The in Vivo Effect of Colchicine on the Addition of Galactose and Sialic Acid to Rat Hepatic Serum Glycoproteins*

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Colchicine inhibits the secretion of plasma protein by rat hepatocytes and causes their intracellular accumulation in Golgi-derived secretory vesicles. This study examines whether colchicine affects secretion before or after galactose and sialic acid have been added to the secretory glycoproteins. D-[G-'H]Galactose was injected into rats and was found to be incorporated into serum glycoproteins contained within Golgi-derived secretory vesicles. The administration of colchicine (25 μmol/100 g, body weight), immediately before the injection of D-[G-'H]galactose, caused an increase in radioactivity of the serum glycoproteins in these cell fractions. D-[G-'H]Glucosamine was incorporated into serum glycoproteins contained within the rough and smooth endoplasmic reticulum and the Golgi cell fractions; however, its incorporation into the sialic acid moieties of these proteins only occurred in Golgi-derived cell fractions. Colchicine administration resulted in an increased incorporation of D-[G-'H]glucosamine into the sialic acid residues of serum glycoproteins contained within the Golgi cell fractions. These data indicate that colchicine inhibits secretion of serum proteins by rat liver after the addition of galactose and sialic acid to the secretory proteins has taken place.

SECRETORY PROTEINS PRODUCED BY RAT HEPATOCYTES ARE SYNTHESIZED ON POLYSOMES ATTACHED TO THE MEMBRANES OF THE ENDOPLASMIC RETICULUM (1-5), AND VECTORIALLY DISCHARGED INTO THE LUMEN OF THE ROUGH ENDOPLASMIC RETICULUM (6-8) AND THEN FOLLOW AN INTRACELLULAR PATHWAY WHICH LEADS THEM TO THE SMOOTH ENDOPLASMIC RETICULUM, TO THE GOLGI COMPLEX, AND TO GOLGI-DERIVED SECRETORY VESICLES WHICH, WHEN FUSED WITH SECRETORY PROTEINS, FUSE WITH THE PLASMA MEMBRANE AND DISCHARGE THE SECRETORY PROTEINS INTO THE SPACE OF DISSE (9-12). THE CARBOHYDRATE MOIEITIES OF THE SECRETORY GLYCOPROTEINS ARE ADDED IN STEPSWISE FASHION (13, 14) AT VARIOUS INTRACELLULAR SITES WITH THE TERMINAL GLYCOSYLATION OCCURRING IN THE GOLGI COMPLEX (15-20).

In recent years many studies have demonstrated that colchicine affects secretion in a variety of tissues (21-28). A similar inhibitory effect of colchicine on plasma protein secretion in rat hepatocytes has also been reported by our laboratory. We have further shown that colchicine does not impede the synthesis of secretory proteins or the movement from the rough endoplasmic reticulum to the Golgi complex, but that it inhibits late events which occur either at the Golgi complex or after these proteins have been packaged into secretory vesicles but prior to fusion of the vesicles with the plasma membrane (29). To determine whether colchicine is blocking the secretion of hepatic glycoproteins before or after the addition of the terminal sugars, we have examined the in vivo effect of colchicine on the addition of the penultimate and terminal sugars, D-galactose and sialic acid.

MATERIAL AND METHODS

Animals—Young male rats (100 to 175 g) were not fed overnight before the experiments. Injections of colchicine, radioactive amino acids, or sugars were given intravenously into the femoral vein while the rats were anesthetized with ether.

Cell Fractionation and Identification of Fractions—The livers were fractionated, as previously described, into Golgi fraction I, fraction II, fraction III, smooth endoplasmic reticulum, and rough endoplasmic reticulum fractions (29). Electron microscopy of the three isolated Golgi fractions had previously shown that G1 consists of rounded secretion vesicles filled with very low density lipoprotein particles embedded in a dense matrix; G2 is composed of a mixture of similar secretion vesicles and cisternal elements of Golgi, while G3 is predominantly composed of flattened Golgi cisternae (28). All isolated cell fractions were also characterized biochemically by assaying for the following marker enzymes: glucose-6-phosphatase (28, 30), succinic dehydrogenase (31), UDP-galactose:N-acetylgalactosamine galactosyltransferase (16), and 5' nucleotidase (32). The results of these experiments indicate that the cell fractionation procedures used yield rough and smooth endoplasmic reticulum and Golgi cell fractions which are comparable in purity to those previously described (20) and those described by others (33, 34).

Immunoprecipitation and Determination of Protein Radioactivity—The radioactive serum proteins contained in the RER, SER, and Golgi fractions were obtained by immunoprecipitation from 0.5% sodium deoxycholate extracts of these fractions using a multivalent rabbit antisera to rat serum (29). All radioactive protein samples

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were dissolved in 1 ml of Soluene and counted in a Packard scintillation radiometer in the presence of 0.1 ml of glacial acetic acid and 10 ml of toluene scintillation phosphor.

Other Procedures—Sialic acid radioactivity in the antibody-antigen complexes was determined by hydrolyzing the samples in 0.1 N H₂SO₄ for 1 hour at 80°, and by isolating the released sialic acid by ion exchange chromatography by the method of Macbeth et al. (35) as modified by Lawford and Schachter (36). The radioactivity in these aqueous hydrolysates was determined by counting in a scintillation counter in the presence of 10 ml of Aquasol.

The antigen-antibody precipitates were dried at room temperature, dissolved in 6 M urea/0.1 M sodium phosphate buffer, pH 7.2, and incubated at 45° for 1 hour prior to gel electrophoresis. Sodium dodecyl sulfate-urea-polyacrylamide gel electrophoresis (37) was performed using 7.5% acrylamide gels/0.1 M phosphate buffer, pH 7.2, and incubated at 45° for 1 hour. The gels were either stained with Coomassie blue or sliced into 1.5-mm thick slices, and then digested at 70-75° in 0.1 ml of hydrogen peroxide and 0.2 ml of perchloric acid followed by counting in 10 ml of Scintisol.

Protein was assayed by the method of Lowry et al. (38) using bovine serum albumin as standard.

RESULTS

Incorporation of d-Glucosamine into Proteins of Various Cell Fractions—Radioactive d-glucosamine was incorporated into trichloroacetic acid-precipitable protein of all cell fractions measured. A time course study showed that there was maximal incorporation into trichloroacetic acid-precipitable protein between 30 min and 1 hour after administration and that the incorporation (per mg of organelle protein) is higher in the Golgi than in the RER and the SER fractions (Fig. 1A).

d-Glucosamine is metabolized in vivo and is converted to other sugars, which are subsequently added to protein (36, 39-41). Incorporation of these radioactive sugars occurs into many different types of cellular proteins, both secretory and nonsecretory. In order to determine the time course of incorporation of d-glucosamine into sialic acid moiety of secretory proteins, the nascent serum proteins were obtained from the various isolated cell fractions by immunoprecipitation and the sialic acid radioactivity of the immunoprecipitates was determined by isolating the sialic acid residues which had been released by treatment with 0.1 N H₂SO₄ at 80°. These experiments showed that, as expected, there is little or no incorporation of sialic acid into serum proteins of the RER and the SER but that it is incorporated into secretory proteins while they are in the Golgi cell fractions (13-20, 36, 42-46). G1 was usually more active in the incorporation of sialic acid into glycoprotein than G2 and G3, although this incorporation was variable and occasionally near equal amounts of incorporation was observed in G1 and G2, but always the incorporation into these two fractions exceeded that into G3. In contrast to the time course of incorporation of d-glucosamine into trichloroacetic acid-precipitable protein, the incorporation of sialic acid into secretory protein showed a sharp maximal peak 30 min after administration and by 90 min it had dropped to a level comparable to that seen at 10 min (Fig. 1B).

Effect of Colchicine on Intracellular Distribution of Both L-Leucine- and d-Glucosamine-labeled Serum Proteins—In order to determine whether colchicine affects intracellular secretion before or after the stepwise addition of sugars to secretory protein has occurred, rats were injected with a mixture of L-[4,5-³H]leucine and d-[¹⁴C]glucosamine immediately after the administration of 25 μmol/l00 g, body weight, of colchicine. Then 1 hour after the administration of the radioactive material the livers were removed and cell fractions were analyzed for sialic acid radioactivity (Fig. 2).

Colchicine caused an increase in both the ³H and the ¹⁴C radioactivity of the serum proteins obtained from G1, G2, and G3. It had little or no effect on the radioactivities of the serum proteins obtained from the RER, the SER, or from the soluble cell fractions (Fig. 2). This suggests that colchicine blocks the secretion of glycoprotein after the addition of glucosamine derived sugars and that, as shown before (29), the nonsecreted proteins accumulate in the Golgi.

To determine, however, whether colchicine blocks secretion before or after the terminal sugar, sialic acid was added, control and colchicine-treated rats were injected with d-
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FIG. 2. Effect of colchicine on the intracellular distribution of L-[4,5-3H]leucine- and D-[14C]glucosamine-labeled serum proteins. Rats were injected with either 25 μmol/100 g, body weight, of colchicine or an equal volume of saline. This was followed by the injection of 65 μCi/100 g, body weight, of L-[4,5-3H]leucine (56 Ci/mmol) and D-[14C]glucosamine (56 μCi/mmol). Each rat received 15 μCi/100 g, body weight, of each radioactive compound and 1 hour after administration the livers were removed and cell fractionation performed as discussed under "Material and Methods." A shows the incorporation of L-[3H]leucine into serum protein isolated from the various cell fractions by immunoprecipitation, while B shows the incorporation of D-[14C]glucosamine into the same cell fractions. The solid bars represent the control rat and the striped bars the colchicine-treated rats. H, homogenate; S, soluble fraction.

FIG. 3. Effect of colchicine on the incorporation of D-glucosamine into the sialic acid moiety of serum proteins isolated from various cell fractions. Rats were injected with 25 μmol/100 g, body weight, of colchicine and the control rats received an equal volume of saline. This was followed by the injection of 65 μCi/100 g, body weight, of D-[6-3H]glucosamine (10.13 Ci/mmol). Then 1 hour after administration the livers were removed and cell fractionation performed as discussed under "Material and Methods." The measurements of incorporation of D-[6-3H]glucosamine into sialic acid of serum protein obtained from these cell fractions is also described under "Material and Methods." The solid bars represent the radioactivity in cell fractions from control rats and the striped bars, from those of colchicine-treated rats. H, homogenate; S, soluble fraction.

Fig. 4. Effect of colchicine on the intracellular distribution of D-[4,5-3H]glucose- and D-[14C]glucosamine-labeled serum proteins. The time course of incorporation of D-galactose into serum proteins within the Golgi vesicles differs from that of D-glucosamine. While the conversion of D-glucosamine to sialic acid and its incorporation into serum protein is maximal at 30 min (Fig. 1) that of D-galactose reaches a peak at 5 to 10 min after intravenous administration and falls rapidly at the end of 30 min. It is possible in this experiment to see a progression of incorporation of D-[4,5-3H]glucosamine into serum protein contained within cell fractions G3 and G2 (at 5 min) and then later, at 10 min, into G1 (Fig. 4). There was little or no incorporation of galactose into the serum protein located within the RER and the SER.

H [3H]glucosamine, and again the liver was fractionated, the nascent serum glycoproteins were isolated from the various cell fractions by immunoprecipitation, and the radioactivity in sialic acid was determined.

These experiments showed that there was little sialic acid-labeled serum protein in any cell fraction other than the Golgi and that the administration of colchicine to rats caused a large increase in sialic acid radioactivity in serum protein. The increase in radioactivity was predominantly seen in serum protein found in cell fractions G1 and G2 with much less accumulation in G3 (Fig. 3).
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Fig. 4. Time course of incorporation of D-[G-SH]galactose into total trichloroacetic acid-precipitable protein and into plasma protein from Golgi fractions. D-[G-SH]Galactose (specific activity, 0.6 Ci/mmol) was injected intravenously (14 μCi/100 g body weight) into the femoral vein of male rats. The animals were killed at different time intervals, the livers removed, and Golgi prepared as described under "Material and Methods." A shows the radioactivity found in serum proteins obtained from the Golgi fractions by immunoprecipitation with antiserum against rat serum proteins and B shows the incorporation of [14C]galactose into total trichloroacetic acid-precipitable protein.

Detect any effect by colchicine in the protein of these cell fractions (Fig. 5).

Identification of Galactose and Glucosamine-labeled Proteins within Golgi Fractions—Since in these studies a multivalent rabbit antiserum against rat serum protein has been employed, it is of interest to determine whether the radioactivity measured is incorporated into one or more serum glycoproteins and whether colchicine is affecting the retention within the Golgi vesicles of all secretory glycoproteins or of only a few selected ones. Colchicine-treated and control rats were injected with D-[14C]galactose for 10 min or with D-[14C]glucosamine for 30 min prior to cell fractionation of the livers, and isolation of the radioactive glycoprotein from cell fractions Gl, G2, and G3. The proteins obtained by immunoprecipitation from these cell fractions were electrophoresed on sodium dodecyl sulfate-urea-polyacrylamide gels and the mobility of the radioactive proteins on the gel compared with that of proteins obtained from rat plasma by immunoprecipitation. Both D-[14C]galactose and D-[14C]glucosamine labeled a large number of proteins. The distribution of protein and radioactivity derived from D-[14C]glucosamine in the polyacrylamide gels is shown in Fig. 6. A similar pattern was observed with the D-[14C]galactose-labeled proteins, except that with D-galactose as a radioactive precursor there was less incorporation into the protein. In both cases colchicine administration did not change the radioactive pattern on the gels. The only effect of colchicine was to increase the amount of radioactivity obtained in each of the gel areas. Radioactivity was seen in several places of the gel, and they coincided with the appearance of plasma proteins as shown by Coomassie blue staining. The area of gel in which albumin appeared had little or no radioactivity.

Discussion

Previously we showed that in rat liver colchicine inhibits secretion at a late stage, after the Golgi vesicles have been filled with secretory proteins but prior to discharge of the secretory proteins into the Space of Disse (29). Some of the sugars, such as N-acetylglucosamine and mannose, are added to the nascent plasma glycoprotein soon after polypeptide
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![Diagram of cpm/SLICE](Fig. 6. Sodium dodecyl sulfate-acrylamide gel electrophoretic patterns of radioactive proteins isolated from Golgi fractions by immunoprecipitation after injection of colchicine-treated rats with p-[14C]glucosamine. Colchicine-treated rats (25 nmol/100 g body weight) were injected with 100 µCi/100 g, body weight, of p-[14C]glucosamine. One hour after injection the liver was fractionated into various cell fractions and the radioactive serum protein from Golgi fractions I and II collected by immunoprecipitation. The washed precipitate was electrophoresed in urea-sodium dodecyl sulfate-polyacrylamide gels as described under "Material and Methods." The gels were stained with Coomassie blue and then 1.5-mm slices were cut, digested, and the radioactivity determined. Coomassie blue-stained areas which appear when the antibody is used to precipitate plasma protein presumably represent the heavy (H) and light (L) immunoglobulin chain and albumin (Alb). The other stained bands are plasma proteins.)

Colchicine does not affect any of the steps in the assembly of secretory proteins but that it inhibits the discharge of Golgi-derived secretory vesicles which contain fully completed secretory protein. The inhibition of secretion by colchicine is often ascribed to its interaction with microtubules (21-28). In hepatocytes this remains open to question because these cells contain few microtubules and these microtubules do not show consistent depolymerization in vivo levels of colchicine which give maximal inhibition of secretion (29). It is possible, however, that colchicine may have a limited effect on microtubules which may not be detected morphologically but which is sufficient to impair the normal movement of secretory vesicles which would place them at the point of fusion with the plasma membrane.

Another possibility stems from studies with a variety of different cells, such as lymphocytes, fibroblasts, polymorphonuclear leukocytes, and Tetrahymena pyriformis, which suggest that colchicine-sensitive structures may be involved in the regulation of the mobility of various membrane receptors (47-49). Thus, colchicine may cause a dispersal of binding sites on the plasma membrane, or in Golgi-derived vesicles which may, in turn, inhibit fusion. The interaction of colchicine with membranes has been noted (50-52). While it has been shown that colchicine does not affect a large number of physiological functions of the hepatocyte (24, 25, 29), it is still possible that it may be having a direct effect on a parameter of hepatocyte metabolism which has not yet been measured. At present these possibilities are conjectural and the mechanism by which colchicine inhibits secretion remains to be explained by further studies on the process of exocytosis.

REFERENCES

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