MILD PRESSURE INDUCES RESISTANCE OF ERYTHROCYTES TO HEMOLYSIS BY SNAKE VENOM PHOSPHOLIPASE A_2

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ABSTRACT It is generally assumed that mild pressure of a few atmospheres, such as that applied to blood cells during routine centrifugation, does not affect cell function. The results of the present study refute this notion. To explore the effect of mild pressure on cell function we examined its effect on the susceptibility of red blood cells (RBC) to hemolysis by snake venom phospholipase A_2 (PLA_2). Rat RBC were subjected to pressure of up to five atmospheres, returned to ambient pressure and interacted with PLA_2 to induce hemolysis. The hemolysis was markedly decreased with increasing the pressure applied before induction of hemolysis. Application of such a pressure induces the shedding of a chemical factor, as yet uncharacterized, which facilitates the action of PLA_2 on RBC.

INTRODUCTION

Application of high pressure, in the order of hundreds or thousands of atmospheres, has been shown to affect cell functions and properties, such as depolymerization of microtubules in cells (1) and in vitro (2), ion transport (3, 4), membrane-lipid phase transition (5, 6), release of membrane proteins (7), receptor-protein dissociation (8), and platelet aggregation (9). Yet, it is generally assumed that mild pressure in the range of a few atmospheres, does not affect cell function. Such pressure is applied to blood cells during routine centrifugation, or to organisms under sea. The present study was undertaken to examine the effect of mild pressure on cell function. For this purpose, we applied pressure of several atmospheres to red blood cells (RBC). The cells were then returned to ambient pressure and hemolysis was induced by exposing the cells to snake venom phospholipase A_2 (PLA_2). This enzyme hydrolyses phospholipids at the cell membrane to produce lysophospholipids and fatty acids. When this action is followed by the addition of fatty acid-free albumin, this protein scavenges the membranal fatty acids produced by PLA_2 and the cell lyses (10). It is well accepted that the induced hemolysis is a measure of the hydrolysis of the cell membrane phospholipids by the exogenous PLA_2 (10, 11). This system was employed here to examine the effect of mild pressure on the cell membrane, since the action of PLA_2 is sensitive to changes in chemical and physical properties of the membrane (12–14).

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EXPERIMENTAL PROCEDURE

Application of Pressure

Cells at the bottom of a spinning tube are subjected to hydrostatic pressure p, generated by the centrifugal field (w^2R), as expressed in the equation:

$$\Delta P = \int_{R_0}^{R} \rho_s w^2 R dR = \tfrac{1}{2}\rho_s w^2 (R^2 - R_0^2),$$

where ρ_s is the aqueous phase density, w is the angular velocity of the spinning rotor, R and R_0 are the distances from the center of rotation to the bottom of the tube and the air-water miniscus, respectively.

Additional pressure is applied to the cell surface due to the acceleration of the cell mass, but this is negligible compared with the hydrostatic pressure. Thus, the total pressure on the spinning cell is:

$$P = P_0 + \tfrac{1}{2}\rho_s w^2 (R^2 - r_0^2),$$

where P_0 = the atmospheric pressure.

Correspondingly, pressure was applied to RBC by the following three methods: (*a*) Changing the angular velocity of the centrifugation (w). (*b*) Changing the height of the aqueous column in the spinning tube (R_0). (*c*) Application of a direct pressure to the cell suspension, in a tube connected to an argon cylinder (changing P_0 in the equation).

After these treatments, the cells were returned to ambient pressure, and snake venom PLA_2 was added to induce hemolysis.

Induction of Hemolysis

Rat RBC were collected from heparinized blood, drawn from the aorta, and washed twice with the reaction buffer (140 mM NaCl, 5 mM KCl and 5 mM Tris-HCl at pH = 7.4), by spinning at 500 $g \times 5$ min. A solution of 0.4 mg/ml Naja mocambica PLA_2 (Makor Chemicals, Jerusalem, Israel) and 2 mM CaCl was prepared from concentrated stock solutions in the reaction buffer, and left at room temperature for 5 min. To start the reaction, equal volumes of 5% RBC suspension and the PLA_2-$CaCl_2$ solution were mixed (to obtain final concentrations of 5%

RBC and 1 mM $CaCl_2$) and incubated for 20 min at 37°C, in a shaking bath. For termination, EDTA from a stock solution of 200 mM in the reaction buffer, was added to a final concentration of 2 mM and the tubes were cooled in ice. To obtain hemolysis the samples were returned to room temperature and fatty acid-free albumin solution was added to a final concentration of 0.5%. The samples were spun and the absorbance of the supernatant was measured at 540 nm (10).

Determination of PLA_2 Activity

To determine the enzyme's activity, the hydrolysis of phospholipids was measured by the amounts of lysophospholipids produced. RBC were interacted with PLA_2 for the desired time and the reaction was terminated by the addition of EDTA as described above. Similar to previous reports (10, 11), the addition of EDTA blocked the PLA_2 activity and the subsequent hemolysis. The cells were then subjected to lipid extraction and two-dimensional thin layer chromatography on silica gel plates (Kieselgel 60, Merck & Co., Inc., Los Angeles, CA) as described by Roelofsen and Zwaal (11). The desired phospholipids were extracted from the silica and their content was measured by phosphate determination (11).

RESULTS AND DISCUSSION

To examine the effect of mild pressure on PLA_2-induced hemolysis, pressure was applied to RBC by either of the three methods described in the Experimental section. The cells were then returned to ambient pressure and interacted with PLA_2. As shown in Fig. 1, the hemolysis was inhibited proportionally to the hydrostatic pressure applied to the cells, using any of the three methods outlined above. Application of ~5 at. for 15 min. already reduced the hemolysis to ~5%. It should be noted that application of higher pressure did not produce a considerable increase in the inhibition of hemolysis, beyond that obtained by ~5 at. The reduction of hemolysis was dependent also on the duration of the pressure application, as a similar effect was obtained by centrifugation of 15 min at 2,000 *g* (4.8 at) or 30 min at 900 *g* (2.75 at) (Fig. 1).

It should be noted that when pressure was applied to cells by centrifugation (methods [a] and [b]), RBC were placed at the bottom of the tube and the buffer was layered on top, before spinning. This was carried out in order to avoid mixing the cells and to rule out possible effect(s) of shear force applied to the cells during sedimentation (15).

To substantiate the relation of the reduced hemolysis to PLA_2 activity, we examined the pressure effect on the production of lysophospholipids by the interaction of the enzyme with RBC. Similar to the experiment of Fig. 1, cells were pretreated with pressure, returned to ambient pressure, and interacted with PLA_2. The cell suspension was then subjected to lipid extraction and determination of lysophospholipid produced. The only one produced was lysophosphatidylcholine (LPC). In accord with previous reports (11), other lysophospholipids were not detected. As shown in Table I, the amount of LPC was increased by the action of the snake venom PLA_2 at the expense of PC. This is indeed expected since this enzyme acts on the outer leaflet of the membrane, which is composed almost exclusively of PC and sphingomyelin (10, 11).

Table I shows that application of pressure to RBC before interaction with PLA_2, reduced the production of LPC, concomitantly with the reduction of hemolysis, shown in Fig. 1.

RBC are deformable cells and may alter their shape or form aggregates in response to changes in chemical and physical factors, such as blood flow and shear rate, albumin concentration, or haematocrit (15–19). A possible explanation for the pressure-induced reduction of hemolysis is formation of aggregates which might reduce the accessibility of the enzyme to the cell membrane. Another possibility is that the cells change their shape, and their susceptibility to the enzyme is subsequently reduced. These possibilities were ruled out by examination of the RBC under the microscope. The cells were singly dispersed, and had a spheric shape as expected in the absence of albumin (19). These characteristics were not altered by application of pressure. The pressure-induced inhibition of hemolysis

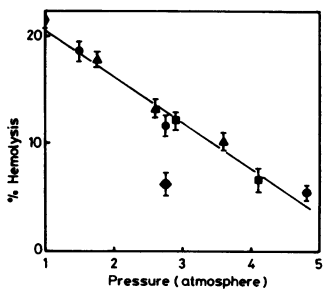

FIGURE 1 Effect of pressure on lysis of rat RBC by snake venom PLA_2. Pressure was applied to RBC by (*a*) varying *w*, the centrifugation RPM (●); (*b*) varying *Ro*, the height of the aqueous column on the cells in the centrifuge tube (▲); (*c*) connecting the cell-containing tubes to an argon cylinder (■). Rat RBC were collected, washed, and suspended at 5% hematocrit in the reaction buffer as described in the Experimental Section. In *a* and *b*, the RBC suspensions were centrifuged for 15 min. at the indicated pressure. The cell pellet was then suspended in fresh buffer to the same concentration and interacted with PLA_2. In *c*, a 5% RBC suspension was placed under argon pressure for 15 min, returned to ambient pressure, and interacted with PLA_2. ( ), Centrifugation at 900 *g* (2.75 at.) for 30 min. The ordinate expresses percent of total hemolysis, obtained by subjecting an equal amount of RBC to hypoosmotic shock. Each datum is mean ± SD for four replications. The correlation coefficient, $r = 0.987$.

TABLE I
EFFECT OF PRESSURE ON FORMATION OF LYSOPHOSPHATIDYLCHOLINE (LPC) BY SNAKE VENOM PLA_2

Treatment		LPC
pressure	PLA_2	%
–	–	9.7 ± 1.3
+	–	11.4 ± 0.8
–	+	28.8 ± 1.1
+	+	16.2 ± 1.7

RBC suspension were prepared as in the experiment of Fig. 1. Pressure was applied by spinning at 2,000 *g* for 15 min and the pressurized cells were returned to ambient pressure before interaction of PLA_2. The reaction was terminated as described in Methods and the cell suspension was then subjected to lipid extraction and thin layer chromatography as described in Methods. PC and LPC were extracted from the silica and determined. The amount of LPC is expressed as mole percent of total PC + LPC (11). The average total PC + PLC was 1.1 μmol/ml packed cells.

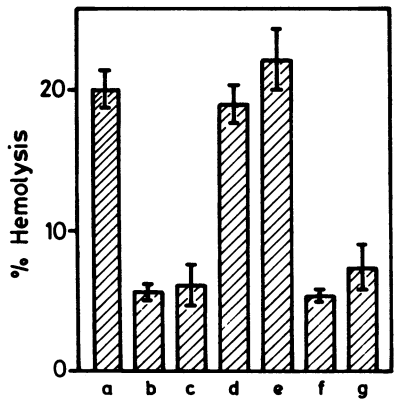

FIGURE 2 Examination of the nature of the pressure effect on RBC lysis by snake venom PLA_2: Rat RBC were collected, washed, and suspended at 5% hematocrit in the reaction buffer as described in the Experimental section. In *a*, the control experiment, the cells were subjected to the action of PLA_2 with no further treatment. In experiment *b* the cells were further exposed to hydrostatic pressure by spinning at 2,000 *g* for 15 min. the supernatant was removed, the cell pellet was suspended in fresh buffer and immediately interacted with PLA_2; In experiments *c* to *f* the cells were further exposed to hydrostatic pressure by spinning at 2,000 *g* for 15 min and subjected to the following treatments before interaction with PLA_2: (*c*) The pelleted cells were suspended in the fresh buffer and preincubated at room temperature for 20 min. (*d*) The pelleted cells were resuspended in their own supernatant, and preincubated at room temperature for 20 min. (*e*) The pelleted cells were suspended in the supernatant of another portion of cells which were spun at 2,000 *g* for 15 min (conditioned medium), and preincubated for 20 min at room temperature. (*f*) The pelleted cells were resuspended in their own supernatant (same as in *d*) but were preincubated on ice for 20 min. (*g*) The pelleted cells were suspended in conditioned medium and immediately interacted with PLA_2 (without preincubation).

was obtained at various RBC concentrations, ranging from 2.5 to 50%. It appears therefore that the reduction of PLA_2 activity was not due to cell-cell interaction or modification of the cell shape.

To further explore the nature of the pressure effect, we examined the possibility that application of pressure produces a chemical (rather than physical) modification of the cell, such as shedding of a cellular factor necessary for the action of PLA_2 on RBC. For this purpose the experiments described in Fig. 2 were designed. In these experiments pressure was applied to RBC by centrifugation. The pelleted cells were then resuspended either in their own supernatant (*column d*) or in conditioned medium collected from other treated cells (*column e*). These cell suspensions were preincubated for 20 min at room temperature before the addition of PLA_2. As shown in Fig. 2 (*column d and e*) preincubation of the cells in supernatant of pressure-treated cells reversed the pressure effect, and restored the PLA_2 activity, as the degree of hemolysis was similar to that of nontreated cells (*column a*). However, the pressure-induced inhibition of hemolysis persisted when the supernatant was removed after spinning, and the pelleted cells were suspended in fresh buffer (*column b and c*). We conclude, therefore, that application of pressure to RBC induces shedding of a factor, as yet not characterized, which facilitates the action of PLA_2. It appears that this labile factor readily incorporates into the cell and subsequently increases the PLA_2 activity (*column d and e*). The incorporation is temperature dependent, as it does not occur when the cell suspension is incubated on ice (*column f*).

The mechanism of PLA_2 action on membrane lipids involves rapid binding to the membrane surface after its activation (12–14). The activated enzyme remains bound and hydrolyses lipids along the membrane surface (10–13). In the light of this mechanism, it was of special interest that the pressure effect was not reversed if the conditioned medium was applied after the enzyme had been introduced to the cells (*Fig. 2, column g*). Apparently, the restoration of PLA_2 activity is not obtained once the enzyme has interacted with the RBC.

As already noted in the introduction the fact that high pressure alters cell properties is well known (1–9). In the present study we applied pressure which is orders of magnitude smaller than that used in the above mentioned reports and more relevant to physiological states. An indication that application of mild pressure might affect cell function was already presented by Murayama (20), who demonstrated that decompression of platelet-rich plasma (to 380 mm Hg for 3 h at 38°C) produced platelet aggregation. The surprising finding here is that a short-term application of a few atmospheres to RBC, before interaction with PLA_2, produces a marked alteration in their susceptibility to hemolysis, by shedding of a PLA_2 cofactor from the cells. Such pressures are routinely applied to cells in research and clinical laboratories. Our findings suggest that the effect of mild pressure on cells cannot be ignored and might be pertinent to cell physiology and function in general.

We thank Dr. Rami Rahamimov and Dr. Dov Lichtenberg for their helpful discussion and critical review, and Mr. Eli Hovav for his skillful technical assistance.

This work was supported by grants from the Israeli Academy of Sciences, the Szold Foundation for Research, and the Ministry of Commerce, Jerusalem, Israel.

Received for publication 25 February 1988 and in final form 12 May 1988.

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