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IN VIVO EFFECTS OF COLCHICINE ON MILK FAT GLOBULE MEMBRANE *

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Milk secretion in lactating goats was suppressed reversibly by infusing colchicine (2.5 to 5 mg) into one half of the udder via the teat canal. Fat globules were isolated from milks before, during and after (96 h post-infusion) this suppression. Protein, phospholipid, cholesterol (free and esterified), 5′-nucleotidase activity and peptide patterns by gel electrophoresis of these globule samples were determined. Association of [^{14}C]colchicine with milk fat globules in vivo and in vitro also was investigated. Amounts of protein, phospholipid and free cholesterol per g of globule and 5′-nucleotidase per mg of globule protein fall following colchicine infusion. The nature of these changes suggests that the supply of membrane for milk secretion is restricted as a result of the drug treatment. Patterns of globule peptides by gel electrophoresis were qualitatively similar during the experimental period. However, a major globule glycoprotein, $M_r = 52\,000$, showed a significant (3-fold) increase relative to the other principal peptide bands during the period of reduced milk flow. Analysis of milks for radioactivity following infusion of [^{14}C]colchicine revealed that a portion of activity returning in milk is associated with fat globules. This activity peaked at 72 h post-infusion. Evaluation of [^{14}C]colchicine binding to milk fat globules in vitro yielded evidence that the drug binds to the cytoplasmic, but not the exterior surface of the globule membrane. Colchicine's inhibition of milk synthesis and secretion is discussed.

Introduction

Colchicine, the plant alkaloid and microtubule antagonist, suppresses exocytosis in a variety of tissues and cells including liver [1,2], pancreas [3], parotid gland [4] and lactating mammary gland [5,6]. The suppression of milk secretion involves not only the exocytotic mechanism by which the aqueous phase (proteins, lactose, salts, water, etc.) is secreted but also the unique budding process by which milk fat droplets are enveloped in plasma membrane and released from the cell. For review of these two mechanisms, which appear to be coupled [7,8], see Ref. 9.

As in other cells, microtubules are an essential element of the cytoskeleton in the lactating cell [10]. Both colchicine and the vinca alkaloids cause disassembly of microtubules [11] and suppression of milk secretion [12,13]. It is not known whether microtubules are directly involved in the mechanisms of milk secretion, for example, in moving secretory vesicles, or more indirectly in maintaining cell size and structural organization. For further evidence and discussion of this matter, see Refs. 14–17.

We have been attempting to reveal the means by which colchicine has its unique inhibiting effect on lactation. Initial studies showed reversible sup-

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pression of milk flow when the alkaloid was infused into the lactating goat gland via the teat canal [6]. This has been confirmed in the goat [18] and cow [19]. Subsequently an ultrastructure and morphometric investigation of colchicine-induced changes in lactating tissue (rat and goat) revealed extensive accumulation of secretory products within cells of drug treated-glands [14]. It was noted that secretory vesicles, though abundant and crowded in the apical (secretory) region of the cell, were decidedly smaller than normal. One would have expected them to be larger as a result of continuing synthesis and arrested secretion. Indeed, this was the case with milk fat droplets [8]. As one possibility, these conditions suggested a membrane defect in that the vesicles had reduced capability for either vesicle-vesicle fusion to increase size or vesicle-plasma membrane fusion in exocytosis. In light of this observation we investigated effects of the drug on membrane issuing from the cell in the secretion of milk fat globules.

Materials and Methods

The general experimental plan was to infuse colchicine into goat lactating glands, to collect milkings before, during and after the milk flow suppresion period, to isolate milk fat globules (the bearers of secretory membrane), from these milks, and to analyze the globules for various membrane properties and constituents.

Colchicine infusions and milk collections. An experimental herd of 6 to 8 goats with daily milk productions from 1 to 3 liter per day per animal were used. They were fed hay and grain with water available ad libitum. Colchicine (Sigma Chemical Co., St. Louis, MO, U.S.A.), 2.5–5 mg dissolved in 5 ml of distilled H_2O was the infusate in each of four similar experiments. This solution was drawn into a 5 ml syringe and infused with the aid of a cannula into either the right or left gland via the teat canal. Infusions were always made after completely milking both glands. For 12 to 24 h before (2 or 3 milkings) and up to 96 h after an infusion, complete milkings from both glands were collected separately, measured for volume and held for various fractionations and analyses described below.

Radioactive colchicine was employed in one infusion experiment and in several in vitro binding experiments. This was [*ring C-methoxy*-^{14}C]colchicine with specific activity of 57.08 mCi/mol (New England Nuclear, Boston, MA, U.S.A.). One day prior to infusion the goat was placed in a portable wooden metabolism stall for the 7 days of the experiment. The infusion was 50 μCi of [^{14}C]colchicine with 4 mg of unlabeled colchicine in 5 ml of H_2O.

Preparation of globules and membrane therefrom. Fat globules were isolated by centrifuging milk in 38-ml plastic tubes at $1000 \times g$ for 20 min in a Sorvall refrigerated centrifuge, Model RC-5B, using rotor SS-34 at room temperature. The skim milk was decanted from under the layer of globules. The layer was gently resuspended in 0.25 M sucrose to 38 ml and the suspension centrifuged as before. Washing with sucrose solution at room temperature was repeated twice. After the final centrifuging fat layers were hardened in ice for 60 min, the sucrose phase decanted and the layers resuspended with mixing to 38 ml with distilled H_2O adjusted to 37°C. When not used immediately, the globule suspensions were stored at −18°C for subsequent analyses.

To prepare membrane, washed globules from 120 ml of milk were suspended in a small volume of distilled H_2O and churned in a Polytron (Brinkman Instruments, Westbury, NY, U.S.A.). The aqueous phase was decanted from the butter and centrifuged at $40\,000 \times g$ and 4°C for 1 h. The resulting membrane pellet was suspended in distilled H_2O by vortex agitation and used in colchicine binding experiments.

Analysis of milk and milk fat globules. Total fat and protein in milk samples were estimated by turbidity and dye binding measurements, respectively, in the automatic Milko-Tester analyzer (Foss Electric Company, Hillerod, Denmark). Globule lipids were extracted by the Roese-Gottlieb method [20]. Extracts were then evaporated to dryness under vacuum at 40°C and total lipids were quantitated by weighing. Since milk fat globules are 98–99% lipid, measurements of total lipids are equivalent approximations of globule weights.

Neutral lipids were separated in 15-g silicic acid columns with 150 ml of diethyl ether, and polar lipids were eluted from columns with 150 ml of methanol. Lipid fractions were stored in chloroform/methanol (19 : 1, v/v) at −18°C. Individual

classes of phospholipids were determined in duplicate using two-dimensional thin-layer chromatography and phosphorus analysis of spots [21]. Total and unesterified cholesterol in neutral lipids were assayed by the method of Searcy et al. [22]. Analysis of unesterified cholesterol required preliminary separation of neutral lipids by thin-layer chromatography. For this purpose precoated silica gel G plates (Merck) and a solvent system of petroleum ether/ethyl ether/acetic acid (75 : 25 : 1, v/v) were employed. Areas on plates corresponding to free cholesterol were scraped and analyzed together with suitable controls. Total protein in globule and membrane dispersions was quantitated by the method of Lowry et al. [23] after removing excessive lipid by extraction with ice-cold *n*-butanol. Bovine serum albumin (Sigma Chemical Co., St. Louis, MO, U.S.A.) was used as a standard. Assays for 5′-nucleotidase were performed by the procedure of Touster et al. [24] using adenosine monophosphate as substrate and 20-min incubation time at 37°C. The inorganic phosphorus formed in the reaction was determined by the method of Cheng et al. [25].

Washed milk fat globule samples (freeze-dried and without removal of lipid) were prepared for sodium dodecylsulfate (SDS) polyacrylamide gel electrophoresis, with SDS, mercaptoethanol and heating as prescribed by Laemmli [26]. The electrophoresis was performed in 8% polyacrylamide slab gels 1.5 mm thick, in the presence of SDS using the Bio-Rad Model 220 electrophoresis apparatus. The gels were stained either with Coomassie blue [26] or with the periodic acid-Schiff reagent (PAS) for glycoproteins [27]. Gels were photographed and sliced into bands which were scanned in a Gilford Model 250 spectrophotometer at 560 nm (Coomassie blue-stained slices only).

Colchicine binding studies. Association of colchicine with milk fat globules in vivo was studied by infusing [^{14}C]colchicine and collecting 12-h milk samples as described above. Radioactivities in milkings from both sides of the udder were determined. [^{14}C]Colchicine activity of globules was derived as the difference between activity of the whole milk and that of the skim milk prepared (see above) therefrom. Colchicine binding studies in vitro were conducted with washed milk fat globules and milk fat globule membreane prepared as previously stated. All incubations and radioactivity countings were done in duplicate. Into 100 μl of membrane suspension (0.5 mg of protein), 100 μl of [^{14}C]colchicine in 10^{-6} M solution was added and the final volume was made to 1 ml with water. The suspensons were incubated at 37°C with gentle shaking. After various intervals of incubation, suspension samples were centrifuged at $50\,000 \times g$ for 20 min at 4°C, and 100-μl aliquots were taken from the clear infranatant of each sample for assay of radioactivity. To study binding to intact globules, 900 μl of the globule suspension (0.5 mg of protein) was incubated with 100 μl of [^{14}C]colchicine in 10^{-6} M solution as in the case of membranes. After the incubation the globules were centrifuged at $1000 \times g$ for 20 min at 4°C and a 100-μl aliquot was taken from the clear aqueous layer to estimate the amount of free radioactivity in the solution. All determinations were done in duplicate.

Radioactivity was determined with a Packard Tri-Carb liquid scintillation spectrometer (Model 3330, Packard Instrument Co., Downers Grove, IL, U.S.A.). Sample activities were counted in 10 ml of Quantafluor Scintillar (Malinckrodt, Inc., St. Louis, MO, U.S.A.).

Results

Data representative of the effect of colchicine on milk yield from infused and uninfused glands are shown in Fig. 1. The reversible suppression of milk secretion from the infused side with minimum yield at 36 h agrees closely with previous observations [6,12]. Globules isolated from milkings for the experiment, Fig. 1, were analyzed for protein, phospholipid and cholesterol. Data for the infused side, Fig. 2, showed significant depressions in all three parameters with lowest values for the 48-h post-infusion milking. Data for milkings from the uninfused side (not shown) revealed only minor fluctuations from pre-infusion levels. The depressions in protein and phospholipid, Fig. 2, were of similar magnitude, to about 50% of the pre-infusion concentrations. The observed drop in phospholipid agrees with earlier values [8] for lipid phosphorus of globules under such conditions. The maximum drop in unesterified cholesterol was 19%. Total cholesterol values were essentially the same

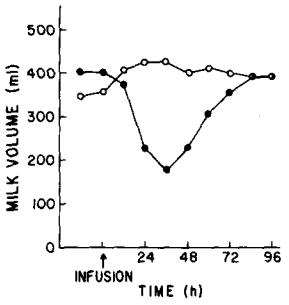

Fig. 1. Effect of an intramammary infusion of colchicine on volumes of complete milkings at 12-h intervals from infused (●———●) and uninfused (○———○) sides of a goat's udder. The infusion was 2.5 mg of the alkaloid in 5 ml of water into the gland via the teat canal immediately following milking at time zero.

as those for unesterified cholesterol through the 48-h milking. The former increased decidedly thereafter. Total cholesterol values for the 60-, 72-, and 84-h milkings were 2.6, 3.9 and 3.0 mg/g of globules, respectively. This implies that cholesterol destined for milk was being esterified more extensively during the suppression period. It was also noted that fat globules from milkings during the recovery period, 36 to 60 h post infusion, had more of a tendency to churn during washing than those from earlier or later milkings.

In a previous investigation [8] it was found that the proportion of sphingomyelin in milk fat globule phospholipids fluctuated during the colchicine-induced suppresion. We analyzed phospholipid distribution in milkings from two of our four colchicine infusion experiments and no such variation was observed. The mean and range for sphingomyelin as a percent of total phospholipid for 0-h through 84-h milkings were 22.4 (18.7–27.6). The other phospholipids also appeared to be within normal ranges, as previously shown [8].

The activity of 5'-nucleotidase, an enzyme frequently used as a marker for plasma membrane [28], was determined for globules from three of our infusion experiments. It is known that milk fat globules are relatively rich in this enzyme and that it is exposed in the globule outer surface [29]. In all three experiments a drop in this activity was associated with colchicine infusion, Fig. 3.

Further evaluation of colchicine's effect on globule composition was afforded by SDS-polyacrylamide gel electrophoresis of the globule peptides. A typical stained gel for globule samples following colchicine infusion is shown in Fig. 4. Peptide patterns for samples throughout the experimental period generally were very similar although some aberrations in minor bands could be seen. Density scans were prepared of stained gels from two infusion experiments and the contribution of the four major components (at M_r = 155000, 70000, 52000 and 49000) to total peak

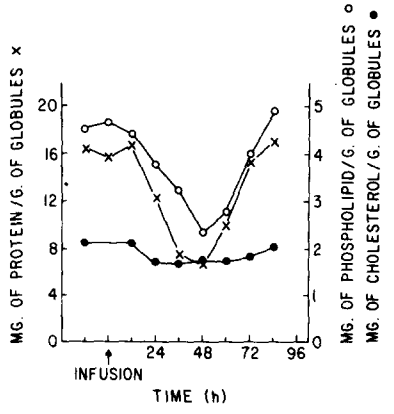

Fig. 2. Effect of an intramammary infusion of colchicine on protein, phospholipid and cholesterol (unesterified) of milk fat globules obtained from milkings of the infused gland. For infusion conditions and yields of milk, see Fig. 1.

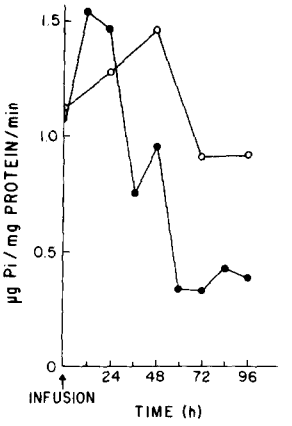

Fig. 3. 5'-Nucleotidase activities of milk fat globules from milkings of the infused (●———●) and uninfused (○———○) sides of the udder at intervals following intramammary infusion of colchicine. The values are means from three infusion experiments.

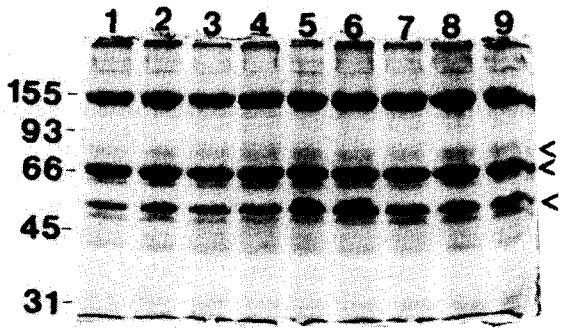

Fig. 4. SDS-polyacrylamide gel electrophoresis of milk fat globules obtained from milkings collected at intervals before and after infusing the gland with colchicine (for infusion conditions and yields, see Fig. 1). The peptide bands were revealed by first staining with Schiffs reagent and then with Coomassie blue. Sample numbers refer to hours before or after infusion as follows 1(−12), 2(0), 3(12), 4(24), 5(36), 6(48), 7(60), 8(84), and 9(156). Molecular weight reference points in thousands are to left of the gel and positions of the three major glycoprotein bands (Schiff's positive) are indicated (arrow heads) on right.

area for the four were calculated. Only one of these, a glycoprotein ($M_r = 52\,000$), showed a reproducible trend; i.e. an increase peaking in the 48-h sample, Fig. 5. According to other experiments (Patton, S. and Hubert, J., unpublished data) in which ^{125}I and lactoperioxidase were used to label the globule surface, this component is the major exteriorized protein (a glycoprotein) of the goat milk fat globule. The gel shown in Fig. 4 was first stained with Schiff's reagent (to detect carbohydrate) by which three dominant bands were revealed (arrow heads, Fig. 4). The strongest Coomassie blue-staining band ($M_r = 155\,000$) showed essentially no staining with Schiff's reagent.

In the [^{14}C]colchicine infusion experiment we observed that some of the activity released into post-infusion milkings was associated with fat globules. In Fig. 6 this activity can be discerned as the difference between the two trend lines, one for whole milks and the other for corresponding skim milks. The fat globules achieved a peak of 513 cpm/ml of milk at 72 h post-infusion followed by a decline to 26 cpm/ml at 144 h. A similar trend at lower activities was seeen from the uninfused side. We did not ascertain whether any activity in these samples was due to degradation products of [^{14}C]colchicine although in a similar experiment [12] activity in the 12-h post-infusion skim milk was defined as [^{14}C]colchicine.

In a preliminary experiment (Patton, S. and Trams, E.G., unpublished data) it had been noted that [^{14}C]colchicine does not bind to fat globules in goats milk but that binding does occur to the globule membrane after it has been removed from globules. We conducted two further experiments on this matter. The results, Fig. 7, confirm that colchicine binds to globule membrane following its

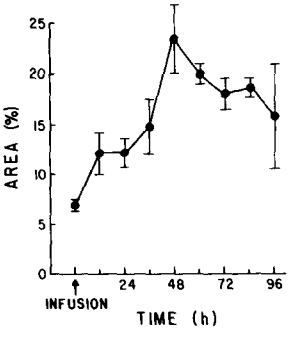

Fig. 5. Peak areas from gel scans for a glycoprotein (lower arrow head, Fig. 4) of milk fat globules as a percentage of the total area for the four major globule peptide bands (155, 70, 52 and 49 kDa) from globules in milkings at intervals following intramammary infusions of colchicine. Data are means with ranges for two infusion experiments.

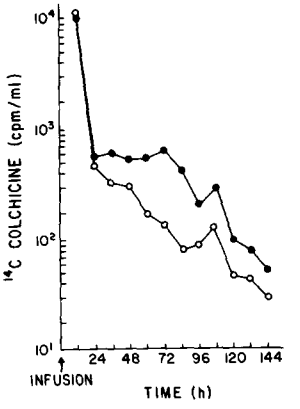

Fig. 6. Specific activity of milkings (●———●), and skim milks (○———○) therefrom, collected at 12-h intervals following intramammry infusion of [^{14}C]colchicine. The infusion was 50 μCi of [*ring C-methoxy*-^{14}C]colchicine with 4 mg of unlabeled colchicine in 5 ml of water into the gland via the teat canal immediately following the zero-time milking.

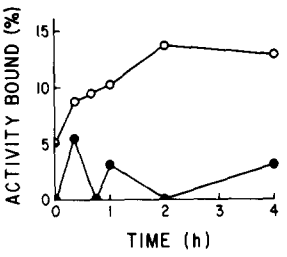

Fig. 7. Percentage of added [^{14}C]colchicine activity bound to goat milk fat globules (●———●) and to membrane (○———○) released therefrom as a function of incubation time at 37°C. Each sample contained 0.5 mg of protein and 0.04 ng of colchicine in 1 ml of water.

release from goat milk fat globules. While globules did at times show some binding of activity, possibly due to physical changes induced in them during their isolation and incubation, most of these samples were negative and the limited activity produced no coherent pattern.

The binding results (Figs. 6 and 7) do not shed light on how the [^{14}C]colchicine is associated with the globules (in vivo) or the globule membrane (in vitro). However an affinity is clearly involved because as the radioactivity of both globules (Fig. 6) and membranes (Fig. 7) went up that in the dispersing medium went down.

Discussion

Our investigation has shown that colchicine infused into the lactating goat gland induces a number of changes in milk fat globules. These were reversible for the most part and related to reversible supression of milk flow by the drug. They included decreases in protein, phospholipid and unesterified cholesterol per g of globule, decreases in 5′-nucleotidase activity per mg of globule protein and an increase in the relative proportion of a major membrane glycoprotein. Beyond this latter, the patterns of globule peptides recorded at intervals during the colchicine-induced suppression of milk flow were similar. To some extent these results imply a reduction in the quantity of membrane available to envelope globules at secretion. The amounts of protein, phospholipid and cholesterol per g of globule were falling most precipitously, Fig. 2, at the same time that milk, and thus globule secretion, was most drastically reduced by the drug, i.e., 12 to 36 h post infusion. These decreases precede the major increase in globule size induced by colchicine which commences at 36 h, when milk flow reverses, and peaks at 60 h post infusion [8]. Thus the fall in the foregoing constituents per gram of globules does not appear to be explained simply by change in the globule surface to volume relationship. The heightened tendency of globules to churn during the post infusion period also suggests inadequate or altered membrane surfaces on globules as milk flow resumed. Increased globule size might also be a factor in this churning.

The drop in 5′-nucleotidase activity on either a mg of globule protein or a per g of globule basis was greater than for the other constituents. To the extent that this enzyme is actually quantitatively related to the amount of plasma membrane, the supply of new plasma membrane for secretion must have been drastically reduced by colchicine. Recent evidence indicates there is a continuous exchange of this enzyme between cell surface and cytoplasmic compartments [30]. Our findings suggest that any such (membrane) cycling was substantially blocked by the colchicine treatment. It seems unlikely that the drug itself interferes with activity of the enzyme because the highest concentrations of alkaloid were present in the first (12-h) post-infusion milking at which time the globule 5′-nucleotidase activity was normal. Whether the loss of activity is due to reduced levels of the enzyme in the membrane or to an inactivation will require further study. In vivo administration of colchicine also lowers 5′-nucleotidase activity and protein and lipid components in rat-liver plasma membrane [31,32].

It was previously proposed [14] that one of the inhibiting effects of colchicine on milk secretion results from a change in properties of secretory vesicle membranes. These vesicles appear to lose their capacity to fuse with each other or with the plasma membrane as a result of the colchicine action. The foregoing evidence herein that plasma membrane replenishment is reduced by the alkaloid coupled with observations that colchicine associates with the internal surface of milk fat globule membrane (Figs. 6 and 7) are consonant with this hypothesis. This membrane is derived

from membrane of secretory vesicles and apical plasma membrane in the mechanisms of milk secretion [7]. Thus, binding of colchicine to them may interfere with their capacity to function in milk secretion. Stadler and Franke [33] have reported binding of colchicine to membranes of the liver cell and Wunderlich et al. [34] have presented evidence that colchicine impairs mobility of components in plasma membrane of *Tetrahymena pyriformis*. Binding of colchicine to plasma and Golgi membranes of lactating tissue have been demonstrated recently by Houdebine et al. [35]. They suggest that tubulin is the molecule responsible for this binding.

An alternative explanation for the reduced fusion of secretory vesicles with each other or with the plasma membrane is that the organized constriction and interfacing of components within the cell is reduced due to disruption of the cytoskeleton (microtubules) by colchicine.

It is increasingly evident that the effects of colchicine on the lactating cell are multiple and complex. Ultrastructural investigations [14,16] have shown clearly that colchicine suppresses milk secretion and causes an accumulation of secretory products in the cell. Suppression of lactose synthesis by the drug has been demonstrated in vitro [36], and this effect has been confirmed in vivo with the lactating goat (Patton, S. unpublished data). A consideration of milk yield in the colchicine-treated goat over several days, Fig. 1 (see also Refs. 6 and 12), establishes that production of milk as a whole is restrained. We conclude that colchicine interferes with both synthesis and secretion of milk.

Our results showing that colchicine binds to milk fat globule membrane support the findings of Houdebine et al. [35] which reveal binding of the drug to Golgi and plasma membranes from lactating tissue. They observe further that this binding interferes with casein gene expression and that the microtubule antagonist, griseofulvin, does not cause such interference. Thus, to understand the effects of colchicine on the lactating cell it becomes important to further characterize its binding to the cell membranes. We are at present attempting to specify this binding with respect to the milk fat globule membrane.

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