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Effects of Gliclazide—a New Antidiabetic Agent—on Blood Platelet Function *In Vitro* and *In Vivo*

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ABSTRACT

The effect of gliclazide on platelet activity was investigated and compared to that of tolbutamide and glipizide.

In vitro studies: Adenosine diphosphate induced human platelet aggregation was inhibited by gliclazide in high concentrations (0.5–2.0 mg/ml). In the same concentration range tolbutamide exhibited a slightly less pronounced inhibitory effect. Collageninduced human platelet aggregation was inhibited by gliclazide, glipizide and tolbutamide in a concentration range of 0.5–2.0 mg/ml. Gliclazide had a similar effect on rabbit platelets.

In vivo studies: Both acute and seven days administration of gliclazide to rabbits resulted in prolonged primary and total hemostatic plug formation time both in arterioles and venules. Similar results were obtained with tolbutamide and glipizide.

Experiments with laser-induced platelet plug formation in the rabbit ear chamber demonstrated that 100 mg gliclazide/kg body weight and 25 mg gliclazide/kg body weight during 7 days significantly reduced the number of platelet emboli formed during 10 minutes after laser injury.

INTRODUCTION

Vascular and thromboembolic problems in diabetes mellitus are well recognized. These can in part be explained by an increased platelet adhesiveness and aggregability in diabetic patients. An antidiabetic agent which also effects platelet function could potentially be of value in the treatment of diabetes and in the prophylaxis of vascular complications. There is one report (14) which suggests that 1-(4-methylbenzene-sulfonyl)-3,3- azabicyclo--(3.30) acetyl - urea (gliclazide, Diamicron) has such properties.

This study was undertaken to further evaluate the effect of gliclazide on platelet activity in vitro and in vivo. A comparison was also made with other antidiabetic agents (glipizide and tolbutamide).

MATERIAL AND METHODS

In vitro experiments

Platelet aggregation

Human blood was collected into Acedex^{R}, (Pharmacia AB, Uppsala, Sweden) in the proportion (9:1.5) and centrifuged at 144 x g for 15 minutes to obtain platelet rich plasma (PRP). For the aggregation experiments, PRP was diluted with platelet poor plasma (PPP) to obtain a platelet count of ~ 375.000 platelets per μl.

Rabbits were anaesthetized with 30 mg/kg body weight of Sodium pentobarbital (Abbot Lab., North Chicago, Ill., USA). The blood was collected from the central ear artery and otherwise anticoagulated and processed as described for human blood.

Platelet aggregation was carried out according to Born (1962). The aggregation mixture was 2.4 ml PRP, 0.2 ml THAM-buffer (pH 7.4, 0154 M) and either 0.2 ml of a saline solution of the antidiabetic agent to be tested of 0.9% saline. Aggregation was induced either by 0.2 ml of 10 μg/ml adenosine diphosphate (ADP) (Sigma Chem Co., St. Louis, USA) or 0.2 ml of a 0.2 mg/ml collagen solution (Hormon-Chemie, Munich, West Germany).

The aggregation experiments were carried out in a Vitatron photometer UC 200 at 37°C and with magnetic stirring. The photometer was connected to a Vitatron recorder model UR 400 and the aggregation curve recorded in log. mode. The initial ratio of aggregation (Vi) was measured as the steepest slope on the aggregation curve, i.e. the largest decrease in optical density per sec. The extent of aggregation (ΔOD) was also determined.

In vivo experiments

Hemostatic plug formation

The method has been described in detail by Bergqvist (7) and Bergqvist and Arfors (8). New Zealand white rabbits of both sexes, weight 2.4 $\pm$ 0.4 kg, fed on a standard diet (Teknosan pellets, Ferrosan AB, Malmö, Sweden) were used. Under urethane anaesthesia (12) a short segment of distal ileum was exteriorised through a midline incision and the mesentery laid over a siliconised glass plate mounted in an electrically heated microscope table. The mesenteric microcirculation was observed through a Leitz Biomed intravital microscope using a Leitz Ultrapak 6.5 x objective at a total magnification of 81 x. The preparation was continously superfused with Tyrodes solution at 37.5 - 39°C and pH 7.4.

Arterioles and venules were each devided according to size into two groups, 20 - 40 μm and 40 - 60 μm, and four arterioles and five venules in every rabbit were cleanly transectedwith a disposible Gillette scalpel blade (shape E 11).

The time from transection until the bleeding first stopped was recorded and called primary haemostatic plus formation time (PHT). The frequency and duration of rebleeding were recorded and the sum of PHT and all the rebleedings times was called total haemostatic plug formation time (THT).

TABLE 1. The following groups were studied.

Group I	
Control saline 10 ml i.v.	n = 5
Gliclazide 100 mg/kg	n = 5
Gliclazide 25 mg/kg x VII	n = 5
Group II	
Glipizide 6.25 mg/kg	n = 5
Glipizide 1.56 mg/kg x VII	n = 5
Group III	
Tolbutamide 100 mg/kg	n = 5
Tolbutamide 25 mg/kg x VII	n = 5
Group IV	
Control saline 10 ml	n = 5
Gliclazide 100 mg/kg	n = 5
Gliclazide 25 mg/kg x VII	n = 5

Group IV was also used for calculation of the frequency of rebleeding from haemostatic plugs. The study was made blind.

Laser-induced microvascular injury

The methodology has been described previously (2,3). Regenerative titanium ear chambers were mounted in Sandy-Lop rabbits. When fully developed, the regenerative tissue was 800 - 100 μm thick and an extensive network of arterial, venous and lymphatic vessels formed. The microvasculature was observed through a Leitz Ortholux microscope using a x 23 Ultrapak intravital water immersion objective. Arterioles with a luminal diameter of 15 - 25 μm were injured in conscious animals with a single pulse ruby biolaser (TRG 153, New York, USA). The input energy to the laser can be adjusted by means of a power supply unit and in these experiments an input energy of 200 J was used. This corresponds to an output energy of 7.3 mJ measured with a calibrated substage monitor.

After laser induced endothelial trauma, the number of emboli from the site of injury was counted and the cumulative number in 10 minutes has been used as a measure of platelet activity. In all experiments the mean number of emboli after 4 laser injuries was determined before the intravenous administration of different doses of gliclazide. The effect of these materials on platelet activity in vivo was then examined.

The following groups were studies, each with four rabbits.

1. Control saline 10 ml i.v.
2. Gliclazide 25 mg/kg
3. Gliclazide 25 mg/kg x VII days

Statistical methods

For haemostatic experiments the choice of statistical methods has been based on the fact that arterioles and venules differ in their haemostatic pattern and that the haemostatic plug formation time has a skew distribution (7). For details, see Arfors et al. (4). When testing the frequency of rebleeding the rank-sum test was used (15). Otherwise Student's t-test has been used.

RESULTS

Platelet aggregation in vitro

In table 2a and 2b the results from the experiments with gliclazide, tolbutamide and glipizide on human platelets are summarized. These results demonstrate that both gliclazide and tolbutamide markedly inhibited ADP-induced human platelet aggregation. This applied both to the rate and extent of aggregation. The inhibitory effects of tolbutamide were somewhat less marked than those of gliclazide.

Experiments with collagen-induced aggregation gave analogous results with gliclazide and tolbutamide. Glipizide was also tested and gave similar results to gliclazide and tolbutamide. Glipizide tended to produce a slightly stronger inhibitory effect than gliclazide on a concentration basis.

The effect of gliclazide on rabbit platelet aggregation is shown in table 3. A marked inhibition of both ADP- and collagen-induced aggregation was observed in concentrations similar to those found to be effective against human platelet aggregation.

It should be noted that collagen-induced rabbit platelet aggregation was inhibited by lower concentrations of gliclazide than what was necessary to inhibit the collagen-induced aggregation of human platelets.

The effects of the antidiabetic agents on platelet aggregation were not related to changes in pH caused by the drugs since pH was similar in the control and experimental aggregation mixtures.

TABLE 2a. Effect of antidiabetic agents on aggregation of human platelets.

a) Aggregation by ADP

Test substance	Conc. mg/ml	No. of exp	Vi ± SD[1] as % of control	OD ± SD[1] as % of control
Gliclazide	2.0	5	32 ± 4	24 ± 6
	1.0	4	69 ± 5	60 ± 7
	0.5	4	87 ± 2	85 ± 5

	Conc. mg/ml	No.of exp	Vi ± SD [1] as % of control	OD ± SD [1] as % of control
Tolbutamide	2.0	6	52 ± 6	45 ± 6
	1.0	5	84 ± 5	75 ± 5
	0.5	5	98 ± 4	99 ± 5

1) Expressed as the arithmetic mean of the designated number of experiments.

TABLE 2 b. Aggregation by collagen.

Test substance	Conc. mg/ml	No. of exp	Vi ± SD [1] as % of control
Gliclazide	2.0	5	13 ± 5
	1.0	5	33 ± 7
	0.5	5	70 ± 3
	0.25	4	90 ± 3
Tolbutamide	2.0	3	17 ± 2
	1.0	5	43 ± 8
	0.5	5	65 ± 7
	0.25	4	87 ± 4
Glipizide	1.33	3	26 ± 1
	0.67	4	63 ± 1
	0.33	4	90 ± 5

1) Expressed as the arithmetic mean of the designated number of experiments.

TABLE 3. Effects of gliclazide on ADP- and collagen-induced aggregation of rabbit PRP [1]

Gliclazide in reaction mixt.	ADP-induced aggregation Vi as % of	OD as % of	Collagen-induced aggregation Vi as % of	Comments
0.25 mg/ml	-	-	39 (34-43)	
0.50 mg/ml	-	-	10 (0-21)	
1 mg/ml	73 (71-74)	57 (55-59)	0	Slow chape change
2 mg/ml	51 (43-59)	24 (20-27)	0	No chape change

1) Mean of 2 experiments. Figures within brackets represents range.

In vivo experiments

Haemostatic plug formation

In Table 4 are shown primary (PHT) and total (THT) haemostatic plug formation times from haemostatic experiments with the different antidiabetic agents used. In some of the groups some vessel types and vessel sizes show borderline significant differences in plug formation times in treated and non-treated groups. Group I and Group IV received the same treatment with acute and chronic doses for 7 days respectively. In Group IV we have significant ($p < 0.05$) differences in plug formation time in both arterioles and venules although Group I shows no difference in any of the vessel types used. This emphasize the small differences between control and treated groups. Gliclazide given over

7 days shows a prolonged haemostatic plug formation time although not significant. This is due to the varying individual response in the rabbits which is documented by a very large standard deviation.

Table 5 gives the frequency of rebleeding for gliclazide treated animals (Groups IV). No difference from control is found in the rebleeding pattern.

Laser induced microvascular injury

Table 6 shows the results from the laser experiments in four rabbits. Gliclazide markedly inhibits platelet embolus formation both after one single administration and after 7 days of repeated i.v. administration.

DISCUSSION

Platelet aggregates in diabetic patients with retinopathy or neuropathy are known to be more stable than in normal people (16, 24). It is also known that intravascular ADP-induced platelet aggregation can induce vascular damage (18).

The inhibitory effects of several antidiabetic drugs including gliclazide on *in vitro* platelet reactivity have been reported in the literature (14, 20, 21, 22, 26). It has also been shown previously that the inhibitory effects of such drugs on platelet aggregation and adhesion are not proportional to their hypoglycemic effect. The present study confirms these results and also the result of Desnoyers et al(14). However, results obtained in *in vitro* systems do not necessarily provide accurate information concerning the effect in the more complex *in vivo* situation.

It is therefore important to study any *in vitro* effects on platelet function in relevant *in vivo* models. The demonstrated *in vitro* antiplatelet activity of gliclazide was further tested in two *in vivo* situations where a normal platelet function is necessary and where the development of platelet aggregates is well defined.

Transection of microvessels in the transparent tissues of anaesthetized animals forms a valuable experimental model for intravital microscopic studies of the formation of haemostatic plugs. This method has been used by Apitz (1), Zucker (27) and Hugues (17), and the rabbit mesentery preparation has been further developed to investigate parameters of importance in the formation of an efficient haemostatic plug (5, 6, 8, 9, 10).

A ruby laser has been used to inflict precise endothelial trauma in mesenteric vessels (19). A combination of biolaser-induced endothelial injury with rabbit ear chamber technique has been found to give a simple and precise method of quantitating platelet activity *in vivo*. Preliminary observations of the reproducibility and application of this method have been presented (2). Both these *in vivo* techniques have previously been shown to be primarily an expression of ADP-induced platelet aggregation (11, 23).

TABLE 4. Effects of antidiabetic agents, gliclazide, glipizide, tolbutamide on haemostatic plug formation 1) $p<0.05$

	Arteriole 20-<40 µm		Arteriole 40-<60 µm		Venule 20-<40 µm		Venule 40-<60 µm	
Group I	THT	PHT	THT	PHT	THT	PHT	THT	PHT
Control n = 5	50 ± 6	49 ± 8	101 ± 31	96 ± 23	251 ± 56	186 ± 66	203 ± 115	179 ± 94
Gliclazide 100 mg/kg n = 5	80 ± 40	77 ± 40	127 ± 67	105 ± 49	281 ± 67	173 ± 61	243 ± 80	216 ± 82
Gliclazide 25 mg/kg x VII n = 5	153 ± 165	142 ± 163	216 ± 215	211 ± 218	283 ± 65	254 ± 43	258 ± 89	255 ± 93
Group II								
Glipizide 6.25 mg/kg n = 5	79 ± 22[1]	70 ± 27	110 ± 45	110 ± 45	224 ± 53	159 ± 76	150 ± 27	113 ± 55
Glipizide 1.56 mg/kg x VII n = 5	89 ± 45	89 ± 45	134 ± 37	131 ± 36	214 ± 91	197 ± 75	212 ± 91	197 ± 93
Group III								
Tolbutamide 100 mg/kg n = 5	116 ± 49[1]	109 ± 49[1]	166 ± 105	139 ± 73	317 ± 69	293 ± 91	326 ± 78	276 ± 99
Tolbutamide 25 mg/kg x VII n = 5	84 ± 26[1]	75 ± 33	155 ± 77	155 ± 77	219 ± 44	164 ± 48	241 ± 18	216 ± 9
Group IV								
Control n = 5	52 ± 44[1]	34 ± 26[1]	110 ± 45[1]	104 ± 39	197 ± 54	136 ± 66[1]	249 ± 118	153 ± 49[1]
Gliclazide 100 mg/kg n = 5	111 ± 39[1]	104 ± 37[1]	196 44[1]	165 ± 55	401 ± 110	334 ± 165[1]	344 ± 104	283 ± 78[1]
Gliclazide 25 mg/kg x VII n = 5	135 ± 143	131 ± 146	220 ± 112	196 ± 117	283 ± 135	234 ± 155	393 ± 296	306 ± 302

TABLE 5. Frequency of rebleeding from haemostatic plugs.

Group IV	Arteriole 20 - <40 μm	Arteriole 40 - < 60 μm	Venule 20 - < 40 μm	Venule 40 - <60 μm
Control n = 5	0.25	0.25	0.68	0.80
Gliclazide 100 mg/kg n = 5	0.35	0.70	0.28	0.64
Gliclazide 25 mg/kg x VIII days n = 5	0.10	0.30	0.52	0.84

The frequency of rebleeding is given by dividing the total number of rebleeding episodes by the number of transected vessels.

TABLE 6. Laser induced microvascular injury.

	Number of emboli Mean ± SD	
Control n = 4	8.4 ± 3.7	1)
Gliclazide 100 mg/kg n = 4	2.8 ± 2.2	
Control n = 4	9.5 ± 3.2	1)
Gliclazide 25 mg/kg x VII n = 4	3.7 ± 2.8	

1) $p<0.05$

Using the haemostatic plug formation model gliclazide gives results which do not differ from the other antidiabetic agents. The prolongation of the bleeding time is not marked and in many instances insignificant. After treatment with gliclazide for one week the time until haemostasis occurs is longer although not significant because of the large standard deviation.

The laser model using awake animals with titanium ear chambers showed a significant depression of intravascular platelet aggregation after an acute dose of gliclazide.

The in vitro and in vivo experiments in this study all demonstrate that platelet activity is decreased by the hypoglycemic drugs. This warrant in depth studies on the effect of such agents on platelet activity in vivo in patients.

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