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RENAL AND MICROVASCULAR EFFECTS OF AN ALDOSE REDUCTASE INHIBITOR IN EXPERIMENTAL DIABETES

BIOCHEMICAL, FUNCTIONAL AND ULTRASTRUCTURAL STUDIES

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Abstract—Aldose reductase inhibitors, and particularly sorbinil, have been reported to prevent glomerular basement membrane thickening (GBMT) and albuminuria development in diabetic rats, but contradictory observations have been published. The aim of this study was to answer the following questions (i) is the corrective effect of sorbinil on GBMT, if confirmed, associated with an effect on collagen metabolism alterations? (ii) Is it associated with an effect on microvascular functional alterations? We therefore studied the influence of sorbinil on glucosyl-galactosyl-hydroxylysyl-glucohydrolase activity (GGHG; EC 3.2.1.107 which is involved in the catabolism of collagen disaccharide units), 3- and 4-hydroxyproline content and GBMT by ultrastructural morphometry in the kidney cortex of streptozotocin-diabetic rats after 5 months of disease. In parallel, the effects on albumin renal clearance and another functional alteration, the microvascular response to norepinephrine, were evaluated. We confirmed a corrective effect of sorbinil on both renal albumin clearance and GBMT. In the diabetic rats, sorbinil diminished the 3-hydroxyproline (but not the 4-hydroxyproline) content, whether expressed per mg protein or per total kidney cortex relative to body weight. Sorbinil reduced GGHG activity measured in the dialysed 10,000 *g* supernatant whether expressed per mg protein or per total kidney cortex; this activity has been shown to be increased in diabetes. Sorbinil also corrected the microvascular response to norepinephrine which is altered in diabetes.

Key words: diabetic microangiopathy; glomerular basement membrane; albuminuria; collagen disaccharide unit glucosidase; microvascular response to norepinephrine; sorbinil

Diabetic microangiopathy is characterized by BMT§ related to type IV collagen accumulation [1]. This collagen unique to BM is particularly rich in disaccharide units and 3-hydroxyproline-[2]. GGHG (EC 3.2.1.107) is involved in the catabolism of collagen disaccharide units [3] and has been shown to be inhibited by high glucose concentrations in diabetic kidneys [4]. This decrease in activity is associated with a high GGHG concentration which may be secondary to substrate accumulation [5].

Diabetic microangiopathy is also characterized by functional alterations of the microcirculation. After 1 or 2 months of diabetes, a decrease in blood flow to the skin and some skeletal muscles, but not to the kidney, has been reported for the rat [6]. Increased capillary permeability to albumin has also been reported [7, 8].

The most studied ARI, sorbinil, has been shown to inhibit cataract formation [9, 10] and, in some [7, 11, 12] but not all [13, 14] reports, albuminuria development in diabetic rats. Correction of GBMT has also been described [13] but this effect has not been found by all authors [8]. To our knowledge, the effects of sorbinil on the metabolism of renal collagen in diabetes have not been studied. Thus the aim of this study was to answer the following questions:

(i) Is the corrective effect of sorbinil on GBMT, if confirmed, associated with an effect on collagen metabolism alterations?

(ii) Is it associated with an effect on microvascular functional alterations?

Therefore, we studied the influence of sorbinil on GGHG activity, 3-Hyp and 4-Hyp content and GBMT in the kidney cortex of streptozotocin-diabetic rats after 5 months of disease. In parallel, the effects on albumin renal clearance and another functional alteration, microvascular response to norepinephrine, were evaluated. Laser Doppler

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§ Abbreviations: ARI, Aldose reductase inhibitor; b, rat subgroup used for biochemical studies; BM, basement membrane; BMT, basement membrane thickening; D, diabetic rat; DS, sorbinil-treated diabetic rat; GBM, glomerular basement membrane; GBMT, glomerular basement membrane thickening; GGHG, glucosyl-galactosyl-hydroxylysyl-glucohydrolase; Hyp, hydroxyproline; N, normoglycemic rat; NS, sorbinil-treated normoglycemic rat; p, rat subgroup used for pharmacological functional and ultrastructural explorations; STZ, streptozotocin.

blood flowmetry has been adapted for a dynamic exploration of the skin's microvascular response to norepinephrine in long term diabetic rats.

MATERIALS AND METHODS

Animals

Sixty-six 7-week-old male Wistar rats (IFFA-CREDO, l'Arbresle 69210 France), 170 ± 5 g body weight, were divided into four groups. Two groups were made hyperglycemic after two intramuscular injections at days D 0 and D 7 of streptozotocin (STZ, Sigma) 55 mg/kg in citrate buffer pH = 4.5 and two groups received buffer alone. One diabetic group (DS, n = 18) and one control group (NS, n = 14) were treated with sorbinil (generous gift from Pfizer) 12 mg/kg body weight/day, dissolved in 0.5% carboxymethylcellulose and administered through an oesophageal cannula, starting 2 days before D 0 and continued until death. The other diabetic (D, n = 18) and control (N, n = 16) groups were treated with the vehicle alone. Each group (N, NS, D, and DS) was divided in two subgroups as follows: a subgroup *p* for pharmacological functional and ultrastructural explorations, and a subgroup *b* for biochemical studies. For instance, normoglycemic rats (N) were divided in two subgroups: Np and Nb. The animals were kept in an air-conditioned room with natural light cycle, and with free access to food and water. A standard 24% protein diet was given. The animals were weighed daily in the first month and weekly thereafter. The presence of cataract was evaluated by clinical examination.

The animals were killed after 22 weeks of diabetes. The Np, NSp, Dp and DSp subgroups were heparinized after the study of microvascular response to norepinephrine and urine collection. Blood was taken from the left carotid and the rats were killed by exsanguination. The kidneys were removed for ultrastructural studies. The Nb, NSb, Db and DSb subgroups were killed by decapitation and blood was taken in tubes with EDTA and the kidneys were immediately frozen and kept in liquid nitrogen.

Pharmacological functional explorations

The rats were anaesthetized by chloral hydrate (Prolabo, Paris; 300 mg/kg IP).

Microvascular response to norepinephrine was studied in the skin. Norepinephrine (Winthrop, U.K.) was injected intravenously at increasing doses: 2.9, 5.8, 14.4, 28.8 and 57.6 nmol/kg body weight. The resulting changes in blood flow were measured by a laser Doppler flowmetry method. The Periflux PF2B (Perimed KB, Stockholm, Sweden) apparatus and the technique have been described in detail by Nilsson *et al.* [15]. The probe was applied on the internal side of the thigh shaved 24 hr previously. The sensitivity of the monitor was set at 3 or 10 × 12 kHz. This technique allows the measurement of blood flow variations (expressed in volts) only in microvessels (diameter < 50 μm) [15].

Renal albumin clearance. The pudendal vein was cannulated and the ureter was ligated. After emptying the bladder by punction and injecting 2.1 mL/kg saline intravenously in the N and 3.75 mL/kg in the D rats, the urine produced in 1 hr was obtained by bladder puncture. Blood was taken after the urine collection. Urinary albumin excretion and albuminemia were determined and renal clearance of albumin calculated.

Ultrastructural morphometry

After death, a fragment of the renal cortex was cut into 1 mm^3 blocks, which were immediately fixed for 2 hr in a solution of 2.5% glutaraldehyde in phosphate buffer (0.1 M; pH = 7.4) at 4°. After fixation with a solution of 1.5% osmium tetroxide in veronal buffer and dehydration with graded alcohols, 10 blocks/rat were embedded in epoxy resin and left for 48 hr at 60° for polymerization. Semi-thin sections stained with toluidine blue were made on each block in order to locate the glomerular structures. Ultra-thin sections were stained with uranyl acetate and lead citrate and observed with a Siemens Elmiscope IA electron microscope. Ten electron micrographs of the most well-defined and linear glomerular basement membranes (GBM) were taken randomly. Each group of 10 micrographs/rat was preceded by a micrograph of a calibrated reference grid in order to standardize the measures, at a magnification of × 10,000. The GBM thickness was directly measured on electron micrographs projected on a screen. Several measures of GBM were made at regular intervals (0.5 cm) and the mean measure was calculated after correction with the calibrated reference grid. A conversion factor, calculated from the characteristics of the electron microscope, allowed the results to be expressed in nm.

Biochemical studies

Weekly, then monthly, measurements of blood *glucose* were carried out on capillary blood from the tail by a glucose oxidase micromethod. *Fructosamine* was measured in plasma at sacrifice with a kit (Roche, France). These parameters were determined in all animals (*b* and *p* subgroups).

3 and 4-Hyp were measured in homogenized renal cortex after hydrochloric acid hydrolysis by thin layer chromatography [16]. *GGHG activity* was measured in the dialysed 10,000 *g* supernatant of renal cortex homogenates, using 0.75 mM GGH-peptide as substrate, at pH 4.4; in the conditions of the assay, glucose release has been shown to be proportional to the enzyme concentration; glucose levels were insignificant in control tubes containing only the dialysed enzyme fraction from N or D rats; enzyme activity was expressed in units per mg protein or per total kidney cortex; *proteins* were measured by the Lowry method; one unit corresponds to 1 nmol of glucose released per hour at 37° [3]. These parameters were determined in the *b* subgroup.

Plasma and urinary *albumin* were measured in the *p* subgroup by electro-immunodiffusion with an anti-rat albumin antiserum (Nordic I.L. Tilborg, The Netherlands) and rat albumin (Sigma, St Louis, MO, U.S.A.).

Statistical methods

Results are generally expressed as mean ± SEM. The hyperglycemic rats were compared to N and NS rats by two-way analysis of variance followed by a

Table 1. General characteristics of the animals at 22 weeks of diabetes

Group	Body weight (g)	Blood glucose (mmol/L)	Plasma fructosamine (mmol/L)	Cataract (%)
D; n = 18	259.5 ± 9.21*	39.21 ± 1.14*	2.52 ± 0.10*	100*
DS; n = 18	273.0 ± 8.88*	38.27 ± 1.17*	2.66 ± 0.13*	0†
N; n = 16	473.4 ± 10.0	5.86 ± 0.21	1.60 ± 0.05	0
NS; n = 14	473.4 ± 9.85	5.55 ± 0.12	1.67 ± 0.09	0

All values are determined on subgroups ***b*** + ***p*** and expressed as mean ± SEM except for cataract expressed as percentage of diseased eyes.
* $P < 0.001$ D vs N, or DS vs NS.
† $P < 0.001$ DS vs D, or NS vs N.

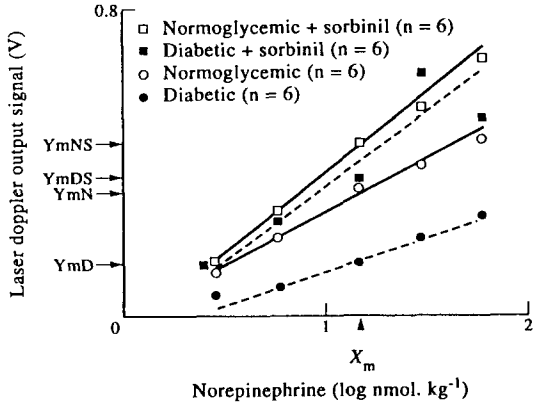

Fig. 1. Skin microvascular blood flow variations in response to norepinephrine measured by laser Doppler blood flowmetry. Individual dose–response regression lines were calculated for the linear portion of the individual experimental curves. For each ***p*** subgroup, the mean slope and the mean response amplitude Y_m (corresponding to the mean norepinephrine dose $X_m = 1.12$ nmol/kg) were calculated and compared by variance analysis. The mean dose–response line is presented for each experimental group together with the mean experimental points for each norepinephrine dose. Y_m is significantly lower in untreated D (●) when compared with untreated N (○), $P < 0.05$) and with DS (■, $P < 0.01$) or NS (□, $P < 0.01$). The slope of the regression line is increased in DS vs untreated D ($P < 0.02$); $P < 0.1$ between NS and untreated N.

Table 2. Renal clearance of albumin at 22 weeks of diabetes

Group	Renal clearance of albumin (nL/min)	Renal clearance of albumin (nL/min/kg body weight)
D; n = 6	182.5 ± 15.5*	728.8 ± 91.9*
DS; n = 6	120.5 ± 24.3*‡	432.5 ± 96.5†§
N; n = 6	10.7 ± 2.21	23.95 ± 5.17
NS; n = 6	15.8 ± 3.94	35.34 ± 9.17

All values are determined on ***p*** subgroups and expressed as mean ± SEM.
* $P < 0.001$, † $P < 0.01$ D vs N, or DS vs NS.
‡ $P < 0.01$, § $P < 0.05$ DS vs D, or NS vs N.

Student's *t*-test corrected for multiple comparisons. When a variance difference was found by the Fisher *F* test, a Mann–Whitney's *U* test was carried out. Cataract frequency was expressed as percentage of diseased eyes and compared using a χ^2 test. Aberrant values were detected and discarded by the maximum deviation from the mean test [17]. The significance threshold retained was $P < 0.05$.

RESULTS

The animal characteristics

These are shown in Table 1. In all untreated D rats, a bilateral cataract was observed 3 months after the STZ injection, whereas no cataract was detected in any DS rat. Body weight was decreased, plasma fructosamine and blood glucose levels were higher in the D when compared with the control rats (N, $P < 0.001$) for the three parameters. Sorbinil had no effect on body weight, blood glucose and plasma fructosamine levels in the DS rats.

Microvascular response to norepinephrine (Fig. 1)

The skin microvascular response to norepinephrine was decreased in the D rats in comparison with the N rats as shown by the mean response Y_m values. Sorbinil treatment maintained a normal microvascular reactivity in the DS rats.

Albumin renal clearance

This was increased in D rats ($P < 0.001$) when compared with their N controls (Table 2). Sorbinil treatment significantly decreased albumin renal clearance in the DS rats. Albuminemia was decreased in the D rats when compared with their N controls (20.4 ± 0.93 g/L for Dp vs 26.0 ± 0.97 g/L for Np; $P < 0.001$). Under sorbinil treatment, albuminemia was no longer significantly decreased (23.0 ± 0.84 g/L for DSp vs 25.3 ± 1.35 g/L for NSp; $P < 0.1$ between DSp and NSp and $P < 0.1$ between DSp and Dp). Albuminuria was increased in the D rats (12.07 ± 2.06 μg/min/kg body weight vs 0.63 ± 0.15 in the Np group; $P < 0.001$). Sorbinil treatment decreased albuminuria in the DSp group but not

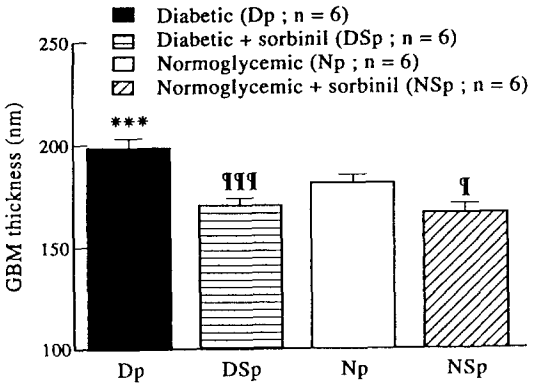

Fig. 2. GBM thickness in the diabetic and normoglycemic rats with or without treatment by sorbinil (*p* subgroups). ***, $P < 0.001$ Dp vs Np; ¶, $P < 0.05$ NSp vs Np; ¶¶¶, $P < 0.001$ DSp vs Dp.

significantly (9.78 ± 2.08 μg/min/kg) and did not change it in the NSp group (0.84 ± 0.15 μg/min/kg).

Ultrastructural morphometry (Fig. 2)

Diabetes resulted in an increased GBM thickness ($P < 0.001$ between Dp and Np) with an irregular aspect. Sorbinil treatment prevented this GBM thickening. GBM was regular; its thickness was significantly lower in the DSp group when compared with the Dp group ($P < 0.001$) and not different from the Np group ($P > 0.1$). Also, in NSp rats, sorbinil treatment decreased GBM thickness ($P < 0.05$ between NSp and Np).

Renal collagen metabolism (Table 3)

Kidney cortex weight and total protein content were moderately increased in the D rats when compared with the N controls after 5 months of disease, as previously reported in long-term diabetes with severe hyperglycemia [5]. The increase in kidney cortex total protein was significant only when expressed relative to body weight. GGHG specific activity was significantly increased in the dialysed 10,000 *g* supernatant of renal cortex homogenates in the untreated D rats when compared with their N controls, whether expressed per mg protein or per total cortex or per cortex relative to body weight ($P < 0.001$). Sorbinil treatment significantly decreased GGHG specific activity in the DSb group vs the Db group ($P < 0.05$ if expressed in any of the three ways). The 3- and 4-Hyp contents were significantly increased in the kidney cortex of the untreated D rats ($P < 0.001$, Db vs Nb) when expressed per cortex relative to body weight. The increase in the amount of 3-Hyp, but not of 4-Hyp, per cortex relative to body weight was significantly reduced in the DS rats ($P < 0.05$, DSb vs Db). The cortex total protein content relative to body weight was not influenced by sorbinil. The concentration per mg protein of 3-Hyp, but not of 4-Hyp, was significantly reduced in the DS rats only.

DISCUSSION

In this study we observed a preventive effect of sorbinil treatment on GBMT after 5 months of STZ-induced diabetes. We also found a decrease in GBM thickness in the N controls treated by sorbinil. Thus, we confirm the results of Mauer *et al.* concerning the effects of sorbinil on GBM thickness in STZ-D rats and also in N controls [13]. Sorbinil and other ARI have been reported to prevent BMT in the

Table 3. Kidney cortex biochemical characteristics at 22 weeks of diabetes

Group	D (n = 11)	DS (n = 12)	N (n = 8)	NS (n = 8)
Kidney cortex weight				
g	1.02 ± 0.05	1.07 ± 0.03	0.94 ± 0.04	0.95 ± 0.03
Kidney cortex total proteins				
mg/cortex	126 ± 7.90	139 ± 4.70	116 ± 5.21	120 ± 7.25
mg/cortex/Kg body weight	528 ± 20*	531 ± 13*	233 ± 10	245 ± 10
3-Hydroxyproline				
nmol/mg total protein	2.40 ± 0.09	2.00 ± 0.08†‡	2.59 ± 0.24	2.52 ± 0.12
nmol/cortex	301 ± 21	276 ± 12	301 ± 30	302 ± 22
nmol/cortex/kg body weight	1247 ± 89*	1065 ± 57*‡	603 ± 55	617 ± 34
4-Hydroxyproline				
nmol/mg total protein	38.2 ± 2.3	35.2 ± 1.4	36.9 ± 3.9	37.7 ± 1.2
nmol/cortex	4746 ± 300	4886 ± 245	4299 ± 494	4520 ± 304
nmol/cortex/kg body weight	19,777 ± 1570*	18,709 ± 898*	8604 ± 896	9224 ± 439
GGHG activity§				
U/mg protein §§	3.13 ± 0.14*	2.68 ± 1.18*‡	1.59 ± 0.11	1.57 ± 0.70
U/cortex	328 ± 16*	280 ± 14*‡	149 ± 14	174 ± 8
U/cortex/kg body weight	1391 ± 95*	1066 ± 71*‡	327 ± 25	342 ± 14

All values are determined on *b* subgroups and expressed as mean ± SEM.
* $P < 0.001$, † $P < 0.01$ D vs N, or DS vs NS. ‡ $P < 0.05$ DS vs D, or NS vs N.
§ 1 unit (U) corresponds to 1 nmol glucose released per hour at 37°.
§§ Determined in the 10,000 *g* supernatant of kidney cortex homogenate; total supernatant proteins were as follows: 103 ± 4 mg/cortex in the D; 110 ± 5 mg in the DS; 93 ± 3 mg in the N and 104 ± 8 mg in the NS group.

retina or muscle capillaries in D or galactosemic rats [18–26]. However, no effect of sorbinil on BMT has been found by others in the glomerulus of D [8] or galactosemic rats [27]. Moreover, the absence of effect on BMT with another ARI, statil, has also been reported [28].

The discrepancy between the various observations in the literature may be due to different factors: (i) difficulties in quantifying and comparing BM thickness statistically [29]; (ii) the mode of administration of ARI: most authors have used ARI incorporated in the diet. The absorbed dose can then vary with time and differs between N and D rats. Perhaps drug stability may be altered under these conditions. In our study sorbinil was administered freshly daily through an oesophagal cannula. (iii) The type of diet: no effect was found Mauer *et al.* in D rats fed with a 50% protein diet, whereas an effect was observed in rats fed with a 20% protein diet [13]. In our study, a standard 24% protein diet has been given. (iv) The anatomical localization of the capillaries studied: Chakrabarti and Sima found a corrective effect of statil on the BMT of deep but not superficial capillaries of the retina in BB diabetic rats [26]. Sorbinil treatment has been reported to prevent the BMT in the retina but not the kidney glomerulus of galactosemic rats [27].

In this study, the effect of sorbinil on GBMT has been found to be associated with effects on alterations of collagen metabolism: decrease in 3-Hyp concentration in the diabetic kidney cortex and correction of the increase in GGHG activity in the dialysed diabetic kidney cortex preparation. The effect of sorbinil on the increase in 3-Hyp content relative to body weight was not associated with an effect on the relative contents of 4-Hyp or total protein. Type IV collagen, which is the main BM collagen, is particularly rich in 3-hydroxyproline and disaccharide units, in contrast with interstitial collagens. GGHG appears to be involved in a late-lysosomal-stage of collagen disaccharide unit catabolism [3]; the high GGHG concentration described in diabetes may be secondary to substrate (disaccharide unit) accumulation [5]. Thus sorbinil seems selectively to influence BM collagen metabolism in the diabetic state. Das *et al.* have reported a beneficial effect of sorbinil on type IV collagen and laminin accumulation in galactose-induced retinal capillary BM [30]. One mechanism for the preventive effect of sorbinil on GBMT may be the inhibition of advanced glycation end product formation which induces cross-linking between GBM proteins and consecutive resistance to protease degradation [31, 32]

In our study the corrective effect of sorbinil treatment on GBMT has also been found to be associated with an effect on microvascular functional alterations. The decrease in the microvascular response to norepinephrine observed in the diabetic rats is in agreement with the decrease in blood flow to the skin and some skeletal muscles reported in the literature [33]. Sorbinil has been shown here to prevent these alterations. This corrective effect may be due to an action on the microvessel wall and/or blood viscosity. On the one hand sorbinil partially prevents decreased erythrocyte deformability in STZ diabetic rats [34]. On the other hand, we have demonstrated here an effect of sorbinil treatment on GBMT and on collagen metabolism.

Albumin renal clearance alterations have also been significantly reduced by sorbinil in this study. Conflicting results have been reported concerning the effects of ARI on proteinuria in diabetic animals. Positive effects on proteinuria have been found by Beyer-Mears *et al.* [11, 12], Tilton *et al.* [7], and Chang *et al.* [35]. In contrast, no effect on albuminuria has been observed by Mauer *et al.* [13] and Körner *et al.* [14].

Thus, in this study a parallel beneficial effect of sorbinil treatment has been found on GBMT and albuminuria whereas a dissociation between the effect on GBMT and the absence of effect on albuminuria has been reported by Mauer *et al.* [13]. The relationship between the effects on GBMT and albuminuria is not clearly understood. No effect of sorbinil on the decrease in anionic charges of the diabetic glomerular vascular wall has been reported. Indeed no effects on glomerular heparan sulfate proteoglycan [36] or on kidney cortex sialic acid [37] contents have been described.

In conclusion, we have confirmed the corrective effect of sorbinil on the GBMT and the increase in renal albumin clearance observed in diabetic rats. We have shown that sorbinil also corrects the microvascular response to norepinephrine which is altered in diabetes. We have also described some effects on collagen metabolism, particularly on 3-hydroxyproline concentration and GGHG activity, in the diabetic kidney cortex.

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