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The relative roles of advanced glycation, oxidation and aldose reductase inhibition in the development of experimental diabetic nephropathy in the Sprague-Dawley rat

T. Soulis-Liparota, M. E. Cooper, M. Dunlop, G. Jerums

Department of Medicine, University of Melbourne, Heidelberg Repatriation, Austin and Royal Melbourne Hospitals, Melbourne, Victoria, Australia

Summary Advanced glycation is an important pathogenic mechanism in the development of diabetic complications. However, other biochemical processes, such as the polyol pathway or lipid and protein oxidation which can interact with advanced glycation can also yield tissue fluorescence and may also be implicated in the genesis of diabetic microangiopathy. Aminoguanidine is an inhibitor of advanced glycation, but it is not known if all of its effects are mediated by this mechanism. The present study explores the relative contributions of aldose reductase, oxidative stress and advanced glycation on the development of aortic and renal fluorescence and urinary albumin excretion in streptozotocin diabetic rats. The study groups included non-diabetic (control), streptozotocin diabetic rats and diabetic rats receiving aminoguanidine, the anti-oxidants butylated hydroxytoluene and probucol and the aldose reductase inhibitor, ponalrestat. Serial measurements of glycaemic control and urinary albumin excretion were performed every 8 weeks. At 32 weeks, animals were killed, tissues removed and collagen extracted for measurement of fluorescence. Diabetic rats had increased fluorescence in aorta, glomeruli and renal tubules. Aminoguanidine prevented an increase in fluorescence at all three sites suggesting that diabetes-related tissue fluorescence is predominantly due to advanced glycation. Ponalrestat retarded fluorescence in aorta only and butylated hydroxytoluene attenuated fluorescence at the renal sites but not in the aorta. Diabetic rats had increased renal cortical sorbitol levels. Ponalrestat normalized renal cortical sorbitol levels but aminoguanidine did not affect this parameter. The only agent to decrease plasma thiobarbituric acid reactive substances was butylated hydroxytoluene. Diabetic rats developed albuminuria over the 32-week period. This increase in urinary albumin excretion was only attenuated significantly by aminoguanidine therapy, but not by probucol or ponalrestat. The effects of butylated hydroxytoluene on albuminuria were intermediate between aminoguanidine-treated and untreated diabetic rats. The failure of either antioxidants or aldose reductase inhibition to reproduce the renal effects of aminoguanidine suggest that aminoguanidine may act predominantly via inhibition of advanced glycation and not via the alternative biochemical processes evaluated in this study. [Diabetologia (1995) 38: 387–394]

Key words Advanced glycation, oxidation, aldose reductase inhibition, diabetic nephropathy, aminoguanidine, ponalrestat, probucol, butylated hydroxytoluene.

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Corresponding author: Dr. T. Soulis-Liparota, Department of Medicine, Heidelberg Repatriation Hospital, West Heidelberg, Victoria 3081, Australia

Abbreviations: AGE, Advanced glycated end products; AG, aminoguanidine, TBARS, Thiobarbituric acid reactive substances; BHT, butylated hydroxytoluene.

The development of diabetic nephropathy may be related not only to formation and subsequent accumulation of advanced glycation end products (AGEs), but also to other biochemical processes including oxidation [1] and activation of the polyol pathway [2]. AGEs are derived from the non-enzymatic glycation of proteins such as collagen with subsequent further rearrangement to yield glucose-derived cross-links,

and fluoresce at specific wave lengths. However, the observed fluorescence in diabetic tissues could result from other processes that are increased in diabetes mellitus such as fructosylation. Fructosylation occurs after glucose is converted by aldose reductase to sorbitol and then to fructose. The reaction of fructose with protein yields fluorescent products at a rate ten times faster than glucose [3] and may be more potent in damaging proteins [4]. Oxidation has been shown to produce fluorescent products and to interact with the advanced glycation process [5]. Phospholipids, for example, react directly with glucose to form AGEs which subsequently initiate lipid oxidation. Phospholipid-linked AGEs are able to mimic the absorbance and fluorescence of AGEs [1]. Hyperglycaemia itself may contribute to oxidative events by forming glycation products that can propagate free radical events [6].

Aminoguanidine, a phenylhydrazine compound, has been previously shown by our group [7] to reduce collagen-related fluorescence in the aorta as well as the kidney (renal tubules and glomeruli) after 32 weeks of streptozotocin diabetes. Aminoguanidine also attenuated the rate of development of albuminuria and prevented mesangial expansion in diabetic rats. The present study explores the relative contributions of advanced glycation, oxidative stress and the polyol pathway in the pathogenesis of experimental diabetic nephropathy. Since the exact nature of AGEs is poorly characterised and the links between advanced glycation and other biochemical processes have been poorly elucidated, specific inhibitors of the putative biochemical processes were used in a rat model of experimental diabetes over a 32-week study period. Major endpoints included albuminuria and markers of the individual biochemical processes studied.

Materials and methods

Male Sprague-Dawley rats, 6 weeks of age, weighing between 200 and 250 g were randomized into control or diabetic rats. Diabetes was induced by intravenous injection of streptozotocin (60 mg/kg body weight in citrate buffer, pH 4.5) after an overnight fast and all animals with plasma glucose levels greater than 20 mmol/l after 1 week were included in the study. The control group was sham injected with citrate buffer alone. Diabetic animals were further randomized to five groups which received no therapy, aminoguanidine hydrogen carbonate (Fluka Chemica, Buchs, Switzerland) 1 g/l in drinking water, the anti-oxidant, Probucol (Marion Merrell Dow, Cincinnatti, Ohio, USA) 1 % in the diet [8], the anti-oxidant Butylated hydroxytoluene (BHT) (Aldrich, Castle Hill, Australia) 0.4 % in the diet [9, 10] or the aldose reductase inhibitor Ponalrestat (ICI, Macclesfield, UK) 0.03 % in the diet [11]. Two lipid-soluble antioxidants were used; BHT and probucol, the latter possessing cholesterol-lowering as well as antioxidant properties [10]. All rats were given free access to food throughout the study (GR2 rat cubes, Clark King and Co, Melbourne, Australia). Probucol, BHT and ponalrestat were coated onto the pellets. Diabetic rats received 2 IU ultralente insulin (Ultratard HM, Novo Industries, Bagsvaerd, Denmark) every second day to maintain body weight and improve survival over the 32-week period of the study. Rats were caged in groups of three. At eight weekly intervals, rats were weighed and placed in metabolic cages (Iffa Credo, L'Abresele, France) for collection of 24-h urine samples used for measurement of albuminuria. Blood was collected from the tail vein for measurement of plasma glucose and glycated haemoglobin at 8-week intervals.

Plasma glucose was measured by a glucose oxidase technique [12] and glycated haemoglobin was measured by a high-performance liquid chromatography technique [Bio-Rad, Richmond, Calif., USA) [7]. Urine from diabetic rats was tested on a regular basis for the presence of ketones (Ketostix, Miles, Mulgrave, Australia) but ketonuria was not detected in any diabetic animal throughout the study. Urinary albumin concentration was measured by radioimmunoassay [13]. The interassay coefficient of variation was 7 % ($n = 50$) at a concentration of 180 ng/ml and the detection limit of the assay was 31.2 ng/ml.

At 32 weeks rats were anaesthetised with pentobarbital sodium (Nembutal, Bomac, Asquith, Australia) and the left kidney was excised followed by the removal of the lower abdominal aorta and right and left common iliac arteries. These tissues were immediately snap frozen in liquid N_2 and stored at −80 °C. The preparation of tissues for measurement is outlined below and is based on previously published methods [7]. In brief, the left kidney was finely minced and was processed to isolate glomeruli and tubules. Glomeruli and tubules were separated by differential sieving with stainless steel mesh with pore sizes of 250–75 μm yielding 15–20,000 glomeruli/kidney. The aorta was homogenized by a Polytron homogenizer (Ultra-Tumax, Janke and Kunel, Staufen, Germany). The isolated renal glomeruli and tubules as well as the homogenized aorta were suspended in phosphate buffered saline (pH 7.4) followed by centrifugation of 15,000 rev/min for 30 min at 4 °C. Lipid extraction of the pellet was performed by addition of 5 ml of chloroform/methanol (2:1 vol/vol) followed by gentle shaking and standing overnight at 4 °C. The upper layer was removed and the pellet was washed three times each with methanol and distilled water. The pellet was then resuspended in 0.5 mol/l acetic acid and 1 mg/ml pepsin, incubated for 18 h at 4 °C to remove non-collagenous proteins, and washed twice with 0.1 mol/l calcium chloride, 0.02 mol/l Tris-HCl (pH 7.5) and 0.05 % toluene. No specific fluorescence could be detected in the supernatants at this stage of the extraction procedure. The pellet was then digested with type IV collagenase 0.1 mg/ml, (Sigma, St Louis, Mo., USA) and proteinase K, 0.1 mg/ml, (Sigma) by gentle shaking at 37 °C for 72 h and centrifuged at 15,000 rev/min for 30 min at 4 °C. The resultant supernatant was used to measure collagen-related fluorescence with excitation/emission at 370/440 nm. The fluorescence of an enzyme blank (type IV collagenase and proteinase K) was subtracted from the tissue fluorescence measurements. A colorimetric technique was used for the measurement of protein content [14] of the supernatants from each of the various tissue samples.

At 32 weeks, the renal cortex sorbitol content was measured by a specific radioisotopic assay [15]. Sorbitol was assayed in a two-step procedure which measured the amount of NADPH produced by sorbitol dehydrogenase.

Assessment of oxidative stress was carried out in plasma at 32 weeks by the measurement of thiobarbituric acid reactive substances (TBARS) [16]. In addition, TBARS were measured in renal homogenates from control and diabetic rats.

Serum lipid levels were measured at 32 weeks. Total cholesterol was assessed by an enzymatic colorimetric method

Table 1. Body weight and glycaemic control of rats studied

Group	(n)	Weight (g)	Glucose (mmol/l)	HbA_{1c} (%)
Control	12	715 ± 27*	7.8 ± 0.3*	2.1 ± 0.1*
Diabetic	10	403 ± 33	33.8 ± 2.9	4.7 ± 0.4
Diabetic + AG	11	442 ± 28	31.7 ± 4.8	4.8 ± 0.3
Diabetic + BHT	9	356 ± 27	36.3 ± 0.5	4.6 ± 0.3
Diabetic + prob	12	422 ± 36	33.3 ± 2.8	4.7 ± 0.3
Diabetic + pon	10	377 ± 44	33.2 ± 5.2	4.8 ± 0.5

Results are mean ± SEM for data at 32 weeks, [a] $p < 0.01$ vs all other groups. AG, aminoguanidine; BHT, butylated hydroxytoluene; prob, probucol; pon, ponalrestat

(Monotest, cholesterol and triglycerides GP-PAP; Boehringer Mannheim, Mannheim, Germany). HDL-cholesterol was measured following precipitation with polyethylene glycol 6000 [17].

Statistical analysis

Logarithmic transformation of urinary albumin data was performed to yield a normal distribution for this parameter. Comparison of normally distributed variables between the different groups during the study was performed by analysis of variance with or without repeated measures using the Statview SE Program (Brainpower, Calabasas, Calif., USA) on a Macintosh IIsi personal computer (Apple, Cupertino, Calif., USA). Comparisons between groups were performed by Fisher's least significant difference method. A p value of less than 0.05 was viewed as statistically significant. All data are shown as mean ± SEM unless otherwise specified.

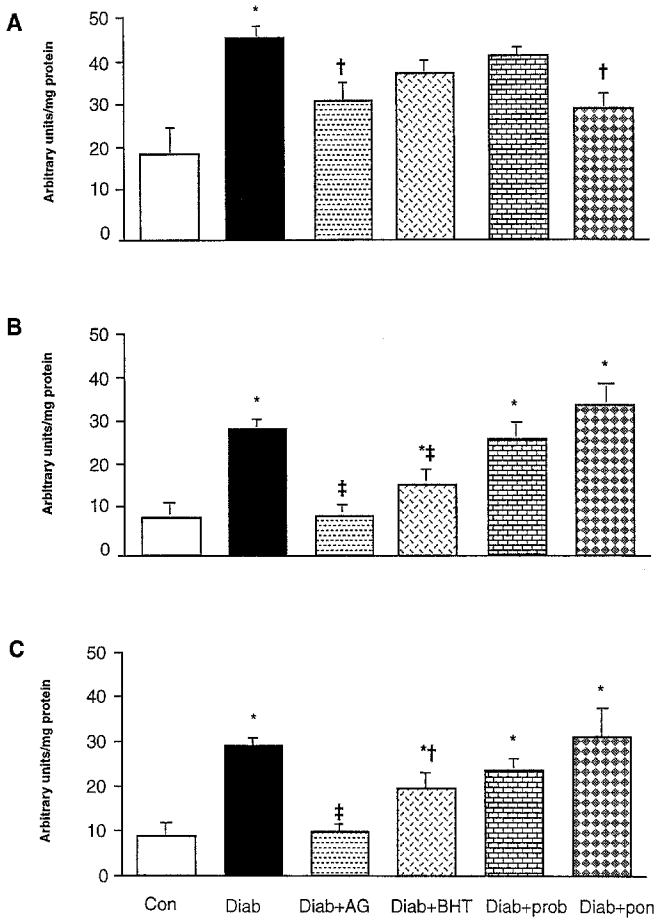

Fig. 1. (**A–C**) Fluorescence corrected for protein (arbitrary units/mg protein) in aorta (**A**), renal glomeruli (**B**), and renal tubules (**C**). Results are shown as mean ± SEM at 32 weeks in control (Con), diabetic (Diab), diabetic + aminoguanidine (Diab + AG), diabetic + butylated hydroxytoluene (Diab + BHT), diabetic + probucol (Diab + prob), and diabetic + ponalrestat (Diab + pon) rats. * $p < 0.01$ vs control; † $p < 0.05$; ‡ $p < 0.01$ vs diabetic

Results

Body weight and glycaemic control at week 32 are shown in Table 1. After 16 weeks diabetic animals had lower body weight than control animals [control, 644 ± 19; diabetic, 342 ± 36; diabetic + aminoguanidine, 385 ± 26; diabetic + BHT, 247 ± 27; diabetic + probucol, 321 ± 30; diabetic + ponalrestat, 282 ± 43 g, $p < 0.01$ control vs other groups]. At 32 weeks diabetic rats had significantly lower body weight, higher plasma glucose and higher HbA_{1c} levels when compared to control rats (Table 1). Treatment with aminoguanidine, probucol, BHT or ponalrestat did not influence any markers of glycaemic control or body weight in diabetic animals. Mean plasma glucose levels over the study period performed every 8 weeks were increased to a similar degree in all the diabetic groups [control, 7.4 ± 0.3; diabetic, 32.5 ± 2.4; diabetic + aminoguanidine, 32.5 ± 1.6; diabetic + BHT, 36.9 ± 1.0; diabetic + probucol, 36.4 ± 1.3; diabetic + ponalrestat, 34.2 ± 3.0 mmol/l, $p < 0.01$ control vs other groups]. A similar elevation in mean HbA_{1c} performed every 8 weeks was also observed in all the diabetic groups irrespective of treatment [control, 2.1 ± 0.02; diabetic, 4.5 ± 0.2; diabetic + aminoguanidine, 4.2 ± 0.2; diabetic + BHT, 4.6 ± 0.2; diabetic + probucol, 4.8 ± 0.2; diabetic + ponalrestat, 5.0 ± 0.4 %, $p < 0.01$ control vs other groups].

Tissue fluorescence data corrected for protein content in the aorta and kidney are shown in Figure 1. There was a significant increase in aortic fluorescence in diabetic rats. Aminoguanidine as well as ponalrestat therapy retarded the increase in aortic fluorescence in diabetic animals. Treatment of diabetic rats with probucol or BHT did not influence aortic fluorescence. In isolated renal tubules and glomeruli, fluorescence was significantly increased in diabetic animals. Treatment of diabetic rats with aminoguanidine totally prevented the increase in fluorescence observed in diabetic renal tubules and glomer-

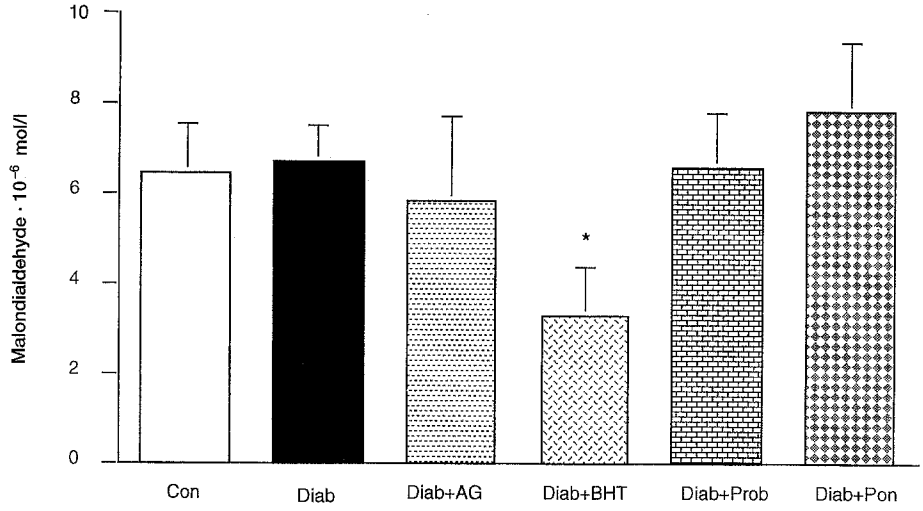

Fig. 2. Malondialdehyde content in plasma samples is shown for the various groups abbreviated as described in the legend to Figure 1. Results are shown as mean ± SEM at 32 weeks. * $p < 0.01$ vs diabetic

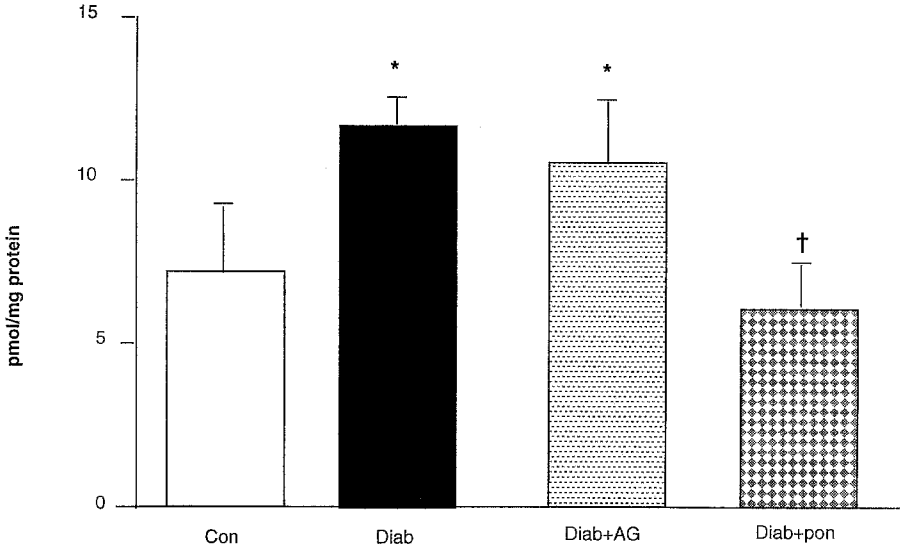

Fig. 3. Renal cortex sorbitol levels in control (Con), diabetic (Diab), diabetic + aminoguanidine (Diab + AG) and diabetic + ponalrestat (Diab + pon) rats. Results are shown as mean ± SEM at 32 weeks. * $p < 0.01$ vs control; † $p < 0.05$ vs diabetic

uli. BHT reduced, but did not normalise, renal fluorescence levels. In contrast to the effects in the aorta, treatment of diabetic animals with ponalrestat did not affect fluorescence in renal tubules or glomeruli. Probucol did not reduce renal fluorescence.

Oxidative stress was assessed by measurement of plasma TBARS as shown in Figure 2. Diabetes itself did not affect plasma TBARS, nor did treatment with aminoguanidine, probucol or ponalrestat. The only reduction in plasma TBARS was observed in diabetic rats receiving BHT. The levels of renal TBARS were similar in control and diabetic rats (control 2.8 ± 0.6 vs diabetic 2.9 ± 1.2 nmol malondialdehyde/mg protein).

Renal aldose reductase activity as assessed by renal cortex sorbitol levels, at 32 weeks, is shown in Figure 3. Renal sorbitol levels were significantly increased in diabetic rats. Treatment of diabetic animals with ponalrestat normalised renal sorbitol levels. Aminoguanidine therapy did not influence renal cortex sorbitol levels in the diabetic rats.

Diabetic rats had elevated total cholesterol (control, 1.53 ± 0.15; diabetic 2.83 ± 0.25 mmol/l, $p < 0.05$) and HDL-cholesterol (control, 0.87 ± 0.19; diabetic 2.16 ± 0.16 mmol/l, $p < 0.05$). Treatment of diabetic rats with probucol significantly reduced total cholesterol diabetic + probucol 2.37 ± 0.14 mmol/l, $p < 0.05$ vs diabetic) and the reduction in HDL-cholesterol approached statistical significance (diabetic + probucol 1.85 ± 0.12 mmol/l, $p = 0.055$ vs diabetic).

Urinary albumin data are shown in Figure 4. Diabetic animals had a progressive increase in urinary albumin excretion over the study period with levels elevated compared to control rats from week 8 onwards. Aminoguanidine (AG) retarded the rate of rise in albuminuria in diabetic rats ($F = 8.39$, $p = 0.014$, Diab vs Diab + AG; week 32 albuminuria; Diab, 30.9 vs. Diab + AG, 5.6 mg/24 h; geometric means shown) with urinary albumin excretion approaching but not reaching the levels observed in the control rats ($F = 4.9$, $p = 0.06$, Con vs Diab + AG, week 32 albu-

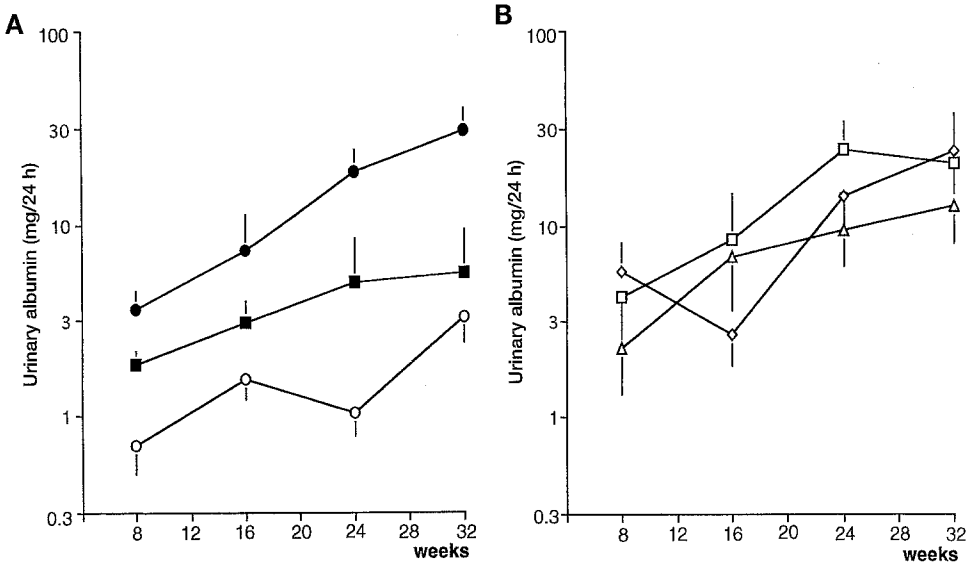

Fig. 4. (A, B) Serial data for albuminuria (y-axis, logarithmic scale) at 8-week intervals over 32 weeks are shown in the left panel (**A**) for control (○), diabetic (●) and diabetic + aminoguanidine (■) rats. In the right panel (**B**), data are shown for diabetic + probucol (◇), diabetic + ponalrestat (□) and diabetic + butylated hydroxytoluene (△) groups. Results are shown as geometric means and tolerance factors. Statistical differences among groups are as below: Con vs Diab, Diab + BHT, Diab + pon, Diab + prob, $p < 0.01$; Con vs Diab + AG, $p = 0.06$; Diab vs Diab + AG, $p = 0.014$; Diab vs Diab + BHT, $p = 0.09$; Diab vs Diab + pon, Diab + prob, NS; Diab + AG vs Diab + pon, $p = 0.02$; Diab + AG vs Diab + prob, $p < 0.01$; Diab + AG vs Diab + BHT, $p = 0.09$. (ANOVA with repeated measures)

minuria, Con 3.3 mg/24 h). Probucol and ponalrestat did not reduce urinary albumin excretion when compared to untreated diabetic rats (week 32 urinary albumin excretion, Diab + prob, 24.5; Diab + pon, 21.4 mg/24 h). Furthermore, aminoguanidine-treated rats had less albuminuria over the 32-week study period than either probucol- ($F = 12.5, p < 0.001$) or ponalrestat- ($F = 7.6, p = 0.02$) treated rats. The effects of BTH on albuminuria in diabetic rats were intermediate between untreated and aminoguanidine-treated rats (week 32; 12.9 mg/24 h, Diab + BHT vs Diab; $F = 2.7$, $p = 0.09$; Diab + BHT vs Diab + AG, $F = 2.69, p = 0.09$).

Discussion

The present study has confirmed that the diabetes-related increase in tissue fluorescence is prevented by an inhibitor of advanced glycation. Inhibition of oxidation and aldose reductase produced only minor effects on tissue fluorescence and on albuminuria, suggesting that these processes are not the major components in the pathogenesis of diabetic nephropathy.

An acceleration of the Maillard reaction as reflected by increased collagen-related fluorescence has been well-documented both in experimental [18, 19] and human diabetes [20, 21]. It has been suggested that the products of this reaction, AGEs, are involved in the pathogenesis of experimental diabetic nephropathy. Our previous study has shown a significant increase in collagen-related fluorescence over a 32-week period of streptozotocin diabetes. Aminoguanidine, an inhibitor of advanced glycation [7] prevented increases in fluorescence in aorta as well as in renal tubules and glomeruli. In addition, aminoguanidine was able to prevent structural as well as functional aspects of diabetic nephropathy in the same rats by preventing mesangial expansion and retarding the development of albuminuria, respectively. Since our initial studies a similar effect on albuminuria with aminoguanidine therapy has been observed in diabetic SHR [22] and diabetic Wistar rats [23]. It has also been shown that aminoguanidine therapy prevents increases in AGE products as assayed by a specific ELISA AGE assay [24]. Furthermore, aminoguanidine therapy has been observed to inhibit increases in haemoglobin-AGE in diabetic patients using a specific ELISA, consistent with its presumed action as an inhibitor of advanced glycation [25].

In the current study, aminoguanidine therapy did not have any effect on body weight, plasma glucose or HbA_{1c} in diabetic rats. After 32 weeks, rats receiving aminoguanidine had fluorescence levels similar to control rats in the aorta as well as in renal glomeruli and tubules. Throughout the 32-week study period, aminoguanidine was able to retard the diabetes-associated increase in urinary albumin excretion. However, aminoguanidine therapy did not affect renal cortex sorbitol levels in these rats, suggesting that aminoguanidine does not act as a potent aldose reductase inhibitor. These findings are confirmed by a recent study in which sorbitol levels in peripheral nerves were not affected by aminoguanidine treatment after 2 months of streptozotocin diabetes although there was an increase in nerve conduction velocity and a decrease in AGE levels in the same rats [26]. In contrast, previous studies have shown aminoguanidine to have aldose reductase inhibitory properties [27, 28] but the effects are weak and seen at concentrations much higher than would be observed in this study. Aminoguanidine was also not able to decrease oxidative stress as assessed by plasma TBARS after 32 weeks in diabetic rats. This suggests that aminoguanidine is not acting primarily as an antioxidant and confirms previous findings in diabetic rats [29]. In the latter

study, 16 weeks of aminoguanidine therapy did not affect oxygen free-radical indices as measured by conjugated dienes and hydroperoxides.

In contrast to aminoguanidine, ponalrestat was a potent aldose reductase inhibitor as evidenced by normalisation of renal cortex sorbitol levels in diabetic rats. After 3 weeks of treatment, ponalrestat has been shown to attenuate urinary albumin excretion in diabetic rats [30]. In another study, ponalrestat-treated diabetic Bio-Breeding (BB) rats had decreased proteinuria after 16 weeks of treatment but after 24 weeks, proteinuria was indistinguishable from levels in untreated diabetic rats [31]. In another study by Daniels and Hostetter [11], ponalrestat failed to show any effect on albuminuria in diabetic rats. In the present study, urinary albumin excretion was not ameliorated with ponalrestat, despite effective aldose reductase inhibition in the kidney. Interestingly, ponalrestat was able to prevent increases in collagen-related fluorescence in the aorta but not in glomeruli or renal tubules of diabetic rats. Previous long-term studies have also suggested a tissue-specific effect of aldose reductase inhibition on tissue fluorescence. For instance, aldose reductase inhibitors have been shown to retard development of fluorescence in the skin [2, 3] but not in the kidney [32] in diabetic rats. The inability of aldose reductase inhibition to attenuate fluorescence in the kidney while preventing increases in fluorescence in the aorta and skin may relate to differences in the predominant collagen in the various tissues. Kidney basement membranes contain type IV collagen whereas aorta and skin contain type I collagen [33, 34].

Collagen-linked fluorescence and pentosidine (an advanced glycation end product) have been shown to be essentially unresponsive to aldose reductase inhibition by sorbinil in the skin from galactosaemic rats whereas fluorescence was somewhat inhibited in the aortic tissue of the same rats [35]. The inhibition of aortic fluorescence in the galactosaemic rat is similar to the findings observed in the present study. However, the lack of an effect of aldose reductase inhibition on skin fluorescence in galactosaemic rats may be due to differences in the experimental models. Aldose reductase activity is significantly increased in the kidney of diabetic rats and ponalrestat reduces aldose reductase activity in the kidney as observed in the present study and by Ghahary et al. [36]. The lack of an effect on fluorescence in the kidney by ponalrestat despite effective aldose reductase inhibition may suggest that kidney fluorescence is unlikely to be related to the polyol pathway through fructosylation. Since there are well-documented beneficial effects of aldose reductase inhibition on the lens [37] and on experimental neuropathy [38], the present study provides further evidence that not all diabetic complications can be explained by a single pathogenic mechanism.

Recently, it has been suggested that diabetic complications may be partly attributed to oxidative stress [5, 39, 40] although clear-cut evidence of oxidative stress in relation to diabetes remains to be shown. It has been proposed that increased glycation may predispose proteins to oxidative damage [41]. The exact sequence of events in this interaction between glycation and oxidation remains to be ascertained, with the classical view being that the oxidative changes occur subsequent to the initial glycation of proteins [41]. In contrast, it has been suggested that oxidative mechanisms are already operative before formation of the amadori products [42]. Recently, Hunt and Wolff [43] have proposed that glucose forms an enediol radical anion under physiological conditions which is further oxidised to a ketoaldehyde. This "autoxidation" of glucose yields the free radicals hydrogen peroxide and hydroxy radicals. This reaction may be accelerated in diabetes and the above compounds may then mediate the reaction of glucose with membrane proteins. Products of such 'glucoxidation' reactions also participate in the process of advanced glycation and result in the formation of carboxymethyl lysine which does not fluoresce [44, 45] and pentosidine [46, 47] which does fluoresce. Other in vitro studies have confirmed a link between glycation and oxidation since the addition of glucose or glycated collagen catalysed the peroxidation of polyunsaturated lipid vesicles [48]. Therefore, the processes of advanced glycation and lipid peroxidation may interact with each other, each process yielding fluorescent products. In our study, the antioxidants butylated hydroxytoluene (BHT) [9, 10] and probucol, a lipid-lowering agent with anti-oxidant properties [49, 50] were evaluated for their effects on tissue fluorescence and oxidative stress in relation to the development of diabetic complications.

Treatment of diabetic animals with probucol did not influence body weight, glucose or HbA_{1c} levels. Probucol therapy did not affect fluorescence in either aorta or the kidney nor did it influence oxidative stress as measured by plasma TBARS. However, there was evidence of a biochemical effect of this drug since at 32 weeks probucol significantly reduced cholesterol and HDL-cholesterol levels. Previous studies have confirmed the lipid-lowering effects of probucol [8, 51] and its ability to reduce HDL-cholesterol in rodents [51].

In diabetic rats, there was no increase in plasma or renal TBARS. The specificity of this assay as a marker of oxidative stress remains controversial. Recently, a lack of an increase in renal TBARS has been observed by two other groups evaluating this parameter in diabetic rats [52, 53]. In the study by Parinandi et al. [52], TBARS were also measured in diabetic hearts and were actually reduced by about 50%. Anti-oxidant treatment with BHT reduced oxidative stress, as shown by decreased plasma TBARS. Fur-

thermore, there was a modest decrease in fluorescence and a possible anti-albuminuric effect. However, aminoguanidine was more effective in preventing increases in both parameters in experimental diabetes. This suggests that products of advanced glycation are the predominant source of fluorescence in the aorta and kidney. However, the partial effects of BHT on tissue fluorescence may indicate that oxidation accelerates the Maillard process. Nevertheless, without detailed characterisation of the nature of the fluorescent products in diabetic tissues, the contribution of oxidative products to the genesis of diabetes-associated fluorophores and albuminuria cannot be ascertained. The lack of an effect of probucol on plasma TBARS may indicate that BHT is a more effective anti-oxidant than probucol in this experimental model or that the dose of probucol was not optimized. Probucol therapy seemed to lower urinary albumin excretion for the first 16 weeks of treatment, but after this albuminuria approached levels similar to untreated diabetic rats. One possibility is that probucol had additional actions such as an anti-diabetic action [54]. This was not the case in the present study, since there was no evidence of lower blood glucose levels in the probucol-treated group. The failure of any of the other drugs to reproduce the effects of aminoguanidine, despite these agents acting as inhibitors of oxidative stress or aldose reductase, suggests that aminoguanidine is acting predominantly although probably not exclusively, by inhibition of advanced glycation. However, the recent in vitro data suggesting that aminoguanidine may be a nitric oxide inhibitor [55] indicate that other actions of aminoguanidine need to be investigated. It should be stressed that the present study has examined the in vivo effects of advanced glycation by indirect methods, using antioxidants and aldose reductase inhibitors as probes. Definitive analysis of the mechanisms involved in the in vivo action of advanced glycation awaits direct measurement of advanced glycation product levels.

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