Aminoguanidine inhibits the development of accelerated diabetic retinopathy in the spontaneous hypertensive rat

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Summary Arterial hypertension has been identified as a major secondary risk factor for diabetic retinopathy. However, the mechanisms by which hypertension worsens retinopathy are unknown. Inhibition of advanced glycation product formation prevents the development of experimental diabetic retinopathy in normotensive diabetic rats. In this study the effect of hypertension on the rate of diabetic retinopathy development and the formation of arteriolar thrombosis was evaluated. We also evaluated the effect of aminoguanidine, an inhibitor of advanced glycation end product formation on retinal pathology of diabetic hypertensive rats. After 26 weeks of diabetes, hypertension accelerated the development of retinopathy despite a lower mean blood glucose level than in the non-hypertensive group (diabetic spontaneous hypertensive rats (SHR) 16.00 ± 6.83 mmol/l; diabetic normotensive Wistar Kyoto rats (WKY) 34.9 ± 3.64 mmol/l; $p < 0.0001$). Diabetic SHR had nearly twice as many acellular capil-

laries as diabetic WKY (SHR diabetic: 91.9 ± 7.5 acellular capillaries per mm^2 of retinal area vs WKY diabetic: 53.7 ± 8.5 acellular capillaries per mm^2 of retinal area), and a 3.8-fold increase in the number of arteriolar microthromboses (SHR diabetic 23504 ± 5523 μm^2 vs SHR non-diabetic 6228 ± 2707 μm^2). Aminoguanidine treatment of SHR diabetic rats reduced the number of acellular capillaries by 50%, and completely prevented both arteriolar deposition of PAS-positive material and abnormal microthrombus formation. These data suggest that hypertension-induced deposition of glycated proteins in the retinal vasculature plays a central role in the acceleration of diabetic retinopathy by hypertension. [Diabetologia (1994) 37: 32–35]

Key words Diabetic retinopathy, rat model, hypertension, SHR, aminoguanidine, glycation.

Over the past few years, hypertension has been identified as a major secondary risk factor for diabetic microvascular complications [1–3]. Hypertension accelerates the progression of diabetic nephropathy, and antihypertensive treatment has been shown to reduce declining renal function even in normotensive diabetic subjects [4].

Systolic hypertension is also a risk factor for diabetic retinopathy [5–7] and in patients with unilateral carotid

artery stenosis, retinopathy is less pronounced in the affected eye [8].

The mechanisms by which hypertension worsens diabetic retinopathy are unknown. In non-hypertensive diabetic animals, inhibition of advanced glycation product formation (AGE) by aminoguanidine prevents the development of experimental diabetic retinopathy [9]. Since AGEs induce a procoagulatory state in endothelial cells in vitro [10], we hypothesized that increased deposition of glycated plasma proteins in the microvasculature of hypertensive diabetic animals would accelerate diabetic retinopathy by inducing microvascular thrombus formation.

In order to test this hypothesis, we examined the effect of aminoguanidine treatment on the retinal pathology of diabetic spontaneously hypertensive rats.

Table 1. Physical, metabolic and retinal morphometric parameters of non-diabetic and diabetic normotensive (WKY), spontaneously hypertensive rats (SHR) and aminoguanidine-treated diabetic SHR

Groups	WKY N	WKY D	SHR N	SHR D	SHR D-AG
Number	7	7	10	7	5
Blood glucose (mmol/l)	11.1 ± 1.5	34.9 ± 3.6	9.55 ± 1.0	16.0 ± 6.8	19.0 ± 10.9
Body weight (g)	459 ± 28	327 ± 54	440 ± 32	279 ± 79	241 ± 68
BP systolic (mm Hg)	119 ± 12	135 ± 13	171 ± 21	179 ± 17	157 ± 17
Microthrombosis (μm^2 of arteriolar area)	ND	ND	6228 ± 2707	23504 ± 5223	4689 ± 4102
Vascular PAS-pos. deposits	0– +	+ – + +	0– +	+ + – + + + +	0– + +
Microaneurysms (no of retinae affected)	0	2	0	5	0
Acellular capillaries (no/mm^2 of retinal area)	13.4 ± 4.1	53.7æ8.5	4.5æ0.6	91.9æ7.5	49.3æ14.5

ND, Not detectable; WKY N, non-diabetic Wistar-Kyoto rats; WKY D, diabetic Wistar-Kyoto rats; SHR N, non-diabetic spontaneous hypertensive rats; SHR D, diabetic spontaneous hyper- tensive rats; SHR D-AG, aminoguanidine-treated diabetic spontaneous hypertensive rats

Materials and methods

Animals

Male 6-week-old spontaneous hypertensive rats (SHR) weighing 75–90 g and their normotensive counterparts (Wistar Kyoto rats, WKY, Charles River, Wilmington, Mass., USA), were rendered diabetic by i. v. injection of 60 mg/kg body weight streptozotocin (Sigma, St. Louis, Mo., USA) after an overnight fast. SHR with blood glucose levels greater than 15 mmol/l at 1 week after injection were randomized to receive either no treatment, or 50 mg/kg body weight aminoguanidine HCl (Aldrich, Rochester, NY., USA) i. p. once daily.

Systolic blood pressure was 170–180 mm Hg in SHR, measured by indirect tailcuff plethysmography in a Gould apparatus (measurements 3–5 times within 1 day). Blood pressure was also measured after 12 weeks and at the end of the study, i. e. after 26 weeks.

Rats were fed a normal diet and water ad libitum. Weekly measurements of body weight and monthly blood glucose levels after an overnight fast were determined in triplicate (Beckman Glucose Analyser, Brea, Calif., USA). A detailed description of the study design has been given previously [11].

Retinal preparations

At the end of the study, the eyes of the animals of each study group (non-diabetic Wistar Kyoto rats (WKY N): $n = 7$; diabetic Wistar Kyoto rats (WKY D): $n = 7$; non-diabetic spontaneous hypertensive rats (SHR N): $n = 10$; diabetic spontaneous hypertensive rats (SHR D): $n = 7$; diabetic spontaneous hypertensive rats, treated with aminoguanidine (SHR D-AG): $n = 5$) were removed under deep anaesthesia and immediately fixed in 4 % buffered formalin.

After retinal isolation, the samples were washed in distilled water for 75 min. A combined digestion – 5 % pepsin in 0.2 % hydrochloric acid for 1 h, then 3 % trypsin in 0.2 mol/l Tris for 3.5 h

– was used to isolate the retinal vessel system. The preparations were placed on glass slides, washed with distilled water, air-dried and stained with periodic acid/Schiff reagent (PAS)/haematoxylin [9, 12]. One retina per animal was used for morphometric evaluation.

Microthromboses were quantitated by interactively measuring the arteriolar areas containing thrombotic material (i.e. homogeneous, fibrinous, weakly PAS-positive material without cellular elements) using an image analysing system with a morphometric software program (CUE 2; Olympus Opticals Europe, Hamburg, Germany) and the total for each retina was expressed as μm^2 of the vessel area.

Quantitation of AGE was performed as described previously [9] with the exception that a DMRXE microscope (Leica, Wetzlar, Germany) was used instead of an Aristoplan microscope.

Precapillary arteriolar PAS-positive deposits at locations corresponding to fields with accumulated AGEs were graded from 0 (none) to + + + + (very prominent) on the basis of staining intensity and size.

Measurements of AGEs, thromboses and PAS-positive deposits were restricted to the arteriolar sites of the retinal network because these changes did not occur in capillaries or venoles.

Acellular capillaries were quantitated by a modification of the method of Engerman and Kern [13]. Using a grid of 100 fields, 10 microscopic fields covering a total area of 6.76 mm^2 of retinal area were scored for the presence of acellular occluded vessels (integration ocular Olympus/400 × magnification). Each field containing acellular capillary segments was recorded as positive, and values were normalized to mm^2 of retinal area.

Retinae were scored for the presence or absence of saccular microaneurysms.

The numbers of endothelial cells and pericytes were counted in ten randomly-selected fields of each retina using the morphometry software program of the image analysing system. Cells were differentiated according to the criteria of Kuwabara and Cogan [12]. Fields covering an average of 45 000 μm^2 of capillary area per retina examined were selected to contain only capillaries (no larger vessels) at a relative capillary density of 20–35 % to avoid area underestimation by capillary overlap. The numbers

of both cell types were automatically corrected to the relative capillary density by the morphometric software program (CUE 2) (number of cell per mm^2 of capillary area). All morphometric evaluations were performed independently by two observers unaware of the identity of the samples being examined.

Statistical analysis

All parameters are given as mean ± SD. The significance of differences between groups was tested using one way analysis of variance and the Student-Newman-Keuls test [14].

Results

The physical, metabolic and retinal morphometric parameters of the rats studied are given in Table 1. After 26 weeks of diabetes, treated (SHR D-AG) and untreated diabetic (SHR D) animals had similar glucose levels (SHR D 16.0 ± 6.8 mmol/l vs SHR D-AG 19.0 ± 10.9 mmol/l; p NS). Mean blood glucose in WKY D was significantly higher (34.9 ± 3.6 mmol/l; $p < 0.0001$ vs SHR D).

Aminoguanidine treatment had no effect on the weight gain of the diabetic groups (SHR D 279 ± 79 g vs SHR D-AG 241 ± 68 g; p NS).

No difference in systolic blood pressure was found (SHR D 179 ± 21 mm Hg vs SHR D-AG 157 ± 17 mm Hg; p NS).

Multiple microthromboses in small, precapillary arterioles were observed in diabetic SHR, covering a total of 23 504 ± 5223 μm^2 of arteriolar area, in contrast to non-diabetic SHR (6228 ± 2707 μm^2; $p < 0.001$) and normotensive WKY diabetic rats, where microvascular thromboses were only found occasionally. Aminoguanidine treatment completely normalized microthrombus formation (SHR D-AG 4689 ± 4102 μm^2; p NS vs SHR N).

PAS-positive deposits were prominent (+ + − + + + +) in diabetic SHR, and 0 − + in SHR N. Precapillary arteriolar autofluorescence was 59.5 ± 4.2 arbitrary units (AU) in SHR N and 153 ± 19.5 AU in SHR D ($p < 0.0001$). Aminoguanidine treatment markedly reduced these deposits in corresponding regions of precapillary arterioles (0 − + +).

Retinae from diabetic SHR showed an almost 20-fold increase in acellular, occluded capillaries compared to non-diabetic SHR (SHR D 91.9 ± 7.5 vs SHR N 4.5 ± 0.6; $p < 0.0001$). Although the small number of acellular capillaries found in SHR N was less than the small number of acellular capillaries observed in WKY N, diabetes increased the number of acellular capillaries in hypertensive animals to a level that was nearly two times greater than in WKY. Aminoguanidine treatment in SHR reduced capillary closure by 50% (49.3 ± 14.5; $p < 0.001$ vs SHR D).

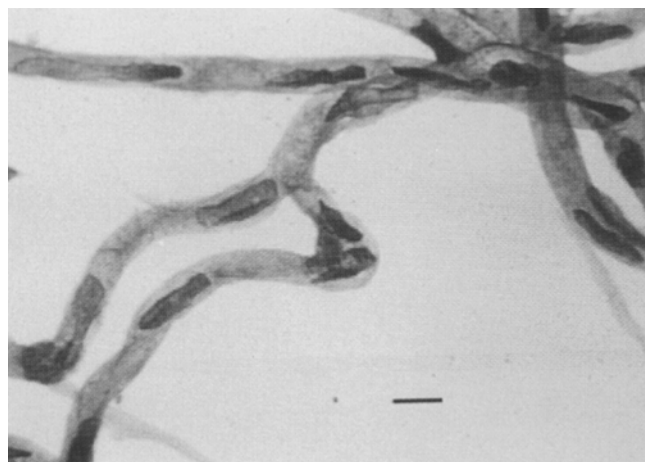Diabetes-typical microaneurysmic capillary lesions (Fig. 1) were observed in five of seven untreated

Fig. 1. Pepsin/trypsin digest of the retinal vasculature prepared from an untreated diabetic SHR showing a capillary microaneurysmatic lesion. Periodic acid-Schiff and haematoxylin stain (bar = 10 μm)

diabetic SHR. No such changes were present in non-diabetic or in aminoguanidine-treated diabetic SHR.

Quantitatively, hyperglycaemia induced a 27% increase in endothelial cell numbers (SHR D 3920 ± 93 vs SHR N 3090 ± 124; $p < 0.0001$) after 26 weeks and a 26% decrease in pericyte numbers (SHR D 1786 ± 35 vs SHR N 2255 ± 86; $p = 0.0003$). Aminoguanidine-treated diabetic SHR (D-AG) showed a significant reduction in endothelial proliferation compared to SHR D (SHR D-AG 2770 ± 74; $p < 0.0001$). Diabetes-associated pericyte loss also appeared to be less but was not significant at a level of 0.05 (SHR D-AG 1860 ± 41; p NS).

Discussion

This study provides evidence that coexisting hyperglycaemia and hypertension result in an acceleration of diabetic retinopathy when compared to non-hypertensive diabetic rats. Retinal changes observed in the diabetic SHR model are congruent with previous findings of Robison et al. [15] concerning vascular PAS-positive deposits, pericyte loss and endothelial proliferation.

The abundance of microthromboses in diabetic SHR suggests that hypertension significantly aggravates the procoagulatory vascular state that exists in diabetes as a result of endothelial cell surface changes induced by AGE-protein accumulation [16].

Hypertension-induced increases in deposition of AGE proteins, reflected by the prominent PAS-positive deposits in the vasculature of diabetic SHR, may play a central role in this process. Recent evidence supporting this hypothesis has been provided by Panagiotopoulos et al. [17] using streptozotocin-diabetic SHR and their non-diabetic and non-hypertensive diabetic

counterparts. They showed that the combination of diabetes and hypertension increases retinal vascular permeability by more than 80%, while diabetes or hypertension alone do so by only 26% and 34%, respectively.

Increased vascular plasma protein deposition and vascular occlusion such as that observed in the SHR model produce retinal neuroinfarcts that occur both in hypertensive and in diabetic primates and humans [18, 19].

Vascular AGE accumulation, confirmed as autofluorescence by measurement in the vasculature of SHR D, has been shown in vitro to promote increased vascular thrombogeneity via mechanisms including (i) a rapid suppression of thrombomodulin activity, which normally promotes the anticoagulatory activity of the protein C pathway and (ii) increased endothelial production of a tissue-factor like procoagulant [10, 20].

Inhibition of AGE formation by aminoguanidine prevented both accelerated diabetic retinopathy and thrombus formation without affecting hypertension.

The data presented in this report demonstrate that in rats as in humans, hypertension accelerates diabetic retinopathy. Further, hypertension is required to produce arteriolar thrombosis in diabetic animals. Both of these effects, which may be causally related, are prevented by the AGE inhibitor aminoguanidine. This result suggests that hypertension-induced deposition of AGE-proteins in the retinal vasculature play a central role in the acceleration of diabetic retinopathy by hypertension.

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