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MICROFILAMENT ACCUMULATION AND THE TRANSPORT OF AMINO ACIDS AND GLUCOSE IN ADULT RAT HEPATOCYTES CULTURED ON COLLAGEN GEL/NYLON MESH

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Summary: Transport of α-aminoisobutyric acid in cultured hepatocytes is temperature- and energy-dependent, whereas transport of 2-deoxy-D-glucose is not energy-dependent. In early cultures of hepatocytes (day 2) on a collagen gel/nylon mesh, the cells contain few microfilaments and the transport of amino acids and glucose is 5-7 times more than in late cultures of hepatocytes (day 6) which contain an apical, extensive accumulation of microfilaments. Cytochalasin D has little effect on the transport of amino acids and glucose in day 2 cultures of hepatocytes, but enhances transport of both compounds in day 6 cultures. These findings suggest that the microfilament accumulation in cultured hepatocytes inhibits transport of amino acids and glucose.

Introduction

Adult rat hepatocytes have been successfully maintained in culture on a floating collagen gel substratum (1) or a collagen gel/nylon mesh substratum (2) with the retention of a variety of hepatic differentiated functions. In addition, such cultured hepatocytes exhibit, as a function of time in culture, certain fetal properties (2) and accumulate numerous microfilament structures (2,3). The accumulation and the properties of these microfilaments have been described (4). Electron microscopy revealed that the microfilaments accumulate as a compact network almost exclusively beneath the plasma membrane interfacing with the culture medium. These microfilaments contain actin. Their extensive accumulation is due in part to the synthesis of actin and

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Abbreviations used are: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; AIB, α-aminoisobutyric acid; dG, 2-deoxy-D-glucose; PBS, Dulbecco's phosphate-buffered saline; PBS-AI, PBS containing bovine serum albumin (2 mg/ml) and 0.1 μM insulin; PBS-A, PBS containing bovine serum albumin (2 mg/ml) only; PBS-A/W, PBS-A supplemented either with 1 mM AIB or 1 mM dG for washing of cells incubated with [^{14}C]AIB or [^{3}H]dG, respectively.

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the formation of new microfilaments. However, the function(s) of the network of microfilaments in the hepatocytes of this culture system have not been elucidated. Their role could be contractile, resulting in increased tension in the epithelial cell sheets or in cell migration. Furthermore, microfilaments might play a cytoskeletal role, involving such activities as endocytosis, secretion, transport processes, and regulation of the topographical distribution of membrane proteins.

The transport of nutrients, e.g., amino acids and glucose, into the hepatocytes is an important and vital cellular process. Amino acids not only provide the building blocks for protein synthesis and substrates for gluconeogenesis under certain conditions, but also may play an important role in controlling DNA synthesis and cell multiplication (5). The metabolism of glucose provides energy for various cellular activities. Since microfilaments accumulate to a significant level in hepatocytes after 6 days in culture but not in those of 2 days in culture (4), this culture system provides a unique tool in studying the role of microfilaments on the tranpsort processes.

Materials and Methods

Adult rat hepatocytes were isolated from normal male albino rats (Holtzman Co., Madison, WI; 180-220 g) by collagenase (Type I; Worthington Biochemical Corp., Freehold, NJ) perfusion and maintained in monolayer cultures as described previously (4). Cultures were maintained on collagen gel/nylon meshes at 37°C in Leibovitz (L-15) medium, pH 7.4, supplemented with Hepes[2] (18 mM), albumin (2 mg/ml), penicillin (100 units/ml), streptomycin (100 µg/ml), insulin (0.5 µg/ml), and glucose (1.5 mg/ml) (hereafter referred to as L-15 culture medium).

The hepatocytes were maintained in cultures for 2 or 6 days before the transport processes of α-[^{14}C]-aminoisobutyric acid ([^{14}C]AIB, specific activity: 52 mCi/mmol; New England Nuclear, Boston, MA) or 2-[1,2-^{3}H]-deoxy-D-glucose ([^{3}H]dG, specific activity: 40 Ci/mmol; NEN) were measured. The cells were washed 3 times with PBS containing 2 mg/ml bovine serum albumin and 0.1 µM insulin (PBS-AI) prewarmed at 37°C. The cells on the gel/meshes were then transferred to culture dishes containing PBS-AI supplemented with either [^{14}C]AIB (1 mM, 0.1 µCi/ml) or [^{3}H]dG (1 mM, 0.5 µCi/ml) and incubated at 37°C for various periods of time with or without other reagents. At the end of the incubation, the cells on the gel/meshes were quickly transferred to dishes containing ice-cold PBS-A supplemented either with 1 mM AIB or 1 mM dG (PBS-A/W). The cells were washed 5 times with PBS-A/W at 0°C before being harvested by treatment with a solution of PBS-A/W containing collagenase (1 mg/ml) and soybean trypsin inhibitor (0.1 mg/ml) at 37°C for 5 min. The cells were collected by centrifugation at 200 X g for 5 min at 4°C and washed 2 more times with PBS-A/W at 0°C. The radioactivity in all washes was monitored

Table 1. Effects of Low Temperature and Cyanide Ion on the [^{14}C]AIB Uptake and [^{3}H]dG Uptake by Adult Rat Hepatocytes

Treatments	[^{14}C]AIB		[^{3}H]dG	
	nmol/mg DNA	% of Control	nmol/mg DNA	% of Control
Control	15.5 ± 0.7	100	55.9 ± 2.6	100
+CN^- (2 mM)	11.3 ± 1.0*	73	64.3 ± 4.7	115
+CN^- (20 mM)	8.3 ± 0.6*	53	58.7 ± 3.7	105
4°C, 5 min.	0.6 ± 0.1*	4	20.4 ± 1.9*	37
4°C, 10 min.	0.5 ± 0.1*	3	25.0 ± 1.8*	45

Day 2 cultures of hepatocytes were incubated at 37°C for 10 min, or at 4°C for 5 and 10 min, in PBS-AI containing [^{14}C]AIB or [^{3}H]dG. KCN was added to the incubation medium to concentrations of 2 and 20 mM, with necessary pH adjustments. The average uptake of [^{14}C]AIB or [^{3}H]dG over the 10-min incubation period was measured in these cultures as described in Materials and Methods. Results were expressed as nmol AIB or dG taken up/mg DNA, and shown as mean ± s.d. from 3 separate experiments.

*values significantly different from the control value by Student's t-test; $p < 0.01$.

and in general found to decrease exponentially to a very low level in the last wash (less than 0.002% of total radioactivity). The cells after the final wash were in some cases monitored for their ability to exclude trypan blue; 90-95% of the cells were unstained. A "zero-time" control sample, which was dipped into the incubation mixture, immediately withdrawn, and washed as described above, was included for each set of experiments.

The washed cell pellets were resuspended with 0.5 ml ice-cold water and immediately precipitated with 0.5 ml 20% (w/v) trichloroacetic acid; 0.9 ml of the supernatants after centrifugation was neutralized with NaOH and counted in a liquid scintillation counter. The pellets were redissolved in 1N NaOH, precipitated with an equal volume of a 15% (w/v) trichloroacetic acid/2N HCl solution, and the DNA contents were determined by a technique modified from that described by Giles and Myers (6). The precipitates were washed twice with 5% (v/v) perchloric acid and hydrolyzed at 70°C for 20 min in 0.6 ml 5% (v/v) perchloric acid. From the hydrolyzed sample, 0.5 ml of the supernatant obtained by centrifugation at 1000 X g for 10 min was removed. To this were added 0.1 ml of 60% (v/v) perchloric acid, 0.6 ml of a 4% diphenylamine/glacial acetic solution, and 30 µl of an aqueous acetaldehyde solution (1.5 mg/ml). The well-mixed solutions were kept in the dark overnight, and the absorbance values were then determined at 595 nm. A standard curve with calf thymus DNA was prepared.

Results

The uptake of nutrients by adult rat hepatocytes cultured on collagen gel/nylon meshes was investigated in this study using nonmetabolizable analogs of an amino acid and glucose, namely, AIB (7) and dG (8) respectively. Table 1 shows that in hepatocytes cultured for 2 days the average

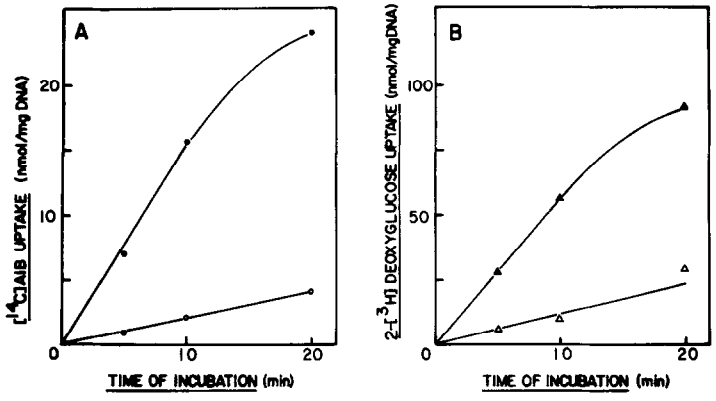

Fig. 1. Time dependence of (A) [^{14}C]AIB and (B) [^{3}H]dG uptake in adult rat hepatocyte cultures. Hepatocytes after 2 days (●,▲) and 6 days (○,△) in culture were incubated at 37°C in PBS-AI containing [^{14}C]AIB or [^{3}H]dG for different periods of time. The uptake of radioactivity in these cultures was determined as described in Materials and Methods.

rates of uptake of AIB and dG were 15.5 nmol/mg DNA and 55.9 nmol/mg DNA per 10 min respectively. The uptake of AIB was severely inhibited by low temperature (4°C) and was reduced 30-50% in the presence of a metabolic inhibitor, cyanide ion. Similarly, the uptake of dG was diminished by low temperature, although to a lesser degree. However, cyanide ions at concentrations of 2 and 20 mM did not exert any inhibitory effect on the uptake of dG. When the time course of both AIB and dG uptakes was followed at 37°C in the cultured hepatocytes, the uptake of either compound in day 2 cultures of hepatocytes was linear with time for the first 10 min in incubation, and leveled off after 10 min of incubation (Fig. 1). Surprisingly, the uptakes of both compounds in the day 6 cultures of hepatocytes were linear up to 20 min of incubation, and the slopes of these curves were all smaller than those for the day 2 cultures of hepatocytes. During 10 min of incubation, the average uptakes of AIB and dG in day 2 cultures of hepatocytes were 5- and 7-fold, respectively, greater than those in day 6 cultures of hepatocytes.

In light of the significant accumulation of microfilaments in hepatocytes cultured for 6 days but not those cultured for 2 days (4), the micro-

Table 2. Effects of Cytochalasin D on the [^{14}C]AIB Uptake and [^{3}H]dG Uptake by Adult Rat Hepatocytes

Treatments	Day 2 cultures		Day 6 cultures	
	nmol/mg DNA	% of Control	nmol/mg DNA	% of Control
[^{14}C]AIB Uptake				
Control	15.5 ± 0.7	100	2.1 ± 0.1	100
+ Cytochalasin D (1 μM)	14.3 ± 0.9	92	3.0 ± 0.2*	143
[^{3}H]dG Uptake				
Control	55.9 ± 2.6	100	9.9 ± 0.8	100
+ Cytochalasin D (1 μM)	52.5 ± 3.8	94	13.1 ± 0.7*	132

Hepatocytes after 2 and 6 days in culture were incubated at 37°C for 10 min in PBS-AI containing [^{14}C]AIB or [^{3}H]dG, with or without the addition of 1 μM cytochalasin D. The average uptake of [^{14}C]AIB or [^{3}H]dG over the 10-min incubation period was measured as described in Materials and Methods. Results were expressed as nmol of AIB or dG taken up/mg DNA, and shown as mean ± s.d. from 3 separate experiments.

*values significantly different from the control value by Student's t-test; $p < 0.005$.

filament-disrupting drug, cytochalasin D, was employed to perturb the microfilaments in order to investigate the possible relation between microfilament accumulation and the transport process. In Table 2, the presence of 1 μM cytochalasin D during the transport of both AIB and dG brought about a slight inhibition in the day 2 cultures of hepatocytes, but a significant enhancement in the day 6 cultures of hepatocytes. Cytochalasin D increased the uptake of both AIB and dG by 30-40% in day 6 cultures of hepatocytes, which had a significant accumulation of microfilaments prior to the cytochalasin D treatment (4).

Discussion

The transport of amino acids and glucose into adult rat hepatocytes cultured on collagen gel/nylon mesh appears to proceed by different mechanisms. Transport of neutral amino acids, as studied by measurements of AIB uptake, is both energy- and temperature-dependent and exhibits a high temperature quotient (Q_{10}), suggesting a facilitated diffusion mechanism,

as found in other cell types (9). Transport of glucose, as studied by measurements of dG uptake, is indepedent of cellular energy production, dependent on temperature but with a low Q_{10}, suggesting a simple diffusion mechanism (8,9) in liver tissue (10). AIB is mainly transported by the A, or alanine-preferring and Na^+-dependent, system for neutral amino acids in a variety of cell types (9,11), including cultured hepatocytes (12) and hepatoma cells (12,13). Hormonal regulation of AIB transport in these two cell types has also been reported (11-14).

The transition of nonproliferating hepatocytes cultured on collagen gel/nylon mesh from an early (day 2) to a late (day 6) age brings about a series of changes (2), one being the prominent accumulation of microfilaments (4). When the transport processes of low molecular weight nutrients are studied in the early and late cultures, an immediate difference is apparent (Fig. 1). The transport of either AIB or dG in a late culture of hepatocytes is only one-fifth and one-seventh respectively, of that in an early culture of hepatocytes. The inhibition, observed in both cases to a similar degree, is evidently independent of the transport mechanism and may likely be a manifestation of some general changes associated with the accumulation of microfilaments. One possible site where such changes may occur is at the plasma membrane, with which both an interaction and association of microfilaments have been well documented (15). By using cytochalasin D, which can induce depolymerization of microfilaments in cultured hepatocytes (Mak and Pitot, unpublished observations) and other cells (16) by binding with high affinity to a membrane protein complex (17), the interaction and association between the plasma membrane and microfilaments could be perturbed. Indeed, cytochalasin D treatment of day 6 cultures of hepatocytes brought about an increase rather than an inhibition of the transport processes. The drug had little, if any, effect on the transport processes in the day 2 cultures of hepatocytes. It has been shown that cytochalasin B did not inhibit AIB uptake in cells (18), and cytochalasin D did not inhibit sugar

transport (19). Our results with the cytochalasin D enhancement of transport may conform to the hypothesis proposed by Lever (20), in which the association and interaction of microfilaments with the plasma membrane exert primarily conformational effects on the mobility and topography of membrane proteins (especially transport carrier proteins), secondarily affecting transport. At least in the adipocytes, a positive correlation between membrane fluidity and glucose transport has been demonstrated (21). In cultured hepatocytes, it was suggested that the microfilaments play an important role in maintaining the integrity of the cell surface (22) and that AIB transport increased with the transmembrane potential (23). A plausible explanation for the cytochalasin D enhancement of transport is that the binding of cytochalasin D to the plasma membrane (16) induces the dissociation and disruption of microfilaments and subsequently alters the fluidity of the membrane, resulting in an increase in transport. In fact, a similar finding was observed in this culture system for cellular endocytosis (24). These data could also be explained by the hypothesis proposed herein.

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