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Vascular action of the hypoglycaemic agent gliclazide in diabetic rabbits

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Summary ATP-dependent potassium channel blockers used as hypoglycaemic agents may have effects on vascular disease in diabetes mellitus beyond their effect on blood glucose control. This study was designed to determine the effects of treatment with gliclazide on the isolated abdominal aorta of diabetic rabbits in which endothelium-dependent relaxation is impaired by a mechanism involving oxygen-derived free radicals. After induction of diabetes with alloxan, there was no effect of gliclazide (10 mg · kg^{-1} · day^{-1} orally) on blood glucose or insulin levels over a 6 week period. Hence, this permitted an examination of the vascular effects of gliclazide in diabetic rabbits exclusive of metabolic effects. Acetylcholine- and nitric oxide-induced relaxation in aortae from rabbits treated with or without gliclazide were measured in the absence or presence of the nitric oxide synthase inhibitor, N^G-nitro-L-arginine (L-NAME). Diabetes was associated with significant impairment of acetylcholine-induced endothelium-dependent relaxation of the abdominal aorta which was not significant in diabetic rabbits treated with gliclazide in vivo. Aortae from diabetic rabbits studied in the presence of L-NAME showed an exaggerated contraction to acetylcholine which was prevented in rabbits treated with gliclazide. Gliclazide treatment did not affect the response to acetylcholine of normal rabbit aorta, and gliclazide when added in vitro had no effect on the response of diabetic rabbit aorta, suggesting that the effect of gliclazide was specific to the abnormality arising with diabetes and was not due to an acute effect of the drug. These data indicate that gliclazide, aside from either a direct antioxidant action or an effect on insulin or glucose levels, may ameliorate diabetic endothelial cell dysfunction. [Diabetolgia (1998) 41: 9--15]

Keywords Diabetes mellitus, aorta, endothelium, hypoglycaemic agents.

ATP-dependent potassium channel blockers of the sulfonylurea class are widely used to treat non-insulin-dependent diabetes mellitus. These drugs produce their hypoglycaemic action by blocking ATP-dependent potassium channels of the pancreatic beta cell resulting in increased insulin release. In contrast to their well-documented beneficial action to improve glycaemic control, the long-term effects of these agents on cardiovascular disease are controversial with a history of potential adverse effects [1]. Because ATP-dependent potassium channels mediate a variety of functions in heart and blood vessels [2] it is possible that sulfonylurea hypoglycaemic agents would have a variety of effects on the cardiovascular system aside from their effects on glycaemic control.

Gliclazide is a widely used, second generation sulfonylurea [3]. It has been reported to potentially benefit the vasculature through improvements in plasma lipids and in platelet function [3]. In diabetic animal models, these effects have been confirmed and include the ability to inhibit atherosclerosis in

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Abbreviations: L-NAME, L-nitroarginine methyl ester; ANOVA, analysis of variance.

rabbits [4]. The mechanism may involve the ability of the drug to increase tissue plasminogen activator or its properties as a free radical scavenger which have been demonstrated both in vivo and in vitro [5, 6]. Some of these effects have been reported in patients who were switched from another sulfonylurea drug or who were insulin-dependent [3]. Thus, it is possible that gliclazide has vascular effects which are unrelated to its hypoglycaemic action and which may be potentially beneficial for vascular disease.

Diabetes is associated with dysfunction of endothelial cells including decreased nitric oxide-mediated endothelium-dependent relaxation. This has been demonstrated in man and animal models of the disease including the diabetic rabbit [7]. Disruption of nitric oxide function is associated with vascular disease processes including increased platelet aggregation and accelerated atherosclerosis [8, 9]. The main purpose of this study was to determine the ex vivo vascular effects of in vivo treatment with gliclazide of alloxan-induced diabetic rabbits. Because gliclazide would not be expected to decrease glucose levels in this model of type 1 diabetes [10], this study design allowed an investigation of vascular effects of gliclazide exclusive of its hypoglycaemic actions.

Materials and methods

Materials were reagent grade or better. Plasma insulin was measured with a radioimmunoassay (Kabi Pharmacia Diagnostics AB, Uppsala, Sweden). Blood glucose was measured with a glucose monitor (Accu-ChekII, Boehringer Mannheim Diagnostics, Indianapolis, Ind., USA). All chemicals were purchased from Sigma Chemical Co. (St. Louis, Mo., USA) unless otherwise specified.

Methods. Male New Zealand White rabbits 2.5--2.8 kg were used. To induce diabetes [11], rabbits were injected with alloxan (150 mg/kg i.v.) via an ear vein 7 days prior to entry into the study. Only those rabbits with a blood glucose level greater than 16.5 mmol/l 7 days following the injection entered the 6 week treatment phase of the study. Data were obtained from 7 untreated and 6 gliclazide-treated diabetic rabbits whose blood glucose was greater than 16.3 mmol/l at the conclusion of the study. The dose of gliclazide ($10\ mg \cdot kg^{-1} \cdot day^{-1}$ orally) was chosen because it increased insulin and lowered blood glucose in control rabbits [10] and resulted in a plasma level of gliclazide similar to that achieved in man. The same dose was reported to prevent atherosclerosis in rabbits [4]. Two groups of normal rabbits were housed for 7 weeks under the same conditions. One group was fed a normal diet (8 rabbits); the other was treated with gliclazide ($10\ mg \cdot kg^{-1} \cdot day^{-1}$) for the last 6 weeks (7 rabbits). The protocols of animal use comply with the Principles of Laboratory Animal Care of the National Institutes of Health.

Gliclazide (sodium salt) was provided by Institut de Recherche Internationale Servier and administered as fixed daily doses in the food. The drug was solubilized in water, sprayed on the food, dried, and administered in approximately 125 g of food (50 g/kg for a 2.5 kg rabbit). Thus, the gliclazide dose of $10\ mg \cdot kg^{-1} \cdot day^{-1}$ for a 2.5 kg rabbit was made by including 25 mg of drug in 125 g of rabbit food. The amount of food administered to the rabbit was determined by administering the appropriate weight of food according to the weight of the rabbit upon entry into the study. The food containing the daily dose of drug was kept in individual plastic bags for each rabbit. Each rabbit was weighed every 2 weeks and the weight of food and dose of drug were adjusted accordingly.

Abdominal aortae were removed after killing the rabbit which was anaesthetized with sodium pentobarbital (50 mg/kg i.v.) and isometric tension of the aorta was measured as previously described [12]. Five millimeter rings of abdominal aorta were mounted for isometric tension recording. Rings were prepared in bicarbonate buffer (4 °C) containing (mmol/l): 118.3 NaCl, 4.7 KCl, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 2.5 $CaCl_2$, 25 $NaHCO_3$, 0.026 Na_2PO_4, 5.5 glucose, and then equilibrated and maintained in this buffer at 37 °C throughout the experiment. The arteries were contracted with phenylephrine and the relaxation to acetylcholine (10^{-8}--3×10^{-6} mol/l) or nitric oxide (10^{-8}--10^{-6} mol/l) was determined according to published methods [12]. As previously reported, there were no significant differences in contractions to phenylephrine in the aorta from diabetic and normal rabbits [11], and gliclazide treatment did not affect the contractions. Prior to adding acetylcholine, contractions to phenylephrine were 8.7 ± 0.4, 9.0 ± 0.8, 9.1 ± 0.8, and 8.4 ± 0.3 g in normal, gliclazide-treated, diabetic, and gliclazide-treated diabetic rabbit groups, respectively. The negative logarithm of the concentration of phenylephrine used to achieve the contractions in the groups was 6.8 ± 0.2, 6.7 ± 0.2, 6.7 ± 0.1, and 6.3 ± 0.1, respectively.

To determine its effects in vitro, arterial rings were pretreated with gliclazide (5×10^{-6} mol/l) for 40 min before being exposed to acetylcholine. The rings were then treated with L-NAME (3×10^{-5} mol/l) for an additional 40 min in the presence or absence of gliclazide. L-NAME had no effect on resting tension of normal or diabetic aortic rings. The rings were then contracted with phenylephrine to 40--50 % of maximal tone, and the relaxations to acetylcholine repeated. All relaxations to nitric oxide were obtained in rings treated with L-NAME in order to eliminate effects of endothelium-derived nitric oxide on the response. Nitric oxide (Matheson Gas Products Co., Gloucester, Mass., USA) was prepared as a saturated solution in helium de-oxygenated water at 4 °C (approximately 1 mmol/l) and diluted in gas tight tubes and syringes.

Plasma insulin and gliclazide, and blood glucose were determined at the start and end of the study. On days 1 (at 16.00 hours) and 2 (at 08.00 hours), 7 and 8 days following alloxan, and days 41 (at 16.00 hours) and 42 (at 08.00 hours), blood was sampled for measurement of glucose and following centrifugation, plasma insulin and gliclazide. Plasma urea nitrogen, plasma total cholesterol, plasma HDL, plasma triglycerides, urinary protein, urinary creatinine, and urinary glucose were measured in blood and urine samples, taken only at the time of killing, by University Diagnostics Services (Boston, Mass., USA).

Analyses of data. Relaxation concentration-response curves to acetylcholine or nitric oxide are expressed as a percentage of the contraction to phenylephrine. Summary data were compiled using a Quattro Pro spread sheet and are presented graphically with the Slide Write program. Two-way analysis of variance for repeated measures (ANOVA) was performed using the SAS software package. Paired or un-paired Students' *t*-test comparisons of the data were performed with the Slide Write program. An asterisk indicates statistical significance ($p < 0.05$).

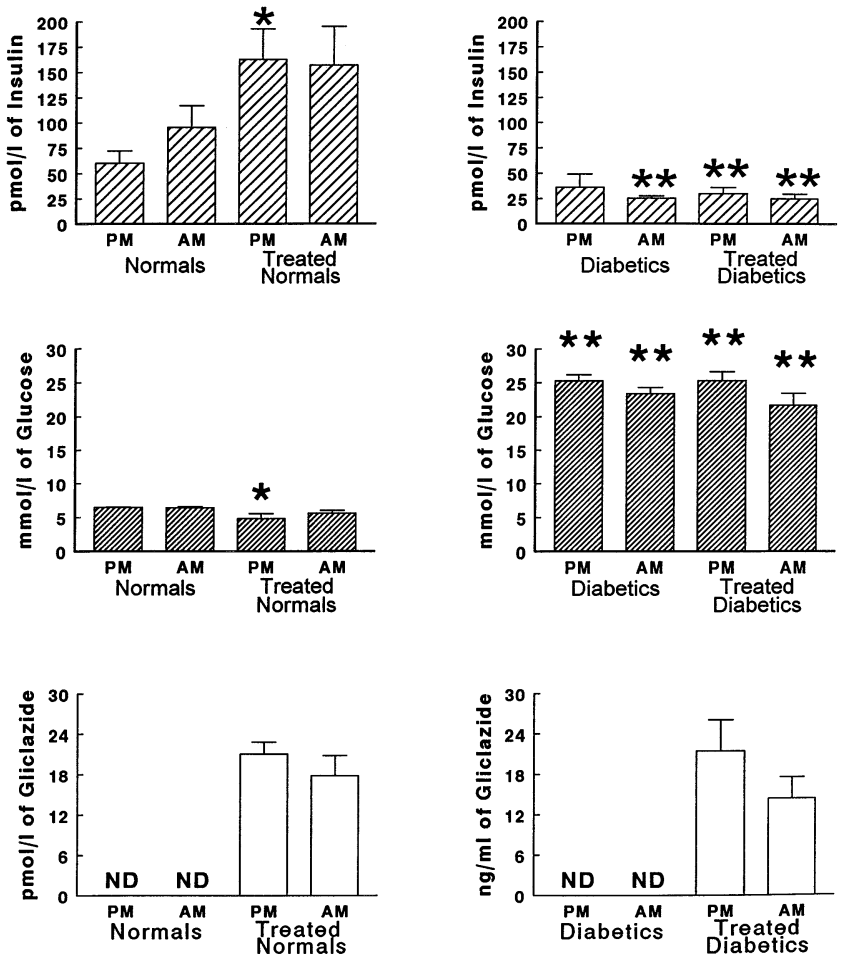

Fig. 1. Comparison of the effect of oral administration of 10 mg · kg^{-1} · day^{-1} gliclazide on plasma insulin, blood glucose, and plasma gliclazide in normal and diabetic rabbits. Data shown were obtained at the end of the study. PM represents a 16.00 hours sample of blood on day 41; AM represents an 08.00 hours sample on day 42. * Significantly ($p < 0.05$) elevated insulin and decreased glucose level in treated normal compared with untreated normal rabbits: ** Difference between normal and diabetic rabbit groups ($p < 0.05$) ND, Non-detectable

Results

Blood chemistries and body weight. Plasma insulin and glucose levels did not change significantly in untreated normal rabbits over the 6 week course of the study (initial data not shown) and were not significantly different between the 16.00 and 08.00 hours blood samples at the conclusion of the study (Fig. 1). Insulin levels at 16.00 hours at the conclusion of the study were significantly higher in gliclazide-treated normal rabbits compared with untreated normal rabbits (Fig. 1). At the conclusion of the study, glucose levels were significantly less only at 16.00 hours in gliclazide-treated normal rabbits compared with untreated normal rabbits.

Compared with normal rabbits, glucose levels were significantly elevated and did not change significantly over the 6 week course in untreated diabetic or in gliclazide-treated diabetic rabbits (Fig. 1). Insulin levels were lower in treated and untreated diabetic rabbits compared with normal rabbits (Fig. 1), and did not significantly change over the course of the study (initial data not shown). Gliclazide levels rose in both gliclazide-treated groups (Fig. 1). There was no significant difference in the levels of gliclazide between the normal and diabetic groups. No significant effect of gliclazide was noted on blood glucose or plasma insulin in diabetic rabbits (Fig. 1).

In diabetic rabbits, plasma urea nitrogen, plasma creatinine levels, plasma triglyceride and urinary glucose levels were significantly higher than in normal rabbits. Plasma urea nitrogen, triglycerides, and urinary glucose were also significantly increased in gliclazide-treated diabetic rabbits (Table 1). A decrease in urinary protein concentration was statistically significant in the untreated diabetic rabbits, but not in the treated normal or treated diabetic rabbits. There was no significant difference from normal in total cholesterol level in the diabetic groups. A reduction in HDL which did not reach statistical significance in the diabetic rabbits, was significant in the gliclazide-

Table 1. Effects of diabetes and gliclazide treatment on biochemical parameters

(n)	Normal (8)	Normal treated (7)	Diabetic (7)	Diabetic treated (6)
Plasma urea nitrogen (mmol/l)	6.6 ± 0.5	5.9 ± 0.4	9.1 ± 0.3[a]	9.5 ± 1.1[a]
Plasma total cholesterol (mmol/l)	1.17 ± 0.16	0.85 ± 0.04	0.95 ± 0.17	1.53 ± 0.50
Plasma HDL (mmol/l)	0.84 ± 0.17	0.56 ± 0.05	0.48 ± 0.05	0.37 ± 0.07[a]
Plasma triglycerides (mmol/l)	0.07 ± 0.01	0.06 ± 0.01	0.16 ± 0.04[a]	0.33 ± 0.14[a]
Plasma creatinine (μmol/l)	79 ± 8.8	77 ± 8.8	100 ± 2[a]	91 ± 8.8
Urinary protein (g/l)	0.19 ± 0.03	0.12 ± 0.01	0.08 ± 0.01[a]	0.10 ± 0.03
Urinary creatinine (mmol/l)	8.75 ± 1.31	6.34 ± 0.78	6.02 ± 0.50	7.04 ± 1.47
Urinary glucose (mmol/l)	0.78 ± 0.28	0.37 ± 0.09	0.31 ± 0.04[a]	0.29 ± 0.08[a]

[a] Significantly different from normal untreated rabbits. Data were collected at day 42 of the study. Data are expressed as mean ± SEM

Table 2. Initial and final body weights

	(n)	Initial	Final
Normal	(8)	3.0 ± 0.1	3.8 ± 0.2
Treated normal	(7)	2.9 ± 0.1	3.6 ± 0.1
Diabetic	(7)	2.9 ± 0.1	3.2 ± 0.1[a]
Treated diabetic	(6)	2.9 ± 0.1	2.9 ± 0.2[a]

[a] Body weights (kg) of diabetic and gliclazide-treated diabetic rabbits were significantly less than normal at the conclusion of the study. Data are expressed as mean ± SEM

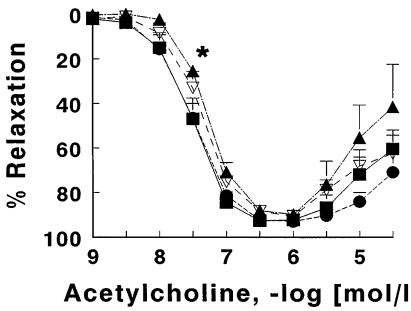

Fig. 2. Acetylcholine-induced relaxations of phenylephrine-contracted rabbit abdominal aortic rings from normal and diabetic rabbits. ANOVA showed a significant difference between normal (■) and untreated diabetic rabbits (▲) from 10^{-8} to 10^{-6} mol/l acetylcholine ($p = 0.022$). * Significance by Student's
t-test vs normal rabbits ($p = 0.018$). Normal rabbits treated with gliclazide (●); diabetic rabbits treated with gliclazide (△)

treated diabetic group. Urinary protein levels were lower in diabetic than in normal rabbits and were unchanged in gliclazide-treated diabetic rabbits (Table 1).

Weight gain was observed in normal rabbits over the 6 week course of the study, and a similar gain occurred in the gliclazide-treated normal rabbits. Diabetic and gliclazide-treated diabetic rabbits both showed a failure to gain weight (Table 2). The final weights in these two diabetic groups were not significantly different from each other (Table 2).

Aortic relaxations. Acetylcholine (10^{-8}--10^{-6} mol/l) caused relaxations which were significantly less in aortae from diabetic rabbits than in those from normal rabbits ($p = 0.022$, ANOVA, Fig. 2). Comparing relaxations to acetylcholine (3×10^{-8} mol/l), a smaller relaxation was observed in diabetic aortae compared with the normal aortae (25 ± 3.0 vs 47 ± 6.9% $p = 0.018$, Fig. 2). The responses to acetylcholine (3×10^{-8} mol/l) of aortae from gliclazide-treated diabetic rabbits (33 ± 7.5%) were intermediary between those of aortae from normal and diabetic rabbits, but there was no significant difference from the responses of aortae from normal or diabetic rabbits (Fig. 2). Furthermore, there was no effect of gliclazide treatment on relaxation in response to acetylcholine in aortae from gliclazide-treated normal rabbits (Fig. 2).

The addition of gliclazide (5×10^{-6} mol/l) in vitro to aortae from diabetic rabbits had no significant effect on relaxation in response to acetylcholine (10^{-8}--10^{-6} mol/l, Fig. 3).

After the addition of L-NAME (3×10^{-5} mol/l) in vitro, acetylcholine caused reduced relaxations, and contractions occurred at concentrations greater than 10^{-6} mol/l which exceeded phenylephrine-induced tone. These contractions were significantly greater in diabetic, compared with normal rabbits (Fig. 4). Comparing responses to acetylcholine (10^{-6}--3×10^{-5} mol/l) in the presence of L-NAME, the contractions were significantly less in aortae from diabetic rabbits treated with gliclazide than in those from untreated diabetic rabbits ($p = 0.041$, Fig. 4). The response of diabetic rabbits treated with gliclazide was not significantly different from that of normal rabbits.

Nitric oxide-induced relaxation was significantly less in aortae from diabetic compared with normal rabbits (Fig. 5, ANOVA $p < 0.03$). The effect of diabetes was not influenced by gliclazide treatment, as a significant difference in nitric oxide-induced relaxation in aortae from treated diabetic rabbits compared with normal rabbits remained (Fig. 5, ANOVA $p < 0.01$). A significantly reduced response to the highest concentration of nitric oxide (10^{-6} mol/l, *t* test $p < 0.01$) was also observed in aortae from gliclazide-treated normal rabbits compared with untreated normal rabbits, but the full concentration-response to ni-

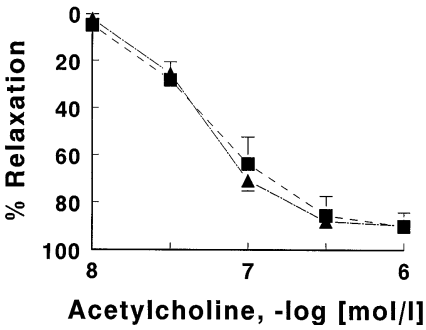

Fig. 3. Acetylcholine-induced relaxations of phenylephrine-contracted rabbit abdominal aortic rings from diabetic rabbits (▲). In vitro addition of gliclazide (5×10^{-6} mol/l) to rings from diabetic rabbits (■) had no significant effect

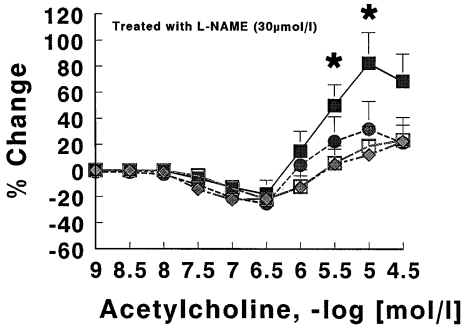

Fig. 4. Acetylcholine-induced changes in tension in phenylephrine-contracted abdominal aortic rings treated with L-NAME (3×10^{-5} mol/l) in vitro. Normal (●); normal-treated with gliclazide (□); diabetic (■); diabetic treated with gliclazide (◆). ANOVA indicates significance ($p = 0.041$) comparing responses to acetylcholine (10^{-6} mol/l to 3×10^{-5} mol/l) in diabetic vs gliclazide-treated diabetic. * Differences at 3×10^{-6} mol/l and 10^{-5} mol/l acetylcholine between rings from diabetic and gliclazide-treated diabetic rabbits by Student's *t*-test

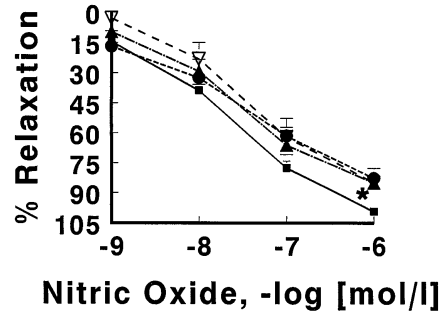

Fig. 5. Nitric oxide-induced relaxation of phenylephrine-contracted abdominal aortic rings. ANOVA indicates significant difference between normal (■) and diabetic (▲) ($p = 0.029$), and between normal and gliclazide-treated diabetic groups (▼) ($p = 0.01$). There was no significant difference by ANOVA between normal and gliclazide-treated normal rabbit aortae (●) responses to nitric oxide. * Significant differences in response to nitric oxide (10^{-6} mol/l) between normal and all other groups by Student's *t*-test ($p < 0.01$)

tric oxide was not significantly different by ANOVA ($p < 0.23$, Fig. 5).

Discussion

Diabetes induced by alloxan in the rabbit is associated with significant inhibition of acetylcholine-induced endothelium-dependent relaxation and augmented contraction of the abdominal aorta [13]. The reduced relaxations are reversed by free radical scavengers [14] and have been ascribed to vasoconstrictor prostanoids which stimulate thromboxane A_2/prostaglandin endoperoxide receptors [7, 13]. The present results show that the abnormal contractile responses are significantly reduced, and the relaxant responses are partially improved, by concurrent treatment with gliclazide. The effect of gliclazide treatment was specific for an aberration arising with diabetes, because gliclazide added in vitro had no significant effect on the response to acetylcholine of diabetic rabbit arteries, and chronic treatment with gliclazide for 6 weeks had no effect on acetylcholine-induced relaxation of the aorta of normal rabbits. The lack of effect of gliclazide in vitro on the responses of diabetic rabbit aortae indicates that a direct and immediate free radical scavenging effect [5] or thromboxane A_2/prostaglandin endoperoxide receptor blocking action [15] both of which have been described for gliclazide, is unlikely to account for the improvement in diabetic endothelial cell dysfunction. The effect of gliclazide in vivo could, however, be due to a chronic influence which counters the vascular effect of diabetes, such as an increase in endothelium-derived vasodilators, a decrease in vasoconstrictors, a reduction in free radical generation or an increase in free radical scavenging. In diabetic patients taking gliclazide, an increase in plasma reduced thiols, a decrease in plasma lipid peroxides, and an increase in erythrocyte superoxide dismutase activity were noted and unrelated to an amelioration in the diabetic metabolic state [6]. Thus, it is possible that similar effects on antioxidant systems in the diabetic rabbit resulted in improvement of endothelium-dependent relaxation. It is also possible that the effect of gliclazide was via K_{ATP} channels which were blocked at least in the pancreas by the dose used in vivo, as evidenced by the increase in insulin and decrease in blood glucose in normal rabbits. However, the concentration of gliclazide used on the aorta in vitro which also blocks K_{ATP} channels [3], failed to acutely affect the relaxation to acetylcholine. Thus, if blockade of K_{ATP} channels played a role in the ex vivo effects of gliclazide observed in this study, it was through some unexplained mechanism related to chronic treatment with the drug. This mechanism is apparently not metabolic as indicated by the lack of an effect of gliclazide on body weight, plasma glu-

cose and insulin, or urinary glucose levels in diabetic rabbits.

Compared with previous findings by our group and by others [13, 16] there was a lesser effect of diabetes on ex vivo aortic relaxations observed in this study. A three- to fourfold rightward shift in the acetylcholine-induced relaxation occurred in previous studies [11, 13, 16], whereas for unknown reasons a smaller twofold shift occurred in this study. Nonetheless, diabetes did cause a significant reduction in relaxation which was not significant in diabetic rabbits treated with gliclazide in vivo. This effect of diabetes on aortic tone was more apparent when examining contractions of the same aortae after treatment with the nitric oxide synthase inhibitor L-NAME. In this case, treatment with gliclazide in vivo caused a significant reversal of the effect of diabetes.

Nitric oxide-induced relaxations were inhibited as a result of diabetes suggesting that, at least in part, the relaxations to acetylcholine which are mediated by nitric oxide are reduced as a result of a diminished response to the endothelium-derived vasodilator. However, relaxations to nitric oxide were not significantly affected by in vivo treatment with gliclazide. This may indicate that gliclazide treatment affects the release of, or response to the prostanoid vasoconstrictors which have been implicated in the decreased relaxations to acetylcholine observed in diabetic rabbits, rather than the release of, or response to nitric oxide. The pronounced difference observed in aortae of diabetic and normal rabbits comparing the contractile response to acetylcholine after nitric oxide synthase was inhibited with L-NAME strongly supports this contention. Indeed, there is evidence for a greater production of endothelium-derived prostanoid contracting factor(s) such as prostaglandin endoperoxide [13, 17, 18] in aortae from diabetic rabbits. Free radical scavengers inhibit contractions to prostaglandin endoperoxide [19] as well as ameliorate impaired endothelium-dependent relaxations of diabetic rabbits [14], providing a mechanism by which the long-term antioxidant effects of gliclazide could ameliorate endothelial function.

The fact that responses to sodium nitroprusside are normal in diabetic rabbit aorta as found by us and others [13, 16] compared with aberrant nitric oxide-induced responses in diabetes is perhaps explained by the different mechanism of action of sodium nitroprusside. That is, it enters the smooth muscle cell and then releases nitric oxide at the site of action [20], thereby avoiding any interstitial barriers, including superoxide anion.

The results of this study suggest that in vivo treatment with gliclazide is associated with improved vascular function in diabetic rabbits. The effect of drug treatment was independent of a metabolic influence. This is commensurate with studies in patients showing that effects of gliclazide on antioxidant systems or platelet aggregation are not correlated with an effect on the severity of diabetes, and have been demonstrated after switching from another sulfonylurea to gliclazide [6]. Plasma HDL was significantly decreased in gliclazide-treated diabetic rabbits, a change not seen clinically [3]. It is evident, however, that the improvement in aortic endothelial responsiveness was not due to this change because such a change would be expected to worsen rather than improve the response [21].

Because the most striking alteration in vascular function in the diabetic animal are the contractions to acetylcholine observed after inhibiting nitric oxide synthase, and because these were prevented by gliclazide, the results suggest that treatment with gliclazide decreases the release of, or response to vasoconstrictor prostanoids, or the production or scavenging of superoxide anion which contributes to these contractions in diabetic rabbit arteries [13, 14, 18, 19]. Whether or not there are similar effects of gliclazide in diabetic patients will have to await clinical studies.

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