Short communication

n-Heptane and *n*-Hexane Enhance in a Dose-Dependent Manner Insulin Binding to Erythrocytes and Its Degradation

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n-heptane has been effectively used to prepare arteficial bilayer membranes. The viscoelastic and/or mechanical properties of the arteficial membranes vary with changes in the lipid/organic solvent ratio (Hianik et al. 1984). Also changes in cell membrane fluidity are known to correlate with the activities of certain membrane-bound enzymes (Yuli et al. 1981; Stubbs and Smith 1984), membrane receptor endocytosis (Elguindi et al. 1985) or hormone receptor binding (Kolena and Ondriaš 1984). In our previous experiments (Hianik et al. 1986) we could observe that small amounts of insulin added to artificial lipid bilayer changed the viscoelastic properties of the latter. Prompted by these results we tried to examine how would insulin binding and degradation in isolated human erythrocytes change following the addition of *n*-heptane in vitro, since this solvent can be assumed to affect viscoelastic characteristics of cell membranes in non-arteficial systems as well. Since also *n*-hexane can be used to prepare model membranes (Montal et al. 1981; Ayala et al. 1985), we tested the effects of both above mentioned aliphatic hydrocarbons in our system. We present herewith the original observation that following the addition of n-heptane or *n*-hexane in vitro, insulin binding to human erythrocytes became enhanced; this was associated with similar changes in insulin degradation as measured in the extracellular medium. Moreover, the effect of the organic solvents is likely mediated through processes in the membrane.

Blood for receptor analyses was taken in the morning from fasting healthy volunteers (8 males, mean age: 30.9 ± 1.2 yrs, mean body mass index BMI: $22.9 \pm 0.6\,\mathrm{kg.m^{-2}}$) with normal glucose tolerance. Insulin binding to erythrocytes was studied using a slightly modified technique described originaly by Gambhir et al. (1978). The effects of the organic solvents on specific insulin binding, expressed as the difference between total and non-specific binding with $1.10^{-5}\,\mathrm{mol.l^{-1}}$ native insulin (NOVO, Denmark) were tested by adding increasing amounts (6.8—153.2 μmol) of *n*-heptane (LOBA Chemie, Austria) or *n*-hexane (7.7—153.2 μmol) (PARK Scientific Ltd., UK) to 500 μl of isolated

human erythrocyte suspension (2.10^9 cells) in HEPES-TRIS buffer, pH 8 at 15 °C. Insulin degradation was measured in the extracellular medium by TCA precipitation. Supernatants (200 µl), obtained by centrifugation of isolated erythrocyte suspension through dibutylphthalate oil (SYNTHESIA Kolín, Czechoslovakia) after incubation with insulin in the presence or absence of the organic solvents, were mixed with 200 µl 2 % BSA (SEVAC, Czechoslovakia) and 400 µl ice-cold 14 % TCA. The mixture was then centrifuged at 2,500 rpm for 10 min in a Beckman TJ 6 centrifuge (horizontal rotor TH 4). The degree of degradation was expressed in terms of dimensionless figures computed as the ratio of the degraded (D) supernatant radioactivity to non-degraded (ND) sediment radioactivity insulin (D/ND). Superficial membrane proteins, including insulin receptor proteins on erythrocytes, were digested by preincubation with trypsin (SERVA, Switzerland). The samples were preincubated with 45 mg trypsin each (specific activity 4 U . mg^{-1}) at 37 °C for 30 min. The reaction was stopped by the addition of 25.7 mg of lime bean trypsin inhibitor (SIGMA, USA) per sample. Results of biochemical determinations were statistically processed using the paired *t*-test and expressed as mean ± SEM.

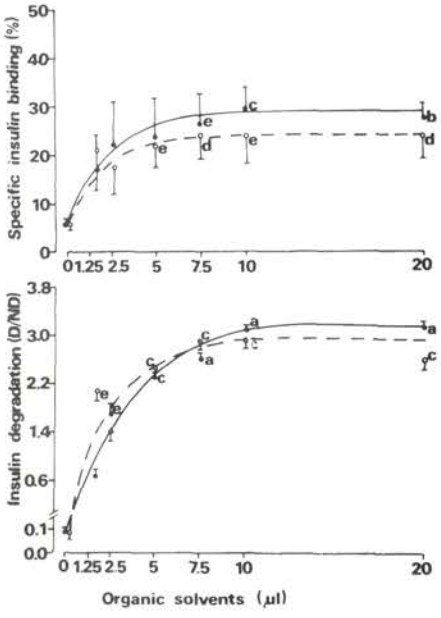

Fig. 1. Relationships between specific insulin binding (*top*) and insulin degradation (*bottom*), and increasing amounts of *n*-heptane (○---○) and *n*-hexane (●——●) added to 500 µl of erythrocyte suspension. Each value represents the mean of 6 experiments performed in 6 individuals. Lower case letters are used to indicate statistical significance of differences as compared to control values obtained without the addition of solvents (b, c, d, e correspond to $p < 0.002$, 0.01, 0.02 and 0.05, respectively).

The addition of increasing amounts of *n*-heptane or *n*-hexane to human erythrocyte suspension in vitro resulted in a dose-dependent enhancement of specific insulin binding (Fig. 1), characterized by a high sensitivity (ED_{50} of approx. 1.25 µl per 500 µl erythrocyte suspension) to both organic solvents

tested. Non-specific insulin binding remained unchanged over the entire range of the concentrations used. Statistically significant enhancement of specific insulin binding was observed following the addition of 5—7.5 µl of each of the solvents tested to 500 µl of erythrocyte suspension, and saturation was reached with 10—20 µl of the solvents. Stimulation of insulin degradation as measured in the extracellular medium following the addition of *n*-heptane or *n*-hexane showed a similar dose-dependent pattern (Fig. 1). The sensitivity of the system was however slightly lower (ED_{50} approx. 2.5 µl per 500 µl erythrocyte suspension); on the other hand, a statistically significant enhancement of insulin degradation was observed with as little as 2.5—5 µl of the solvent per sample.

To establish the participation of insulin receptors in the mechanism of changes in insulin binding and degradation following *n*-heptane and *n*-hexane, isolated erythrocytes were preincubated with trypsin. The enzyme concentration used digests the superficial membrane proteins, including 85—95 % of membrane insulin receptors (Weiner et al. 1985), leaving the erythrocyte per se intact. The treatment of the red blood cells with trypsin effectively eliminated specific insulin binding, and — as expected — *n*-heptane or *n*-hexane induced no effect (Fig. 2). Insulin degradation by trypsin-pretreated erythrocytes (i.e. in absence of the organic solvent) increased statistically significantly (as compared with the control erythrocytes) in the *n*-heptane series only. It nevertheless remains open whether this very small difference has some practical significance. Much more important, to our opinion, was the observation that the addition of *n*-heptane or *n*-hexane enhanced insulin degradation as measured in the extracellular medium of trypsin pretreated erythrocytes statistically significantly in both situations, as compared to control erythrocytes (Fig. 2). These insulin degradation increments represented, however, but a seventh part of those measured under normal conditions following addition of n-heptane or *n*-hexane to intact isolated human erythrocytes. Consequently, it can be assumed that, in contrast to specific insulin binding, the effect of the organic solvents tested on insulin degradation follows at least two different paths, with an approximately 15 % participation of insulin receptors independent mechanism.

To our knowledge, the effects of *n*-heptane and *n*-hexane on stimulation of specific insulin binding to and insulin degradation in isolated human erythrocytes have so far not been studied. We assume that these effects are independent of the tissue or cell type tested, since we could observe similar effects in our preliminary experiments on rat liver membranes. Organic solvents may interfere with the lipid components of the erythrocyte membrane to change the ordering of the latter, resulting in a better accessibility of insulin receptors and/or exposure of s.c. "masked" receptors. Changes in the red blood cell membrane ordering, even as little as involving the receptor area only, can increase the receptor affinity as well. We thus believe that the observed effects on insulin

binding and degradation can be explained by changes in the erythrocyte membrane ordering; this however, awaits direct evidence provided by direct measurements of membrane fluidity.

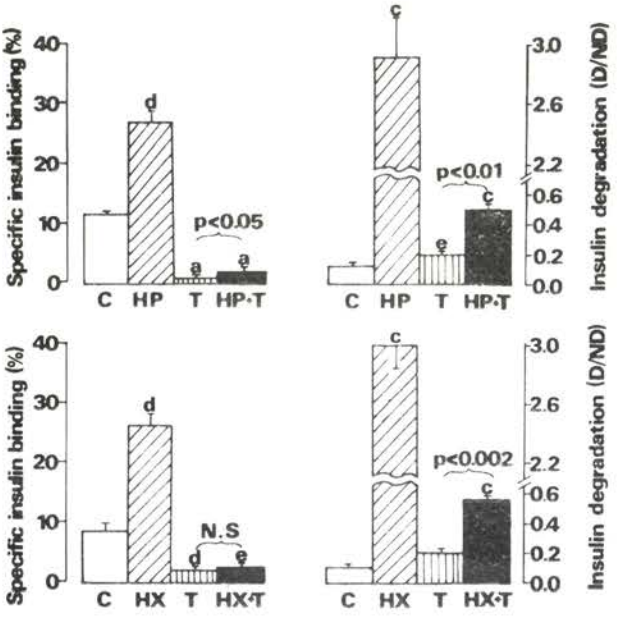

Fig. 2. Effects of the addition of 10 μl of *n*-heptane (*top*) or *n*-hexane (*bottom*) to 500 μl of intact isolated erythrocytes (HP and HX) or to erythrocytes preincubated with trypsin (HP + T and HX + T) on specific insulin binding (*left*) and insulin degradation (*right*). C = initial values without solvent addition, T = trypsin pretreated erythrocytes without solvent addition. Each value represents the mean of 3 experiments in 3 different individuals. Lower case letters are used to indicate statistical significance of differences as compared to group C (a, c, d, e correspond to $p < 0.001$, 0.01, 0.02 and 0.05, respectively).

References

Ayala G., Nascimento A., Gómez-Puyou A., Darszon A. (1985): Extraction of mitochondrial membrane proteins into organic solvents in a functional state. Biochim. Biophys. Acta **810,** 115—122

Elguindi I., Redziniak G., Metezeau P., Meybeck A., Goldberg M. E. (1985): Modulation of membrane receptor endocytosis by chemical effectors of membrane fluidity. Biol. Cell. **54,** 199—206

Gambhir K. K., Archer J. A., Bradley C. J. (1978): Characteristics of human erythrocyte insulin receptors. Diabetes **27,** 701—708

Hianik T., Kvasnička P., Štroffeková K. (1984): Mechanical properties of lipid bilayers with varying cholesterol content. Stud. Biophys. **100,** 23—32

Hianik T., Zórad Š., Kavečanský J., Macho L. (1987): Effect of insulin on lipid bilayer viscoelasticity. Gen. Physiol. Biophys. **6,** 163—172

Kolena J., Ondriaš K. (1984): Age-dependent changes in rat testicular LH/hCG receptors in relation to the membrane fluidity. Gen. Physiol. Biophys. **3,** 89—92

Montal M., Darszon A., Schindler H. (1981): Functional reassembly of membrane proteins in planar lipid bilayers. Q. Rev. Biophys. **14,** 1—79

Stubbs C. D., Smith A. D. (1984): The modification of mammalian membrane polyunsaturated fatty acid composition in relation to membrane fluidity and function. Biochem. Biophys. Acta **779,** 89—137

Weiner R. N., Schneider E., Haest C. W. M., Deuticke B., Benz R. Frimmer M. (1985): Properties of the leak permeability induced by a cytotoxic protein from Pseudomonas aeruginosa (PACT) in rat erythrocytes and black lipid membranes. Biochim. Biophys. Acta **820,** 173—182

Yuli I., Wilbrandt W., Shinitzky M. (1981): Glucose transport through cell membranes of modified lipid fluidity. Biochemistry **20,** 4250—4256

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