Biochemical Pharmacology, Vol. 43, No. 6, pp. 1185–1194, 1992.
Printed in Great Britain.

0006-2952/92 $5.00 + 0.00

THE EFFECTS OF GLUCOSE, INSULIN AND METFORMIN ON THE ORDER PARAMETERS OF ISOLATED RED CELL MEMBRANES

AN ELECTRON PARAMAGNETIC RESONANCE SPECTROSCOPIC STUDY

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(*Received* 24 *October* 1991; *accepted* 26 *December* 1991)

Abstract—Human red blood cell (RBC) membranes (RBC ghosts) were treated with glucose, insulin and metformin. The order parameters of RBC membranes were determined by 5- and 16- doxyl-stearic acid spin labels. Metabolic effects were excluded using an isolated system of RBC membranes. The membranes were incubated with glucose in physiological (5 mM), renal threshold (10 mM) and manifested diabetic (20 mM) concentrations for limited times. High concentrations of glucose (10, 20, 100 mM) increase the order parameters of RBC membranes significantly. Insulin by itself has a similar effect which is, however, not strictly concentration-dependent. By contrast, metformin at therapeutic concentrations (0.5 and 5.0 μM) decreases the order parameters. At 50 μM concentration the metformin effect is expressed less and recurs at 100 μM concentration. The effects are significant with 5-doxyl-stearic acid, but are not significant with the 16-doxyl derivative. When RBC membranes are co-incubated with 20 mM glucose and metformin at 0.5 and 5.0 μM concentrations the order parameters as determined by 5-doxyl-stearic acid remain normal (= control values). Higher concentrations of metformin (50 and 100 μM) cause an overshoot to very low order parameters. Insulin at 10, 100 and 200 mU/L does not influence significantly the effects of metformin. Addition of physiological amounts of bovine serum albumin does not abolish the effects of metformin. Metformin, at therapeutic concentrations (0.5 and 5.0 μM), maintains the normal fluidity at the polar interface of isolated RBC membranes by counterbalancing non-enzymatic glycosylation with 20 mM glucose *in vitro*.

The introduction of insulin therapy in the 1920s was thought to provide the final solution in the treatment of diabetes mellitus. However, the survival of diabetic patients made evident the variety of long-term complications connected with this disease which cannot be prevented by oral sulfonylurea or by s.c. insulin administration. Even the application of human insulin has not yet brought about a breakthrough.

The most outstanding bunch of complications in a variety of organs is based on vascular alterations concerning non-insulin-dependent cells and tissues. In diabetic patients metabolic influences on membranes are versatile and the constant overdosage of insulin by the s.c. route of administration obviously enhances the endothelial proliferation [1].

On the other hand, non-enzymic glycosylation of proteins and lipoproteins plays a major role in long-term diabetic complications [2–5]. This was reported also using red blood cells (RBCs‡) and the RBC membrane [6–8].

Besides its antidiabetic efficiency [9–12] metformin has been reported to exhibit antiatherosclerotic effects in animals [13, 14] and in diabetic patients [15–18].

The question arose as to whether the above metabolic metformin efficacy is also corroborated in isolated membranes, in other words, whether there is a physico-chemical metformin–membrane interaction leading to alterations in membrane fluidity.

The model of the isolated RBC membrane excludes metabolic effects, hence direct interactions can be studied. In order to provide correlation to antidiabetic therapy, pharmaceutical insulin preparations (ampoules containing zinc-protamine suspensions of human insulin) were used in our investigations.

Although the uptake of glucose into RBCs appears to occur in a non-insulin dependent manner, insulin was shown to influence glucose transport in RBC membranes [19] and an insulin receptor has also been found in RBC membranes [20].

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‡ Abbreviations: RBC, red blood cell; EPR, electron paramagnetic resonance; SASL, stearic acid spin label.

Electron paramagnetic resonance (EPR) spectroscopy is one of the methods used to study alterations in biological membranes [21, 22]. The insertion of spin markers into the membrane mirrors the environmental status. Stearic acid derivatives have been used to determine the order parameters in biological membranes [23] and one of the thermotropic transitions in RBC membranes [24]. 5-Doxyl-stearic acid reports from the more polar interface of the membrane whereas 16-doxyl-stearic acid reflects the hydrophobic core of the membrane [25].

MATERIALS AND METHODS

The spin labels 5-doxyl- and 16-doxyl-stearic acid (5-doxyl-SASL and 16-doxyl-SASL) were purchased from Aldrich (Steinheim, Germany). Depot-H-Insulin was a product of Hoechst AG (Frankfurt-Hoechst, Germany), obtained from a public pharmacy store. MetforminHCl was a gift of Lipha (Lyon, France). All other substances were achieved at the purest grade available either from Sigma (Steinheim, Germany) or from Merck (Darmstadt, Germany).

Preparation of the RBC membranes. RBC concentrate from 500 mL of fresh blood (O, Rh +) was obtained from the Blutspendedienst Hessen of the University of Frankfurt. The RBCs were hemolysed, washed and lyophilized as described elsewhere [26, 27]. The centrifuges and rotors were a Sorvall RC 2-B and at first a GSA rotor at 12,000 rpm and then a SS 34 rotor at 14,000 rpm. The temperature was held between 2 and 5°; 15 and 10 mM sodium phosphate buffers, pH 7.4 were used for hemolysis and washing. Finally, the white membranes were suspended in a small amount of distilled water and lyophilized in a Christ Beta I freeze-dryer.

Spin labeling experiments. The RBC ghosts were suspended in phosphate-buffered saline at 4 mg membranes/mL. Glucose, insulin, metformin or albumin was added from PBS solutions at the concentrations indicated, so that the final amount of ghosts was 2 mg/mL. Final concentrations were: glucose 5, 10, 20, 100 mM; insulin 10, 100, 200 mU/L; albumin 1 mM; metformin 0.5, 5, 50, 100 μM. All incubations were for 5 min at room temperature, with vortexing for 10 sec every 30 sec. The suspension was then centrifuged in an Eppendorf centrifuge 3200 at maximum speed for 2 min and the pellet was resuspended in 100 μL of the supernatant. Subsequently, 1 μL of a 5 mM ethanolic solution of the spin label was added, vortexed for 10 sec and centrifuged as above. The supernatant was discarded in order to remove spin label which had not penetrated into the membrane. The pellet was again resuspended in 100 μL of the supernatant from the first centrifugation step. The signals were recorded from 50 μL capillaries in a Bruker B-R 70 Digital EPR spectrometer with a B-E 25 magnet. The spectra were evaluated as described elsewhere [28, 29].

All the results of this study have been evaluated statistically (Mann–Whitney/Wilcoxon) by the Centre of Biomathematic and Medical Statistics of the University Clinics in Frankfurt. The results presented in this study are statistically significant (at least $P < 0.05$ or 0.01), if not, they are designated "not significant".

RESULTS

Studies with unmodified RBC membranes using 5-doxyl- and 16-doxyl-SASLs

Effect of glucose on isolated RBC membranes. The order parameter obtained from 5-doxyl-SASL was 0.749 (Fig. 1) and that from 16-doxyl-SASL was 0.284 (Fig. 2) (= controls). With both spin labels

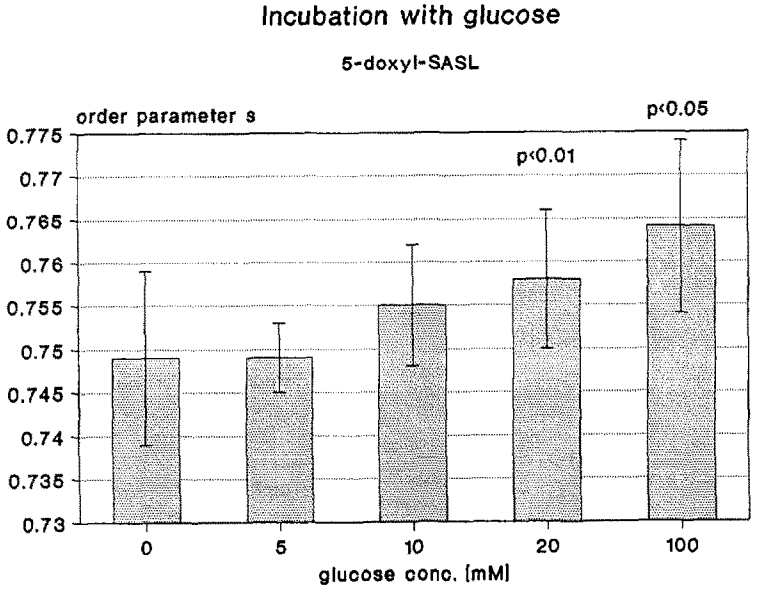

Fig. 1. Influence of glucose on the order parameter of RBC membranes as determined with 5-doxyl-stearic acid. $P < 0.01$ and 0.05 vs control value of $s = 0.749$.

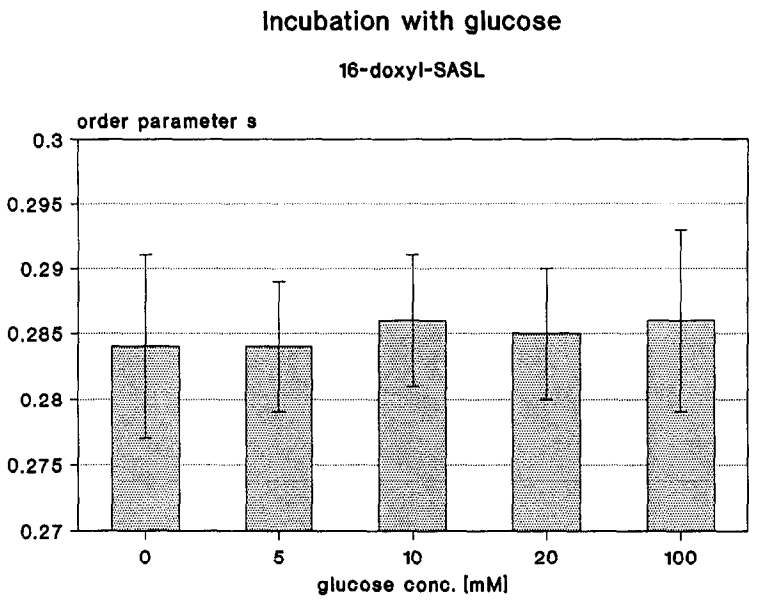

Fig. 2. Influence of glucose on the order parameter of RBC membranes as determined with 16-doxyl-stearic acid.

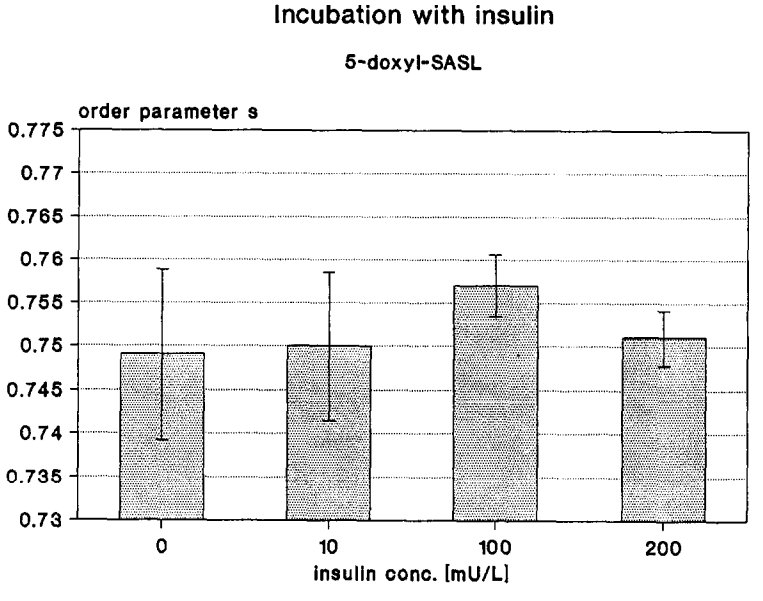

Fig. 3. Influence of insulin on the order parameter of RBC membranes as determined with 5-doxyl-stearic acid.

the order parameters were not altered under the influence of a 5 mM concentration of glucose. At higher concentrations (10, 20, 100 mM) of glucose order parameters increased. This effect was expressed significantly at 20 and 100 mM concentrations with 5-doxyl-SASL (Fig. 1), whereas it was not significant with 16-doxyl-SASL (Fig. 2).

Effect of insulin on isolated RBC membranes. With 5-doxyl-SASL insulin increased the order parameter marginally in a bell-shaped manner (not significant) (Fig. 3). With 16-doxyl-SASL order parameters were also not significantly, but were constantly increased under increasing insulin concentrations ($10 < 100 < 200$ mU/L) (Fig. 4).

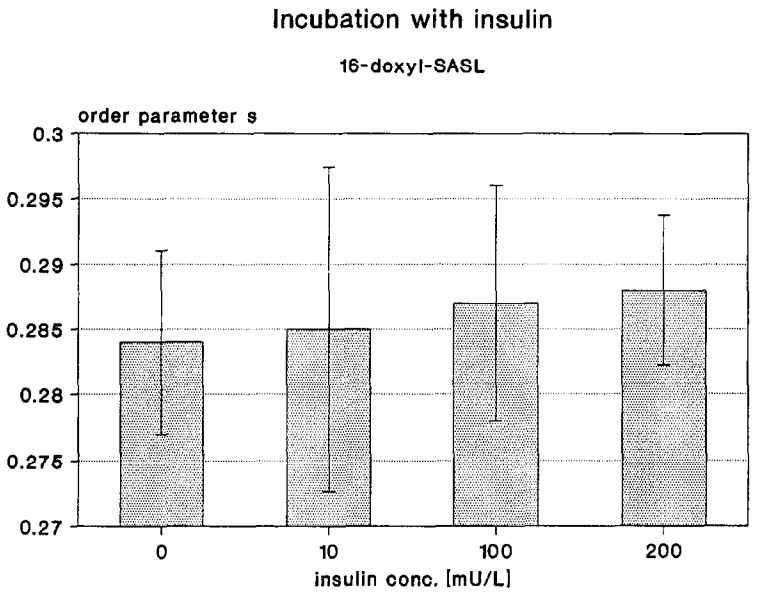

Fig. 4. Influence of insulin on the order parameter of RBC membranes as determined with 16-doxyl-stearic acid.

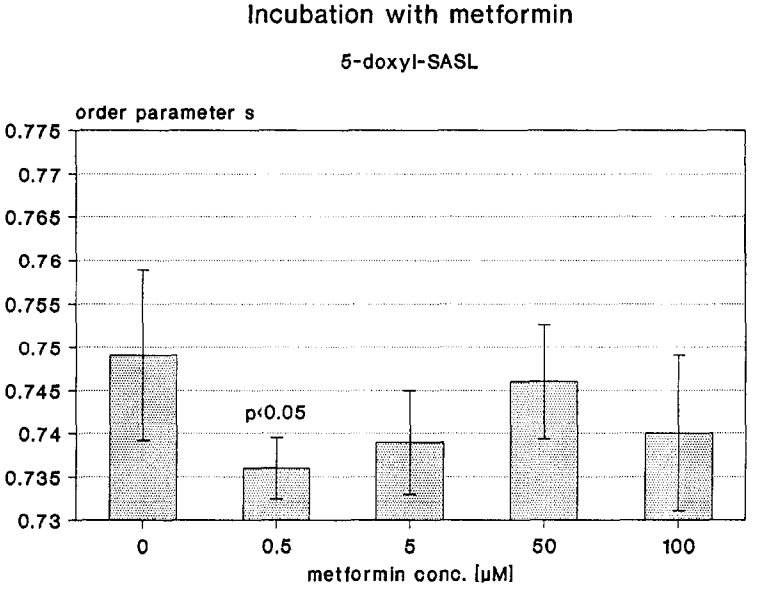

Fig. 5. Influence of metformin on the order parameter of RBC membranes as determined with 5-doxyl-stearic acid. $P < 0.05$ vs control value of $s = 0.749$.

Effect of metformin on isolated RBC membranes. Metformin exerted a bell-shaped effect on the order parameters of both 5-doxyl- (Fig. 5) and 16-doxyl-SASL (Fig. 6). At the lowest concentration used (0.5 µM) metformin decreased the order parameters to 0.736 (5-doxyl-SASL) and to 0.277 (16-doxyl-SASL). The effect was progressively lost with increasing concentrations of metformin (5 and 50 µM) and recurred at a 100 µM concentration of metformin (Figs 5 and 6).

Studies with isolated RBC membrane using 5-doxyl-SASL; interaction between 10 mM glucose, metformin and insulin

The order parameter obtained from 5-SASL was 0.755 after incubation with 10 mM glucose (Table

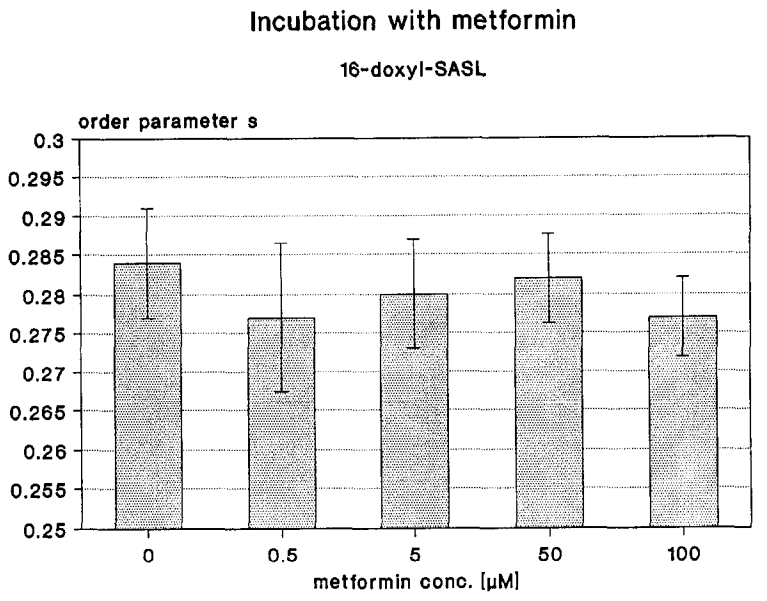

Fig. 6. Influence of metformin on the order parameter of RBC membranes as determined with 16-doxyl-stearic acid.

Table 1. RBC membranes incubated with 10 mM glucose and various concentrations of insulin and metformin

Glucose (mM)	Insulin (mU/L)	Metformin (μM)	Order parameter* (s/SD)
10	0	0	0.755/0.0070
10	100	0	0.747/0.0136
10	0	50	0.746/0.0031
10	100	50	0.746/0.004
10	200	50	0.738/0.0017†
0	100	50	0.748/0.005
0	200	100	0.746/0.0072

* Order parameters obtained from 5-doxyl-stearic acid. $3 < N < 5$, SD = σ_{n-1}, control: s = 0.749, SD = 0.0098.
† $P < 0.05$ vs control value of $s = 0.0755$.

1). Insulin at 100 mU/L reduced the order parameter to 0.747 (SD = 0.0136). The same value ($s = 0.746/0.747$) was obtained with 50 μM metformin and with a combination of 100 mU/L insulin and 50 μM metformin. Insulin at 200 mU/L enhanced the effect of 50 μM metformin to $s = 0.738$ ($P < 0.05$ vs 0.755). This latter overshooting effect was the only significant result obtained in experiments with 10 mM glucose (Table 1).

Studies with isolated RBC membranes using 5-doxyl-SASL; interaction between 20 mM glucose, metformin and insulin

The order parameter of membranes incubated with 20 mM glucose was significantly increased versus unmodified controls ($s = 0.758$ vs 0.749, $P < 0.01$) (Fig. 7).

Effect of insulin. Insulin by itself did not alter the order parameter at 100 or 200 mU/L ($s = 0.758$ in all cases) (Fig. 7).

Effect of metformin. Metformin counteracted the influence of 20 mM glucose on the order parameter ($s = 0.758$) progressively with increasing concentrations. Metformin at 0.5 μM and 5.0 μM reinstated the order parameter of the original unmodified RBC membranes ($s = 0.749$ or 0.748, respectively) (Fig. 7). Metformin at 50 and 100 μM exerted a tremendous overshoot ($s = 0.739$ or 0.738, respectively). Insulin did not influence significantly all these metformin interactions with 20 mM glucose (Fig. 7).

Influence of 1 mM bovine serum albumin on the above effects of glucose, insulin and metformin

Bovine serum albumin did not alter significantly the results of this study. Glucose increased the order parameter (5-SASL) by a maximum of 1.2% without and 0.8% with 1 mM albumin. The maximum metformin effect was a 1.7% reduction versus controls without and 1.6% with albumin. After coincubation with 20 mM of glucose the effects of insulin and metformin were the same, in general, no matter whether albumin was present or not; maximum effects were up to 2.8% versus controls with metformin in both cases (not shown).

DISCUSSION

Discussion of methodology

A variety of methods (i.e. capillary viscosimetry, fluorimetry and EPR spectroscopy) have been used

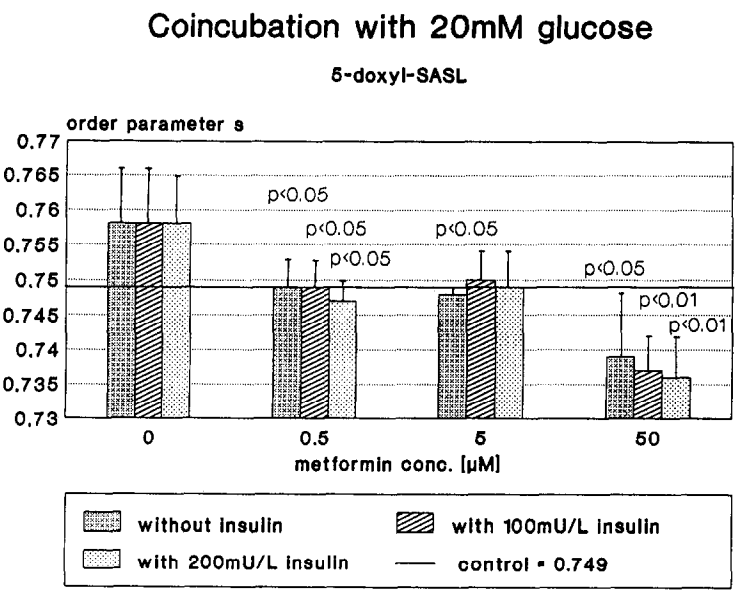

Fig. 7. Influence of metformin at therapeutic concentrations on the order parameter of RBC membranes coincubated with 20 mM of glucose as determined with 5-doxyl-stearic acid, without addition of insulin and with addition of 100 or 200 mU/L of insulin, i.e. no significant interaction between metformin and insulin. $P < 0.01$ and 0.05 vs zero value of $s = 0.758$.

in our group to characterize biological membranes and membrane interactions [30, 31]. Also in this study we first assayed various methods. Each method has advantages and disadvantages [22]. In preliminary studies EPR spectroscopy showed the clearest results versus controls. Among the spin labels used (5-proxyl-nonane, 5-doxyl-decane, stearic acid derivatives) the latter proved the most suitable (5-doxyl- and 16-doxyl-stearic acids). For evaluation of the spectra order parameters were calculated. Coupling constant a_N, rotational-correlation times or other motion parameters [23] were either not applicable or less significant.

In our system of non-metabolic, short-time incubation, EPR spectroscopy mainly yields information on the alterations at the polar interface of the membrane. With metformin, the hydrophobic region exhibits a qualitatively similar modification (only significant at 0.5 and 100 μM concentrations).

In studies with RBC membranes from diabetic patients the hydrophobic region would certainly be involved in alterations to a greater extent [32] via metabolic pathways or long-term influences. Long-term incubations were also carried out in our studies, however, the rapid aging of the membranes gives progressively less specific results. Hence, the model system used in our study appeared the most suitable to investigate direct interactions of glucose, insulin and metformin with the isolated RBC membrane.

Order parameters of isolated RBC membranes have been used to characterize the alteration by drugs and by other biochemical modification i.e. pH [30]. Correlations have been carried out to other methodologies such as 8-anilino-naphthalin-1-sulfonate fluorimetry [33, 34, 22]. The order parameters obtained from 5-doxyl-SASL can be correlated with the polar interface of the membrane, whereas those obtained from 16-doxyl-SASL report on the hydrophobic core. The values found in our study for unmodified membranes are comparable to those reported by Zimmer *et al.* [35]. Furthermore, the order parameters provide evidence of fluidity or rigidity in different regions of the membrane.

Statistical evaluation

Statistical significance may considerably differ from medical relevance. Overshoots as described in this study for high metformin concentrations, are in several cases of high statistical significance but may mirror negative (side) effects rather than a positive therapeutic influence. However, the counterbalance of glycosylation and rigidification of the RBC membrane with 20 mM glucose to "normal" fluidity by 0.5 μM metformin, which must be regarded as the most medically relevant result of this investigation, is also highly significant.

Discussion of results

This study was accomplished in two major parts: in the first part the distinct effects of glucose, insulin and metformin on the unmodified RBC membrane from healthy sources were investigated, whereas in the second part the interactions of insulin and metformin with RBC membranes coincubated with glucose were studied. As glycosylation of the RBC membrane is known to occur not only via metabolic pathways but also via mere chemical reactions, the latter measurements may be interpreted as interference of insulin and metformin with the glycosylation of the RBC membrane. In order to differentiate between the polar interface of the membrane and the hydrophobic core, in the first

part of this study both 5- and 16-doxyl-SASLs were used.

Part I. Glucose at physiological (5 mM) concentration does not alter the order and the fluidity of the membrane in either the polar or the apolar regions. Increasing concentrations to the "renal threshold" (10 mM) to double this concentration (20 mM), which correlates to a manifest diabetes mellitus, and to an experimental over-concentration of 100 mM glucose progressively raises the order and the rigidity at the polar interface of the membrane. These effects are highly significant (i.e. at 20 mM concentration, $P < 0.01$) and cannot be ascribed to mere osmotic effects since addition of metformin in the micromolar range reduces them to control or even lower values.

In the hydrophobic region of the membrane glucose does not influence the order significantly even at higher concentrations ($s = 0.284$–0.286).

Results from studies with RBC or ghosts from diabetic patients vary considerably. This may depend in part on the different methods used, such as EPR spectroscopy, fluorescence polarization and flow cytometry. The parameters of the membrane determined by means of these methods differ, although they are finally taken to draw conclusions from the fluidity or rigidity of the membrane. Hence, some of these studies are summarized and interpreted in terms of fluidity of the membrane. Using EPR spectroscopy a decrease in fluidity was found in RBC and other membranes from diabetic patients by Kamada and Otsuji [36] and in diabetic rats by Kordowiak *et al.* [37]. Similar results were reported by Baba *et al.* [38] and by Bryszewska *et al.* [39] using fluorescence polarisation and by Masuda *et al.* [40] from leukocyte membranes from diabetic rats using flow cytometry. A decrease in fluidity after *in vitro* glycosylation of RBC membranes from healthy individuals was correlated with results from diabetic patients by Bryszewska and Szosland [41]. Lapolla *et al.* [42] investigated the correlation between glycosylation and deformability of RBCs as determined by their filtrability. Watala [43] also found that *in vitro* glycosylation of RBC proteins with high levels of glucose lowers the lipid fluidity of RBC membranes.

On the other hand, fluorescence polarization which reports from the hydrophobic core of membranes also revealed opposite effects. Testa *et al.* [44] found the fluidity of diabetic RBC membranes to be increased, Hill and Court [45] did not find an alteration versus controls and Muller *et al.* [46] found both, not alteration or increased fluidity, depending on the fluorescence chromophore used in their investigations.

Kamada *et al.* [32] detected an increase in order and microviscosity mainly in the hydrophobic region of the RBC membranes from diabetic patients. They determined the order parameters by means of 5-doxyl- and 16-doxyl-SASL and the motion parameter of the latter as well as microviscosity using fluorescence polarization. Although they did not find a significant alteration in the order parameter of 5-doxyl-SASL (i.e. in the polar region) surface electric charge was decreased and cell aggregation was increased in the membranes from diabetic patients. All the parameters obtained from the hydrophobic region indicate an increase in viscosity or rigidity in their study. In parallel, they detected higher concentrations of cholesterol and a decrease in polyunsaturated acyl chains of the phospholipids. These are metabolic effects not detectable in our model and explain the differences between theirs and our study. Metabolic pathways may either counteract chemical alterations at the surface of the membrane by high concentrations of glucose or may enhance the influence on the hydrophobic region.

We had observed counterregulations between the polar and the hydrophobic regions of membranes in other investigations [30]. Insulin at 10 mU/L was used in our study as an equivalent to concentrations normally found in the serum. This concentration does not change significantly the order of the membrane either in the polar or in the apolar regions; 100 mU/L of insulin may occur in the serum postprandially or for a longer period of time after s.c. application. A concentration of 200 mU/L is not reached in the serum under physiological conditions; however, it may occur under s.c. application of insulin.

In our model, insulin tends to indicate a bell-shaped increase in rigidity of the RBC membrane at the polar interface, the highest value being at 100 mU/L. However, this insulin effect is not significant.

Dutta-Roy *et al.* [47] found an S-shaped reduction in the microviscosity of the RBC membrane and in the filtration time of intact cells with "physiological" concentrations of insulin. Use of "supra-physiological" concentrations reversed these effects. They used the method of fluorescence polarization which tends to mirror the viscosity in the hydrophobic region of the membrane. Although they refer to "physiological" blood concentrations ranging between 60 and 150 mU/L, much higher concentrations must be applied for incubation in their system that are not comparable with the concentrations we used. However, the "bell-shaped" influence of insulin on the membrane observed in our study may correlate with the "S-shaped" modifications as reported by Dutta-Roy *et al.* [47]. Farias [48] hypothesized that insulin at 140 mU/L decreases membrane fluidity *in vitro* without modifying its fatty acid composition. Furthermore, he emphasized that there is good agreement between the *in vitro* and *in vivo* ranges of insulin concentration (around 100 mU/L) at which membrane effects (i.e. Hill coefficient, "overall" lipid order) are observable. On the other hand, Baldini *et al.* [49] ruled out an involvement of membrane fluidity in the mechanism of action of insulin on human RBCs.

Metformin exerts a bell-shaped fluidizing effect in both the polar and the apolar parts of the membrane. The strongest influence is observed at the lowest concentration applied (0.5 μM). The influence is lower at a 5.0 μM and at a minimum at a 50 μM concentration. At a concentration of 100 μM the metformin effect is similar to that at one of 0.5 μM. This could be interpreted as there being different sites of interaction with the membrane at different concentrations. Therapeutic concentrations in the serum are in the range of 0.5 to 5 μM. The highest

plasma levels have been found in the hepatic portal vein at about 50 μM at high dosage; however, the concentration never exceeded 100 μM even after oral application of 50 mg/kg [50]. Metformin may modify membrane protein(s) at very low (therapeutic) concentrations and at higher concentrations (about 100 μM up to mM) it may interact preferentially with phospholipids [51]. Similar results were obtained after incubation of RBC membranes with increasing concentrations of phloretin [52].

However, in the case of metformin, the higher concentrations (100 μM up to mM) are beyond therapeutic plasma levels. Concentrations of up to 20 mM of metformin have been used to investigate the interaction with liposomal phospholipids (Dr Teissie, Toulouse, and Dr Wiernsperger, Lyon, personal communication) such that saturation phenomena should not be expected in the range of up to 100 μM applied in our study.

Part II. In the second part of this study we investigated the interference of insulin and metformin with the glycosylation of RBC membranes, i.e. coincubation with 10 or 20 mM glucose.

Treatment of RBC membranes with 10 mM of glucose results in rigidification as compared with unmodified RBC membranes. Insulin at 100 mU/L, metformin at 50 μM and coincubation with insulin and metformin equally counterbalance the glucose effects on the order of the membrane. However, these effects are not significant (Table 1).

The combination of 200 mU/L of insulin and 50 μM of metformin creates an overshoot in fluidity ($P < 0.05$ vs control, but not significant vs the effect of metformin). At lower (i.e. 0.5 and 5.0 μM) concentrations, metformin does not need insulin for its interaction with RBC membranes. As our incubation studies with a 10 mM concentration of glucose yielded results that were either not significant or did not appear relevant to metformin therapy, they will not be further discussed.

In RBC membranes treated with 20 mM of glucose the order in the polar region is increased significantly versus controls ($s = 0.758$ vs 0.749, $P < 0.01$) (Figs 1 and 7).

Coincubation with metformin fluidizes the membrane progressively with increasing concentrations. Metformin at 0.5 μM normalizes the order exactly to the value of unmodified membranes ($s = 0.749$), and the effect of a concentration of 5 μM is similar ($s = 0.748$). Concentrations of 50 and 100 μM exert a tremendous overshoot ($s = 0.739$ and 0.738, respectively) (Fig. 7). In brush-border membrane vesicles an increase in membrane fluidity by 3% by n-aliphatic alcohols correlated with an inhibition of glucose uptake by 80% [53]. Hence a tremendous fluidization of the membrane may be as unphysiological as rigidification. Furthermore, the former may generate metabolic counteraction and increased incorporation of cholesterol *in vivo*.

Insulin does not influence the fluidity of RBC membranes rigidified by incubation with 20 mM of glucose nor does it modify significantly the fluidizing effects of metformin. At therapeutic concentrations metformin counterbalances the effect of 20 mM glucose, i.e. it normalizes membrane fluidity, by itself or in combination with insulin. Rapin *et al.* [54] reported that metformin at a low concentration obviously counteracts the decreased deformability of insulin-treated RBCs from diabetic patients, whereas a 50-fold higher concentration enhances the insulin effect. However, the data in this paper are controversial, especially those concerning metformin concentrations, such that direct comparison is impossible. Nevertheless, the study reveals differences in the interactions of low and high metformin doses with insulin at the RBC membrane.

Albumin does not alter significantly the results obtained from our model investigation. Protein binding of metformin was supposed to be negligible [51]. As we did not find significant interaction between metformin and insulin or albumin, our study with SASLs appears to support the theories of Schäfer [51] on mere unspecific binding of metformin to membrane phospholipids. However, the intensive effect of metformin at the lowest concentration (0.5 μM) suggests a specific binding site. Moreover, we obtained supporting evidence from further experimentation with protein-specific reagents that metformin interacts with proteins in the RBC membrane. This will be within the scope of future investigations.

Conclusions and further aspects

Metformin at therapeutic concentrations regulates the fluidity of the RBC membrane at the polar interface via chemical counteraction of glycosylation. This effect may contribute to the antiatherosclerotic efficiency of metformin reported earlier and may protect diabetic patients against rheological and vascular complications.

Higher concentrations of metformin cause an overshoot in fluidity which in turn is unphysiological and may generate metabolic counterreaction.

The bell-shaped effects of metformin on unmodified RBC membranes may indicate two different sites of interaction: at lower, therapeutic concentrations an influence on membrane proteins is suggested, whereas at higher concentrations interaction with the phospholipids may occur [51].

Proteins such as insulin or albumin do not influence significantly the membrane interaction of metformin in our model. However, *in vivo* metabolic synergism between metformin and insulin as suggested by Wollen and Bailey [55] may occur.

Preliminary experimentation with RBC membranes incubated with glucose or metformin and assayed with a thiol reactive spin label detected signal alterations indicating interactions between glucose and metformin with membrane proteins. However, these interactions can be investigated much more sensitively by other methods such as fluorescence spectroscopy using bromobimanes [56]. This will be within the scope of further investigations.

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