Serum Lipoproteins Modulate Oxygenated Sterol Insertion into Human Red Cell Membranes

Abstract. *The insertion of oxygenated sterol compounds into human red blood cell membranes as well as the consequent transformation of the red cells to an echinocyte shape and the expansion of the membranes are impeded by the presence of serum lipoproteins in the incubation medium. All density classes of human serum lipoproteins bind oxygenated sterol compounds, and lipoproteins can act as acceptors of oxygenated sterols previously inserted into red cells. Since oxygenated sterols have been reported to be atherogenic, the modulating and possibly protective effects of serum lipoproteins on oxygenated sterol-induced derangement of cell membrane structure and function may provide a useful model for further study.*

In addition to their capacity to inhibit cholesterol synthesis in mammalian cells (*1*), oxygenated sterol compounds (OSC) (*2*) are capable of altering both the structure and function of mammalian cell membranes (*3–7*). After a 1-hour incubation of human red blood cells with certain OSC (1.25×10^{-5} to $5 \times 10^{-5} M$), the red cells are transformed into spiny-shaped cells known as echinocytes (*6*). Both echinocyte-forming and non–echinocyte-forming OSC are capable of protecting red cells against osmotic lysis, thus lowering the mean corpuscular fragility (MCF) of the red cells (*8*). The effects of OSC on red cells are a consequence of OSC insertion into the red cell membrane and are proportional to the amount inserted. The amount of an OSC inserted after incubation of red cells with a given concentration of the compound can be reduced by the presence of lipoproteins in the incubation medium, but not by the presence of free cholesterol; free cholesterol does prevent the loss of cholesterol from red cell membranes that occurs under our usual conditions of exposure of red cells to OSC in lipoprotein-depleted medium (LPDM) (*6*). We examined the degree to which lipoproteins can modulate the insertion of OSC into red cells and the consequent secondary effects of OSC on the cells, and we studied the binding of OSC to human serum lipoproteins in an effort to explain this modulation.

We washed freshly drawn human red cells three times in phosphate-buffered saline (0.15*M* NaCl and 0.01*M* PO_4, *p*H 7.4) containing bovine serum albumin (0.25 g/dl). The cells (5 percent hematocrit) were incubated for 1 hour at 37°C in RPMI 1640 containing lipoprotein-depleted serum (20 percent by volume) (*9*) or lipoprotein-containing normal human type AB serum, both of which had been heat-inactivated at 56°C for 30 minutes. The OSC, dissolved in ethanol at 100 times the desired final concentration, were added to the cell suspension just before the start of each experiment. Control cell incubation mixtures contained ethanol (1 percent, by volume). At the end of the incubation period, echinocyte counts were performed where desired (*6*), and the remaining cells were washed three to five times in 100 volumes of cold (4°C) phosphate-buffered saline, a procedure that does not reduce the amount of OSC present in the red cells (*8*). The washed cells were used for determination of red cell osmotic fragility (*8*) and for measurement of the amounts of OSC and cholesterol present in their membranes (*6, 10*). In certain experiments, OSC-containing cells and control cells were subjected to a second similar incubation in various media, then washed and analyzed again.

To determine the capacity of serum lipoproteins to bind OSC, we added the compounds to human serum containing Na_3H-EDTA (0.025 g/dl). The serum was freshly isolated from blood drawn from a fasting type A donor. The final concentrations of OSC and their ethanol solvent were 20 μg/ml ($5 \times 10^{-5} M$) and 2 percent (by volume), respectively. A serum sample containing a similar amount of [^{3}H]cholesterol was also prepared to allow comparison of OSC with a physiological sterol. After incubation for 1 hour at 24°C, the main lipoprotein subclasses—very low density lipoprotein (VLDL) (density < 1.006 g/ml), low density lipoprotein (LDL) (density, 1.019 to 1.063 g/ml), and high density lipoprotein (HDL) (density, 1.063 to 1.21 g/ml)—as

Table 1. The effect of various incubation mediums on the insertion of OSC into red blood cell membranes and their subsequent egress. In experiments 1, 2, and 5, the secondary effects of OSC on red cell membranes, namely, echinocyte formation (*6*) and increased resistance to osmotic lysis due to membrane expansion (*8*), were also monitored. The first incubation medium was LPDM unless otherwise stated. RPMI-AB is RPMI 1640 plus 12 percent AB serum; BSA is bovine serum albumin. The amount of OSC inserted into 0.25 ml of packed red blood cells (OSC inserted) is given as means ± standard deviation. The increased resistance of red blood cells to osmotic lysis is expressed as the difference between the MCF of the OSC-exposed sample and the MCF of the control sample (ΔMCF) and is measured as NaCl concentration. 20α-Hydroxycholesterol and 22-ketocholesterol are non–echinocyte-producing OSC (*3, 6*). The cholesterol content (*15*) of 0.25 ml of packed red blood cells is 333 ± 20 μg. The protein concentration in all mediums, including RPMI-BSA, was 10 mg/ml.

OSC concentration, first incubation medium ($\times 10^{-5} M$)	Medium, second incubation	OSC inserted (μg)	ΔMCF (g/dl)	Echinocytes (%)
		Experiment 1: 7β-hydroxycholesterol		
3.75		50.8 ± 6.5		99
2.5		36.7 ± 6.5		88.5
1.25		20.8 ± 1.4		54.4
6 (RPMI-AB)		30.8 ± 5.1		85.5
3 (RPMI-AB)		13.8 ± 1.8		54
1.5 (RPMI-AB)		9.1 ± 0.9		17.3
		Experiment 2: 22-ketocholesterol		
5			−0.063	
2.5			−0.024	
10 (RPMI-AB)			−0.038	
5 (RPMI-AB)			−0.022	
		Experiment 3: 20α-hydroxycholesterol		
2.5	None	28.8 ± 1.3		
2.5	LPDM	24.9 ± 2.6*		
2.5	RPMI-BSA	22.5 ± 1.8*		
2.5	RPMI-AB	14 ± 1.8†		
		Experiment 4: 7β-hydroxycholesterol		
2.5	None	39.4 ± 3.7		
2.5	LPDM	33.4 ± 3.7†		
2.5	RPMI-BSA	34.8 ± 4.3*		
2.5	RPMI-AB	13.5 ± 5.0‡		
		Experiment 5: 20α-hydroxycholesterol		
5	None		−0.052	
5	LPDM		−0.050	
5	RPMI-AB		−0.016	

*Not significant. †$P < .02$. ‡$P < .001$.

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Table 2. The binding of cholesterol and various OSC to the major lipoprotein subclasses of human serum, given as amount of sterol recovered. The percentages of OSC recovered are expressed with respect to the total amount of sterol recovered. Percentages of total amount of sterol recovered are expressed with respect to the amount originally added to 3 ml of serum (60 μg). Cholesterol percentages were determined by scintillation counting.

Lipoprotein	20α-Hydroxycholesterol		7α-Hydroxycholesterol		25-Hydroxycholesterol		Cholesterol
	Amount (μg)	Percent	Amount (μg)	Percent	Amount (μg)	Percent	Percent
VLDL	6.3 ± 0.9	12.2 ± 1.7	3.0 ± 0.4	8.0 ± 1.1	1.3 ± 0.8	4.5 ± 2.7	4.4
LDL	19.7 ± 1.1	38.2 ± 2.1	14.8 ± 2.7	39.5 ± 7.2	6.3 ± 0.8	21.6 ± 2.7	38.1
HDL	12.6 ± 1.7	24.3 ± 3.3	15.3 ± 2.2	40.8 ± 5.9	7.1 ± 1.5	24.3 ± 5.1	29.7
Bottom fraction	13.1 ± 1.4	25.3 ± 2.7	4.4 ± 2.2	11.7 ± 5.9	14.5 ± 3.1	49.6 ± 10.6	27.8
Total	51.8	86.3	37.5	62.5	29.2	48.7	84.2

well as the bottom fraction (density > 1.21 g/ml) of all 3-ml samples were isolated by sequential ultracentrifugal flotation and isopycnic density gradient ultracentrifugation (*11*). The amount of OSC in each lipoprotein subclass and bottom fraction was determined on its neutral lipid extract by thin-layer chromatography (*6*). Quantification of the amount of added cholesterol distributed among the various fractions was determined by liquid scintillation counting (*11*).

When red cells were incubated with 7β-hydroxycholesterol in LPDM, the amount of the OSC inserted in red cell membranes increases in proportion to the concentration originally present in the incubation medium, and the degree of echinocyte transformation also increases (*6*). Both OSC membrane insertion and red cell echinocyte transformation are impeded, but not abolished, by the presence of lipoproteins in the OSC–red cell incubation medium. Thus, in the presence of lipoproteins, a 2.4-fold increase in the concentration of OSC in lipoprotein-containing medium achieves the same degree of red cell echinocyte transformation as is seen with lower concentrations of OSC in LPDM, and the proportion of the OSC originally present that finds its way into the red cells is one-fourth to one-third of that seen in the absence of lipoprotein (Table 1, experiment 1). Similarly, the protection against osmotic lysis (ΔMCF) afforded red cells by varying concentrations of 22-ketocholesterol in the presence of lipoprotein is only one-half to one-third of the protection afforded by the same OSC when it is exposed to red cells in LPDM (Table 1, experiment 2). Similar results were obtained in experiments with 7β-hydroxycholesterol and 20α-hydroxycholesterol (results not shown).

After OSC is inserted into red cell membranes by incubation in LPDM, the amount of OSC in the cells is reduced only slightly, if at all, by a subsequent 1-hour incubation in LPDM or in RPMI 1640 medium containing an equivalent amount of protein (10 mg/ml) in the form of crystalline bovine serum albumin. In contrast, a second incubation in lipoprotein-containing medium results in the loss from the red cell membrane of 50 to 65 percent of the amount of OSC originally acquired (Table 1, experiments 3 and 4). Concomitant with the loss of inserted OSC, the red cell resistance to osmotic lysis originally acquired and maintained through a second incubation in OSC-free LPDM, is diminished substantially by incubation of the cells in lipoprotein-containing medium (Table 1, experiment 5).

When human serum is incubated with OSC, and the various serum lipoprotein classes are subsequently isolated and analyzed (Table 2), the major portion of the OSC recovered is associated with serum lipoproteins. Among the OSC studied, 25-hydroxycholesterol is found in the largest amount in the bottom fraction (49.6 percent). The largest amount of 20α-hydroxycholesterol found in association with lipoproteins is present in LDL. Approximately equal amounts of 7α-hydroxycholesterol and 25-hydroxycholesterol are present in HDL and LDL. Similar results were obtained for the binding of 7-ketocholesterol by pig plasma lipoproteins (*12*). The elution profile of the various lipoprotein subclasses with respect to protein concentration (determined by absorbance at 280 nm) and total cholesterol content, as well as the position of each lipoprotein class in the density gradient, was not altered by the OSC.

Our experiments suggest that the impedance by serum lipoproteins of both the entry of OSC into red cells and the secondary manifestations of such entry, namely, echinocyte formation and enhanced resistance to osmotic lysis, is a consequence of their capacity to bind 50 to 88 percent of the added OSC and thereby make it unavailable for entry into the erythrocyte membrane. The protection offered by lipoproteins against OSC membrane insertion is only relative; the final equilibrium distribution of OSC between lipoprotein and red cell membrane is such that, if OSC concentrations are increased 2.5- to 3-fold, insertion of OSC into red cell membranes from lipoprotein-containing medium can equal that achieved from LPDM at lower concentrations, and the expected changes in red cell shape and membrane expansion ensue secondarily. In other experiments with purified LDL and with HDL_3, a subclass of HDL having a density of 1.12 to 1.21 g/ml, we have shown that addition of these human serum lipoproteins (100 μg of protein per milliliter of medium) to LPDM reduces 20α-hydroxycholesterol ($2.5 \times 10^{-5} M$) insertion into red cells by 40 and 21.5 percent, respectively (*13*). Furthermore, we have shown that lipoprotein-containing medium and purified human serum lipoproteins can modulate the entry of OSC into long-term human lymphocyte cell lines in a manner precisely analogous to that described for human red blood cells (*13*).

Echinocyte formation by OSC can be reversed by incubating OSC-containing echinocytes in lipoprotein-containing serum (*3*). Similarly, the membrane expansion and resistance to osmotic lysis induced in red cells by insertion of OSC into membranes can be reversed by exposure of the cells to lipoprotein-containing medium. The reversal of these secondary changes induced in red cells by OSC can, on the basis of our studies, be viewed as a consequence of the loss of inserted OSC to serum lipoproteins. Thus, lipoproteins function as OSC acceptors and induce an equilibrium redistribution between cell membranes and medium that is not achievable by other serum proteins or by bovine serum albumin.

The formation of E-rosettes by human lymphocytes can be inhibited by OSC (*3, 5*), as can chemotactic responses by human polymorphonuclear leukocytes (*3, 7*). These functional aberrations of the cell membrane, as well as the lymphocy-

totoxicity of certain OSC (*3, 5*), can be prevented by the presence of serum lipoproteins during exposure of the cells to OSC. Since OSC reportedly possess angiotoxic properties and are suspected of being atherogenic (*14*), the modulating and possibly protective effects displayed by serum lipoproteins toward the entry of OSC into cell membranes and the consequent OSC-induced derangements of cell membrane structure and function may provide a useful model for further study.

R. A. STREULI
Section of Hematology/Oncology, Department of Medicine, University of Chicago, Chicago, Illinois 60637

J. CHUNG
Department of Medicine, University of Chicago

A. M. SCANU
Departments of Medicine and *Biochemistry, University of Chicago*

S. YACHNIN*
Section of Hematology/Oncology, Department of Medicine, Franklin McLean Memorial Research Institute, and *Committee on Immunology, University of Chicago*

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9. The LPDS was prepared by ultracentrifugation flotation of lipoproteins of density < 1.25 g/ml [A. Scanu, *J. Lipid Res.* **7**, 295 (1966)]. The residual bottom fraction containing all other serum proteins was dialyzed extensively against phosphate-buffered saline, heated to 56°C for 30 minutes, and sterilized by membrane filtration before use.
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* To whom requests for reprints should be addressed.

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Natural Explosive Noises

Gold and Soter (*1*) speculate that gas escaping from fractures in the earth is responsible for many unexplained explosive, booming noises. They cite the occurrence of heard but unfelt events prior to and following some major earthquakes as evidence for escaping gas, evidence on which they have erected the hypothesis that large amounts of methane might be found in the lower crust or upper mantle (*2*). In this comment I present evidence that earthquakes too weak to be felt frequently produce loud booming noises, examine the mechanism by which escaping gas can generate loud booming sounds, and critically review some accounts cited by Gold and Soter as supporting their interpretation.

Field observations show that earthquakes too small to be felt sometimes produce loud booming noises. During aftershock studies near the Mojave Desert town of Landers, California (*3*), and in the vicinity of Mammoth Lakes, California (*4*), booming sounds from earthquakes as small as magnitude 1 were transmitted from large bedrock outcrops. Had those conducting the studies not been equipped with seismographs, most of the booming sounds heard would not have been recognized as associated with individual earthquakes. In neither case were signs of escaping gas mentioned by any of the geologists, seismologists, visitors, or residents in the epicentral area.

Perceptions of observers other than seismologists show that unfelt earthquakes can be heard. Earthquakes near Fontana, California (8 January 1980), and Berkeley, California (6 April 1980), were, according to newspaper and police reports, heard rather than felt. In both cases, many citizens telephoned local authorities to report hearing an explosion. In neither case was evidence of an explosive-like discharge of gas reported.

Airwaves associated with the great 1964 Alaska earthquake were recorded on microbarographs thousands of kilometers from the source. Bolt (*5*) demonstrated that a significant part of this signal traveled as an airwave from the epicentral region. Mikumo (*6*) showed that the barograph records, which were distinct from records of large atmospheric explosions, were consistent with the hypothesis that the source of the pressure waves was a sudden vertical displacement of a large area of the earth's crust.

Unless strong evidence to the contrary exists, there is no need to invoke different physical processes for a single phenomenon. Direct transmission of seismic energy from ground to air, sometimes by earthquakes too small to be felt, is clearly adequate to explain booming noises. I will now consider whether gas, high-pressure or combustible, is a good candidate for generating loud booming noises.

Recent events in Kansas and Oklahoma demonstrate what can occur when gas escapes geologic media (*7, 8*). Combustible gas broke through flaws in rock formations, throwing mud and water more than 10 m into the air. Although the gas concentrations were well within explosive limits, the gas did not ignite spontaneously, and no unusual noises were generated. Mud volcanoes and craters were formed and will long mark the sites of these gas eruptions.

High-pressure gas emissions most likely cannot generate loud, low-frequency booms without leaving evidence. Pressure levels for gas in subterranean openings cannot exceed the least principal stress or the cracks will propagate as hydraulic fractures. Weight of overburden thus constrains the allowable pressure for gas at rest in fractures to less than about 300 bars per kilometer of depth. While this value may suggest that explosive, audible discharge of gas is plausible, serious objections must be considered.

It is difficult to conceive of a fractured

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