pendent vasorelaxation. This effect appears to depend on structural damage to the endothelium, because the removal of neutrophils does not restore the ability of ACh to induce vasorelaxation. Our results confirm the data of Varani et al.[5] that H_2O_2 plays the main role in this process, because catalase but not SOD protected the endothelium from damage. It is likely that in general the damage to the endothelium is induced by HO^- generated from H_2O_2[5].

Our results revealed that neutrophils can damage the endothelium to a sufficient extent to provoke a disturbance of vascular reactivity, in particular a decrease in ACh-induced vasorelaxation. Accordingly, pharmacological treatment to counter neutrophil actions on vascular endothelium might be considered as a method for the prevention of vascular disease. The present study shows that calcium channel blockade using nifedipine, and beta-adrenoreceptor blockers like propranolol may be able to reduce the deleterious effects of activated neutrophils on endothelium-dependent vasorelaxation.

In conclusion, while it may be extremely difficult to obtain definitive in vivo evidence for the involvement of neutrophils in processes leading to a disturbance of endothelium-dependent vasorelaxation, our findings are consistent with the suggestion that intravascular activation of neutrophils leads to tissue damage, including direct cytotoxic effects on endothelial cells[4, 5, 9].

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Changes in erythrocyte membrane lipid composition affect the transient decrease in membrane order which accompanies insulin receptor down-regulation

M. T. Santini[a], R. Masella[b], A. Cantafora[b] and S. W. Peterson[c]

[a] *Laboratorio di Ultrastrutture and INFN-Sezione Sanità, Istituto Superiore di Sanità, Viale Regina Elena 299, Rome (Italy);*
[b] *Laboratorio di Metabolismo e Biochimica Patologica, Istituto Superiore di Sanità, Viale Regina Elena 299, Rome (Italy) and*
[c] *Department of Biology, Arkansas College, 2300 Highland Road, Batesville (Arkansas 72501, USA)*
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Abstract. We have recently demonstrated, using electron paramagnetic resonance (EPR) spectroscopy, that insulin receptor internalization in response to insulin incubation (down-regulation) in human erythrocytes is accompanied by a transient decrease in membrane order, as measured by the $2T'_{||}$ order parameter. Since membrane lipids play such an important role in receptor internalization, we investigated the possible effects that an alteration of the normally-occurring lipid profile might have on down-regulation and the concomitant transient decrease in membrane order. Consequently, human erythrocytes enriched with cholesterol and erythrocytes from cirrhotic patients were examined, because both of these groups of cells have a higher cholesterol/phospholipid molar ratio (CH/PL) than controls. The 5-nitroxystearate spin label, which inserts into the lipid bilayer of cell membranes, was used to monitor changes in $2T'_{||}$ for a 3-h period at 37 °C. We report here that both cholesterol-enriched and cirrhotic erythrocytes do not down-regulate, as demonstrated by binding assays, and that they do not show the typical transient decrease in membrane order observed in controls. The results seem to indicate that a more ordered membrane inhibits internalization of the insulin receptor in erythrocytes, and that an increase in membrane disorder is necessary for insulin receptor down-regulation.
Key words. Erythrocyte; insulin receptor; receptor endocytosis; EPR; membrane order; membrane lipids.

We have recently demonstrated by electron paramagnetic resonance (EPR) spectroscopy, using 5-nitroxystearate as spin label, that incubation of human erythrocytes with insulin (1 μg/ml) results in a marked decrease (within the first 90 min) of membrane order as measured by the $2T'_{||}$ order parameter[1]. This parameter, which then returns to its initial value in less than 3 h, completes a cycle that appears to be related to the endocytotic internalization of

insulin receptors. Both down-regulation and corresponding changes in membrane order parameters have been shown to be ATP-dependent[1].

Membrane lipids play an important role in the endocytotic process. This has been demonstrated in both internalization of membrane-bound enzymes and in receptor processing[2,3]. In the case of insulin receptor endocytosis, we were able to show, in another recent study, that pathological and artificially-induced changes in membrane lipid composition are associated with impaired insulin receptor processing[4]. Specifically, the increase in the cholesterol to phospholipid (CH/PL) molar ratio and the decrease in polyunsaturated fatty acid content of phospholipids in erythrocytes of cirrhotic patients resulted in a decrease of down-regulation of insulin receptors. The purpose of the present study was to evaluate the effects that changes in the CH/PL molar ratio induced by either pathological alteration (liver cirrhosis) or in vitro incubation with cholesterol-rich liposomes might have on the behavior of the $2T'_{\parallel}$ membrane order parameter. The results reported here are considered with the previous findings that a reduction in membrane order may be a prerequisite for initiation of the biochemical cascade of events which leads to insulin receptor internalization, and that membrane lipid composition plays an important role in this endocytotic process.

Materials and methods

Normal blood for membrane manipulation studies was a generous gift from the Centro Trasfusionale of Rome University. Five patients with liver cirrhosis and different degrees of alteration in both the CH/PL molar ratio and in down-regulation (DR) were selected for this study.

Fresh human blood was drawn and centrifuged, and the plasma and white cells were then removed. For down-regulation and binding assays, cells were incubated in 50 mM Tris, 50 mM Hepes, 10 mM $MgCl_2$, 2 mM EDTA, 10 mM D-glucose, 10 mM $CaCl_2$, 50 mM NaCl, 5 mM KCl and 0.1 % bovine serum albumin. The saline-adenine-glucose (SAG) solution for red blood cell incubation with cholesterol-rich liposomes contained 1.7, 90.0 and 88.0 mg/ml of adenine, glucose and sodium chloride, respectively. Penicillin G (1000 U/ml) was also added to the solution.

Receptor internalization was induced by incubating human erythrocytes with 1 μg insulin/ml at 37 °C as described by Peterson et al.[5]. After incubation in the presence or absence of insulin, separate aliquots of cells were removed at various times for the insulin binding assays and for EPR measurements. Internalization was determined by cell surface insulin binding assays according to the method of Gambhir et al.[6]. Briefly, samples that had been incubated with 1 μg insulin/ml were removed at various times from the incubating mixture and washed thoroughly. To determine total binding, cells were incubated for 90 min at 15 °C with 1 ng/ml ^{125}I-labeled insulin. To determine non-specific binding, cells were incubated with the same amount of ^{125}I-labeled insulin in the presence of an excess of unlabeled insulin.

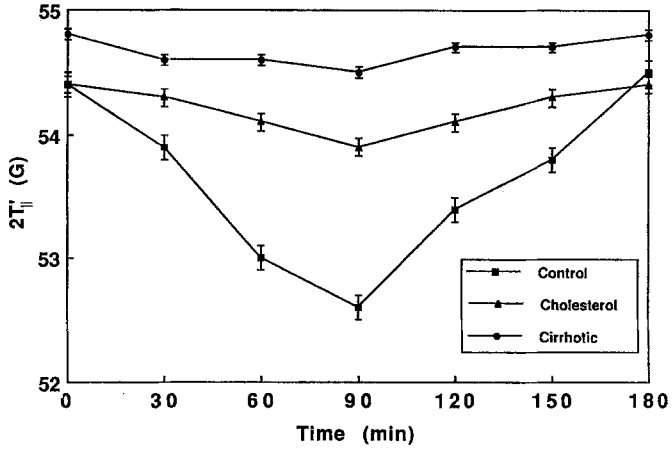
For the EPR studies, about 10^9 erythrocytes were labeled with a solution of 5-nitroxystearate in absolute ethanol to a final spin label concentration of 50 μM as described previously[1,7]. The cell suspension was vortexed and rotation-incubated for 10 min at 37 °C. EPR measurements were carried out on a temperature-controlled Varian E-4 X-band spectrometer. Temperature was monitored by digital thermometer and kept at 37 °C ± 0.5 °C. The spin label 5-nitroxystearate used in this study is believed to orient itself inside cell membranes non-covalently, with its longitudinal molecular axis perpendicular to the membrane surface[8]. The location of the oxazolidine ring at position 5 of the hydrocarbon chain of stearic acid allows the polar position of the hydrophobic tail of the lipid bilayer to be sampled. The ring motion is, therefore, related to the rotation of the adjacent segment of the molecule. The distance between the separation of the outer extrema ($2T'_{\parallel}$) was measured directly from the spectra as described previously[1] and used as an indicator of membrane order.

Erythrocyte membrane lipid composition was modified by incubation with cholesterol-rich liposomes prepared according to Cooper et al.[9]. Briefly, 333 mg of dipalmitoylphosphatidylcholine (DPPC) and 666 mg of cholesterol were dissolved in chloroform-methanol (2:1, by vol.). The mixture was taken to dryness under nitrogen and the residue was suspended in 10 ml of 0.15 M NaCl by 90-min sonication at 45 °C. Subsequently, 40 ml of normal human serum, deactivated by heating at 56 °C for 30 min, was added to the lipid dispersion. The mixture was centrifuged at 21,800 × g for 30 min and the residue was discarded. The cells, diluted to a hematocrit of 10 % in SAG solution, were incubated for 6 h at 37 °C in a water bath shaker. Finally, an equal volume of cholesterol-rich suspension was added to the cells which were then incubated for 12 h in the bath at 37 °C.

All the lipid analyses were conducted on lipid extracts prepared with 0.5 ml of packed erythrocytes. These were hemolyzed for 15 min with an equal volume of water, and 11 ml of isopropanol was then added slowly, while stirring, to the hemolysate. Finally, 7 ml of chloroform were added and the extract was stored at − 30 °C. Total phospholipid was determined by a lipid phosphorous assay according to Bartlett et al.[10]. Cholesterol concentration in the extracts was determined by a modification of a commercial enzymatic method[11].

Results and discussion

The present data confirm our previous finding that normal erythrocytes incubated with insulin show a transient decrease in membrane order as measured by $2T'_{\parallel}$[1]. Specifically, the curve of the $2T'_{\parallel}$ order parameter as a function of time of control erythrocytes (fig.) demonstrates that within 90 min of insulin incubation an maximum decrease in membrane order is observed and that



Human erythrocyte membrane order at 37 °C as a function of time. Membrane order as represented by $2T'_{\|}$ of human erythrocytes to which insulin was added at t = 0 and labeled with the spin label 5-nitroxy-stearate is plotted as a function of time. Membrane order of control erythrocytes (■), cholesterol-enriched erythrocytes (▲) and erythrocytes from cirrhotic patients (●) are shown. Each data point represents the mean and the mean standard deviation from at least three separate experiments.

by about 150 min of this incubation membrane order returns to its original value. This cycle in membrane order was shown to coincide with the endocytotic internalization of insulin receptors[1]. In addition, the data presented in this report demonstrate that alteration of the CH/PL molar ratio of erythrocyte membranes, either by in vitro manipulation (incubation with cholesterol-rich liposomes) or by pathological conditions (liver cirrhosis), affects the membrane order cycle that normally occurs during incubation of erythrocytes with physiological concentrations of insulin (fig.). As can be seen from this figure, neither cholesterol-enriched erythrocytes nor erythrocytes of cirrhotic patients show the typical decrease in membrane order observed in controls.

Control erythrocytes incubated with cholesterol, and cirrhotic erythrocytes, show a significant increase in the CH/PL molar ratio (0.93 and 0.85, respectively versus 0.54) (table), a change that is known to increase membrane stability[12]. Both cholesterol enrichment and cirrhosis also resulted in a significant decrease in insulin receptor down-regulation (20% and − 8% respectively, versus 63%) (table). It was shown previously that alteration of the CH/PL molar ratio induced either by in vitro manipulation or by a pathological state of the blood cells results in an inability of the erythrocyte membrane to internalize insulin receptors, and not in a change in the affinity or number of insulin receptors on the cell surface[4]. In fact, cirrhotic erythrocytes actually show up-regulation (negative value for down-regulation) (table). In these erythrocytes, the insulin receptor is abnormal in structure[13]. It may be possible that the abnormal structure of the receptor, together with the high CH/PL molar ratio in these cells, is responsible for the up-regulation observed. Further studies are necessary in order to test this hypothesis.

It is interesting to note that although both pathological and manipulated erythrocytes show an increase in the CH/PL molar ratio and a decrease in down-regulation capacity, the best correlation of order parameter variation is with down-regulation rather than with the CH/PL molar ratio (Pearson R values for linear regression correlation coefficients of 0.87 and 0.71, respectively). The better correlation of the order parameter with down-regulation than with the CH/PL ratio indicates that changes in the order parameter are the expression of the entire down-regulation process, which may include other factors in addition to the CH/PL ratio. The complete down-regulation process is likely to be affected by other membrane factors such as variations in the degree of unsaturation of phospholipids and differences in the distribution pattern of the different phospholipid classes. In fact, Mahoney et al.[14] have demonstrated, also using EPR, that macrophages whose membranes were enriched with saturated fatty acids are more rigid than controls and have reduced endocytotic activity. Bruneau et al.[15] reported that cultured rat hepatoma cells enriched with polyunsaturated fatty acids show accelerated insulin-induced receptor down-regulation. Thus, a clear relationship between membrane order and endocytosis of insulin receptors appears to exist.

That the order parameter depends upon various membrane factors can also be noted by observation of the absolute values of $2T'_{\|}$ of the three different types of erythrocytes examined. At t = 0, the $2T'_{\|}$ values are 54.4, 54.4 and 54.8 G for control, cholesterol-enriched and cirrhotic erythrocytes, respectively. Although the first two have a very different CH/PL molar ratio, $2T'_{\|}$ is the same and although the CH/PL ratio of cholesterol-enriched and cirrhotic cells is nearly the same, the $2T'_{\|}$ value is quite different. Thus, it is obvious that the order parameter does not depend solely upon this ratio, but also on the more subtle membrane interactions such as phospholipid class distribution or other yet unknown parameters.

Comparison of down-regulation and CH/PL molar ratio in control, cholesterol-enriched and cirrhotic erythrocytes. Down-regulation and CH/PL molar ratio for each experimental group were determined as described in the text. The negative value of down-regulation for cirrhotic erythrocytes indicates up-regulation. Means and mean standard deviations of three separate experiments for control and cholesterol-enriched and of five separate experiments for cirrhotic erythrocytes are given.

Group	Down-regulation (%)	CH/PL
Control	63 ± 10.4	0.54 ± 0.10
Cholesterol-enriched	20 ± 21.3	0.93 ± 0.18
Cirrhotic	− 8 ± 11.5	0.85 ± 0.15

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Effects of the adenylate cyclase activator forskolin and its inactive derivative 1,9-dideoxyforskolin on insect cytochrome P-450 dependent steroid hydroxylase activity

D. P. Keogh, M. J. Mitchell, J. R. Crooks and S. L. Smith*

Department of Biological Sciences, Bowling Green State University, Bowling Green (Ohio 43403-0212, USA)
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Abstract. The adenylate cyclase activator forskolin and its pharmacologically inactive derivative 1,9-dideoxyforskolin were found to inhibit in a dose-dependent fashion the ecdysone 20-monooxygenase activity associated with wandering stage larvae of *Drosophila melanogaster* and fat body and midgut from last instar larvae of the tobacco hornworm, *Manduca sexta*. The concentrations of these labdane diterpenes required to elicit a 50% inhibition of the cytochrome P-450 dependent steroid hydroxylase activity in the insect tissues ranged from approximately 5×10^{-6} to 5×10^{-4} M.
Key words. Adenylate cyclase; cytochrome P-450; ecdysone 20-monooxygenase; forskolin; steroid hydroxylase.

Ecdysone (2β, 3β, 14α, 22R, 25-pentahydroxy-5β-cholest-7-en-6-one) is the secretory product of the insect prothoracic glands (the glandular source of molting hormone) and 20(R)-hydroxyecdysone is the more active metabolite of ecdysone and the predominant hemolymph ecdysteroid during most critical phases of postembryonic development[1]. Not surprisingly, the steroid hydroxylase enzyme system responsible for the conversion of ecdysone to 20-hydroxyecdysone, ecdysone 20-monooxygenase (EC 1.14.99.22), has been studied extensively both for the intrinsic importance of the reaction it catalyzes and as a model for understanding other insect steroid hydroxylases such as those in the prothoracic glands responsible in large part for the biosynthesis of ecdysone from cholesterol or plant sterols[1-3].

Several studies have revealed that ecdysone 20-monooxygenase is an NADPH requiring cytochrome P-450 dependent steroid hydroxylase system similar to the vertebrate cholesterol side chain cleavage system[4-9]. Additional studies have demonstrated that ecdysone 20-monooxygenase activity fluctuates dramatically and in a tissue specific fashion during the insect life cycle[10-17]. Like the vertebrate P-450 dependent steroid hydroxylases[18], the regulation of this insect enzyme system is probably complex and tissue specific. Ecdysone, 20-hydroxyecdysone and the ecdysone agonist RH 5849 have all been shown to enhance ecdysone 20-monooxygenase activity[17,19-21]. Other studies have reported that cyclic adenosine monophosphate (cyclic AMP) and factors which affect its metabolism may contribute to the regulation of ecdysone biosynthesis[26-29]. Accordingly, in the present study we examined the effects of the labdane diterpenes[30] forskolin (7β-acetoxy-8, 13-epoxy-1α, 6β, 9α-trihydroxylabd-14-en-11-one), an adenylate cyclase activator, and its inactive derivative 1,9-dideoxyforskolin on insect ecdysone 20-monooxygenase activity.

Materials and methods

Animals. The animals used in these studies were wandering stage third instar larvae of the Canton S strain of *Drosophila melanogaster*; and day 4 and day 5 (wandering stage) gate II fifth instar larvae of the tobacco hornworm, *Manduca sexta*. Animals were reared and staged as previously described[31,32].

Ecdysteroids and chemicals. The radiolabelled ecdysteroid substrate for the monooxygenase assay was [23,24-^{3}H]-ecdysone (stocks of 45 and 70 Ci/mmol) purchased from New England Nuclear, Boston, MA. Ecdysteroid standards and NADPH were purchased from Fluka Chemical Corp., Ronkonkoma, NY, and Sigma Chemical Co., St. Louis, MO, respectively; salts, organic solvents, and scintillation fluid (Scinti Verse E) were purchased from Fisher Scientific Co., Cleveland, OH; forskolin, its biologically active analog 7-O-hemisuc-