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Aminoguanidine does not inhibit the initial phase of experimental diabetic retinopathy in rats

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Summary We have previously shown that long-term administration of aminoguanidine, an inhibitor of advanced glycosylation product formation, reduces the extent of experimental diabetic retinopathy in the rat by 85 %. In order to determine whether the residual retinopathy that developed despite aminoguanidine was attributable to advanced glycation endproduct formation, a time-course study was performed in three different groups of male Wistar rats: non-diabetic controls (NC), streptozotocin-diabetic controls (DC) and streptozotocin-diabetic rats treated with aminoguanidine HCL, 50 mg/100 ml drinking water (D-AG). Eyes were obtained at 24, 32, 44 and 56 weeks of diabetes/treatment duration and morphologic evaluation was done on retinal digest preparations. At 56 weeks, retinal basement membrane thickness was additionally measured. After 24 weeks of diabetes, the number of acellular capillaries was significantly elevated in DC (44.6 ± 5.7/mm^2 of retinal area, NC 19.6 ± 4.9; $p < 0.001$) and increased continuously over time (DC 56 weeks 87.4 ± 15.1; $p < 0.001$ vs DC 24 weeks). In contrast, acellular capillaries in D-AG increased over the first 24 weeks and then remained constant for the rest of the study (D-AG 24 weeks 35.7 ± 5.18; $p < 0.01$ vs NC 24 weeks and NS vs DC 24 weeks; D-AG 56 weeks 42.0 ± 6.20; p NS vs D-AG 24 weeks). Diabetes-associated pericyte loss (DC 24 weeks 2310 ± 170/mm^2 of capillary area; NC 24 weeks 3120 ± 190; $p < 0.001$; DC 56 weeks 1570 ± 230; NC 56 weeks 2960 ± 50; $p < 0.001$) was significantly prevented by aminoguanidine after diabetic-like changes over the initial 24 weeks (D-AG 24 weeks 2450 ± 75; p NS vs DC 24 weeks; D-AG 56 weeks 2350 ± 90; $p < 0.001$ vs DC 56 weeks). At 56 weeks, aminoguanidine treatment was associated with a 67.4 % reduction in retinal basement membrane thickening. This time-course study demonstrates that aminoguanidine prevents the progression of experimental diabetic retinopathy, and suggests that non AG-inhibitable mechanisms are involved in the initial phase of diabetic retinopathy. [Diabetologia (1995) 38: 269–273]

Key words Diabetic retinopathy, rat model, aminoguanidine, glycation, retinal basement membrane.

Diabetic retinopathy is the most common microvascular disease affecting virtually every patient who has insulin-dependent mellitus diabetes for 15 years or longer [1]. Although the final clinical outcome greatly varies due to factors such as genetic determinants of individual susceptibility, hypertension and lipid status, the severity of retinopathy is associated with the degree of chronic hyperglycaemia [2–4]. From experimental as well as from clinical studies, there is evidence that the glycaemic history of the pre-complication period preceding overt diabetic retinopathy is a major determinant of the clinical outcome, indicating the relevance of hyperglycaemic memory in the pathogenesis of this complication [4–7]. The biochemical basis of this phenomenon may in-

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Abbreviations: NC, Non-diabetic controls; DC, diabetic controls; D-AG, diabetic rats treated with aminoguanidine; AG, aminoguanidine; AGE, advanced glycation end products; STZ, streptozotocin; PAS, periodic acid Schiff.

volve the formation and accumulation of advanced glycation products on long-lived extra- and intracellular molecules, leading to matrix crosslinks, altered cell-matrix interaction and DNA damage [8]. Thus, basement membrane thickening, a generally observed feature of diabetic vasculature, could result from vascular wall advanced glycation end-product (AGE) accumulation.

In support of the possible role of AGEs in the pathogenesis of diabetic retinopathy, it has been shown that long-term administration of aminoguanidine, an inhibitor of AGE formation, for 75 weeks reduces the extent of experimental diabetic retinopathy in the rat by 85 %. This effect is preceded by the inhibition of autofluorescence after 24 weeks [9].

In order to determine whether the residual retinopathy was due to AGE-formation, a time course study was performed in three different groups of rats investigating the effect of aminoguanidine treatment on histological parameters of retinopathy including basement membrane thickening in the streptozotocin (STZ)-diabetic rat model.

Materials and methods

Animals. Male Wistar rats (strain SPF 71, Hoechst-AG, Frankfurt-Hoechst, Germany) weighing 200 ± 2.6 g at the outset were used in this experiment. Diabetes was induced by iv injection of streptozotocin (STZ, 60 mg/kg body weight; Sigma, München, Germany). Rats were fed a regular laboratory rodent chow ad libitum and had free access to drinking water. Animals with blood glucose exceeding 15 mmol/l at 3 weeks after STZ-injection received either no treatment or 50 mg/100 ml drinking water aminoguandine-HCL (Riedel-de Haen, Hannover, Germany). Water consumption was identical in the treated and the untreated diabetic groups.

Blood glucose, body weight and water consumption were checked biweekly and glycated haemoglobin (Glyc Affin GHb, Isolab, DRG, Marburg, Germany) was measured at the time of killing.

Animals were killed at 24 weeks (non-diabetic rats (NC) $n = 6$, diabetic (DC) $n = 5$, diabetic treated (D-AG) $n = 5$), 32 weeks (NC $n = 5$, DC $n = 5$, D-AG $n = 6$), 44 (NC $n = 6$, DC $n = 6$, D-AG $n = 8$) and 56 weeks of diabetes (NC $n = 5$, DC $n = 8$, D-AG $n = 6$).

Retinal digest preparations. Retinae were obtained at the time points indicated above after enucleation of the eyes from the animals under deep anaesthesia and immediately fixed in 4 % buffered formalin.

Retinal vascular preparations were performed using a pepsin-trypsin digestion technique as previously described [9, 10]. Briefly, a combined pepsin (5 % pepsin in 0.2 % hydrochloric acid for 1.5 h) trypsin (2.5 % in 0.2 mol/l Tris for 15–30 min) digestion was used to isolate the retinal vasculature and the specimen were stained with periodic acid Schiff (PAS).

Acellular capillaries were quantitated by a modification of the method of Engerman and Kern [6]. 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.

The total number of pericytes was counted in ten randomly-selected fields of the retina using an image analysing system (CUE 2; Olympus Opt. Inc., Hamburg, Germany). Differentiation of pericytes from endothelial cells was performed according to the criteria of Kuwabara and Cogan [11]. Pericyte identification was facilitated using a polyclonal anti-vitronectin antibody (rb 69, kindly provided by Klaus Preissner, Max-Planck-Institut, Bad Nauheim, Germany).

The numbers of pericytes were normalized to the relative capillary density (numbers of cells per mm^2 of capillary area).

Additionally, retinae were scored for the presence or absence of microaneurysms.

Electron microscopy. At 56 weeks, three additional animals of each group (NC, DC, D-AG) were anaesthetized by i.p. injection of pentobarbital and whole body perfusion via the left ventricle into the ascending aorta ligated with a knob cannula using 2 % glutardialdehyde in phosphate buffer (pH 7.2, 0.135 mol) was performed. Eyes were removed after complete perfusion. Tissues were postfixed in phosphate-buffered 1 % osmium tetroxide, dehydrated through a series of graded ethanol and embedded in Spurr. Semi-thin sections were stained with methylene blue. Ultra-thin sections were mounted on 200 mesh Rb-coated copper grids, stained with uranyl acetate and lead citrate and viewed with a Philips EM 201 transmission electron microscope [12]. Photomicrographs of ten randomly-selected capillaries of the inner nuclear layer were taken at a magnification of 14 500 ×. The width of the retinal capillary basement membrane thickness was measured according to the method of Siperstein, using an image analysing system (Video Plan, Kontron, München, Germany) [13]. Basement membrane proportions in the external circumference of pericytes and between pericytes and endothelial cells were excluded from the examination.

Light and transmission electron microscopic morphometry were performed by two observers blinded to the identity of the samples.

Statistical analysis

All parameters are given as mean ± standard deviation. For statistical analysis, analysis of variance (ANOVA) and the Student-Newman-Keuls test was used [14].

Results

Blood glucose and body weight of treated and untreated rats were essentially identical throughout the study with a slight, but statistically significant, difference between aminoguanidine-treated and untreated diabetic rats at the end of the study. However, Amadori products remained identical and were significantly different from normal controls, as were blood glucose and body weight at each time point (Fig. 1). Mean blood glucose of all measurements was 25.73 ± 1.74 mmol/l in DC, 23.29 ± 3.42 mmol/l in D-AG and 5.48 ± 0.41 mmol/l in NC.

Retinal morphology of non-diabetic rats showed uniform capillary width, an equal distribution of capillary cells and only a few acellular capillaries. The number of pericytes per capillary area and the number of acellular capillaries did not change over time,

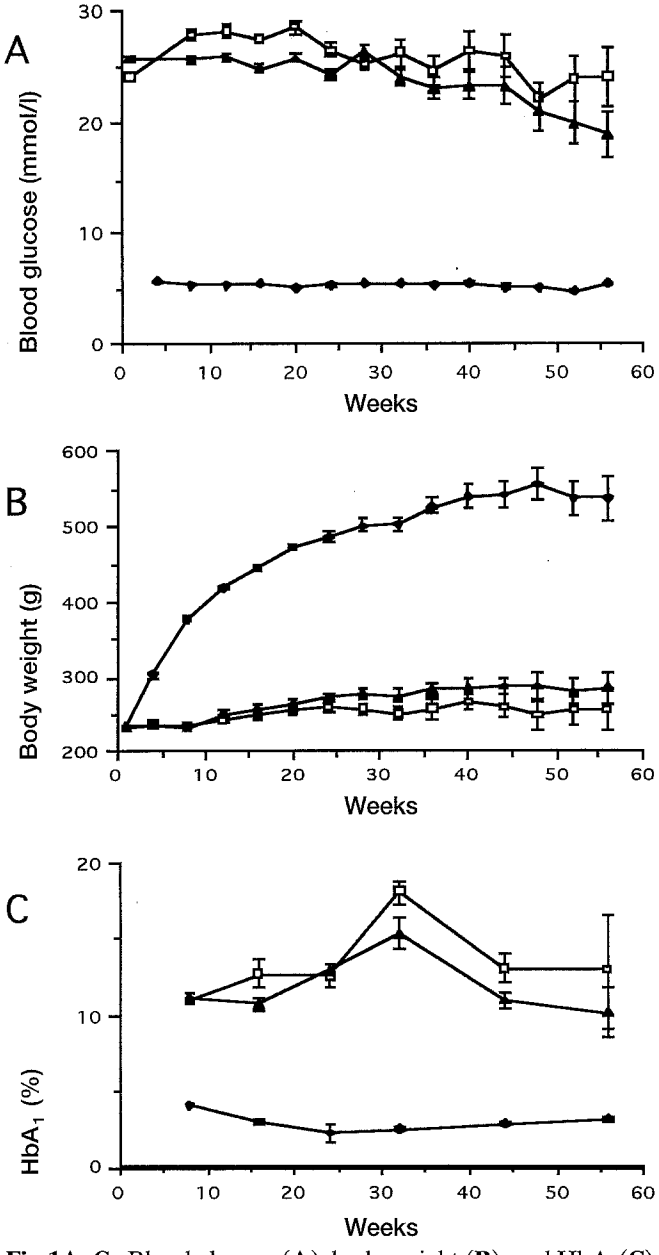

Fig. 1A–C. Blood glucose (**A**), body weight (**B**), and HbA_1 (**C**) in non-diabetic (NC, –◆–), diabetic (DC, –□–), and diabetic rats, treated with aminoguanidine (D-AG, –▲–). Results are shown as mean ± SD

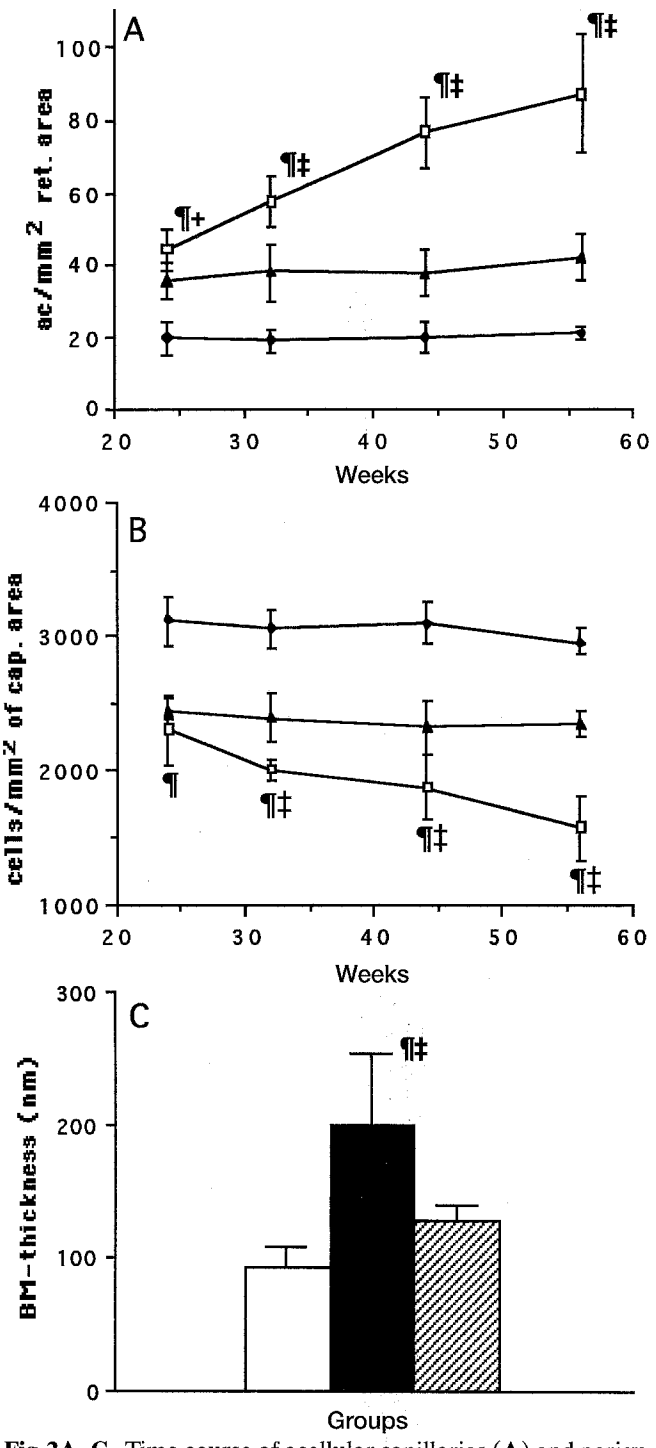

Fig. 2A–C. Time course of acellular capillaries (**A**) and pericytes (**B**) in normal (–◆–), diabetic (–□–) and aminoguanidine-treated diabetic rats (–▲–). Retinal basement membrane thickness (**C**) measured at the end of the study (56 weeks). ¶ $p < 0.001$ vs NC; + $p < 0.05$, ‡ $p < 0.001$ vs D-AG

indicating the absence of age-related retinal changes during the period studied. No microaneurysms were observed in the normal group.

In contrast, retinae from untreated diabetic animals progressively developed striking differences in their morphology compared to normal controls. Extended fields with capillary irregularities, cell loss and capillary occlusions occurred.

Quantitatively, after 24 weeks of diabetes, the number of acellular capillaries in DC was 44.6 ± 5.7 (NC 24 weeks 19.6 ± 4.9 in NC; $p < 0.001$) and increased continuously over time reaching 87.41 ± 15.1 after 56 weeks ($p < 0.001$ vs DC 24 weeks)(Fig. 2 a).

Determined only in areas where capillary occlusions or severe irregularities were absent, chronic hyperglycaemia induced a 47% pericyte loss from 2310 ± 170 after 24 weeks to 1570 ± 230 after 56 weeks while NC had 3120 ± 190 pericytes/mm^2 after 24 weeks and 2960 ± 100 after 56 weeks ($p < 0.001$ vs NC for each time point; Fig. 2 b).

Retinal capillaries of aminoguanidine-treated diabetic animals were strikingly less affected despite exposure to identical levels of chronic hyperglycaemia. Acellular capillaries in D-AG increased over the first 24 weeks to 35.7 ± 5.18 ($p < 0.01$ vs NC 24 weeks) and then remained constant for the rest of the time points studied (D-AG 56 weeks 42 ± 6.20; p NS vs D-AG 24 weeks, $p < 0.001$ vs DC 56 weeks; Fig. 2 a). Aminoguanidine treatment significantly suppressed the progressive pericyte loss after initial diabetic like changes over the first 24 weeks (D-AG 24 weeks 2450 ± 75; p NS vs DC 24 weeks; D-AG 56 weeks 2350 ± 90; $p < 0{,}001$ vs DC 56 weeks; Fig. 2 b).

A cumulative number of 14 out of 24 retinae (58.3 %) of the diabetic group was positive for at least one microaneurysm, the earliest being found after 32 weeks, whereas in aminoguanidine-treated animals only 2 of 25 rats (8 %) had developed such lesions ($p < 0.001$).

After 56 weeks of diabetes, a significant basement membrane thickening of the retinal capillaries was observed in the diabetic group compared to age-matched non-diabetic rats: 199.8 ± 55.2 nm vs 92.9 ± 15.7 nm ($p < 0{,}001$). Basement membrane thickening was significantly diminished by aminoguanidine treatment (127.8 ± 13.0 nm; $p < 0{,}001$ vs DC; Fig. 2 c).

Discussion

This study confirms that aminoguanidine prevents the progression of experimental diabetic retinopathy. The stabilizing effect of aminoguanidine on both pericyte loss and the formation of acellular capillaries at a level reached during the initial 24 weeks suggests either a two-component mechanism or a one-component mechanism consisting of an early and a late phase. The initial component appears to be non-inhibitable by aminoguanidine, whereas the second or late component does.

These time-related characteristics are congruent with essential biochemical features of non-enzymatic glycation [15]. Glucose reacts rapidly with amino groups of proteins forming Amadori products via reversible intermediates (Schiff's base). These early glycation products reach an equilibrium over a period of several weeks [16, 17]. The percentage of Amadori products as measured by HbA_1 in this study was elevated threefold in both diabetic groups irrespective of the treatment compared to the non-diabetic controls. This agrees with the well-known fact that aminoguanidine has no lowering effect on Amadori products [18]. Functional and structural consequences of early glycation on proteins have been extensively studied. Some of these changes have been directly linked to the pathogenesis of diabetic microangiopathy [19].

AGEs are formed from early products in the presence of O_2 through complex biochemical rearrangements involving highly reactive free radical intermediates such as 3-deoxyglucosone [20–22]. It is also known that Amadori products catalyse free radical formation which could subsequently cause direct tissue damage by oxidatively modified plasma constituents such as lipids [23].

In one study, aminoguanidine lacking chelating, reducing or antioxidant properties likewise did not affect glycation levels but effectively inhibited glucoxidation product formation, autofluorescence and collagen crosslinking [22]. Therefore, one general mechanism – non-enzymatic glycation – consisting of two components – formation of Amadori products and formation of AGEs – could underly the initiation of vascular changes in diabetic retinae. Aminoguanidine would intervene with the formation of AGEs at a point distal to the Amadori product formation.

The formation of AGEs is a function of time and glucose (Amadori-product) concentration. AGEs irreversibly accumulate on stable macromolecules forming cross-links and sequester proteins that are leaked through permeable vascular walls in diabetes leading to increased vascular wall thickness [24, 25]. Regular degradation mediated by AGE-receptor bearing macrophages is impaired because of reduced susceptibility of AGEs to enzymatic degradation which, in turn, leads to increased cytokine levels that may serve as additional growth-promoting signals [26, 27].

Finally, there is evidence from this study that vascular basement membrane thickening which is regarded as the ultrastructural hallmark of diabetic microangiopathy [28] is almost normalized by aminoguanidine treatment from a twofold increase in diabetic rats indicating a predominant role for AGEs in the pathogenesis of basement membrane thickening. This suggests that basement membrane thickening is primarily due to AGE formation.

Taken together, this study suggests that aminoguanidine treatment prevents the progression of experimental diabetic retinopathy, and suggests that non-aminoguanidine-inhibitable mechanisms such as Amadori products and reactive oxygen species are involved in the initial phase of diabetic retinopathy.

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