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INCREASE IN POLYMERIZED LIVER TUBULIN DURING STIMULATION OF HEPATIC PLASMA PROTEIN SECRETION IN THE RAT.

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SUMMARY

Involvement of hepatic microtubules in plasma protein secretion by the liver was investigated by stimulating protein secretion in rat liver and then measuring the different forms of tubulin. Total and free tubulin were estimated in liver supernatants by the [^{3}H]colchicine-binding assay. Polymerized tubulin, assumed to reflect the presence of microtubules, was calculated from the difference between total and free tubulin. To enhance liver plasma protein secretion, an acute inflammatory reaction was induced in one group of rats and a nephrotic syndrome in another. In both cases, total liver tubulin increased significantly compared to normal animals, but free tubulin was unchanged. Accordingly, polymerized tubulin rose by 50 % during the inflammatory reaction and by 90 % during the nephrotic syndrome. These results support the hypothesis that hepatic microtubules are involved in plasma protein secretion by the liver and also suggest that enhanced secretion requires additional microtubules.

INTRODUCTION

It has been well established that the liver produces numerous plasma proteins (1). However, although much is known about their synthesis and secretion, some of the mechanisms involved in the different steps of these processes are still not clear (2). In particular, it has been suggested that microtubules could play a part in secretion, since drugs like colchicine or vinblastine, which bind to tubulin and prevent its polymerization into microtubules, inhibit secretion of plasma proteins without affecting their synthesis. This kind of inhibitory effect has been demonstrated *in vivo* and *in vitro* for lipoproteins

Abbreviations : MES, 2-(N-morpholino) ethanesulfonic acid ; GTP, guanosine-5'-triphosphate ; EGTA, ethylene glycol-bis-(β-aminoethyl ether)-N, N'-tetraacetic acid.

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(3, 4), albumin (5, 6), fibrinogen (7) and for many proteins secreted by the liver (5, 6).

Nevertheless, the role of microtubules in liver plasma protein secretion has been inferred from the effects of drugs on hepatic microtubules and not from studies of tubulin itself. In the liver, tubulin is present in two forms : free or monomeric, which is in equilibrium with the second form, polymerized tubulin, assumed to reflect the presence of microtubules (8, 9). The possible changes in this equilibrium when liver plasma protein secretion increases have been poorly investigated.

To gain insight into the relationship between microtubules and plasma protein secretion, we measured the amounts of total and free tubulin in rat livers, both under normal conditions and in two models in which hepatic plasma protein secretion was stimulated. The first model was the acute inflammatory reaction, a condition characterized by increased synthesis and secretion by the liver of certain plasma proteins known as acute phase reactants (10). The second model was the nephrotic syndrome, known to involve enhanced albumin and other plasma protein synthesis and secretion by the liver (11, 12). The amount of tubulin in rat livers was estimated by its colchicine-binding activity ; this method only measures tubulin in the free form (13). In order to measure total tubulin, microtubules were depolymerized.

We show here that in both models, the amount of total liver tubulin rises, whereas the amount of free tubulin remains unchanged, thus strengthening the hypothesis that polymerized tubulin, in the form of microtubules is involved in plasma protein secretion, and that consequently, enhanced secretion requires additional microtubules.

MATERIALS AND METHODS

Drugs and chemicals

Colchicine, MES, GTP, EGTA were purchased from Sigma Chem. Co. (St Louis, Mo., USA), [^{3}H]colchicine (19 Ci/mmol) came from New England Nuclear (Boston, Ma., USA), activated charcoal, from Merck (Darmstadt, West Germany), complete

Freund's adjuvant, from Difco Lab. (Detroit, Mi., USA) and Dimilume, from Packard Inst. Co. (Downers Grove, Il., USA). All other reagents were of analytical grade.

Experimental procedure

1) Rats with an acute inflammatory reaction : an acute inflammatory reaction was induced in male Sprague-Dawley rats weighing 250 to 300g by subcutaneous injection of turpentine (0.5 ml per 100 g body weight) according to the procedure used by Murray et al (14). Rats were studied 24 hours after injection. Normal rats were used as controls.

2) Rats with nephrotic syndrome : six week-old male Lewis rats were injected in the footpads with Fx_1 A fraction isolated from Sprague-Dawley rat convoluted proximal renal tubule and mixed with Freund's complete adjuvant (15, 16). A booster injection was performed 6 weeks later. After 6 weeks, the presence of the nephrotic syndrome was confirmed by severe proteinuria and hypoalbuminemia. All these rats had a glomerulonephritis, as indicated by the presence of typical granular IgG deposits along the glomerular capillary wall when studied by direct immunofluorescence using a fluoresceinated anti-rat IgG antiserum (15). Three types of controls were used : normal Lewis rats, rats injected with adjuvant only and rats injected with antigen and adjuvant, without booster injection ; the latter rats had a glomerulonephritis without nephrotic syndrome. At the time of the study their weight varied from 300 to 430 g.

All the animals were fed *ad libitum* with standard diet.

Tubulin measurements in rat livers

Liver homogenates were prepared according to the method of Patzelt et al (8) with a few modifications. For measurement of total tubulin, livers were perfused with cold MES buffer (0.1 M MES, 1 mM EGTA, 1 mM GTP and 0.5 mM $MgCl_2$, pH 6.5), homogenized in the same buffer (4 ml/g liver) and allowed to stand in ice for 30 min to depolymerize the microtubules. For measurement of free tubulin, livers were perfused and homogenized at room temperature using MES buffer containing 4 M glycerol. These two conditions (temperature and glycerol) preserved the microtubules (8). The glycerol-free homogenates prepared for total tubulin measurement were equalized so as to contain 4 M glycerol. All homogenates were centrifuged (100,000 g, 60 min) at 20° C in a Beckman L 50 ultracentrifuge (Beckman Inst. Inc., Palo Alto, Ca., USA). In the high-speed supernatants, colchicine-binding activity was determined by the charcoal separation method described by Sherline et al (17) with slight modifications. The assay procedure involved the incubation of 100 µl liver supernatant with 10^{-5}M [^{3}H] colchicine (0.2 mCi/mmol) at 37° C for 60 min in the dark. In order to test the influence of colchicine concentration on colchicine-binding activity, in liver supernatants from normal and turpentine-treated rats, different colchicine concentrations ranging from 5 x 10^{-7}M to 10^{-4}M were assayed. As the homogenization buffer used in this study was different from that of Sherline et al., it was verified in preliminary experiments that the maximum colchicine-binding activity was reached after 60 min incubation. Incubation was stopped by adding 1 ml of 0.25 % activated charcoal suspended in distilled water. After 10 min the suspension was centrifuged at 2,200 g for 10 min at 4° C. A 100 µl aliquot of the supernatant was added to 10 ml of Dimilume and radioactivity was measured at 35 % efficiency in an Intertechnique SL 40 liquid scintillation spectrometer (Intertechnique, Plaisir, France). These assay conditions gave a linear response when tested over a range of 0.3 to 3 mg of total protein per 100 µl of the liver supernatant fractions. All measurements were made in duplicate.

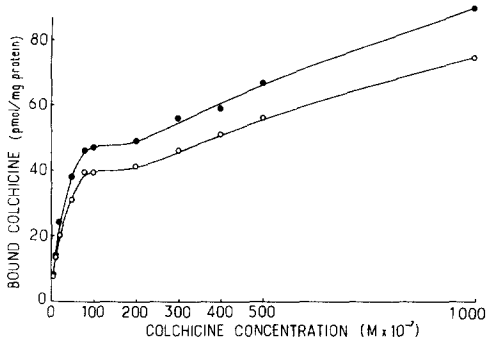

Figure 1. Influence of colchicine concentration on colchicine-binding activity in liver supernatants from normal (o—o) and turpentine-treated rats (•—•). Liver 100,000 g supernatants were prepared in cold MES buffer and incubated with various colchicine concentrations of labeled colchicine as described in Materials and Methods. Each value is the mean of three experiments.

Total protein content of liver supernatants was measured by the method of Lowry et al (18) using bovine serum albumin as standard.

The student's t-test was used for statistical analysis.

RESULTS

Influence of colchicine concentration on colchicine-binding activity

The dependency of colchicine-binding activity of liver supernatants from normal and turpentine-treated rats, upon colchicine concentrations is shown on Figure 1. Liver supernatants from normal rats showed the presence of two binding sites for colchicine, as it had been already reported (19, 20). Saturation of the first, high-affinity site, which corresponds to tubulin, was reached at 10^{-5}M colchicine ; higher colchicine concentrations caused a further rise which corresponds to non-specific, low-affinity binding (19, 20). In liver supernatants from turpentine-treated rats, colchicine-binding activity was higher than in normal ; however, saturation of the high-affinity site occurred at the same colchicine concentration.

Tubulin measurements during the acute inflammatory reaction

Results are shown in Table I. In normal Sprague-Dawley rat livers, free tubulin accounted for about 60 % of total tubulin, the remaining 40 % being

TABLE I

TUBULIN LEVELS IN SUPERNATANTS OF LIVER HOMOGENATES FROM NORMAL RATS AND FROM RATS INJECTED WITH TURPENTINE 24 HOURS BEFORE MEASUREMENT.

	Total tubulin pmol/mg protein	Free tubulin pmol/mg protein
Normal	39.8 ± 3.3	22.4 ± 1.9
Turpentine	48.8 ± 2.9[a]	22.8 ± 4.1[b]

Total and free tubulin were measured by colchicine-binding assay and are expressed as pmol of bound colchicine per mg of total supernatant protein. Each value is the mean ± standard deviation of 6 determinations.

a $p < 0.001$.
b not significant.

polymerized tubulin. During the inflammatory reaction which occurred 24 hours after turpentine injection, total tubulin increased significantly by 23 % but free tubulin remained unchanged. Measurement of the difference between total and free tubulin showed an increase of nearly 50 % in polymerized tubulin.

Tubulin measurements during the nephrotic syndrome

Results are shown in Table II. As in normal Sprague-Dawley rats, the free tubulin in normal Lewis rat livers, amounted to about 60 % of total tubulin. In nephrotic rat livers, total tubulin rose significantly by 30 % while free tubulin remained unchanged. This rise corresponded to a 90 % increase in polymerized tubulin. In control rat livers injected either with adjuvant only or with adjuvant and antigen but which were not nephrotic, total and free tubulin were unchanged compared to the normal level.

DISCUSSION

We studied two animal models in which hepatic plasma protein secretion was stimulated. The acute inflammatory reaction produced in rats by turpentine injection leads to increased synthesis and secretion by the hepatocytes of the group of plasma proteins known as acute phase reactants, whose plasmatic concentration rises (10). The experimental nephrotic syndrome induced in other rats

TABLE II

TUBULIN LEVELS IN SUPERNATANTS OF NEPHROTIC AND CONTROL RAT LIVER HOMOGENATES.

	Total tubulin pmol/mg protein	Free tubulin pmol/mg protein
Normal	37.2 ± 2.8	23.2 ± 2.8
Control 1	36.3 ± 4.4[a]	23.3 ± 2.2[a]
Control 2	39.8 ± 4.3[a]	22.0 ± 4.6[a]
Nephrotic syndrome	48.8 ± 5.0[b,c,d]	22.3 ± 3.0[a]

Total and free tubulin were measured by colchicine-binding assay and are expressed as pmol of bound colchicine per mg of total supernatant protein. Each value is the mean ± standard deviation of 6 determinations.

Control 1 : Rats injected with adjuvant only.
Control 2 : Rats which were injected with antigen and adjuvant but developed no nephrotic syndrome.

a not significant compared to normal.
b $p < 0.001$ compared to normal.
c $p < 0.01$ compared to control 1.
d $p < 0.02$ compared to control 2.

was characterized by severe proteinuria mainly formed by albumin (11) and by a dramatic fall in the plasma albumin concentration. The syndrome enhances albumin and other plasma protein synthesis and secretion by the hepatocytes (11, 12).

Methods using the $[^3H]$ colchicine-binding assay have been employed to estimate either total tubulin or total and free tubulin content in high-speed liver supernatants (8, 9, 17, 19, 20). The limitations of these methods have been extensively discussed elsewhere (8). The 10^{-5}M colchicine concentration used in the assay, saturated the high-affinity site corresponding to tubulin (19, 20), in liver supernatants from both normal and turpentine-treated rats (fig. 1). In these conditions, the non-specific binding which occurs at higher colchicine concentrations, was avoided (19, 20). Although the influence of colchicine concentration was not tested in nephrotic rats, similar results are likely to be obtained. The colchicine-binding assay only takes into account tubulin in the free form (13) and cannot evaluate the amount of polymerized tubulin, i.e. microtubules or aggregated tubulin material, that are measured by immunological methods (21). Using the colchicine-binding assay, total liver tubulin was estima-

ted after depolymerization of the microtubules by cold. The amount of tubulin in the form of microtubules was calculated from the difference between total and free tubulin.

Our results are identical to those of Patzelt et al (8) for normal mouse livers. Thus, normal rat livers contained about 60 % of free tubulin and therefore 40 % of polymerized tubulin. When plasma protein secretion increased, in the acute inflammation and the nephrotic syndrome, the amount of total liver tubulin rose significantly, by 23 % and 30 % respectively, but there was no change in the amount of free tubulin. Accordingly, the amount of polymerized tubulin increased by 50 % during inflammation and by 90 % during the nephrotic syndrome, compared to normal conditions. As the nephrotic model required injection of adjuvant and induction of a glomerulonephritis, we verified that total and free liver tubulin were not different from normal levels, in rats injected either with adjuvant only (control 1, Table II) or with adjuvant and antigen but which were not nephrotic (control 2, Table II). Since polymerized tubulin reflects the presence of microtubules (8), the amount of microtubules rises with plasma protein secretion. This not only supports the hypothesis that hepatic microtubules play a part in plasma protein secretion, but also indicates that increased secretion requires more microtubules.

Our results differ from those of Reaven and Reaven (22) who found no change in the tubulin or microtubule content of hepatocytes when lipoprotein secretion was raised. Nevertheless, our results are in agreement with two investigations whose authors observed a connection between the number of hepatic microtubules and plasma protein secretion by the liver when the number of microtubules was reduced. Reaven and Reaven showed that the number of microtubules and the lipoprotein secretion rate were tightly coupled when microtubules were gradually disrupted by increasing doses of colchicine (23). Similarly, partial disruption of hepatocyte microtubules by alcohol or its metabolites has been shown to lower plasma protein secretion (24, 25).

The rise we observed in the amount of total tubulin implies that tubulin synthesis increased in response tc augmented plasma protein secretion. Although

the possibility cannot be ruled out that enhanced synthesis of the secretory proteins might have induced a general rise in hepatic proteins, including tubulin, it should be stressed that only the polymerized form increased. The absence of change in the amount of free tubulin seems to indicate that hepatocytes require the maintenance of a constant free tubulin pool, perhaps because this form of tubulin interferes with the regulation of tubulin synthesis, as recently suggested by Ben-Ze'ev et al (26). According to these authors the amount of free tubulin would regulate tubulin synthesis, and the regulatory system would maintain a constant free tubulin pool, independently of the polymerized tubulin pool.

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