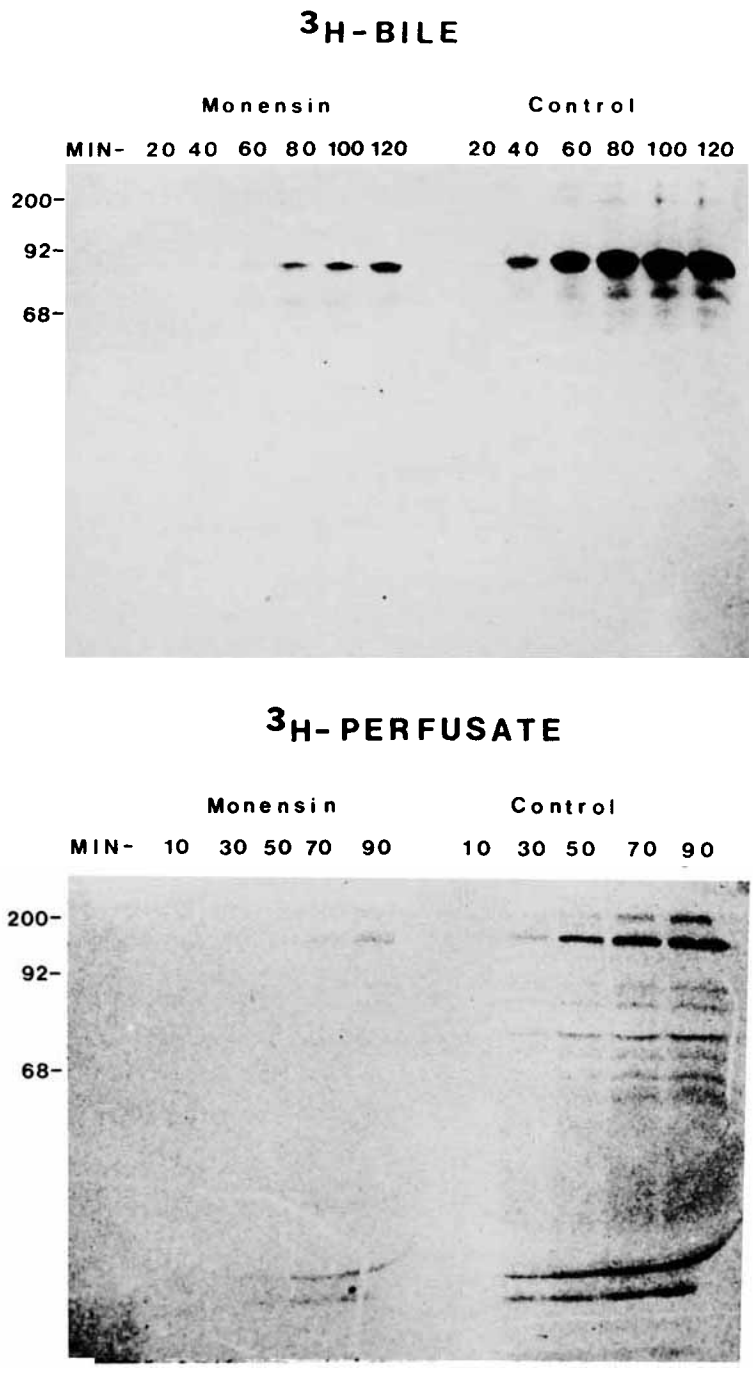

Fig. 4. Secretion of ^{3}H-leucine-labeled proteins into the perfusate medium or into the bile of control monensin-treated perfused livers. Perfused livers were continously labeled with ^{3}H-leucine. Samples of perfusate medium or bile were collected at the designated number of min after injection of isotope into the portal vein. Samples were electrophoresed in non reducing 8.5% SDS gels, and the gels were treated for fluorography. Bands corresponding to SC are depicted (arrow). The distortion and lane fusion in the middle portion of the perfusate samples is due to large amounts of BSA in the perfusion medium. Migration of molecular weight markers ($\times 10^{-3}$) is indicated.

at about 20 min. The ^{3}H-proteins also were secreted into the bile of the control livers, although the time of appearance was delayed compared to plasma proteins (40 min vs 30 min), and the compositional profile of the biliary proteins was different than those proteins secreted into the perfusate; secretory component (SC), the major radiolabeled biliary protein, was absent from the perfusate.

Monensin treatment severely reduced and delayed the secretion of newly synthesized hepatic proteins into both the perfusion medium and the bile (Fig. 4). Densitometric analysis at the 90-min time point for the perfusate and at the 80-min time-point for the bile showed reductions of 90% and 80% respectively. Newly synthesized protein first appeared in the perfusion medium of monensin-treated liver at 90 min (a 60-min delay compared to control livers), secretion of ^{3}H-proteins into the bile was evident at 60–80 min (a 20–40 min delay compared to control livers); (Fig. 4). While release of TCA-precipitable ^{3}H-protein into the perfusate preceded release of precipitable ^{3}H-protein into bile in control livers, in monensin-treated livers, release of precipitable ^{3}H-protein into bile preceded secretion of perfusate proteins (data not shown; see Fig 4). The size of the secreted SC from monensin-treated livers was slightly smaller (approximately 2 kD) than from the control livers; this change presumably represents incomplete terminal glycosylation of SC brought on by monensin treatment [18].

DISCUSSION

The data presented in this report document that the endocytic pathway involved in the degradation of asialoglycoproteins in the perfused rat liver is disrupted by monensin treatment. Control experiments showed that circulating ^{125}I-ASOR was efficiently extracted by the perfused rat liver and partially degraded. A portion of lysosomal products (^{125}I-fragments of ASOR) was released back into the perfusate, and some degradative products were released also into the bile. Some intact molecules of ^{125}I-ASOR also were released into blood and bile, as indicated by TCA precipitability, gel chromatography, and SDS-PAGE experiments. The biliary secretion of a small percentage of intact ASOR has been described, and attributed to ligand missorting [19]. The delivery of other intact asialoglycoproteins to bile [20,21] as well as a diacytosis pathway that delivers endocytosed ligands back into the circulation intact also have been reported [22,23]. That we found a rather high TCA precipitability of radioactivity released back into the perfusate was surprising, as the release of intact diacytosed molecules reportedly requires Ca^{2+} chelation [24]; the perfusion medium used in our expeirments contained physiologic levels of Ca^{2+}.

Our experiments evidently are the first to show that monensin treatment can prevent the degradation of ASOR by the perfused liver, although inhibition of asialoglycoprotein degradation in this model has been achieved through the use of the lysosomal proteinase inhibitor leupeptin [25]. Results similar to ours have been obtained with monensin treatment of cultured hepatocytes. In those experiments, the mechanism of action of monensin has been ascribed to interference with vesicle acidification and consequent interruption of the dissociation of ligand from receptor [26,27]. The major advantage of using the perfused liver is the maintenance of hepatic cell polarity. In our experiments, monensin treatment severely altered the flow of bile and perfusate; the viability of the organ was acceptable, however, since

neither potassium concentration nor oxygen consumption, sensitive indicators of liver dysfunction, were altered.

In addition to showing that monensin treatment interrupted the degradation of ASOR, our experiments showed that monensin decreased the uptake of ASOR, especially when the ligand was injected 10 min after monensin treatment. The latter result supports the contention that monensin blocks recycling of the asialoglycoprotein receptor, thus decreasing the number of receptors on the cell surface [26]. Monensin treatment also blocked the secretion of ASOR into the bile, which suggests that the dissociation of ASOR from its receptor is required before secretion of ASOR into the bile can occur. An additional finding of this study was that monensin, at concentrations that blocked the degradation of ^{125}I-ASOR, markedly delayed the secretion of newly synthesized proteins into the perfusate and bile. We observed, however, that the effect of monensin on secretion of protein into bile was less pronounced. Secretion of the major biliary protein, SC, was reduced, but not halted. SC is synthesized as a transmembrane glycoprotein [28] and is expressed on the sinusoidal plasma membrane of the hepatocyte. Upon binding IgA, the SC-IgA complex is internalized in an endocytic vesicle and transported via vesicles to the bile canalicular membrane, where the SC-IgA complex is released into the bile; the last step involves the proteolytic cleavage of the membrane form of SC [12]. It should be noted that the SC pathway operates in the absence of ligand [29, Kloppel et al, Hepatology, in press]; thus, the presence of polymeric IgA is not required for the proper processing of membrane SC to soluble SC and the subsequent secretion of soluble SC into the bile.

The decrease in the amount of Coomassie-stainable SC that was secreted as a result of monensin treatment might be attributable to a block in the sinusoidal membrane expression of SC. The basis for this opinion is that monensin treatment drastically inhibited the secretion of newly synthesized proteins into the perfusate; a concomitant decrease in the insertion of newly synthesized SC into the sinusoidal plasma membrane could be presumed. Thus, the Coomassie-stainable SC observed in the bile from the monensin-treated liver presumably was derived from vesicles ready for exocytosis, vesicles in transport from the sinusoidal membrane, and pre-existing SC in the sinusoidal membrane. On the basis of other data [30], the monensin block likely is at the level of the Golgi and may not affect the sinusoidal membrane-to-canalicular membrane pathway for SC vesicles.

While hepatic secretory pathways were profoundly inhibited by monensin treatment, the effect of monensin on the secretion of newly synthesized SC into bile was not as dramatic as on the secretion of plasma proteins. There are two possible explanations for this result: first, the pathway for delivery of membrane SC to the sinusoidal plasma membrane may be different from the pathway that delivers secretory proteins, destined for exocytosis, to the sinusoidal face; and this SC pathway may not be inhibited by monensin. Indeed, the expression of constituitive membrane proteins presumably involves a different intracellular route from that for proteins destined for stimulated exocytosis [31]. Second, some SC may be secreted into bile without temporarily residing in the sinusoidal membrane, ie, by direct transport from the Golgi to the bile canalicular surface; such a pathway would be analogous to the direct insertion of membrane proteins into the microvillar surface of polarlized epithelial cells [32]. In this regard, it has been reported that monensin did not inhibit the insertion of viral membrane proteins into the apical surface of epithelial cells but did inhibit their insertion into the basolateral membrane [33]. By analogy, monensin

in the hepatocyte might disrupt the secretory and membrane protein pathways leading to the sinusoidal plasma membrane but not the pathway from the Golgi to the canalicular plasma membrane.

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