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INCREASE IN INSULIN BINDING AND INHIBITION OF THE DECREASE IN THE PHOSPHOLIPID CONTENT OF HUMAN TERM PLACENTAL HOMOGENATES IN CULTURE BY THE SULFONYLUREA GLIPIZIDE

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Abstract—Term placental explants were cultivated for 48 hr without (control) and with various concentrations of glipizide. Maximum binding of [^{125}I]-insulin in the control samples was decreased after 12 and 24 hr returning to initial values after 48 hr. In the presence of glipizide the binding was generally higher, reaching 180% (557 and 1000 nmol/L) of the corresponding control value ($P < 0.01$) after 48 hr owing to the presence of nearly 3-fold more ($P < 0.05$) receptors than in the untreated controls. Tissue cholesterol content was almost unaffected whereas both the phospholipid content and the corresponding phospholipid-to-cholesterol ratios were markedly, and in a time-dependent manner, increased by glipizide as compared to the controls. This was due to decreasing cholesterol and phospholipid concentrations in the controls during the time of culture as compared to initial values, and also to unchanged levels in glipizide-treated cultures. We conclude that glipizide affects placental insulin receptors and the phospholipid content of the tissue.

Sulfonylureas are widely used drugs for the treatment of patients with non-insulin-dependent diabetes mellitus. There is now general agreement that sulfonylurea drugs exert their glucose lowering effects by increasing both extrapancreatic glucose uptake and pancreatic insulin secretion [1]. Moreover, changes in insulin binding to its receptors on the surface of various human cell types after treatment with sulfonylureas have been reported, although some cell lines did not respond [2].

The human placenta contains a large number of insulin receptors [3] which are located in the microvillous plasma membrane of the syncytiotrophoblast [4]. The concentration of these receptors is changed in pregnancies complicated by gestational or overt diabetes mellitus [5]. Information as to whether these receptors are dynamically regulated and affected by exogenous substances *in vitro* is lacking. Such studies have been virtually impossible to undertake so far, since the syncytiotrophoblast can not be isolated and cultivated in the amounts necessary.

Since sulfonylurea drugs are used in some centers in the management of patients with gestational or overt diabetes mellitus [6, 7], we were wondering if treatment with glipizide—a second generation sulfonylurea—can affect placental insulin receptors which, owing to their location, are immediately exposed to the maternal circulation.

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§ Abbreviations: PL/Chol ratio, phospholipid-to-cholesterol ratio; DMEM, Dulbecco's modified Eagle medium.

We took advantage of an *in vitro* tissue culture system of human placenta to measure the effects of glipizide supplementation of the culture medium on placental insulin receptors. In addition, the cholesterol and phospholipid concentrations in the tissue samples were determined, since the affinity and number of insulin receptors are both affected by the lipid composition of the placental membranes [3].

MATERIALS AND METHODS

Materials. Monoiodinated (A14Tyr)-[^{125}I]-insulin (sp. act. approx. 2000 Ci/mmol) and [4-^{14}C]-cholesterol were obtained from Amersham (U.K.); human monocomponent insulin from Novo (Vienna, Austria); bovine serum albumin, 2-propanolol, bacitracin and phenyl-methylsulfonylfluoride from Sigma (Taufkirchen, F.R.G.). Dulbecco's modified Eagle's medium (DMEM§) was purchased from Biological Industries (Beith Haemek, Israel). Glipizide was kindly provided by Pfizer (U.S.A.). Tris and all other reagents and solvents were from Merck (Darmstadt, F.R.G.) at the highest available purity.

Placental tissue culture and incubations. Small placental fragments (about 0.5 g wet wt) were dissected out in a sterile fashion from term (40 weeks of gestation) placental tissue obtained from healthy women with normal body weight after elective cesarean section because of previous cesarean section. The women were non-smokers and did not receive any medication throughout pregnancy. After extensive washings and rinsing, the tissues were

explanted as described [8]. The tissue was cultured without (four placentae) or with (two placentae) various concentrations of glipizide (dissolved in DMEM) in petri dishes containing 2 mL DMEM, 0.5% bovine serum albumin and 1% antibiotic solution. Glipizide concentrations were chosen to include the therapeutic plasma levels observed in man (maximum level 0.6–1.0 μmol/L; [9]). Incubations were carried out at 37° in a 95% air and 5% CO_2 atmosphere and were stopped by placing the dishes on ice. Finally, the explants were frozen at −40° in Eppendorf microtubes and stored until further analysis.

Viability of the explants was assessed as described previously [8–11] based on the following criteria: (1) progressive glucose consumption determined by the glucose concentration in the culture media using a commercially available kit (Sera-Pak, Miles, U.K.); (2) continuous increase in hormone secretion measured by standard radioimmunoassay kits: progesterone (Bio Yeda, Rehovot, Israel), estradiol-17β (Bio Yeda) and human chorionic gonadotropin (MAIA Clone, Serono, Rehovot, Israel); (3) progressive increase in the activity of glucose-6-phosphate dehydrogenase (EC 1.1.1.49) in the tissue measured with a commercially available kit (Sigma Chemical Co., St Louis, MO, U.S.A.); (4) unaltered pH in the culture medium during the incubations; and (5) intact tissue morphology demonstrated by staining of tissue with hematoxylin and eosin.

Measurement of insulin binding. The disintegration of tissue and incubation with insulin have been described in detail elsewhere [12]. Briefly, after addition of 0.2 mL ice-cold buffer (50 mmol/L Tris–HCl + 1 mmol/L phenyl-methylsulfonylfluoride, pH 7.4) the samples were sonicated for 1.5 min under careful cooling (4°) with a precooled microtip (Labsonic 2000, Braun Melsungen, F.R.G.) at 40 W energy setting. The homogenated material was centrifuged at 3000 *g* for 20 min at 4°. The pellet was resuspended in 1 mL ice-cold buffer and centrifuged again at 3000 *g* for 20 min at 4°. After this washing, the homogenate was resuspended in 0.5 mL ice-cold buffer. Subsequently, the homogenates were incubated with [^{125}I]-insulin (approximately 165,000 cpm) for 18 hr without (defining maximum binding) or with six increasing concentrations of unlabeled insulin, ranging from 1.65 pmol/L to 49.5 nmol/L. Each of the different conditions was measured in triplicate. The tubes were then centrifuged, the supernatant decanted and radioactive disintegrations of bound [^{125}I]-insulin (total binding) were counted in a 16 channel counter (Nuclear Enterprise NE 1600) at approximately 65% counting efficiency.

For the determination of non-specific binding [12], the pellets of the identical samples, on which total binding had already been measured, were carefully reconstituted in 0.3 mL ice-cold buffer by multiple vortexing/cooling cycles. The samples were then incubated with unlabeled insulin at a final concentration of 4.95 μmol/L. After centrifugation, the supernatant was discarded and the radioactivity of the pellet was counted. The intra-assay coefficient of variation for the non-specific binding was 10.3%; the inter-experimental coefficient of variation was 12.7%. Both incubations were performed in buffer containing 0.2% bovine serum albumin and 2% bacitracin at a final volume of 0.5 mL under constant shaking at 4°.

Degradation of insulin was measured by precipitation of the higher molecular weight material in duplicate samples of the incubation mixture with ice-cold 10% (w/v) trichloracetic acid before and at the end of the incubation. The loss of precipitable [^{125}I]-insulin during incubation was assumed to reflect its degradation. It never exceeded 2% and was unaffected by glipizide.

After precipitation with 20% (w/v) ice-cold perchloric acid and subsequent alkaline hydrolysis, the protein content in the pellet was measured by the method of Lowry *et al.* [13] using bovine serum albumin as the standard.

Specific binding was calculated as the difference between total binding at each concentration of unlabeled insulin and the corresponding values for non-specific binding, which varied from sample to sample, reflecting the individual degree of tissue disruption [12].

The values for affinity and concentration of the receptors were obtained by non-linear model fitting according to a non-cooperative model comprising one class of binding sites as described [5].

Extraction of lipids. Lipids from placental tissue (approximately 2 mg tissue protein) were extracted according to the method of Folch *et al.* [14] as described [3]. Recovery of [4-^{14}C]cholesterol, added in tracer amounts, was usually 90–95%. The cholesterol content was determined after evaporation of the extract and resolving in 2-propanol by the method of Siedel *et al.* [15].

Phospholipids were precipitated with trichloracetic acid (1.2 mol/L) and the amount of phosphorus was determined by the method of Fiske and Subbarow [16]. Total phospholipid content was calculated by multiplying the lipid phosphorus in mg/100 mL by 25.

After lipid extraction the remaining tissue was precipitated with 20% (w/v) trichloracetic acid and the protein content was measured by the method of Lowry *et al.* [13] after alkaline hydrolysis.

Statistical evaluations. For intergroup comparison the non-parametric Wilcoxon rank sum test was used. Significances were accepted at the level of 95% or more.

RESULTS

The glucose concentration in the culture media continuously decreased throughout the experiments and did not differ between untreated and treated explant cultures (data not shown). The explants contained around 20 mg protein/g placental wet wt. The protein content of the tissue was virtually unchanged during the culture period and was unaffected by treatment with glipizide (Table 1).

The maximum specific binding of [^{125}I]-insulin to placental homogenates in culture decreased from an initial value (at $t = 0$) of 3.8 ± 0.7% to 2.5 ± 0.4% ($P < 0.05$) after 12 hr. Twenty-four hours after the culture was started, the maximum binding was virtually unchanged as compared to the value at

Table 1. Protein, cholesterol (Chol) and phospholipid (PL) contents and the PL/Chol ratio in explants of placental tissue in culture (control) and after treatment with various concentrations of glipizide

Treatment (mmol/L)	hr	N	Protein (mg/g wet wt)	Chol (mg/g protein)	PL (mg/g protein)	PL/Chol
Control	0	8	19.9 ± 3.5	26.9 ± 2.0†	88.2 ± 5.7†	3.24 ± 0.15†
	12	8	21.1 ± 4.1	26.1 ± 2.5†	60.7 ± 4.8†	2.31 ± 0.24
	24	8	20.8 ± 2.8	19.4 ± 2.8	49.2 ± 8.1†	2.54 ± 0.27
	48	8	19.3 ± 4.0	19.6 ± 1.6	20.8 ± 0.5‡	1.10 ± 0.16‡
55.7	24	4	22.4 ± 3.6	25.7 ± 2.3	56.6 ± 7.2	2.25 ± 0.33
	48	4	20.5 ± 1.9	19.7 ± 1.8	37.7 ± 9.3‡	1.93 ± 0.28‡
111	24	4	18.9 ± 5.3	27.0 ± 2.7*	64.5 ± 10.1	2.44 ± 0.41
	48	3	21.3 ± 4.6	20.4 ± 2.1	44.1 ± 7.6‡	2.15 ± 0.39‡
200	12	4	20.3 ± 4.7	24.5 ± 1.9	70.9 ± 5.8	2.89 ± 0.12†‡
	24	4	20.7 ± 3.4	28.0 ± 1.6*‡	73.0 ± 14.7	2.75 ± 0.67
	48	4	19.4 ± 2.6	21.7 ± 2.5	45.9 ± 5.6*‡	2.35 ± 0.13*‡
557	24	4	21.2 ± 5.2	27.5 ± 2.9*	68.7 ± 12.6	2.51 ± 0.32†
	48	4	20.7 ± 3.7	23.5 ± 3.0	73.8 ± 8.7*	3.19 ± 0.48*
1000	12	4	18.8 ± 4.2	26.7 ± 2.4	63.4 ± 6.6†	2.28 ± 0.12†
	24	3	20.0 ± 3.7	27.2 ± 1.8*	75.1 ± 16.3	2.52 ± 0.20*†
	48	4	19.7 ± 2.8	24.5 ± 1.7*	82.5 ± 2.4*	3.38 ± 0.12*

Values are means ± SEM.
* $P < 0.05$ vs control; † $P < 0.05$ vs 48 hr; ‡ $P < 0.05$ vs 1000 nmol/L.
N, number of replicates from four (control) and two (glipizide-treated) placentae.

12 hr (2.3 ± 0.4%). It increased to initial values (4.3 ± 1.1%) after 48 hr ($P < 0.05$ vs 12 and 24 hr). If the culture medium was supplemented with 55.7, 111, 200, 557 or 1000 nmol/L glipizide, the temporal changes in maximum [^{125}I]-insulin binding resembled those in the untreated (control) tissue. The binding values, however, generally tended to be higher at all time points measured. After 48 hr treatment in culture the maximum specific binding was higher, in a dose-dependent manner, as compared to both the control values and the values at all other earlier time points (Fig. 1). The increase between 24 and 48 hr was most pronounced with 557 and 1000 nmol/L glipizide, both leading to maximum specific binding values, which were higher by about 80% than those of the control tissue ($P < 0.01$) (Fig. 1).

In order to investigate whether these differences in binding were due to alterations in the affinity or concentration of the receptors, untreated control tissues as well as tissues treated with either 55.7, 111 or 557 nmol/L were analysed by radioreceptor assays (Fig. 2). The affinity values ranged from about 6 to 7×10^8 L/mol and were virtually unaffected by both the duration of culture and the treatment (Table 2). The concentration of receptors, however, tended to increase after 48 hr treatment in culture in a dose-dependent fashion attaining nearly 3-fold higher values in the presence of 557 nmol/L glipizide ($P < 0.05$) as compared to the untreated control tissue (Table 2).

The cholesterol contents of the placental explant controls remained virtually unchanged for 12 hr and dropped thereafter. The corresponding phospholipid contents and the phospholipid-to-cholesterol (PL/Chol) ratios continuously decreased during the period of culture. After 48 hr the PL/Chol ratio was as low as about 30% of the initial value ($P < 0.05$) (Table 1).

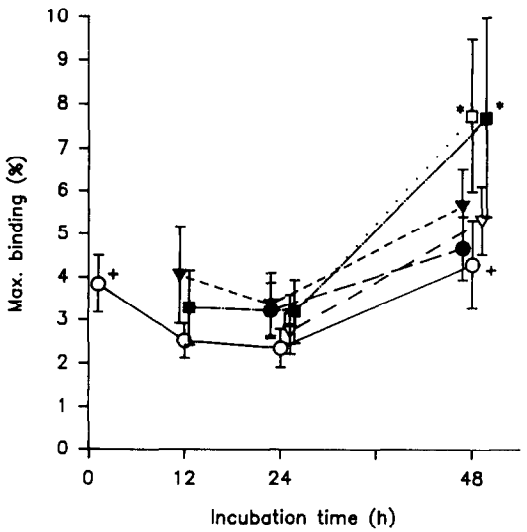

Fig. 1. Maximum specific binding (%) (mean ± SD) of [^{125}I]-insulin to homogenates of human placental tissue after various time periods in culture. Tissue was either untreated [eight replicates from four placentae; control (○—○)] or treated (four replicates from two placentae) with 55.7 (●– –●), 111 (▽----▽), 200 (▼----▼), 557 (□·····□) or 1000 (■·····■) nmol/L glipizide. *$P < 0.01$ vs control values at same time point; +$P < 0.05$ vs control at 12 and 24 hr.

In the presence of glipizide these effects were reduced or even reverted. After 48 hr the PL/Chol ratio had increased over the corresponding control value in a dose-dependent manner by 75% (55.7 nmol/L) to 210% (1000 nmol/L) (Table 1).

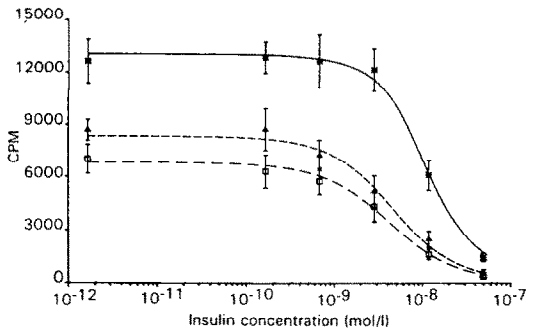

Fig. 2. Mean binding curve of competition experiments with [^{125}I]-insulin and unlabeled insulin on placental homogenates after 48 hr in culture without (□---□) and with 55.7 (△-----△) and 557 (*——*) nmol/L glipizide. The lines represent the best fit of the data by the non-linear regression procedure. Individual binding data were normalized for 165,000 cpm total radioactivity and 2 mg tissue protein per assay tube, and the mean (±SD) cpm value calculated for each concentration of unlabeled insulin.

This was due to the pronounced dose-dependent increase in the phospholipid contents under the influence of glipizide as compared to the control values at the same time points. The cholesterol concentrations were almost unaffected although they tended to be higher in the presence of glipizide than in the untreated controls. Under the influence of 557 and 1000 nmol/L glipizide, the cholesterol and phospholipid concentrations as well as the PL/Chol ratio returned to the initial ($t = 0$) control values (Table 1).

DISCUSSION

Tissue culture systems offer the possibility of studying the effects of exogenous substances supplemented to the culture medium on metabolic and biochemical processes *in vitro* with the tissue maintained in a biochemically well-defined environment. In the present study a well-established [8, 10, 11] explant culture system of human term placenta was used. The viability of the tissue was demonstrated on the basis of a variety of criteria indicating its metabolic activity [8, 10, 11].

The protein content of the tissue did not change under the experimental conditions. This indicates that the observed effects reflect true alterations of the parameters under study and are not due to mere changes in the parameters of reference (protein content). This is also supported by the changes in the PL/Chol ratio, which is independent of the protein content.

The amount of tissue available from tissue explant systems is too small to allow preparation of membranes with an acceptable purity and homogeneity. Therefore, we have measured insulin binding to placental homogenates and not to isolated plasma membranes. We recently described and validated the methodology and the determination of the non-specific binding by consecutive incubation procedures. It proved to lead to reliable results [12]. However, one has to keep in mind that homogenization of the tissue destroys its polarity. Thus, the binding of insulin to the homogenate cannot necessarily be attributed to the microvillous membrane of the syncytiotrophoblast alone.

It is interesting to note the transient decrease in insulin binding to the untreated explants during the first 24 hr in culture. This might reflect the metabolic adaptation of the tissue to the new and artificial environment provided by the culture medium. Similarly, it has been reported recently that the pattern of surface glycans, identified by their lectin binding properties, on isolated placental cytotrophoblast cells was altered during the first 20 hr in culture and was completely re-established after 48 hr [17].

Lipid analysis of the untreated placental tissues revealed a steady reduction in both cholesterol and total phospholipid content during 48 hr of cultivation. The decrease was more pronounced with phospholipids leading to a concomitant decrease in the PL/Chol ratio. Again, this could be due to an increased intracellular utilization of cholesterol and phospholipid for covering metabolic demands in the period of adaptation. A similar breakdown of phospholipids in placental tissue was observed after 48 hr in culture, when the fatty acid content in the tissue phospholipid fraction was lower by about 40% on average as compared to zero time [18].

The addition of glipizide to the culture medium generally resulted in a tendency for higher maximum binding of insulin to the placental tissue, which could

Table 2. Affinity (K; L/mol) and concentration (R; mol/g protein) of insulin receptors in homogenates of placental tissue in culture (control; eight replicates from four placentae) and after treatment (four replicates from two placentae) with various concentrations of glipizide for 24 and 48 hr

	Control		55.7 nmol/L		111 nmol/L		557 nmol/L	
	$K \times 10^8$	$R \times 10^{-13}$	$K \times 10^8$	$R \times 10^{-13}$	$K \times 10^8$	$R \times 10^{-13}$	$K \times 10^8$	$R \times 10^{-13}$
0 hr	6.24 ± 1.02	1.01 ± 0.94	SC	SC	SC	SC	SC	SC
24 hr	5.23 ± 1.13	0.59 ± 0.07	7.03 ± 1.46	0.68 ± 0.11	6.48 ± 1.29	0.58 ± 0.16	6.77 ± 1.92	0.64 ± 0.23
48 hr	6.05 ± 1.67	1.24 ± 0.32	6.31 ± 1.38	1.39 ± 0.28	5.76 ± 1.47	1.97 ± 1.04	7.12 ± 1.57	4.78 ± 1.19*

Values are means ± SD.
* $P < 0.05$ vs control at 48 hr.
SC, see control values.

be observed even after 12 hr. At high doses of glipizide, maximum binding after 48 hr in culture was significantly higher than in the untreated controls. These differences in maximum binding were due to increased concentrations of surface binding sites for insulin.

Other *in vivo* and *in vitro* studies on glipizide action on insulin receptors have led to controversial results. In rats, treatment with glipizide *in vivo* did not alter hepatic [19, 20] or adipose tissue [19] insulin receptors. In mouse liver, however, an increase in total binding of insulin due to an increased number of both high and low affinity receptors was also reported. In this study the affinities of both receptor classes were unaltered [21]. *In vivo* studies in the human found higher insulin binding due to a higher affinity after treatment of insulin-dependent diabetes mellitus patients with glipizide [22], whereas in non-insulin-dependent diabetes mellitus subjects, insulin binding to adipocytes [23] and erythrocytes [24] was unaffected. *In vitro* studies on human red blood cells [22], K562 cells [25] and MOLT4 cells [26] demonstrated increased insulin binding. It was suggested that second generation sulfonylurea drugs, such as glipizide, decrease the receptor-mediated endocytosis of insulin, thereby increasing the number of insulin binding sites [27]. This could involve inhibition of the intracellular enzyme transglutaminase (EC 2.3.2.13), which participates in receptor-mediated endocytosis [28]. Recent data, however, have cast some doubt on this hypothesis, because in erythrocytes glipizide failed to inhibit transglutaminase activity [29].

Treatment with sulfonylureas other than glipizide either increased [27, 30, 31] or did not change [32–34] insulin receptor binding to various tissues (for review see Ref. 35).

This study demonstrates, for the first time, effects of a sulfonylurea on the gross lipid composition of a tissue. Treatment with glipizide maintained the lipid contents of the explants which were higher as compared to control values. The membrane cholesterol concentrations were virtually unchanged. The data do not provide any information on the underlying mechanisms, but an inhibitory effect of glipizide on placental phospholipases may be hypothesized. The results suggest a broader spectrum of action of glipizide, or sulfonylureas, at the cellular levels, than has been assumed. This notion is supported by the recently reported stimulation by sulfonylureas of the proliferative activity of umbilical cord vessel endothelial cells [36].

It has frequently been shown that the membrane lipid composition affects affinity or concentration of insulin receptors in various tissues and cells [37–41] including human placenta [3]. The alteration of the phospholipid content and of the PL/Chol ratio under glipizide influence, parallel to the increase in insulin binding to the insulin receptor, makes it tempting to speculate about a change in receptor concentration mediated indirectly by the modified membrane lipid composition. However, in control cultures, insulin binding at 48 hr was restored to zero time levels in association with a dramatic reduction in PL/Chol ratio. Therefore, a direct relationship between insulin binding and the lipid composition of cultured placental tissue cannot be inferred from these data.

Our results represent the first direct evidence that placental insulin receptors can be dynamically regulated *in vitro* by exogenous substances. The increases in the placental phospholipid content is a consequence of glipizide treatment by which means this drug might also affect the properties and function of other membrane receptors and enzymes *in vitro*. It cannot be predicted, however, whether the binding of insulin to placental insulin receptors after glipizide treatment also increases *in vivo* as has been found under *in vitro* conditions. As long as this is unknown, and as long as effects of insulin on placental metabolism are not fully established, possible impacts on the placenta of glipizide treatment of diabetic women during pregnancy remain enigmatic.

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