0031-3998/84/1808-0773$02.00/0
PEDIATRIC RESEARCH
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Vol. 18, No. 8, 1984
Printed in U.S.A.

Membrane Fluid Properties of Cord Blood Mononuclear Leucocytes: Association with Increased Insulin Receptors

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Summary

Insulin receptors are present on fetal and newborn tissues in significantly greater numbers than on adult tissues. Recent studies have suggested that membrane fluidity, which is dependent upon lipid constituents, is important in regulating the appearance and behavior of insulin receptors. We have compared the lipid composition and fluidity as well as insulin receptor binding to monocytes from normal adults and full term normal infants.

Newborn infants had significantly higher insulin levels than did fasting adults (17.4 ± 2.4 *versus* 9.8 ± 0.6 μU/ml; $P < 0.001$); despite this, cord blood monocytes showed significantly higher ^{125}I-insulin tracer binding than did those of adults (9.5 ± 0.51 *versus* 7.6 ± 0.45%/10^7 cells; $P < 0.02$). From Scatchard analysis, it was evident that cord monocytes had greater numbers of both high (2.94 *versus* 1.25 × 10^{-10} M^{-1}) and low affinity (13.1 *versus* 8.57 × 10^{-10} M^{-1}) receptors than adult monocytes.

Cord mononuclear cells had significantly lower phospholipid concentrations than adult cells (0.085 ± 0.012 *versus* 129 ± 0.012 mg/mg of protein; $P < 0.025$) and significant elevations of cholesterol/phospholipid ratios (0.520 ± 0.045 *versus* 0.354 ± 0.009; $P < 0.005$). Microviscosity determinations were performed using the fluorescent probe 1,6-diphenyl-1,3,5-hexatriene. Cord cells had significantly greater microviscosity values (fluorescence polarization) (0.339 ± 0.030 *versus* 0.186 ± 0.019; $P < 0.005$), compared to adult cells. For all subjects, a highly significant correlation was noted between cell microviscosity measurements (fluorescence polarization) and ^{125}I-insulin tracer binding to mononuclear cells ($r = 0.72$, $n = 15$, $P < 0.005$).

The data support the concept that as membrane fluidity decreases (*i.e.* viscosity increases) intrinsic proteins including receptors are displaced outward, increasing their exposure to the aqueous environment.

Abbreviations

DPH, 1,6-diphenyl-1,3,5-hexatriene
FP, fluorescence polarization
ΔE_{act}, flow activation energy (kcal/mol)
PBS, phosphate-buffered saline

Insulin receptors are present on fetal and newborn tissues in significantly greater numbers than on adult tissues (17, 19, 49). We have previously shown that hepatic insulin receptors increase in the fetal rat during the last part of gestation, in a manner which parallels the increasing insulin content in the fetal pancreas (33, 34). Furthermore, in the presence of maternal diabetes and fetal hyperinsulinemia, paradoxical up-regulation of the insulin receptor has been documented for both monocytes and lung membranes (30–32). Only after birth do insulin receptors decrease in number on erythrocytes and hepatic membranes to levels comparable to those in adults (11, 17, 39, 41, 48, 51).

The factors which regulate the appearance of the insulin receptor in the fetus and newborn are not well understood. One characteristic of fetal development is the accumulation of membrane phospholipid, a process which continues after birth (12, 27). We and others have suggested that membrane lipid composition and particularly membrane fluidity, which is dependent upon lipid constituents, is important in regulating the appearance and behavior of insulin receptors (2, 14, 29). This becomes evident if one considers the membrane to be a mosaic structure composed of zones of irregularly distributed lipid which form the environment for membrane proteins (47).

We have compared the membrane lipid composition and fluidity as well as insulin receptor binding to mononuclear cells obtained from adult and cord blood. Our findings suggest that the appearance of increased insulin receptors on cord blood mononuclear cells is due to reduced membrane fluidity which results from diminished membrane phospholipid. The structure of the plasma membrane on these cells may also prevent insulin receptor translocation by limiting movement of constituents within the membrane. This may result in limitations in insulin action which have been reported to occur in fetal cells (49, 51).

MATERIALS AND METHODS

Na[^{125}I]iodide was purchased from New England Nuclear Corp. (Boston, MA). Enzymo-Beads for lactoperoxidase iodination were obtained from Bio-Rad Corp., Richmond, CA. Purified monocomponent insulin was obtained from Novo Corporation, Copenhagen, Denmark. DPH (95% purity, as determined by gas-liquid chromatography) was purchased from Sigma Chemical Co. (St. Louis, MO). Silica gel-coated plates (25-mm thickness) were obtained from E. Merck, Darmstadt, Germany.

Subjects. Cord blood samples were obtained from 32 full-term normal infants (17 male, 15 female) following uncomplicated vaginal deliveries without anesthesia. Mothers in this group were healthy and none had histories of diabetes or drug usage during pregnancy.

Ten healthy adult subjects (four males, six females) ranging in age from 26–42 years were also studied following overnight fast. All subjects were within 120% of ideal body weight (26) and, at the time of study, none were taking drugs known to affect carbohydrate tolerance. All studies were performed with informed consent according to the guidelines established by the Human Subjects Protection Committee, Cedars-Sinai Medical Center.

Blood was collected in sterile heparinized tubes and mononuclear leukocytes were harvested following separation by Ficoll-

Hypaque gradients as previously described (32). This cell fraction was used for both insulin binding and cell lipid studies. Plasma was saved and stored at −40°C for subsequent glucose and insulin determinations.

Insulin-binding Studies. ^{125}I-Insulin was prepared by the lactoperoxidase method (28). Specific activities of these preparations ranged from 80–280 nCi/μg. The methods for insulin-binding studies employed in this laboratory have been described previously (30–32).

Data Reduction. Insulin-binding data were subjected to Scatchard analysis (42). For further interpretation, the two-site receptor model was chosen and determination of affinity constants and binding capacities was derived by the method of Blanchard *et al.* (4). Statistical analyses were performed using Student's *t* test for independent variables or linear regression analysis using the method of least squares; significance was accepted at levels of 5% or less.

Lipid Extraction and Analyses. Intact mononuclear cells, isolated previously as described above, were washed in Tris buffer (pH 7.4) and lipids were extracted by the method of Folch *et al.* (9). It has been previously demonstrated that very little difference existed between monocyte and lymphocyte lipids for individual subjects (5). Furthermore, since the bulk of the lipid in such cells is predominantly associated with the plasma membrane, no further subcellular purification step was deemed necessary (16, 37, 40). Cholesterol content was determined colorimetrically (10). Phospholipids were separated and species were isolated by thin layer chromatography. Quantitation of phospholipid species was performed as described previously (32).

Fluorescence Polarization Studies. Membrane microviscosity has been shown to correlate with the freedom of rotation within the membrane of the fluorescent dye molecule DPH (6). Resistance to rotation is provided by the surrounding lipid matrix and can be assessed by the relative fluorescent intensities of polarized light emerging parallel and perpendicular from the sample, following excitation by a vertically polarized light (44). As has been demonstrated by other investigators, DPH equilibrates almost exclusively within the plasma membrane of mononuclear leukocytes, and thus its behavior reflects the overall fluid characteristics of this membrane (45). Measurements were obtained using an Elscint MV-1a microviscosimeter (Hackensack, NJ). FP measurements are provided by the equation:

$$FP = I_{\parallel} - I_{\perp}/I_{\parallel} + I_{\perp}$$

where $I_{\parallel}$ and $I_{\perp}$ are the fluorescent intensities emerging parallel and perpendicular to the original light source (44).

For this system, the fluorescence anistropy (r) is defined as:

$$r = 2FP/3 - FP.$$

The relationship between fluorescence polarization and the apparent microviscosity (n) is defined by the Perrin equation:

$$r_0/r = 1 + K(T/n).$$

The coefficient of viscosity (n) is in units of force (dyne-cm^2) × sec. For most systems, viscosity has been shown to decrease with temperature in an exponential fashion:

$$\dot{\eta} = Ae^{\Delta}E_{act/RT}.$$

A plot of log η *versus* $1/T$ provides an estimate of $^{\Delta}E_{act}$, the flow activation energy, which is the kinetic energy required to overcome the intermolecular forces that oppose flow. Value of $^{\Delta}E_{act}$ reflect the order of the system: a system with a value of $^{\Delta}E_{act}$ equal to 10 kcal/mol is considered to be more highly ordered than one with value of 2 kcal/mol (45).

For these studies, mononuclear cells were suspended in PBS, pH 7.4, at a concentration of 2 × 10^6 cells/ml. One ml of cell suspension was mixed with 1 ml of 10^{-3} M DPH in PBS and incubated for 1 h at 25°C. Cells were washed twice in PBS and resuspended in a final volume of 1 ml. The cell suspension was transferred to a fluorescence cuvette, for determination of the FP value at 25°C using the MV-1a microviscosimeter.

Although this method has been used by many investigators to study fluid properties of mononuclear leucocytes (3, 31, 32, 37), we attempted to validate that this method did reflect the fluid state of the monocyte membrane by performing two additional experiments. Using two preparations, further purification of the mononuclear cell fraction was performed using Percoll gradients (15). This method permitted separation of three fractions of cells: Band 1 containing cells more than 90% of which stained for esterase and were thus considered monocytes; Band II, containing 65% esterase-positive cells; and Band III, containing 18% esterase-positive cells, considered predominantly lymphocytes. For these preparations, mean microviscosity measurements were: Band I, 0.310 ± 0.002; Band II, 0.301 ± 0.009; and Band II, 0.286 ± 0.013, and for the whole mononuclear cell preparation was 0.299 ± 0.011; the differences between measurements of Bands I and III as well as the whole cell preparation were not significant.

Furthermore, we prepared plasma membranes from the mononuclear cell fractions of cord blood (37). Membrane fractions obtained in this manner showed a 20-fold enrichment in 5′-nucleotidase content as compared to cell homogenates (34); fluorescence polarization measurements of plasma membranes showed a mean value of 0.314 ± 0.024, which was not different from the mean value of 0.325 ± 0.011 for whole cells, by the null hypothesis (P = 0.103). Additionally, a significant linear relationship between FP values for whole cells compared to plasma membranes was obtained when five preparations were examined simultaneously (r = 0.917, P < 0.02). Thus, we believe that the data obtained with this technique provided an accurate assessment of the physical state of the monocyte membrane. Despite reservations on the part of some investigators, we believe that this technique is useful in clinical studies such as the one reported here.

RESULTS

Comparison of plasma glucose levels from fasting adults and full term infants at birth showed no significant difference (Table 1). Newborn infants did have significantly higher insulin levels than did fasting adults. Despite this elevation in plasma insulin, cord blood monocytes showed significantly higher ^{125}I-insulin tracer binding than did adult monocytes.

Competition studies of insulin binding to monocytes from adult and cord blood are shown in Figure 1. Insulin binding to cord monocytes was significantly higher at all concentrations of insulin. Scatchard analyses of these data are shown in Figure 2. Cord monocytes have greater insulin-binding capacities than do adult monocytes, as determined by the intersections of the curves with the x axis.

The two-site receptor model was employed to interpret the findings from Scatchard analysis (42). Results of these calculations are presented in Table 2. Cord monocytes have 2½ times more high affinity, low capacity receptor sites than do adult

Table 1. *Glucose and insulin measurements from adult and cord blood samples**

	Glucose (mg/dl)	Insulin (μU/ml)	^{125}I-Insulin bound (%)†
Adults	81 ± 5	9.8 ± 0.6	7.6 ± 0.45
	(10)	(10)	(8)
Cord blood	79 ± 17	17.4 ± 2.4‡	9.5 ± 0.5§
	(32)	(30)	(9)

* Data are means ± SE unless otherwise noted; values in parentheses, number of experiments.

† Per 10^7 cells.

‡ P < 0.001 adult *vs.* cord blood.

§ P < 0.02 adult *vs.* cord blood.

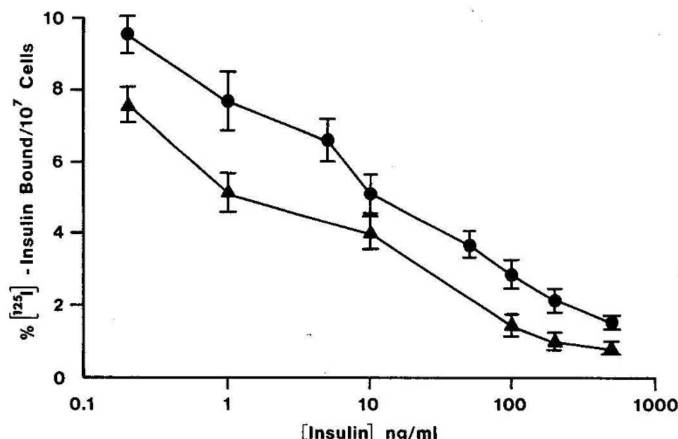

Fig. 1. ^{125}I-Insulin competition binding curves to cord (●) and adult (▲) monocytes. The percentage of ^{125}-I-insulin specifically bound to 1 × 10^7 monocytes is plotted against total insulin concentration. Data represent mean ± SD.

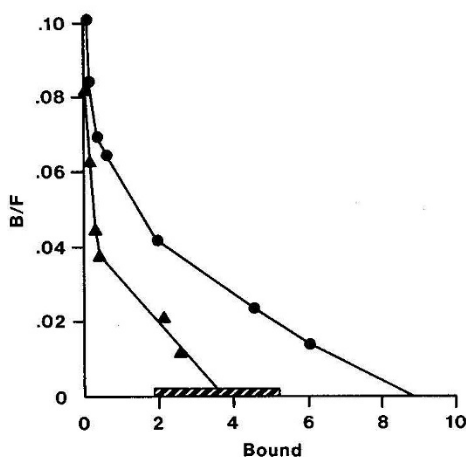

Fig. 2. Scatchard analyses for cord and adult monocytes, derived from Figure 1. The *hatched bar* on the *x* axis represents the normal adult range for maximum binding capacities.

Table 2. *Insulin receptor capacities and affinity constants for monocytes obtained from Scatchard analyses using a two-site model**

	K_{a_1} (M^{-1})	Capacity$_1$ (M/liter)	K_{a_2} (M^{-1})	Capacity$_2$ (M/liter)
Adult	6.17×10^8	1.25×10^{-10}	4.5×10^7	8.57×10^{-10}
Cord	3.25×10^8	2.94×10^{-10}	4.25×10^7	1.31×10^{-9}

* Subscript 1 = high affinity, low capacity site. Subscript 2 = low affinity, high capacity site.

monocytes. The affinity constants (K_D) for these receptors are somewhat lower, but of the same order of magnitude as found on adult cells. In addition, cord monocytes have 50% more low affinity, high capacity receptors than do adult monocytes; the affinity constants for this class of receptors are comparable for both cell types.

Table 3 gives the results from cell lipid analysis for adult and cord mononuclear cell fractions. The latter had significantly lower ratios of phospholipid/protein and significant elevations of cholesterol/phopholipid molar ratios. No significant differences were noted in the ratios of individual phospholipid species.

Microviscosity of mononuclear cell preparations, was determined by the fluorescence polarization technique, using the microviscosimeter, and is noted in Table 3. Cord cells had significantly higher values of FP than did those from adults. Since we have established that FP measurements obtained in this manner reflect the physical state of the plasma membrane and since fluidity is determined to be the reciprocal of the coefficient of viscosity, these data show that cord cells have reduced membrane fluidity.

As shown in Figure 3, a highly significant direct linear relationship was observed between ^{125}I-insulin tracer binding to mononuclear cell fractions and the respective FP values for our subjects ($r = 0.72$, $P < 0.005$).

Studies of microviscosity as a function of temperature was performed for four preparations each of cord and adult monocytes, and the data are presented in Figure 4. At all temperatures, cord monocytes have higher values of n; determination of slopes of these lines gives the values $^{\Delta}E_{act}$, the flow activation energy. Cord monocytes have nearly 2-fold greater values of $^{\Delta}E_{act}$.

DISCUSSION

Cord cells have significantly greater numbers of detectable insulin receptors than adult monocytes, despite elevation in plasma insulin levels; our results show a direct relationship between ^{125}I-insulin tracer binding and membrane microviscosity, and suggest that the physical state of the membrane is a

Table 3. *Cell lipid composition and microviscosity determinations (FP) of mononuclear cells isolated from cord and adult blood*

	Phospholipid/ protein (mg/mg)	Cholesterol/ phospholipid (molar ratio)	Lecithin/ sphingomyelin ratio	FP at 25°C
Adult	0.129 ± 0.012 (10)	0.354 ± 0.009 (10)	4.4 ± 0.23 (10)	0.186 ± 0.019 (8)
Cord	0.085 ± 0.012* (16)	0.52 ± 0.045† (16)	3.7 ± 0.5 (15)	0.339 ± 0.031† (14)

* $P < 0.025$ adult *vs.* cord.
† $P < 0.005$ adult *vs.* cord.

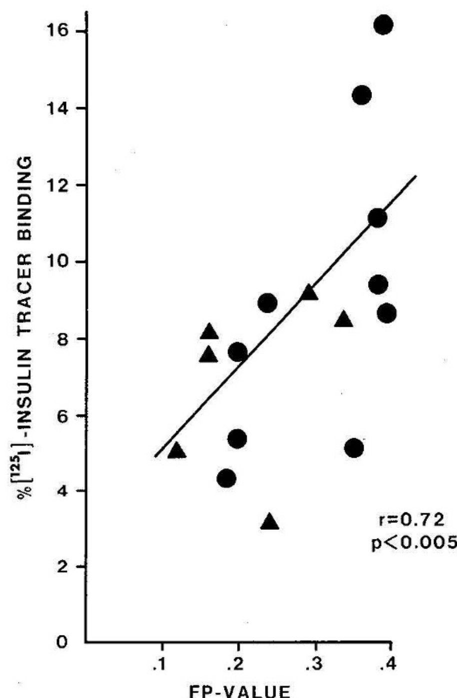

Fig. 3. Scatter diagram depicting the relationship between cell membrane microviscosity (FP value) and ^{125}I-insulin tracer binding to cord (●) and adult (▲) mononuclear leukocytes. Linear regression analysis shows a highly significant r value (0.72; $P < 0.005$) for this *line*, the equation for which is $y = 21.5x + 2.9$).

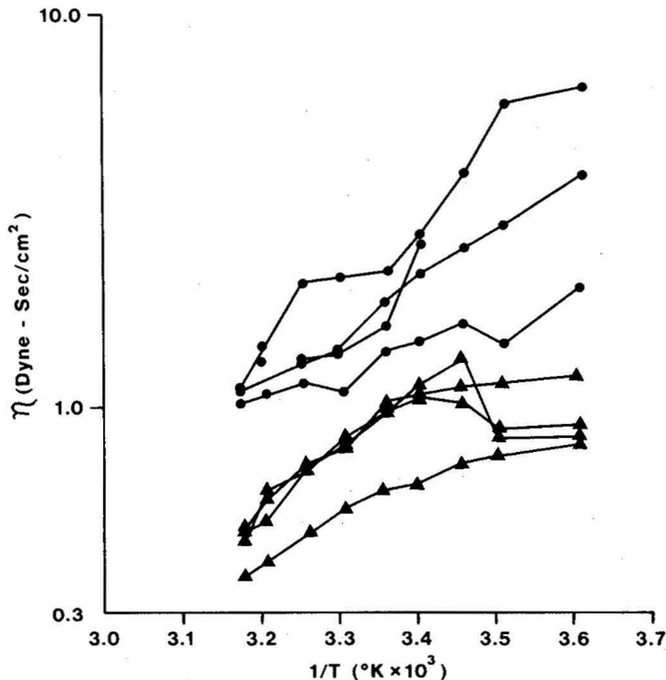

Fig. 4. Variation of microviscosity with temperature for cord and adult monocytes. The *solid lines* represent the values for four cord blood (●) and 4 adult (▲) monocyte preparations. The slopes of these *lines* derived from the equation $\eta = Ae^{\Delta E/RT}$ yield the value for ΔE_{act}, the flow activation energy. For adult cells, the mean value was 3.34 ± 0.36 kcal/mol; for cord cells, the mean value of 6.35 ± 1.26 kcal was obtained, which was significantly greater ($P < 0.05$) than that for adult cell preparations.

major determinant of the differences in receptor behavior noted here. Interpretation of these data rests on two assumptions: 1) that the probe DPH localizes within the plasma membrane of these cells such that the fluorescence changes are determined primarily by differences in the physical properties of the plasma lipid (44, 45); and 2) that the changes in cellular lipid are a reflection of changes in plasma membrane lipid, which comprises, 60% or more of total cell lipid (45). Both of these assumptions were found to be valid in our preliminary experiments.

While regulation of receptor concentration is incompletely understood, it would appear that the receptor behaves according to the physical constraints provided by its immediate environment. Several investigators have suggested that membrane fluidity is an important determinant of the behavior of peptide-hormone receptors (14, 20, 29). The membrane model most frequently considered is that of a lipid-protein mosaic structure, in which zones of highly fluid surround the more sparsely distributed membrane proteins (47). Alterations in the fluid behavior of the surrounding lipid zone would be expected to result in disturbances in the behavior of the interspersed proteins.

The most commonly employed means of altering lipid fluidity is by changing the temperature; lipids become more fluid at higher temperatures and less fluid in the cold. Amatruda and Finch were the first to attribute changes in receptor behavior which accompanied changes in temperature to variations in membrane fluidity, although similar observations had been made by other investigators (2, 13, 51). These authors showed that increasing temperature from 25 to 37°C was associated with a dramatic change in membrane fluidity, resulting in an apparent 2-fold reduction in insulin receptors. This led to a higher ED_{50} for insulin to stimulate glucose transport (2).

Increasing membrane fluidity results from increasing the freedom of molecular motion and has been observed in several situations where lipid composition has been altered: 1) decreasing the cholesterol content relative to the amount of phospholipid; 2) increasing the phospholipid content relative to the amount of protein; 3) increasing the phosphotidylcholine content relative to sphingomyelin; 4) increasing the phosphatidylethanolamine content relative to phosphatidylcholine; 5) increasing the degree of unsaturation of fatty acids; and 6) decreasing the fatty acid chain length (22). From our data, it appears that the reduction in membrane fluidity of cord cells is due primarily to diminished phospholipid/protein ratios, which are also associated with increased cholesterol/phospholipid molar ratios relative to those of adult cells. The measurements of both insulin binding and cell microviscosity were performed on the whole mononuclear cell fraction. Although the data for insulin binding were expressed as a function of the monocyte concentration, which as been shown to be the major cell population that binds insulin (43), such correlations were not performed for the fluidity and lipid data. Based on present observations, as we assume that such differences observed in the entire cell are representative of changes in this subset of cells.

During fetal development, the total phospholipid concentration in membranes increases, a process which continues during the postnatal period (12, 27). The accumulation of phospholipids in the cell membrane after birth would be predicted to increase membrane fluidity. Reduced fluidity in fetal microsomal membranes has been reported; increases in membrane fluidity were observed to occur after birth (23, 25). Since increasing fluidity is related to a reduction of insulin binding, this would explain the results of Hendricks *et al.* (17) and Sinha *et al.* (48) who reported the "disappearance" of insulin receptors on erythrocytes during the postnatal period.

We have recently completed studies on fetal lung membranes from normal and diabetic pregnancies which show an inverse relationship between membrane phospholipid content and increasing receptor exposure with progressive fetal hyperinsulinemia. Data from our laboratory suggest that insulin at high concentrations inhibits some aspects of phospholipid metabolism in fetal lung (35). These findings would explain the up-regulation of insulin receptors that we have previously reported (19) in hyperinsulinemic offspring of diabetic mothers.

A model of membrane structure and receptor configuration has been developed based on these data. As shown in the schematic diagram (Fig. 5), cord cell membranes have only small lipid zones, which results both in high microviscosity and outward displacement of receptors. In adults, accumulation of membrane lipid decreases the microviscosity and permits increased lateral mobility of receptor proteins, causing them to "disappear" within the membrane matrix.

Further support for this model of membrane protein behavior

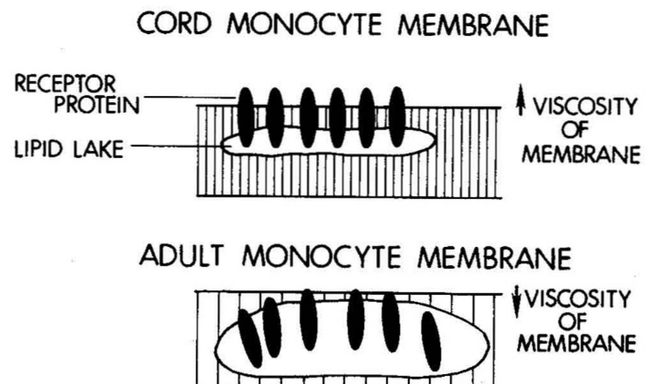

Fig. 5. Schematic diagram of membrane lipid content and insulin receptors in cord and adult monocyte membranes. In cord monocytes, only small lipid zones are detectable; this results in increased viscosity of the membrane and promotes vertical displacement of the receptor. As the lipid zones increase in size in the adult membrane, the receptors are displaced vertically downward.

comes from studies by Shinitzky *et al.* (44–46) in which fluorescent tagging of membrane proteins on cells was performed. Changes in membrane fluidity were produced by cholesterol enrichment of depletion of cell membranes, after which measurements for detectable fluorescence were made. Cholesterol depletion which resulted in increased membrane fluidity was accompanied by decreased visible fluorescence, indicating reduction of surface proteins. Conversely, cholesterol enrichment of membranes resulted in decreased membrane fluidity and was associated with greater detectable fluorescence, suggesting outward displacement of membrane proteins. These data suggested that vertical displacement of membrane proteins was dependent in the fluid state of the membrane (39, 40).

Recently, Sinha *et al.* (49) have reported reduced responsiveness to insulin in fetal tissues, despite the presence of increased numbers of insulin receptors in comparison to adult cells. Originally, it was suggested that postbinding hormone-mediated action required translocation of the hormone-receptor complex to an intracellular site. From our data, it would appear that more than twice the energy for particle (*e.g.* receptor) translocation within the membrane would be required in fetal cells as compared to adult cells; if such energy requirements limit receptor-hormone translocation, this would explain the reduction of insulin action in fetal tissues.

The differences in membrane physical state in fetal and adult cells appear to result from the relatively lower content of phospholipid observed in fetal preparations. It has been shown that postreceptor phenomena induced by hormones may be mediated by membrane phospholipids specifically (8, 24, 36). More recently Yuli *et al.* (52) have demonstrated that alteration of the physical properties of the membrane lipid core by alcohol treatment was associated with changes in binding affinities of chemoattractant membrane receptors. Such treatment had differential effects on postreceptor biological activities. The data suggested that postreceptor effects vary with the affinity of the receptor and can be regulated by the physical state of the membrane.

In this study, we showed a strong correlation between membrane physical state and binding properties of the insulin receptor. It may well be that the diminished responsiveness to insulin exhibited by fetal tissues also directly results from these differences in membrane physical state.

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53. These studies were supported by grants from the National Foundation of the March of Dimes, The Donald E. Simon Foundation, The Juvenile Diabetes Foundation, The National Institutes of Health, National Institute of Arthritis, Diabetes, and Digestive Kidney Diseases Grant R01 AM 33503-01, and the American Diabetes Association of Southern California, as well as a gift from Tosco Corporation. Portions of this study were presented before the Meeting of the Society for Pediatric Research, February 1982 in Carmel, CA.
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55. Received for publication January 2, 1984.

0031-3998/84/1808-0778$02.00/0
PEDIATRIC RESEARCH
Vol. 18, No. 8, 1984
Printed in U.S.A.
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Comparative Monovalent Cation Transport in Neonatal and Adult Red Blood Cells

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Summary

Active transport of Na and K under physiologic and maximally stressed conditions was identical in *normal* adult red blood cells (RBC) and term neonatal erythrocytes. These results are consistent with the previous observation that Na-K ATPase is the same in *normal* adult RBC and term neonatal erythrocytes. These data, however, are at variance with a previous observation that active K transport is impaired in neonatal erythrocytes. The most reasonable explanation for this difference relates to inherent problems with the use of radioisotopes which were used in previous *in vitro* studies of cation transport.

Abbreviations

RBC, red blood cells
GBS, glycylglycine-buffered salt solution

It previously has been reported that active K transport is decreased in term neonatal RBC compared to normal adult erythrocytes (1). The explanation for this altered K transport was that neonatal RBC have decreased Na-K ATPase activity (8). Term neonatal RBC do in fact have decreased Na-K ATPase activity compared with adult reticulocyte-rich RBC; however, there is no difference in enzyme activity when compared with normal adult RBC (8). The studies reported here were designed to resolve the paradox of why term neonatal RBC have decreased active K transport despite the fact that they contain the same Na-K ATPase activity as that seen in normal adult RBC.

MATERIALS AND METHODS

General experimental design. Nonisotopic cation fluxes were measured in normal adult RBC and term neonatal erythrocytes. The cation flux studies were determined under normal physiologic conditions and under conditions where the intracellular sodium concentration was elevated to maximally activate Na-K ATPase (2).

Preparation of RBC. Blood was collected in EDTA from the umbilical cords of healthy term infants and from normal adult volunteers. Erythrocytes were separated from the plasma and buffy coat by centrifugation at 4°C. The red blood cells next were washed three times in Na-GBS, pH 7.4 (37°C) with the following composition: 145 mM NaCl, 5 mM KCl, 1 mM Na_2 HPO_4, 1 mM $MgCl_2$, 5 mM glucose, and 20 mM glycylglycine.

Preparation of high sodium RBC. Na-loaded human RBC were prepared by incubating washed RBC (5% hematocrit in Na-GBS) with amphotericin B sulfate at a final concentration of 5 μg/ml. After 2-h incubation in a shaking water bath (100 oscillations/min) at 37°C, RBC were washed five times with 20 volumes of Na-GBS containing bovine albumin (1 g/dl). Albumin was added to bind and remove amphotericin from the cell suspension.

Measurement of RBC cation transport. Red blood cells were suspended in Na-GBS (hematocrit 20%) and incubated 4 h at 37°C in a shaking water bath (100 oscillations/min). Cell Na and medium K were measured every 30 min utilizing previously described methods (3, 4). Changes in cell K were determined indirectly from the hematocrit and medium K concentration. There was no hemolysis during these studies, and thus more precise measurements of net K efflux could be obtained by measuring medium K changes (increased values above a small number). Active transport was defined as the net cation difference between cells incubated in the presence and absence of ouabain (10^{-4} M).

RESULTS

Na-K active transport at low intracellular sodium concentrations. The fresh or "low" Na content of adult and neonatal RBC was maintained during the period of incubation. The mean cell