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CHARACTERIZATION OF THE SULFONYLUREA-INDUCED POTENTIATION OF THE INSULIN RESPONSE IN CULTURED 3T3 ADIPOCYTES

JOS P. M VAN PUTTEN* and H. MICHIEL J KRANS
Laboratory for Diabetes Research of the Department of Endocrinology and Metabolic Diseases, University Hospital, 2333 AA Leiden, The Netherlands

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Abstract—Studies were carried out to determine the role of sulfonylureas in the regulation of insulin-sensitive hexose uptake in cultured 3T3 adipocytes Exposure (0–72 hr) of cells to the sulfonylurea-derivative tolbutamide (0.05–0.3 mg/ml) induced a time- and concentration-dependent potentiation of the stimulatory effect of insulin on hexose uptake (500 vs 340%) The effect was maximal within 24 hr and completely reversible It was strictly limited to the presence of insulin Basal hexose uptake and insulin binding were not affected by the drug High concentrations of the agent (>0 3 mg/ml) induced a decrease in insulin response, suggesting a concentration optimum Lineweaver–Burk analysis of uptake data revealed that the potentiating effect of tolbutamide was due to enhancement of the insulin-induced increase in apparent V_{max}, i.e. in the number or activity of hexose transporters This enhancement was inhibited by cycloheximide (1 μg/ml), indicating involvement of protein synthesis in the induction of the effect It is concluded that sulfonylureas act by influencing synthesis of protein(s) which potentiate the effect of insulin on hexose uptake

Sulfonylureas are generally used in control of plasma glucose in patients with non-insulin-dependent diabetes mellitus. There has been much controversy about the mechanism of action of these agents. At first, the blood glucose lowering effect of these drugs was attributed to a stimulation of insulin secretion [1–3]. Later on, extrapancreatic actions became apparent [4–7]. Sulfonylureas have been reported to increase insulin binding in blood cells and fibroblasts both *in vivo* and *in vitro* by altering the number and/or affinity of insulin receptors [8–10]. In insulin-sensitive cells, however, this effect has only rarely been found [11–16, cf. ref. 17]. In these cells post-receptor alterations appear to be predominant. Both a potentiation of the insulin response on hexose uptake and lipolysis have been described [11–13, 18, 19]. The apparent difference in the effect of sulfonylureas on insulin binding in various cell types and, moreover, the potential relevance of the ability of these drugs to modulate the insulin response for the understanding of the mechanism of action of these agents as well as that of insulin, prompted us to characterize the effects of tolbutamide on the insulin binding and hexose uptake in cultured 3T3 (mouse) adipocytes. Cultured 3T3 adipocytes [20] have been shown to be suitable for studies on the long-term regulation of insulin effectiveness [21–23].

METHODS

Cell culture. 3T3-L_1 (pre-)adipocytes were grown and differentiated as previously described [21]. The effect of sulfonylureas on insulin effectiveness was determined by culturing fully differentiated 3T3 fat cells in the absence and presence of tolbutamide (Hoechst, Amsterdam, The Netherlands)

Insulin binding [^{125}I]insulin binding was determined as described previously [22, 23]. Briefly, 60 mm culture wells (2×10^6 cells) were incubated (20°) in 1.5 ml 50 mM Tris–HCl buffer (pH 7 4) containing 20 pM mono-A^{14}-[^{125}I]insulin (human) (spec. act. 360 Ci/mole) (Eli-Lilly Nederland, Utrecht, The Netherlands) and various concentrations of unlabelled insulin. Bacitracin (1.5 mM) was added to inhibit insulin degradation. In the presence of bacitracin, insulin degradation was less than 3% in both control and tolbutamide-treated cells during the incubation period, as measured by TCA precipitation [24]. After 3 hr (binding equilibrium) the cells were rapidly rinsed four times with 1 ml phosphate-buffered saline (0°). The amount of [^{125}I] bound to the cells was determined in a gammacounter (Packard Instruments), and aliquots were taken for protein determination [25]. Non-specific binding was defined as the amount of [^{125}I] bound in the presence of an excess of unlabelled insulin (10^{-6} M) and was subtracted in calculation of data

Hexose uptake. Hexose uptake was estimated using the glucose analogue 2-deoxyglucose, which is transported and phosphorylated but not further metabolized. Determination of intracellular concentrations of 2-deoxyglucose and 2-deoxyglucose-6-phosphate and of hexokinase activity has revealed that, the uptake of 2-deoxyglucose reflects transport of the hexose across the membrane under the conditions employed [26] Uptake experiments were performed as described elsewhere [22] In brief, 30 mm culture wells (5×10^5 cells) were incubated (37°) in 0 75 ml 50 mM Tris–HCl buffer (pH 7 4) in the absence and presence of insulin for 30 min Then, 0 05–5 0 mM 2-deoxy-D-[1-^{14}C]glucose (710 cpm/

* To whom correspondence should be addressed

nmole) was added and uptake was monitored for up to 10 min Uptake was stopped by rapidly washing the cells four times with 1 ml phosphate-buffered saline (0°) [^{14}C] was counted in a liquid scintillation counter (Packard Instruments) and aliquots were taken for protein determination Non-carrier mediated 2-deoxyglucose uptake was defined as the amount of label taken up in the presence of 0 1 mM cytochalasin B, and was subtracted in calculation of data

Data analysis All data were corrected for protein content of the wells 10^6 cells contained about 1 mg of protein Tolbutamide treatment did not affect the protein-cell ratio Data were expressed per 10^6 cells. Where appropriate results from different experiments were statistically analyzed by Student's *t*-test for paired comparison

MATERIALS

Culture media and sera were obtained from GIBCO (Grand Island, New York, U.S A.), except for the culture wells (Greiner, Nurtingen, F.R G.) 2-deoxy-D-[1-^{14}C]-glucose was purchased from New England Nuclear (Boston, Massachusetts, U.S A) Bacitracin was from Sigma, (St Louis, Missouri, U S A) and cycloheximide from Serva (Heidelberg, F R G) Demineralized bovine serum albumin was obtained from Organon (Oss, The Netherlands) All other chemicals were of p a grade

RESULTS

The effect of sulfonylureas on insulin binding was examined by culturing fully differentiated 3T3 adipocytes in the absence and presence of tolbutamide. Exposure of the cells to the drug (0.2 mg/ml) for various periods (0–72 hr) did not influence [^{125}I]insulin receptor binding, either at tracer (20 pM) (Fig 1) or at higher insulin concentrations (128 pM, 1 6 nM and 6.6 nM) (N = 4, NS). Similarly, pre-incubation of the cells with various concentrations of tolbutamide (0 05–5.0 mg/ml) for 30 min and 24 hr did not alter [^{125}I]insulin binding (not

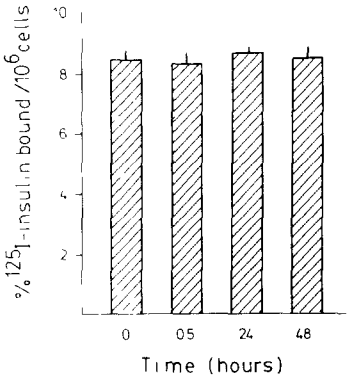

Fig 1 Effect of tolbutamide on the insulin binding Fully differentiated 3T3 adipocytes (2×10^6 cells) were cultured in the absence and presence of tolbutamide (0 2 mg/ml) At the times indicated [^{125}I]insulin binding (20 pM) was determined (3 hr, 20°) Data are mean ± S E M of four experiments

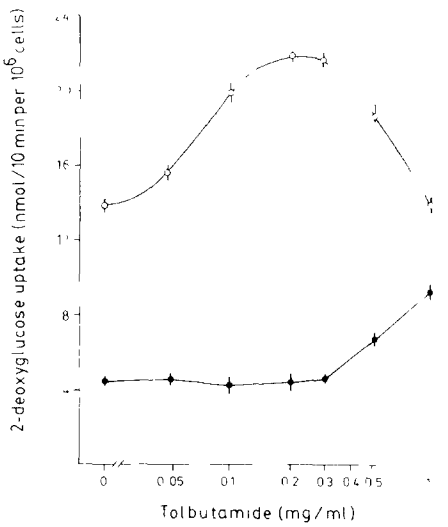

Fig 2 Concentration curve of effect of tolbutamide on the insulin response Fat cells (5×10^5 cells) were cultured in the presence of tolbutamide (0–1 0 mg/ml) for 48 hr Then, basal (●) and maximal-insulin stimulated (10^{-6} M) (○) 2-deoxyglucose uptake were determined Data represent the mean ± S E M of four experiments

shown) Prolonged exposure (24 hr) to concentrations of the drug of more than 0 3 mg/ml, however, did induce morphological alterations of cells they rounded up and were less well-fixed to the culture dishes Cell death however, was not observed. The absence of acute and long-term effects on the insulin binding in 3T3 adipocytes strongly suggest that, at least in this cell system, sulfonylurea do not influence the number or affinity of insulin receptors.

The insulin-sensitive hexose uptake on the other hand, was markedly affected by tolbutamide treatment Exposure of the cells to various concentrations (0 05–1 0 mg/ml) of the drug for 48 hr induced a biphasic response At concentrations between 0.05 and 0 3 mg/ml basal hexose uptake was unchanged, while maximal insulin-stimulated (10^{-6} M) uptake was increased with approximately 60% (Fig 2) Expressed as percentage stimulation of basal hexose uptake, the insulin response increased from 340% up to 500% ($N = 4$, $P < 0.001$) After exposure of the cells to tolbutamide concentrations of more than 0 3 mg/ml a quite different pattern was observed Basal hexose uptake increased, while maximal insulin-stimulated (10^{-6} M) hexose uptake decreased (Fig 2) The insulin response decreased from 340% up to 150% ($N = 4$, $P < 0.005$) The latter effect was accompanied by alterations in cell morphology similar to those observed in the binding experiments

Time course experiments revealed that the stimulatory effect of tolbutamide on the insulin response required several hours of incubation The effect was maximal within 24 hr (Fig 3) Incubation for longer periods (up to 72 hr) did not substantially alter insulin-sensitive hexose uptake further When cells were cultured first in the presence of the drug (0 2 mg/ml) for 24 hr and, subsequently, in the absence of the drug the effect appeared to be completely reversible Within 24 hr after the omission of tolbutamide from

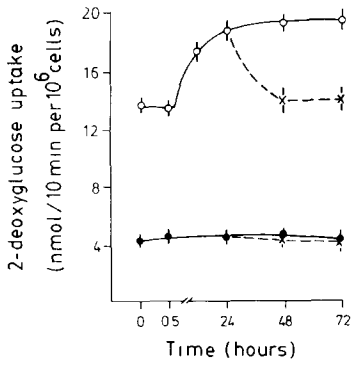

Fig 3 Time course and reversibility of the effect of tolbutamide Fat cells (5×10^5 cells) were incubated with tolbutamide (0 2 mg/ml) for up to 72 hr At the times indicated basal (●) and insulin-stimulated (10^{-6} M) (○) 2-deoxyglucose uptake were determined The reversibility of the effect was tested by omitting the drug from the culture medium after 24 hr of incubation (×—×) Data represent the mean ± S.E M of three experiments

the culture medium original hexose uptake values were obtained (Fig. 3).

In an attempt to acquire more insight into the mechanism by which tolbutamide appears to regulate the stimulatory effect of insulin, we compared the effects of both compounds on hexose uptake. First, concentration curves (0.05–5.0 mM) of hexose uptake in control and tolbutamide-treated (0.2 mg/ml for 24 hr) cells were performed in the absence and presence of insulin (10^{-6} M). Analysis of uptake data according to Lineweaver–Burk revealed that insulin increased the apparent V_{max} of hexose uptake ($N = 3$, $P < 0.005$, Table 1). Tolbutamide treatment resulted in further enhancement of the insulin-induced increase in the apparent V_{max} ($N = 3$, insulin-stimulated hexose transport tolbutamide-treated cells vs control cells· $P < 0\ 05$; Table 1). The apparent K_m of hexose uptake was altered neither by insulin nor by tolbutamide ($N = 3$, NS; Table 1). Considering the difference in time course for the induction of the effects of insulin (min) and tolbutamide (hr) we then tested the protein synthesis dependence of these effects. Exposure of the cells to cycloheximide (1 μg/ml, an inhibitor of protein synthesis) for 16 hr did not affect the stimulatory effect of insulin on hexose uptake (0.30 ± 0.03 nmole hexose/min/10^6 cells vs 0 91 ± 0.08 nmole hexose/min/10^6 cells in the absence and presence of insulin (10^{-6} M), respectively; ($N = 3$, $P < 0.005$). The tolbutamide-induced potentiation of the insulin response, however, was completely abolished when cycloheximide (1 μg/ml) was present in the culture medium (0.87 ± 0.04 nmol/min per 10^6 cells vs 0 91 ± 0.08 nmole/min/10^6 cells in maximal insulin-stimulated (10^{-6} M) tolbutamide-treated (0 2 mg/ml for 16 hr) and control cells respectively; ($N = 3$, NS) These data suggest that protein synthesis is required for the tolbutamide-induced enhancement of the stimulatory effect of insulin on hexose uptake

DISCUSSION

The data presented indicate that in cultured 3T3 adipocytes tolbutamide modulates insulin-sensitive hexose uptake, but not insulin receptor binding, by a time- and concentration-dependent, and reversible process, which requires protein synthesis. The induction of post-receptor alterations only is in concert with observations in isolated fat cells [11] and cultured hepatocytes [12, 16], but contrasts studies *in vivo* and *in vitro* on non-insulin-sensitive cells [8–10]. A number of reports have described a sulfonylurea-induced increase in insulin binding [8–10, 17] Nearly all of these findings, however, were obtained with non-insulin-sensitive cells In target tissues of insulin an increase in insulin binding upon sulfonylurea treatment has only rarely been found [cf. ref. 11–16 with 17]. Therefore our data support the idea that the response to sulfonylureas varies with insulin-sensitivity of cells, which urges caution to the use of non-insulin-sensitive cells in studies on the blood glucose lowering effect of these drugs

The observation in the cultured fat cells of an optimum concentration for the stimulatory effect of tolbutamide on the insulin response has not been reported before, but might explain *in-vivo* observations of an optimum therapeutic range for the blood glucose lowering effect of this sulfonylurea-derivative. In fact, the optimum concentrations *in vivo* (0 08–0.18 mg/ml) [27] closely resemble those *in vitro* (0 1–0.2 mg/ml) The rapid decrease in effect

Table 1 Effect of tolbutamide on hexose transport kinetics*

	K_m (mM)	V_{max} (nmole/min/10^6 cells)
Basal hexose uptake		
control cells	2 0 ± 0 1	6 4 ± 0 3
tolbutamide-treated cells	2 2 ± 0 2	7 0 ± 0 2
Insulin-stimulated hexose uptake		
control cells	2 3 ± 0 2	18 6 ± 0 9
tolbutamide-treated cells	2 0 ± 0 2	26 8 ± 0 7

* Cells were cultured in the absence (control cells) and presence of tolbutamide (0 2 mg/ml) for 24 hr Then, the uptake of 0 05–5 mM hexose in the absence and presence of insulin (10^{-6} M) was monitored for up to 5 min Data were analyzed according to Lineweaver–Burk and represent mean ± S E M of three experiments

of insulin upon exposure of the cultured cells to concentrations of the agent of more than 0.3 mg/ml, which was accompanied by morphological alterations, might indicate toxicity of the drug, although no effect on insulin binding and cell viability could be demonstrated. The decrease in insulin response was probably not due to the rounding up of the 3T3 adipocytes, since EDTA (2 mM) treatment (15 min, 37°), which causes a rounding up of cells, did not affect basal or insulin-stimulated hexose uptake (unpublished observations). *In vivo*, a tolbutamide-induced decrease in insulin response is probably not very common, since therapeutic plasma levels rarely exceed 0.3 mg/ml [27].

The effect of tolbutamide on the hexose uptake was strictly limited to the presence of insulin This might indicate that tolbutamide acts by modulating the stimulatory effect of insulin and not by altering hexose uptake *per se* This concept is strengthened by Lineweaver–Burk analysis of the uptake data, which revealed that tolbutamide enhanced the insulin-induced increase in apparent V_{max}, i.e the number or activity of hexose transporters These data are compatible with those of Salhanick *et al* [12] showing that sulfonylureas enhance the effect of insulin but not that of the insulin mimicker H_2O_2 and, furthermore, with those of Jacobs and Jung [28] demonstrating that sulfonylureas enhance the insulin-induced recruitment of hexose transporters between an intracellular compartment and the plasma membrane The mechanism by which sulfonylureas appear to amplify the signalling of the hormone is still unknown. Our time course experiments indicate that several hours of incubation with tolbutamide are required for induction of the increase in insulin responsiveness. Apparently, tolbutamide does not simply potentiate the rapid effect of insulin. On the contrary, the observation that the effect of the drug was inhibited in the presence of cycloheximide indicates that its potentiation of the effect of insulin requires protein synthesis This finding might be of great interest, since it is the first indication of involvement of protein(s) in the modulation of the sequence of events mediating the insulin response Identification of the locus at which sulfonylureas regulate the coupling process between insulin receptor binding and hexose uptake might therefore not only yield important information about the mechanism of action of the drug but also about that of insulin. In this respect, it might be noteworthy that sulfonylureas have been reported to potentiate the effect of insulin on hexose uptake [11, 12], but to have no effect on the insulin-induced stimulation of amino-acid transport [14], suggesting that the coupling process is not uniformly regulated for all effector systems

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