Phalloidin Alters Bile Canalicular Contractility in Primary Monolayer Cultures of Rat Liver

SUMIO WATANABE, MAMORU MIYAIRI, CHIKARA OSHIO, CHARLES R. SMITH, and M. JAMES PHILLIPS
Departments of Pathology and Pediatrics, Hospital for Sick Children; University of Toronto, Toronto, Ontario, Canada

We observed the motility of bile canaliculi in isolated rat hepatocytes obtained from animals that were pretreated with phalloidin (500 µg/kg body wt) for 1 and 3 days. Time-lapse cinephotomicrographs were taken in each experiment and in controls for 16.25 h. As we previously reported in normal hepatocytes, active contractions of bile canaliculi were observed. The number of contractions was 127.6 in controls, compared with 58.0 in 1-day phalloidin pretreated and 16.2 in 3-day phalloidin pretreated groups. The contraction process itself was not normal in the experimental groups, the contraction phase being slow and sustained. The altered canalicular motility depended on the dose of phalloidin administered. These results suggest that the integrity of actin filaments is necessary for normal bile canalicular motility. Further, dysfunction of actin microfilaments with altered canalicular motility may be involved in the pathogenesis of canalicular cholestasis.

Actin is present in a variety of nonmuscle cells, including hepatocytes. Actin filaments play an important role in the maintenance of cell shape, cell movement, phagocytosis, secretion, cell division, and cell communications (1).

In hepatocytes, actin filaments are especially numerous near the bile canaliculi, as has been shown by numerous techniques (2–8). These pericanalicular actin filaments may play a role in the mechanism of bile flow (2,9), but the precise mechanism is not known. Recently, we observed active contractions of bile canaliculi in freshly prepared monolayer cultures of rat hepatocytes using time-lapse cinephotomicrography (9) and have recently shown that bile canalicular contractility is sensitive to cytochalasin B (10).

Phalloidin, a cyclic peptide isolated from Amanita phalloides, is another agent that alters actin filaments in liver cells. Its administration to rats is associated with a decrease in bile flow and an increase of actin filaments throughout the cytoplasm of hepatocytes, especially around the bile canaliculi (11,12). Therefore, it has been hypothesized that microfilament dysfunction may be responsible for the cholestasis in phalloidin-treated rats. In view of this, it was considered of interest to observe whether phalloidin administration had an effect on the motility of bile canaliculi that can be observed in primary monolayer cultures of rat hepatocytes.

Materials and Methods

Animals

Female Wistar strain rats, each weighing ~250 g and fed ad libitum laboratory pelleted rat diet and tap water, were used for each experiment and control. Each of the experimental groups and the control group consisted of 5 rats.

Phalloidin Pretreatment

There were two experimental groups that underwent pretreatment with phalloidin. The phalloidin (Boehringer Mannheim, West Germany) was dissolved in 0.9% NaCl and a dose of 500 µg/kg body wt was injected into the peritoneal cavity. The first group received a single dose of
Figure 1. Micrographs of rat livers. a. The DACM-HMM staining of normal rat liver shows the typical "polygonal" pattern of the hepatocytes. Specific fluorescence is also seen on bile canaliculi as well as the inner part of blood vessels (×250). b. The DACM-HMM staining of phalloidin 1-day pretreated rat liver shows the increase of specific fluorescence in the region of bile canaliculi and on the submembranous area (×250). c. After 3 days of phalloidin pretreatment, a further increase in fluorescence is shown in the region of bile canaliculi. A few fluorescence spots are seen throughout the cytoplasm (×250).

phalloidin 24 h before liver cell isolation. The second group received a single dose of phalloidin daily for 3 days, and liver cell isolation was performed on the fourth day.

**Liver Cell Culture**

Liver perfusion and dissociation were performed according to a modification of the procedure of Seglen (13) and Laishes and Williams (14). The rats were anesthetized with sodium pentobarbital using 50 mg/kg body wt. An initial washout perfusion through the portal vein was performed with Ca²⁺ and Mg²⁺ free Hanks' balanced saline solution, which contained 0.5 mM ethyleneglycol-bis (β-aminoethylether)-N,N'-tetraacetic acid (EGTA), for 4 min at 40 ml/min. The subhepatic inferior vena cava was severed immediately before this perfusion was begun. A 0.05% solution of type 4 collagenase (Sigma Chemical Co., St. Louis, Mo.) in L-15 medium (15) was then perfused through the portal venous system for ~8 min at 25 ml/min. The solutions, which had been previously gassed with 100% oxygen, were kept at 38°C during perfusion. Upon termination of the perfusion, the liver was excised and placed in a large culture dish. It was held at the porta hepatis and cells were detached by combing with forceps.

The isolated liver cells were centrifuged at 50 g for 3–4 min and resuspended in 50 ml of fresh cold medium. The cells were then filtered through nylon mesh and recentrifuged. The yield of isolated hepatocytes ranged from 1.8 to 4.0 × 10⁶ per rat and there was no significant difference between the controls and the pretreated group. All solutions used for cell isolation were supplemented with 10 mM N-2-hydroxyethylpiperazine-N’-2-ethane sulfonic acid (HEPES). One million cells were inoculated into 60-mm Corning culture dishes (Corning Glass Works, Corning, N. Y.). After an interval of 2 h, during which time the hepatocytes attached themselves to the bottom of the dishes, the culture medium containing unattached cells was withdrawn and the dishes were replenished with fresh medium. In this procedure, the proportion of hepatocytes that attached to the dishes was calculated. It ranged from 80% to 95% of the living cells. There was no difference between the control and the treated group. The culture medium consisted of L-15 supplemented with 10% fetal bovine serum, 10 mM HEPES, penicillin (100 U/ml), and streptomycin (100 µg/ml).

The viability of the hepatocytes was examined both at the start and end of the experimental period. After initial isolation, the proportion of liver cells that were viable
ranged from 78% to 95% of the total cell count, as determined by the trypan blue exclusion test. In addition to the trypan blue exclusion test, the viability and the survival rate of the hepatocytes were judged by the time-lapse cinephotomicrography. In the movies, the living cells showed several types of motile activity throughout the cytoplasm, whereas the dead cells did not show any evidence of motility. Therefore, the viability and survival rate of the hepatocytes could be easily judged using this method. At the beginning of the period of cinephotomicrography, 80%-95% of the inoculated hepatocytes were viable, and there was no significant difference in the viability between the control and the pretreated groups. The survival rates of the hepatocytes were similarly determined at the end of each experiment. Of the hepatocytes that were viable at the start of the movie, 88%-96% were viable at the end of the period of study. There was no significant difference between the controls and the groups pretreated with phalloidin.

**Time-Lapse Cinephotomicrography**

An inverted camera microscope (ICM 405, Carl Zeiss Inc., New York, N. Y.) with phase contrast optics, 16 mm movie camera (H16, RX-5 Bolex), 16 mm reversal films (Plus-X 7276 Kodak), and a motor drive system with time-lapse controller (Nikon and Hommel Electronics) were used. All movies were taken at the speed of 1 frame per 15 s. The time-lapse movies were started 1 h after completion of the final medium change described above.

**Analysis of Movies**

Movie fields containing three representative bile canaliculi from each treated or control animal were photographed over a 16.25-h period (3900 movie frames). Each group consisted of 5 animals, for a total of 15 bile canaliculi from each treated or control animal were photographed over a 16.25-h period (3900 movie frames). Each group consisted of 5 animals, for a total of 15 bile canaliculi. Using an analytic movie projector (Photo-optical Data Analyser 2204A, MK-V, L-W International, Woodland Hills, Calif.), the diameter of each of the canaliculi was studied in a frame-by-frame fashion. Canalicular contraction was characterized by a discernable decrease in the size of the canalicular lumen. For each bile canaliculus, the movie frame numbers of the starting point and end point of every contraction were recorded. From this data, it was possible to determine the number of contractions of each bile canaliculus during the period of photography and the length of time required for each bile canaliculus to contract.

**Statistical Analysis**

The statistical analysis was performed using analysis of variance and a \( \chi^2 \) method.

**Electron Microscopy and Fluorescence Microscopy**

Representative samples of treated rat livers and isolated hepatocytes were examined by electron microscopy. Standard methods of tissue processing were used (16,17). The specimens were examined in a Philips FM 300 electron microscope (Phillips Electronic Instruments, Inc., Mahwah, N. J.).

In order to observe the distribution of actin filaments, we used the N-(7-dimethylamino-4-methylcoumarinyl) maleimide (DACM) labeled heavy meromyosin (HMM) method according to the procedure of Namihisa et al (6). The specimens were examined with a Leitz fluorescent microscope (E. Leitz, Inc., Rockleigh, N. J.).

**Morphometric Analysis**

For quantitation of the fluorescence staining of actin filaments of the liver cells, two frozen blocks were randomly selected from each rat and four pictures were taken from randomly chosen areas of each of the two blocks. Therefore, a total of eight pictures were taken from each rat and a total of 40 pictures were taken from the 5 animals studied in each group. The original pictures were taken at a magnification of \( \times 250 \) and printed at a final magnification of \( \times 4000 \).

For quantification of transmission electron microscopic studies, two blocks were randomly selected from each rat and 15 pictures of bile canaliculi were taken from randomly selected areas of each block. Therefore, a total of 30 pictures were taken from each rat and a total of 150 pictures were taken from the 5 rats studied in each group. The original pictures were taken at a magnification of \( \times 1500 \) and printed at a final magnification of \( \times 12000 \).

Morphometric analysis was performed according to the method of Wiebel(18).

**Results**

**Fluorescence Microscopy of Rat Liver**

The DACM-HMM staining of normal rat liver tissues showed the typical "polygonal" pattern of the hepatocytes (Figure 1a). Specific fluorescence of bile canaliculi as well as of the endothelium of blood vessels was seen. Bile canaliculi had a small dot or short band shape. After 1 day of pretreatment with phalloidin, the polygonal pattern of hepatocytes became obvious and the amount of peripheral fluorescence increased significantly (Figure 1b). After 3 days of phalloidin pretreatment, a further increase in fluorescence was shown, especially in the region of the bile canaliculi. A few fluorescent spots appeared throughout the cytoplasm in addition to strong peripheral fluorescence (Figure 1c).

**Electron Microscopy of Cultured Hepatocytes**

In the cultured hepatocytes of untreated rats, the ultrastructure was similar to that reported previously (19) (Figure 2a). The cultured hepatocytes...
pretreated with phalloidin for 1 day showed a mild thickening of actin filaments around the bile canaliculi, and bile canaliculi showed dilation with loss of microvilli (Figure 2b). In cultured hepatocytes pretreated for 3 days, the pericanalicular actin filament zone appeared thicker than that of the hepatocytes pretreated for 1 day. Bile canaliculi showed dilation with loss of microvilli (Figure 2c). Such morphologic changes were still recognizable at the end of the experimental period.

Morphometric Analysis

Fluorescence microscopy. The relative volume of the specific fluorescence of actin filaments per unit of the liver was 15.2% in the control, 32.3% in the phalloidin 1-day pretreated group, and 53.9% in the 3-day pretreated group. The difference between the three groups was significant at a probability of p < 0.001 (analysis of variance).

Electron microscopy. The relative volume of the pericanalicular actin filaments per unit of the hepatocytic cytoplasm was 1.1% in the control, 3.4% in the phalloidin 1-day pretreated group, and 7.3% in the 3-day pretreated group. The difference between the three groups was significant at a probability of p < 0.001 (analysis of variance).

Time-lapse movies of cultured hepatocytes. As we reported previously, several types of motile activity were observed throughout the cytoplasm of control hepatocytes. Some of these movements were of random, Brownian movement-like nature, while other pericanalicular phase-contrast-positive vacuolar movements were present. In some phalloidin

Figure 2. Micrographs of hepatocytes. a. This electron micrograph of isolated normal hepatocytes from one of the monolayer cultures was taken 6 h after isolation. The pericanalicular zone of actin filaments is not prominent, and tight junctions are well preserved (×8000). b. An electron micrograph of isolated phalloidin 1-day pretreated rat hepatocytes prepared from one of the monolayer cultures taken 6 h after isolation. This micrograph shows a mild thickening of actin filaments around the bile canaliculus. The lumen of this bile canaliculus shows dilation. There is a reduction in the number of microvilli (×8000). c. In 3-day pretreated cultured hepatocytes, the pericanalicular actin filament zone appears thicker than that at 1 day of pretreatment (compare with Figure 2b). A dilated bile canaliculus with loss of microvilli can be seen in this micrograph which was taken 18 h after isolation. Small vesicles are seen in the dilated bile canaliculus (×8000).
pretreated hepatocytes, we observed protrusions of the plasma membrane as have been noted by others (20).

To assess bile canalicular function, we analyzed the number of contractions that occurred and the length of time required for the canaliculi to contract (canalicular systole) (19). The mean numbers of observed contractions in 16.25 h were 127.6 per rat (three bile canaliculi) in untreated controls, 58.0 per rat (three bile canaliculi) in the phalloidin 1-day pretreatment group, and 16.2 per rat (three bile canaliculi) in the phalloidin 3-day pretreatment group. Each number is a mean total contraction number of three bile canaliculi of 1 rat. The difference is statistically significant at 1 and 3 days against controls and at 3 days against 1 day (analysis of variance).

Figure 4. The mean numbers of observed contractions in 16.25 h per rat are 127.6 in untreated controls, 58.0 in the phalloidin 1-day pretreated group, and 16.2 in the phalloidin 3-day pretreated group. Each number is a mean total contraction number of three bile canaliculi of 1 rat. The difference is statistically significant at 1 and 3 days against control and at 3 days against 1 day. As reported previously, canalicular systole of normal hepatocytes was 60±30 s (mean±SD) (19); in >95% of instances, it was <120 s. Therefore, we called the contractions that did not end within 120 s "abnormal contractions" (Figure 6). Using this criterion, we analyzed each contraction in the phalloidin pretreated and control groups. In the 1-day pretreated groups, 55% of the contractions were normal, as were 34% in the 3-day pretreated group (Figure 5); the controls were similar to previously reported results (95%).

In the control group, normal contractions are 95%. In the 1-day pretreated group, 55% of the contractions are normal; in the 3-day pretreated group, only 34% are normal. The difference is statistically significant at 1 and 3 days against control and at 3 days against 1 day (χ² method).
Figure 6. A phase-contrast micrograph of freshly isolated phalloidin 3-day pretreated rat hepatocytes in monolayer culture (printed from 16 mm movie film). a. Beginning of canalicular contraction (systole). Dilated bile canaliculus is shown by arrow. b. Fifteen minutes after a, the diameter of bile canaliculus decreased (arrow). c. Twenty-five minutes after a, contraction is completed (arrow) (×800) NOTE: This is a very slow and sustained contraction taking 25 min to complete; in normal cells, canalicular systole lasts 1 min.

Discussion

Microfilaments (actin filaments) are particularly abundant in the pericanalicular region of hepatocytes, and they may play an important role in bile formation. Two kinds of cytopharmacologic agents that affect actin filament structure and function, cytochalasin B and phalloidin, produce cholestasis in rats. In the liver, it was shown that cytochalasin B altered pericanalicular microfilaments in addition to reducing bile flow. Microfilament dysfunction was suggested as a possible explanation for the intrahepatic cholestasis (21). Bile canaliculus-enriched membrane fractions derived from cytochalasin-treated animals are devoid of attached actin filaments, as found in vitro as well (4). Because membrane attachment is necessary for the generation of force or tone, filament detachment from the canalicular wall would effectively remove their contractile action on the canaliculus and would also remove any support function these filaments may subserve. Since time-lapse cinephotomicrography of cultured hepatocytes showed that cytochalasin B markedly decreased the number of amplitude of bile canalicular contractions and resulted in a progressive increase in canalicular diameter, we proposed that cytochalasin-induced intrahepatic cholestasis may be an actin filament-based subcellular motility disorder of hepatocytes (10).

That phalloidin should affect canalicular function is not an unreasonable hypothesis. The liver cell has
a specific receptor for this compound (22). Phalloidin has a direct effect on actin filaments to which it binds, preventing depolymerization (22). The administration of phalloidin to isolated hepatocytes results in rapid bleb formation on the surface of the cells (20). When administered in small, repeated doses in vivo as in our experiments, the hepatocytic cytoplasm gradually fills with filaments which form a thick encasement around the bile canaliculi (11). This is accompanied by a reduction in bile flow (12). The precise mechanism for this phalloidin-induced cholestasis is unknown, but effects on tight junction permeability and the paracellular route of ion and water movement into bile have been suggested (23). Microfilament dysfunction has also been suggested as a possible mechanism to explain the cholestasis (11,12,24).

The results reported here show there was a significant decrease in the number of bile canalicular contractions and that the contraction process itself was not normal. This decrease in motility depended on the dose of phalloidin administered. Wehland et al. (25) reported that phalloidin-induced actin polymerization in the cytoplasm of cultured cells interfered with cell location and growth. We conclude that the decreased motility of bile canaliculi in phalloidin-treated hepatocytes is a result of the increase in polymerized actin. When considered with respect to the secretion of bile into the bile canaliculi, it is remarkable that the bile canalicular lumen did not widen progressively in the phalloidin-treated hepatocytes in the face of the diminished contractions. This lack of progressive canalicular filling may reflect a decrease in bile secretion by the hepatocytes. It is likely that the increased polymerized actin in the hepatocyte has effects on cellular transport processes that could not be analyzed in the present study but which might influence bile formation or secretion, or both. An alternate explanation for our results is that the canalicular encasement by filaments or their altered contraction pattern, or both, prevents canalicular distention and perhaps impedes bile flow.

In the present study, we showed that phalloidin administered to rats for 1 and 3 days before isolation was associated with a decrease in contractility of bile canaliculi and an increase in number of pericanalicular actin filaments. These phalloidin results, taken in conjunction with the cytochalasin time-lapse studies cited (10) and the reported work on the cholestatic effects of both of these agents (11,12,21,23), provide strong support for the hypothesis that normal liver function is dependent on intact actin-containing microfilaments. Furthermore, microfilament dysfunction with impaired canalicular motility may be important in the pathogenesis of intrahepatic cholestasis.

References