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The effects of monensin on membrane lipids of cultured human skin fibroblasts

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We have investigated the effects of monensin, a monovalent cationophore, on the metabolism of neutral lipids, fatty acids, ceramide and phospholipids in cultured human skin fibroblasts. Treatment with 1 μM monensin for 18 h reduced the cellular cholesterol ester content to less than one-third of untreated cells, and incorporation of [^{3}H]acetate into cholesterol ester was also reduced, to less than one-fifth. Concomitantly, a greater conversion of [^{3}H]acetate into free cholesterol occurred. There was a moderate increase in free fatty acids, but no change in triacylglycerol content, although the content of the latter appeared to increase in the presence of fetal calf serum in the culture medium. Phosphatidylcholine decreased in content and phosphatidylserine increased among the phosphatides, but ceramide remained unchanged after monensin treatment. These findings suggest that monensin influences the metabolic interrelationships of structural lipids in fibroblasts.

Monensin, a monovalent cationophore that alters cellular monovalent cationic trans-membrane flow, has been shown to disturb the Golgi apparatus and to slow down the intracellular transport of newly synthesized proteoglycans, secretory proteins and plasma membrane glycoproteins, and to inhibit some of the post-translational modifications of proteins which are believed to occur in the Golgi apparatus [1]. Monensin also has been shown to disrupt lysosomal function [2] and to inhibit recycling of LDL receptors to the plasma membrane [3] and also to reduce pinocytotic activity [4].

Sites of biosynthesis and the mechanism of intracellular translocation of newly synthesized lipids have recently gained widespread attention, but have not been fully elucidated. Since monensin inhibits translocation of macromolecules between subcellular organelles, it may be a good tool for the study of lipid biosynthesis and intracellular translocation mechanisms. Previously, we have reported that monensin inhibits glycosylation of polyglycosylated glycosphingolipids and induces accumulation of simple glycosphingolipids, which suggests different intracellular sites of glycosylation of lower and higher glycosphingolipids in human fibroblasts [5,6]. Similar results have been obtained by others in cultured neurotumor cells [7]. The effects of monensin on myelin galactolipids have also been studied [8]. Recently, Lipsky and Pagano [9] have reported that monensin inhibits the translocation of analogues of the phosphosphingolipid, sphingomyelin, and the glycosphingolipid, glucocerebroside, from the Golgi apparatus to the plasma membrane. It has also been reported that synthesis and transport of phosphatidylethanolamine is not inhibited by monensin [10]. We describe in the current study the effects of monensin on the cellular composition of these lipids. A remarkable influence of this cationophore on cholesterol metabolism was observed.

Cultured human skin fibroblasts (GM3440) were obtained from the Institute for Medical Research (Camden, NJ). The cells were cultured in 10 ml of growth medium consisting of 83% Dulbecco's modified Eagle's medium (Gibco), 15% fetal calf serum (Gibco) (FCS) and 2% penicillin-streptomycin (5000 I.U. and 5 mg per ml, respectively, Flow Laboratories) in 75 cm^2 plastic tissue culture bottles (Falcon) at 37°C in a 5% CO_2 atmosphere in air. Fibroblasts used in this study were between the eighth and fifteenth passage. In order to observe the effects of monensin (Calbiochem), confluent fibroblasts were incubated in 1 μM monensin and sodium [^{3}H]acetate (NEN, 685 mCi/mmol) at a concentration of 10 μCi/ml. To observe the effects of monensin under a serum-free arrangement, confluent fibroblasts (cultured in the growth medium for 10–14 days after seeding) were washed with 5 ml of Dulbecco's modified Eagle's medium and incubated in medium containing 98% Dulbecco's modified Eagle's medium and 2% penicillin-streptomycin. After 24 h, 1 ml of fetal calf serum or monensin (final concentration, 1 μM) or both was added to the culture medium with [^{3}H]acetate (ICN, 20 Ci/mmol) at a concentration of 2.5 μCi/ml. Cellular protein was determined by the method of Hartree [11].

The method of analysis of neutral lipids, fatty acids, ceramide, and phospholipids is based on the method of Macala et al. [12] modified as described below.

Total lipids were extracted from the cells harvested from a 75 cm^2 – culture bottle with 2 ml each of chloroform/methanol (2 : 1, v/v) three times. The extract was applied on a Unisil (activated silicic acid, Clarkson Chemical Co.) column prepared by using a Pasteur pipet with 2 cm of silicic acid. Neutral lipid and fatty acid were eluted from the column with chloroform (three times with 2 ml) and then phospholipids with methanol (three times with 2 ml) after redissolving the sample in methanol and reapplying to the column. The isolated neutral lipids were spotted on a 10 × 20 cm high-performance silica gel G thin-layer chromatography (HPTLC) plate (E. Merck) along with 5, 10, 15 and 20 μl of a standard neutral lipid mixture (consisting of 0.4 μg/μl cholesterol, 0.06 μg/μl oleic acid, 1 μg/μl triacylglycerol and 0.8 μg/μl cholesterol ester dissolved in dioxane (all chemicals obtained from Sigma)). The plate was developed in diethyl ether/hexane/acetic acid (35 : 65 : 2, v/v) until the solvent front ascended to about 4–5 cm above the bottom edge of the plate. After the solvent was evaporated, it was rechromatographed using diethyl ether/hexane/acetic acid (2 : 98 : 1, v/v) until the solvent front ascended to 1 cm from the top of the HPTLC plate. The lipids on the plate were charred for densitometry with 3% cupric acetate (w/v) in 8% phosphoric acid (v/v) solution [13] and scanned according to Macala et al. [12] in a spectrodensitometer (Kratos). The lipids were then quantitated by comparison with standards. The amount of each lipid was normalized to the cellular protein content. One-fourth of the amount of the phospholipid fraction was separated on a HPTLC plate along with 5, 10, 15 and 20 μl of a standard mixture (consisting of 2 μg/μl sphingomyelin, 3 μg/μl phosphatidylcholine, 0.1 μg/μl phosphatidylserine, 0.05 μg/μl phosphatidylinositol, and 0.5 μg/μl phosphatidylethanolamine dissolved in dioxane (all chemicals obtained from Sigma)) developed in chloroform/methanol/water (60 : 35 : 4, v/v). The visualization and quantification of each phospholipid was done as described for neutral lipids. For the determination of ceramide, the total lipids were applied to a Unisil column and eluted three times with 2 ml of chloroform/methanol (9 : 1, v/v). This fraction was applied to a HPTLC plate along with standard ceramide (Sigma) and developed in chloroform/methanol/water (60 : 25 : 8, v/v) until the solvent front reached to about 3–3.5 cm from the bottom edge of the plate according to Selvan and Radin [14]. After evaporating the solvent, it was rechromatographed using chloroform/methanol/acetic acid (90 : 2 : 8, v/v) until the solvent front ascended to 1 cm from the top of the HPTLC plate. Visualization and quantification of the ceramide was done as described for neutral lipids and phospholipids. For analysis of the radioactivity incorporated into each lipid, the lipids were separated on HPTLC plates as described above and the patterns of radioactivity were obtained by scraping each 2 mm wide strip from TLC plates, transferring the samples into scintillation vials, adding 5 ml of Aquasol (NEN), sonicating and

counting in a Beckman scintillation spectrometer. The amounts of radioactivity were normalized to the cellular protein content.

Table I shows the effects of monensin on the major lipids of cultured human skin fibroblasts. Confluent fibroblasts were incubated in growth medium containing 1 μM monensin and [^{3}H]-acetate for 18 h. The cells were harvested with a rubber policeman and their lipids and the radioactivity incorporated were analyzed. Monensin reduced the content of cholesterol ester to one-third of control, and labelled acetate incorporation into cholesterol ester was reduced to one seventh of the control. Radioactivity incorporated into each lipid increased in all except cholesterol ester, ceramide and sphingomyelin. The increase in labelled free cholesterol was most prominent (5-fold) with monensin treatment. The amount of free fatty acids increased moderately, and a reciprocal increase in the content of phosphatidylserine and decrease in phosphatidylcholine occurred among the phosphatides. The content and the radioactivity of ceramide remained unchanged. Table II shows the effect of fetal calf serum and monensin on neutral lipids and fatty acids. The cells were incubated in serum-free medium for 24 h. Then, 10% fetal calf serum or 1 μM monensin, or both, were added to medium containing [^{3}H]acetate, and the cells were further incubated for 18 h, and their lipids analyzed. The results are presented as percent of control (containing neither fetal calf serum nor monensin). Fetal calf serum enhanced both the content of cholesterol ester and the radioactivity incorporated into this lipid as reported previously [15], and radioactive acetate incorporation into free cholesterol decreased. Monensin abolished the effects of fetal calf serum on cholesterol and cholesterol ester. The cellular content of triacylglycerols, which increased in the presence of fetal calf serum, was not influenced by monensin treatment. Although the triacylglycerol content increased, augmented incorporation of labelled acetate into triacylglycerol did not occur in the presence of fetal calf serum.

Previously, we reported the effects of monensin on the anabolism of glycosphingolipids of cultured human skin fibroblasts [5,6]. In the present study, we investigated the effects of monensin on the other major lipids of fibroblasts, since biosynthesis and intracellular translocation of lipids might be related to those systems known to be disrupted by monensin. Remarkable effects of monensin are observed on cholesterol metabolism. Monensin reduced the content of cholesterol ester

TABLE I

EFFECT OF MONENSIN ON FIBROBLAST LIPIDS

Confluent fibroblasts were incubated in 1 μM monensin and sodium [^{3}H]acetate (10 μCi/ml) for 18 h and the cells harvested and their lipids and the radioactivity incorporated analyzed. Each value represents mean of three separate trials ± S.D. + Monensin represents monensin-treated cells: whereas, − monensin represents non-monensin-treated cells. * $P < 0.05$; ** $P < 0.01$ as compared with non-monensin treated cells using Student's two-tailed t-test.

	Amount (μg/mg of protein)		Radioactivity (dpm/mg of protein)	
	+ monensin	− monensin	+ monensin	− monensin
Neutral lipids				
Cholesterol	60.49 ± 7.42	76.67 ± 12.24	14313 ± 4004 **	2948 ± 844
Free fatty acids	2.65 ± 0.46 *	1.70 ± 0.38	10341 ± 799 *	3663 ± 1185
Triacylglycerols	39.55 ± 3.44	50.64 ± 11.26	61834 ± 12726	38700 ± 12892
Cholesterol ester	4.15 ± 2.18 **	14.91 ± 3.84	482 ± 111 **	3275 ± 790
Phospholipids				
Sphingomyelin	65.10 ± 6.85	78.33 ± 29.12	12723 ± 3.234	11125 ± 2947
Phosphatidylcholine	136.23 ± 6.67 *	224.13 ± 44.41	137013 ± 7730 **	55424 ± 13277
Phosphatidylserine	14.99 ± 1.33 **	4.76 ± 2.28	10909 ± 1214 **	5012 ± 1880
Phosphatidylinositol	5.16 ± 0.65	4.83 ± 2.56	1259 ± 298 *	627 ± 248
Phosphatidylethanolamine	235.33 ± 52.14	247.52 ± 67.81	18657 ± 3074 **	7677 ± 1716
Ceramide	0.45 ± 0.20	0.41 ± 0.07	44.94 ± 17.33	46.32 ± 15.00

TABLE II

EFFECT OF MONENSIN AND FETAL CALF SERUM ON NEUTRAL LIPID AND FATTY ACIDS

Confluent fibroblasts were incubated in serum-free medium for 24 h, and then in fresh medium with [^{3}H]acetate (2.5 μCi/ml) for 18 h. To fresh medium, either fetal calf serum (FCS) (9%) or monensin (1 μM) or both were added. The cells were harvested and their free cholesterol, free fatty acid, triacylglycerol and cholesterol ester were analyzed. The content or the radioactivity of each lipid was measured, normalized to the cellular protein content and presented as percent of control (which was incubated without fetal calf serum or monensin). The values are means ± S.D. for triplicate cultures.

	Content of lipid (% of control)			Radioactivity (% of control)		
	FCS	FCS + monensin	Monensin	FCS	FCS + monensin	Monensin
Cholesterol	97.7 ± 13.2	113.2 ± 26.8	124.1 ± 10.5	38.6 ± 12.3	105.2 ± 25.0	249.3 ± 35.1
Free fatty acids	81.6 ± 14.8	79.4 ± 13.0	85.0 ± 8.3	103.1 ± 8.5	135.9 ± 27.6	162.9 ± 25.4
Triacylglycerols	188.1 ± 19.0	210.0 ± 20.9	92.8 ± 25.0	90.9 ± 21.4	52.4 ± 6.2	89.9 ± 12.4
Cholesterol ester	342.8 ± 81.9	104.4 ± 25.1	84.4 ± 22.0	439.4 ± 43.0	46.2 ± 8.8	92.4 ± 23.8

and the radioactive acetate anabolically incorporated into it, and stimulated the incorporation of labelled acetate into free cholesterol. An LDL-mediated regulatory mechanism for cholesterol metabolism has been extensively studied in human fibroblasts [16]; inhibition of recycling of LDL receptors by monensin was reported [3]. It is probable that some of the effects of monensin on cholesterol metabolism that we have observed were induced by the inhibition of recycling of LDL receptors, and additionally, inhibition of lysosomal activity, which was also reported to be induced by monensin [2]. In spite of the great increase in the incorporation of [^{3}H]acetate, the level of cholesterol remained unchanged, probably because LDL-bound cholesterol could not be supplied after monensin treatment. A similar effect to monensin was observed in the absence of LDL in serum-free medium. Nevertheless, triacylglycerols were not affected by monensin. Fetal calf serum increased the content of triacylglycerols, but did not increase incorporation of labelled acetate. The cells may therefore acquire triacylglycerols indirectly from the fetal calf serum by a mechanism which is not influenced by the effects of monensin on recycling of LDL. The decrease in phosphatidylcholine by monensin may relate to the activation of phospholipases by an increase in the availability of intracellular Ca^{2+} following upon monovalent cation flux by this cationophore as suggested previously [17]. The level of ceramide was unchanged by monensin although glucosylceramide accumulated in the cells [5]. We have investigated the composition of neutral lipids and fatty acids in neuronopathic hereditary lysosomal β-glucosylceramide β-glucosidase deficient (Gaucher disease) fibroblasts and found a normal amount of cholesterol, but a 50% decrease in cholesterol ester. Fatty acid composition in cholesterol ester was different from that of normal fibroblasts (Salgia, R., Saito, M. and Rosenberg, A., unpublished results). Likewise, a metabolic block in esterification of cholesterol was reported in a genetic disorder in BALB/c mice associated with storage of the ceramide-containing sphingolipids, sphingomyelin and glucosylceramide [18]. The accumulation of glucosylceramide and reduction of cholesterol ester induced by monensin may provide a model system for study of the metabolic relationships of complex lipids in these disorders.

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