Biochimica et Biophysica Acta, 643 (1981) 182–190

BBA 79211

EFFECTS OF CHOLESTEROL ON THE PROPERTIES OF THE MEMBRANES OF ISOLATED SHEEP LIVER NUCLEI AND NUCLEAR ENVELOPES

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(Received October 13th, 1980)

Key words: Cholesterol; ESR; Membrane property; (Sheep liver, nucleus)

Summary

The exchangeability of cholesterol between sheep liver nuclear membranes and liposomes, and the effect of cholesterol on the fluidity of the membrane lipid were studied. In intact nuclei, the cholesterol/phospholipid ratio increased from 0.102 to 0.145 mol/mol on incubation with cholesterol-rich liposomes, with a time for half-maximal uptake of 4.2 h. In isolated envelopes under the same conditions, the ratio increased from 0.110 to 0.266 mol/mol with a time for half-maximal uptake of about 1.9 h. Moreover, the approximate order parameter of the spin label 5-(*N*-oxyl-4′,4′-dimethyloxazolidino)-stearic acid was 0.677 in intact nuclei and 0.723 in isolated envelopes prior to exchange; after exchange, these values increased to 0.717 and 0.756, respectively. These differences between the preparations could not be attributed to differences in the capacity for cholesterol uptake between the two nuclear membranes, or to a slow rate of exchange between them; the presence of an intact nuclear matrix appeared both to disorder the lipid partially and to inhibit cholesterol uptake. The differences indicate that conclusions based on physical studies of the membrane lipid in isolated envelopes are not necessarily applicable to the intact nucleus.

Introduction

Cholesterol can readily be exchanged between membranes and can also be added or removal from them [1]. Modification of the level of cholesterol in a

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membrane can be used both as a probe for the role of cholesterol within that membrane and as a probe for the function of the membrane itself, since it is known that cholesterol plays a role in modifying the physical state of both model and biological membranes [1,2].

The mammalian liver nuclear envelope comprises inner and outer membranes, pore complexes and a fibrous lamina [3—5]. Removal of the two membranes by extraction with Triton X-100 leaves essentially lipid-free material [3—5] which suggests that the lipid is virtually confined to the membranes. The lipid composition of the membranes resembles that of liver microsomes [6—7], except that the proportion of saturated fatty acids may be greater in nuclear membranes [7,8]. Cholesterol is present in amounts of about 1 mol per 10 mol phospholipid [6,9,10]. No definitive method for the separation of the two membranes has yet been reported. There are therefore no reliable data on the differences in the composition of the two membranes.

There is some evidence that the microviscosity of the nuclear envelope lipid may be important in the regulation of nucleo-cytoplasmic RNA transport, at least in *Tetrahymena* [11—13] and possibly in mammalian liver [14], though this point is controversial [15,16]. Alteration of the nuclear envelope fluidity by modification of the cholesterol content [16,17] might provide a method for evaluating this point critically.

In the present paper, we describe the effects of altering the cholesterol content of isolated nuclear envelopes and of intact nuclei on lipid fluidity and on the activities of two enzymes of the nuclear envelope.

Materials and Methods

Dimyristoyl phosphatidylcholine, cholesterol, aminophenazone gentisate, ATP (disodium salt, grade II) and NADH were obtained from Sigma London, Poole, U.K. Cholesterol oxidase was obtained from Boehringer Mannheim (Lewes, U.K.). [4-^{14}C]cholesterol was obtained from the Radiochemical Centre, Amersham, U.K. All other reagents were of analytical grade.

Isolation of nuclei and nuclear envelopes. Nuclei were isolated from sheep liver by a modification [17] of the method of Blobel and Potter [18], and nuclear envelopes were prepared by the method of Harris and Milne [19]. A concentration of 1 mg nuclear protein per ml was used during the lysis stage of nuclear envelope isolation, but in some experiments this was decreased to 0.1 mg nuclear protein per ml. Envelopes isolated under the latter conditions are outer-membrane-depleted [20]. Nuclei were lysed in 1 mM sodium bicarbonate [19] and were used in some cholesterol exchange experiments without further treatment.

Isolation of nuclear envelope-matrix complex. Nuclear matrices were isolated from nuclei by a modification of the method of Berezney and Coffey [21]. Briefly, this modification involves (a) a decrease in the number of centrifugation steps in the procedure, and (b) omission of the Triton X-100 extraction stage. The omission of the detergent extraction results in the preparation of matrices with the nuclear membranes virtually intact [12].

Modification of nuclear envelope cholesterol content. Nuclei or envelopes (about 0.3 μmol phospholipid/ml) were incubated for 16—20 h at 37°C in a

total volume of 2.2 ml containing 0.25 M glucose, 10 mM Tris-HCl, 5 mM spermidine, 5 mM 2-mercaptoethanol, 2 mM magnesium chloride, 0.5 mM calcium chloride and 0.3 mM manganese chloride, pH 7.6. Nuclei are stable in this medium for long periods [14,16,22]. The medium was supplemented with liposomes (about 3 μmol phospholipid/ml, sonicated for 60 s at 5 μm peak-to-peak amplitude using a MSE ultrasonic disintegrator with a 3 mm diameter probe), containing either dimyristoyl phosphatidylcholine alone or dimyristoyl phosphatidylcholine and cholesterol in equimolar ratios. After incubation, the suspension was mixed with 50 ml 30% (w/v) sucrose, 1 mM sodium bicarbonate and centrifuged at 40 000 $\times g$ for 30 min; the liposomes were floated and the nuclei and envelopes were recovered in the pellet.

Time-course of cholesterol uptake. Incubations were performed as described above except that (a) only dimyristoyl phosphatidylcholine liposomes were used, and [4-^{14}C]cholesterol was added to a final concentration of 25 μCi/ml, and (b) the total incubation volume was 15 ml and 2 ml aliquots were taken at 0, 1, 2, 3, 4, 8, and 18 h after the start of the incubation and the nuclei or envelopes were recovered as described above. The pellets were dispersed in a Triton X-100-based scintillator solution [23] and counted.

Chemical determinations. Protein was determined by a modification [24] of the method of Lowry et al. [25]. DNA was estimated by the method of Burton [26], RNA by the method of Ashwell [27] and phospholipid by the phosphate content [28] of a 70% (v/v) perchloric acid digest of a chloroform/propan-2-ol (2/1, v/v) extract of the envelopes or nuclei. Cholesterol was determined by incubating the chloroform/propan-2-ol extract with 1.25 ml 50 mM Tris-HCl, pH 7.0, containing 0.3% (w/v) *m*-aminophenazone, 5 μg peroxidase/ml and 4-μg cholesterol oxidase/ml at 37°C for 30 min and reading the A_{400} against a cholesterol-free blank.

Enzyme assays. Nucleoside triphosphatase activity (EC 3.6.1.15) was determined by the method of Agutter et al. [29] and NADH-ferricyanide reductase activity (EC 1.6.99.3) by the method of Zamudio et al. [30]. The former enzyme is a pore-lamina component [31,32] and the latter is localised in the hydrophobic part of one or both membranes [7,20].

Order parameter of fatty acid spin label. 100 μl suspensions of envelopes or nuclei (0.3—0.5 μmol phospholipid) were incubated for 30 min to 1 h at 20°C over a film of 50 nmol 5-doxyl stearate (5-(*N*-oxyl-4′,4′-dimethyloxazolidino)-stearic acid, (Syva, Palo Alto, CA, U.S.A.)). Spectra were recorded at 20°C or at 30°C on a Varian E4 ESR spectrometer. Order parameters (S_{app}) were calculated from the spectra by the method of Griffith and Jost [33].

Results

Incubation of nuclear preparations with liposomes

Intact nuclei, lysed nuclei and nuclear envelopes were incubated with liposomes prepared from dimyristoyl phosphatidylcholine alone or dimyristoyl phosphatidylcholine and cholesterol. The recovery of nuclear or nuclear envelope protein after incubation was 64 ± 5% (mean ± S.E. of three measurements). The properties of the membranes obtained after separation of the liposomes at the end of a 16-h incubation are shown in Table I. The results show

TABLE I

EFFECTS OF INCUBATION WITH LIPOSOMES ON COMPOSITION OF NUCLEAR ENVELOPES

Nuclei, lysed nuclei or envelopes were incubated (a) with phosphatidylcholine liposomes, (b) with phosphatidylcholine-cholesterol liposomes, or (c) without liposomes for 16 h. Results are means ± S.E. of four duplicate determinations. S_{app} = approximate order parameter.

Sample	Incubation conditions	μg DNA/mg protein	μmol phospholipid/ mg protein	μmol cholesterol/ μmol phospholipid	S_{app}
Nuclei	a	262 ± 34	0.29 ± 0.05	0.083 ± 0.005	0.661
	b	252 ± 23	0.26 ± 0.02	0.145 ± 0.010	0.717
	c	275 ± 27	0.30 ± 0.05	0.102 ± 0.007	0.677
Lysed nuclei	a	222 ± 21	0.49 ± 0.05	0.093 ± 0.003	0.676
	b	229 ± 28	0.47 ± 0.03	0.161 ± 0.010	0.723
	c	209 ± 24	0.44 ± 0.04	0.107 ± 0.004	0.681
Nuclear envelopes	a	44 ± 10	1.36 ± 0.14	0.094 ± 0.006	0.692
	b	44 ± 13	1.34 ± 0.20	0.266 ± 0.038	0.756
	c	41 ± 9	1.28 ± 0.13	0.110 ± 0.011	0.723 ± 0.015

that (a) no significant contamination of the recovered material by phospholipid from the liposomes had occurred. Exchange of phospholipid between the liposomes and the membranes may have occurred, but the nearly constant phospholipid/protein ratios indicate that there was no net dimyristoyl phosphatidylcholine accretion; (b) incubation with liposomes did not markedly affect the relative amounts of DNA and protein or phospholipid and protein when compared with an incubated preparation, suggesting that no component of the nucleus or envelope was selectively released under the conditions used; (c) more cholesterol from the cholesterol-rich liposomes entered isolated envelopes than entered the membranes of intact nuclei. The cholesterol/phospholipid ratio in untreated envelopes and nuclei was consistent with those reported by Kleinig [9] and Keenan et al. [10]; and (d) for each type of sample, the approximate order parameter of the fatty acid spin probe increased with increasing cholesterol content, which is consistent with the generally held view that cholesterol increases the order of a phospholipid bilayer [2].

Since the net uptake of cholesterol after 16 h was greater in the isolated envelopes than in the lysed nuclei, and in lysed nuclei than in intact nuclei (Table I and (c) above), it is possible that the cholesterol uptake followed a different time-course in the three different preparations. Fig. 1 shows the time-course of uptake of cholesterol for intact nuclei and for envelopes. In the case of the envelopes, the process was half complete after 1.9 ± 0.9 h but for the nuclei, 4.2 ± 1.3 h was required for half-complete uptake to take place. The difference in times is significant ($p < 0.05$).

These unexpected differences between intact nuclei and isolated envelopes in terms of rate of uptake and extent of uptake of cholesterol might be explained in three ways. First, it is possible that in isolated envelopes both membranes are exposed to the liposomes, but in intact and lysed nuclei, only the outer membrane is exposed. If the rate of cholesterol exchange between the two membranes is much slower than that between membrane and liposomes, and if the

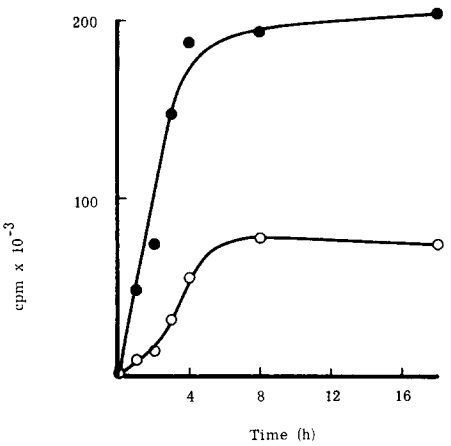

Fig. 1. Time-course of uptake of [^{14}C]cholesterol from liposomes into nuclear membranes, using isolated nuclear envelopes (•) and intact nuclei (○). Values are means of three duplicate determinations.

inner and outer membranes are both similar in lipid composition and in the exchangeability of their lipids, then the rate and extent of cholesterol uptake should be approximately twice as great in the envelopes as in the intact or lysed nuclei.

Second, the inner membrane, but not the outer, may be able to take up cholesterol and therefore marked uptake of cholesterol can only occur in isolated envelopes.

Third, although lipid exchange between membranes and liposomes may be very rapid in isolated envelopes, in intact nuclei the membranes may be so constrained, for example by their attachment to the nuclear matrix [12,13], that lipid exchange is retarded.

The following experiments were performed to attempt to distinguish between these possibilities. First, nuclei were incubated for 4 or 16 h with cholesterol-rich liposomes, and after each incubation the washed nuclear pellet was divided. Nuclear envelopes were prepared in the normal way from one part of the pellet, and outer-membrane-depleted envelopes were prepared from the remainder. The cholesterol/phospholipid ratios of the envelope preparations (Table II) suggest that, although the rate of uptake into the inner membrane

TABLE II

CHOLESTEROL/PHOSPHOLIPID RATIOS IN NORMAL AND OUTER-MEMBRANE-DEPLETED ENVELOPES

Values are means ± S.D. of triplicate determinations.

Incubation time (h)	Cholesterol/phospholipid ratio (mol/mol)	
	Normal envelopes	Outer-membrane-depleted envelopes
0 (control)	0.108 ± 0.012	0.099 ± 0.011
4	0.139 ± 0.013	0.117 ± 0.013
16	0.158 ± 0.020	0.141 ± 0.022

TABLE III

UPTAKE OF CHOLESTEROL INTO INTACT AND OUTER-MEMBRANE-DEPLETED NUCLEAR ENVELOPES

Values are means of two determinations.

Envelope preparation	Incubation conditions	μg phospholipid/ mg protein	μmol cholesterol/ μmol phospholipid
Intact	No liposomes	309	0.122
	Cholesterol-rich liposomes	354	0.280
Outer-membrane-depleted	No liposomes	182	0.117
	Cholesterol-rich liposomes	197	0.295

may be slower than incorporation into the outer membrane (as indicated by the differences after 4 h), there is no significant difference between the membrane after 16 h. This is not consistent with the first hypothesis above. Second, a preparation of nuclear envelopes prepared in the normal way was incubated with cholesterol-rich liposomes as before and compared with a sample of outer-membrane-depleted envelopes incubated under the same conditions. The results of this experiment are presented in Table III. It can be seen that the extent to which cholesterol is taken up by the two membrane preparations is identical. This is not consistent with the second hypothesis above. Third, an attempt was made to assess the effect of the nuclear matrix on the envelopes by comparing the order parameter of 5-doxyl stearate in isolated envelopes with that in a preparation of the envelope-matrix complex. The value for the isolated envelopes was found to be 0.723 and that for the envelope-matrix complex was 0.662, similar to that for whole nuclei (0.677). This lower degree of order in the envelope-matrix complex may reflect a disordering effect of the matrix on the membrane lipid which would be consistent with the third possibility. It is noteworthy that the values of the order parameters (Table I) before and after cholesterol exchange were greater for isolated envelopes than for the intact nuclei. In conjunction with the value for the envelope-matrix complex, this indicates that the nuclear membrane lipid in situ is somewhat disordered because of the attachment of the envelope to the matrix fibrils.

TABLE IV

EFFECTS OF MODIFICATION OF CHOLESTEROL CONTENT OF ISOLATED ENVELOPES ON ACTIVITIES OF NUCLEAR ENVELOPE ENZYMES

a, b and c, as in Table I. Results are means ± 1 S.E. of four duplicate determinations.

Incubation conditions	μmol cholesterol/ μmol phospholipid	Enzyme activity (nmol substrate/min per mg protein)	
		Nucleoside triphosphatase	NADH-ferricyanide reductase
a	0.094 ± 0.006	17 ± 4	253 ± 22
b	0.266 ± 0.038	16 ± 4	222 ± 19
c	0.110 + 0.011	19 ± 5	261 ± 24

Effect of cholesterol enrichment on nuclear enzymes

Table IV shows that the activity of nucleoside triphosphatase, a pore-lamina component, was not affected by the addition of cholesterol. However, the NADH-ferricyanide reductase activity was slightly lowered ($p < 0.05$) in a preparation where the cholesterol content was increased to 0.266 mol/mol phospholipid. Since this enzyme is integral to the nuclear membranes, this result may be consistent with a small effect of the more ordered membrane on the enzyme activity.

Discussion

The clear differences in the extent and rate of uptake of cholesterol into the various preparations is an indication that there are major differences in character between them. These differences may be important in studies which are concerned with the properties of the envelope as a whole and not with an individual component. It is possible that in the present case there is little contact between the two nuclear membranes resulting in the slow exchange of cholesterol observed between them (Table II), a process which in other membrane systems can be rapid. For example, cholesterol has been shown to exchange into the inner mitochondrial membrane from rat corpora lutea when intact mitochondria are incubated with liposomes [34], and cholesterol can also penetrate into the intracellular membranes of the platelet when these cells are incubated with liposomes [35]. However, the rate of incorporation of cholesterol into the platelet intracellular membranes is significantly less than the rate of incorporation into the plasma membrane. Since these systems show a relatively facile transfer of cholesterol between two functionally related membranes in a cell or organelle, the differences found here suggest that the inner nuclear membrane may be quite substantially protected from access by materials in solution and from the outer membrane by contact with the nuclear matrix on the one hand and by lack of contact with the outer membrane on the other.

Cholesterol has various effects on the properties of membrane proteins. It has been shown to inhibit the $(Ca^{2+} + Mg^{2+})$-ATPase of the sarcoplasmic reticulum [36], and its effect on the electron transport chain of rat liver microsomes seems to depend upon the method by which extra cholesterol is incorporated [37,38]. In the red cell, cholesterol inhibits the $(Na^{+} + K^{+})$-ATPase and has been shown to interact specifically with the band 3 protein [39]. These differences presumably reflect the environment of these proteins and may reflect their own characteristic affinity for a specific lipid environment. The present results show a small effect of added cholesterol on one nuclear membrane enzyme (NADH-ferricyanide reductase) which implies that it is slightly sensitive to the nature of its lipid environment. In contrast, the pore-lamina component, nucleoside triphosphatase, was not affected by the addition of cholesterol which is consistent with its being located in a protein assembly which is not sensitive to direct effects of its lipid environment.

The results described in this paper indicate that the physical properties of the nuclear membrane lipid, and the exchangeability of cholesterol between the nuclear membranes and liposomes, depend to a large extent on the nature of

the nuclear envelope preparation. The observations suggest that the presence or absence of an intact nuclear matrix contributes largely to these differences. In *Tetrahymena*, the matrix is contractile, and its state of contraction has been shown to affect the fluidity of the membrane and the amount of intrinsic membrane protein [12,13]. Although the mechanism whereby the matrix influences the state of the membrane lipid in liver nuclei is not yet clear, these findings suggest that matrix contraction might determine the physical state of the envelope without there being any change of membrane composition, and this in turn could affect nucleo-cytoplasmic exchange of macromolecules [11,13]. Whether or not this speculation is valid, the present findings show that conclusions drawn from physical studies on the lipid of isolated nuclear envelopes cannot be assumed to apply to the envelope in situ.

Acknowledgements

We are indebted to the Cancer Research Campaign and to the Medical Research Council for support of this work, and to Mr. C.D. Gleed for excellent technical assistance.

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