

## Aminoguanidine has an anti-atherogenic effect in the cholesterol-fed rabbit

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### Abstract

Advanced glycosylation endproducts (AGEs) which result from the non-enzymatic interaction of proteins and glucose are implicated in the vasculopathy of diabetes and aging. Since aminoguanidine (A) inhibits the accumulation of AGEs, we explored its effects on the development of atherosclerosis. Male New Zealand white cross rabbits fed a high cholesterol (1%) diet were randomized to control (C) or increasing doses of A treatment (25, 50 and 100 mg/kg A body weight). The animals were sacrificed after 12 weeks. Sudan IV was used to stain the lipid containing plaques of the aortic arch, thoracic and abdominal aorta and the surface area occupied by atheroma was assessed. Increasing doses of A treatment were associated with reduction in plaque formation in the aorta. At a dose of 100 mg/kg A, there was a 30, 49 and 48% reduction in plaque formation in the aortic arch, thoracic and abdominal aorta, respectively. There was a correlation between AGE levels and the degree of atheroma in these cholesterol fed rabbits (control,  $r = 0.75$ ,  $P < 0.01$ ; 100 mg/kg A,  $r = 0.59$ ,  $P = 0.02$ ). These data suggest that advanced glycation may participate in atherogenesis and raise the possibility that inhibitors of advanced glycation may retard this process. © 1998 Elsevier Science Ireland Ltd.

**Keywords:** Aminoguanidine; Glycation; Atherosclerosis; Cholesterol

### 1. Introduction

Advanced glycation endproducts (AGEs) are generated from a series of non-enzymatic chemical reactions involving the attachment of reducing sugars to proteins, lipids and nucleic acids [1]. The final reaction step, which is essentially irreversible, gives rise to AGE products which are chemically heterogeneous and incompletely characterized. Clinically, increased levels of AGEs are associated with aging, hyperglycemia and diabetic complications [1] and end-stage renal failure [2]. In experimental diabetes, the accumulation of fluorescent AGEs in the aorta from 16 week alloxan-di-

abetic rats was approximately 5 times greater than age-matched non-diabetic rats [3]. Furthermore, this accumulation of AGEs was prevented by aminoguanidine, an inhibitor of advanced glycation [3].

Numerous studies have implicated AGEs in the pathogenesis of atherosclerosis in addition to the micro- and macrovascular complications of diabetes. These actions of AGEs are thought to be mediated in part by endothelial [4] and macrophage [5] receptors for AGEs, one of which has been termed RAGE [4]. There is indirect evidence linking advanced glycation to atherogenesis, based on experiments which have shown that AGEs accumulate in the vascular wall and increase mechanical stiffness of collagen fibres [6], increase vascular permeability [7], inactivate nitric oxide [8], elicit a chemotactic response for monocytes [9], cause cytokine

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and platelet-derived growth factor release [10], induce the expression of vascular cell-adhesion molecule-1 in vivo [11] and in cultured human endothelial cells [12] and covalently trap low-density lipoprotein (LDL) [13].

Circulating lipoproteins, including the apolipoprotein and lipid moieties, are susceptible to advanced glycation [14]. Glycated LDL is thought to generate foam cells more readily than non-glycated LDL [15]. Furthermore, advanced glycated LDL behaves like oxidized LDL with respect to uptake and degradation by foam cells [16] and stimulation of platelet aggregation [17]. Combined glycation and oxidation ('glycooxidation') of lipoproteins also has the potential to form atherogenic products [18].

The role of AGEs in micro- and macrovascular disease is further supported by studies using aminoguanidine, a nucleophilic hydrazine derivative which inhibits advanced but not early glycation [3]. Aminoguanidine has been previously studied in animal models of diabetes and shown to prevent retinopathy [19], nephropathy [20] and neuropathy [21] without the normalization of hyperglycemia. These studies showed that aminoguanidine attenuated the diabetes-induced accumulation of tissue AGEs as assessed by measuring the specific fluorescence in tissue digests [19–21].

The aim of this study was to examine the effects of aminoguanidine on the development of atherosclerosis in the non-diabetic cholesterol-fed rabbit. This model has been used extensively in studies of atherosclerosis since it is characterized by a lipoprotein profile [22], histomorphology and distribution of plaques which resemble atherosclerosis in man.

## 2. Materials and methods

### 2.1. Pilot study

In a separate pilot study, 12 New Zealand White rabbits were randomly assigned to two groups (control or aminoguanidine treatment: 50 mg/kg body weight/day). All rabbits were fed a 2% cholesterol diet with or without aminoguanidine for 8 weeks. The aims of this study were to determine how the animals tolerated the diet, the effect of aminoguanidine on lipid peroxidation and to quantify the degree of absorption of aminoguanidine. Lipid peroxidation was assessed by measurement of plasma thiobarbituric acid-reactive substances (TBARS) [23] and plasma aminoguanidine levels were assayed colorimetrically [24].

### 2.2. Animals and study design

Studies were performed on 48 male New Zealand white rabbits aged 12 weeks which were obtained from Walter Meadows (Victoria, Australia). Following a 2

week stabilization period, rabbits aged 12 weeks were randomly assigned to four treatment groups and received 0, 25, 50 or 100 mg aminoguanidine/kg body weight, respectively. All animals were fed standard rabbit chow (Clarke King, Melbourne, Australia) enriched with 1% cholesterol (Sigma, St. Louis, MO) in 9% peanut oil with or without aminoguanidine (Alteon, Ramsey, NJ) for 12 weeks. Rabbits were housed individually and given 130 g of rabbit chow per day. All animal experiments were conducted in accordance with the guidelines of the Austin Hospital Ethics Committee.

### 2.3. Determination of lipid profiles

At weeks 0 and 12, rabbits were fasted overnight and 10 ml of blood was collected from the central ear artery for measurement of plasma cholesterol (total and HDL) and triglycerides. Plasma cholesterol (total and HDL) and triglycerides were analyzed using commercially available diagnostic kits (Sigma, St. Louis, MO). Phosphotungstic acid with magnesium was used to precipitate non-HDLs [25].

### 2.4. Tissue preparation

At the end of the treatment period, the rabbits were injected with an intravenous overdose of pentobarbitone (325 mg/kg body weight). The entire aorta, from the aortic arch to the iliac bifurcation, was excised and rinsed in 0.9% NaCl (4°C) before removing the adhering adipose and connective tissue. The surrounding adventitia was then carefully dissected away and the artery cut in half longitudinally; one half to be stained for plaque visualization and the other half to be assayed for tissue AGEs. One half of the aorta was fixed in 10% buffered formalin for 48 h. The atherosclerotic plaques were stained with Sudan IV [26] and mounted in Kaiser's Glycerol Jelly. The lesioned areas were directly traced from the mounted sections using an image analyzer (MCID, Imaging Research, Ont, Canada). A blinded observer, using computer-aided planimetry, determined the percentage of the total area occupied by Sudan positive plaques. Representative segments of atherosclerotic plaques were removed for examination by light microscopy. The remaining half of the aorta from five rabbits in each group was frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  for later analysis of tissue AGEs. Tissue AGEs were analyzed in five samples randomly chosen from the high dose group. Thawed tissues were finely minced prior to extraction of lipids by acetone/chloroform overnight at 4°C. Arterial AGEs were measured by competitive ELISA following lipid extraction (acetone/chloroform) and collagenase (type VII) digestion as previously described [27].

### 2.5. Statistical analysis

The effect of diet and treatment on body weight, plasma lipids and plaque formation were analyzed by analysis of variance. Variables before and after 12 weeks of treatment with aminoguanidine were compared by Student's paired *t*-test. A *P* value of <0.05 was considered significant. The data were analyzed using the StatView S.E. + Graphics Program (Abacus Concepts, Berkeley, CA) on an Apple Macintosh Quadra 605 (Apple, Cupertino, CA). Data are shown as mean  $\pm$  S.E.M. unless otherwise specified.

## 3. Results

### 3.1. Pilot study: plasma aminoguanidine and TBARS

The plasma aminoguanidine level in the treated group was  $9.9 \pm 1.9$   $\mu$ g/ml plasma, indicating that aminoguanidine was being absorbed to the systemic circulation. Plasma TBARS, expressed as relative absorbance to malondialdehyde, was not affected by aminoguanidine treatment ( $0.18 \pm 0.02$ , untreated versus  $0.19 \pm 0.03 \times 10^{-5}$  mol/l treated). These findings confirmed that aminoguanidine was adequately absorbed and that it did not have any significant anti-oxidant effects at the dose used in the pilot study.

### 3.2. Effect of diet and aminoguanidine on rabbit survival and weight

One of 12 rabbits from groups B (25 mg/kg A) and D (100 mg/kg A), and three of 12 rabbits from group C (50 mg/kg A) died during the study. This was attributed to starvation in the majority of cases. On post-mortem examination, fur balls found in the stomach may have accounted for loss of appetite in two rabbits. There were no signs of recent myocardial infarcts and there was no significant difference in the number of rabbits completing the study amongst the assigned treatment groups.

The rabbit body weights (Table 1) were similar in the treated and untreated groups both before and after the study period. There was no significant difference in the food intake among groups.

### 3.3. Changes in plasma lipids

The mean serum cholesterol and triglyceride levels increased significantly in all rabbits fed the atherogenic diet (*P* < 0.001). HDL-cholesterol decreased with administration of the atherogenic diet in both treated and untreated groups. However, there was no difference between animals receiving placebo or aminoguanidine with respect to total cholesterol, HDL-cholesterol or triglycerides at week 12 (Table 1).

### 3.4. Effect of diet and aminoguanidine on plaque formation

Extensive plaque formation was seen in the entire aorta following 12 weeks of feeding with a 1% cholesterol diet (Fig. 1). However, there were marked regional differences with respect to the distribution of plaques. The largest sudanophilic area was in the aortic arch ( $85.6 \pm 3.0\%$ ), which was at least double that seen in the thoracic ( $39.0 \pm 4.2\%$ ) or abdominal aorta ( $28.7 \pm 4.7\%$ ). Atherosclerotic plaque formation was significantly reduced by high dose aminoguanidine therapy in the aortic arch, thoracic and abdominal aorta. The percentage of the aortic arch occupied by sudanophilic lesions was  $59.8 \pm 6.1\%$  in the high aminoguanidine treatment group (*P* < 0.01). Furthermore, high dose aminoguanidine treatment halved plaque formation in the thoracic ( $19.6 \pm 2.8\%$ , *P* < 0.05) and abdominal aorta ( $14.7 \pm 4.8\%$ , *P* < 0.05). Plaque formation was not reduced with either of the lower doses of aminoguanidine.

### 3.5. Effect of diet and aminoguanidine on tissue AGEs

The level of tissue AGEs was higher in the aortic arch from untreated rabbits compared to the high dose aminoguanidine treatment group (Fig. 2). The effect of the high dose aminoguanidine on AGE formation was not observed in the thoracic or abdominal aorta. In the aortic arch, the lower doses of aminoguanidine were associated with a tendency to decreased AGE levels but this did not reach statistical significance (25 mg/kg A,  $0.77 \pm 0.28$ , 50 mg/kg A  $0.92 \pm 0.53$  arbitrary U/mg protein). There was no statistically significant effect of the 25 mg/kg and 50 mg/kg A doses of aminoguanidine

Table 1  
Body weight and serum lipids in the various treatment groups

	Control	25 (mg/kg)	50 (mg/kg)	100 (mg/kg)
(n)	(12)	(11)	(9)	(11)
<i>Body weight</i>				
Week 0	$3.3 \pm 0.1$	$3.5 \pm 0.1$	$3.4 \pm 0.1$	$3.4 \pm 0.1$
Week 12	$3.2 \pm 0.2$	$3.3 \pm 0.1$	$3.3 \pm 0.2$	$3.2 \pm 0.1$
<i>Cholesterol</i>				
Week 0	$2.2 \pm 0.2$	$1.9 \pm 0.1$	$1.7 \pm 0.1$	$1.9 \pm 0.1$
Week 12	$31.4 \pm 5.3$	$35.9 \pm 4.8$	$24.3 \pm 2.6$	$29.3 \pm 3.8$
<i>Triglyceride</i>				
Week 0	$1.2 \pm 0.2$	$1.2 \pm 0.1$	$1.2 \pm 0.1$	$1.1 \pm 0.1$
Week 12	$5.3 \pm 0.9$	$5.4 \pm 0.7$	$4.9 \pm 0.5$	$4.8 \pm 0.5$
<i>HDL-cholesterol</i>				
Week 0	$1.2 \pm 0.1$	$1.1 \pm 0.1$	$1.1 \pm 0.2$	$1.0 \pm 0.1$
Week 12	$0.5 \pm 0.1$	$0.3 \pm 0.1$	$0.5 \pm 0.1$	$0.5 \pm 0.1$

Values expressed as mmol/l.  
Data are shown as mean  $\pm$  S.E.M.

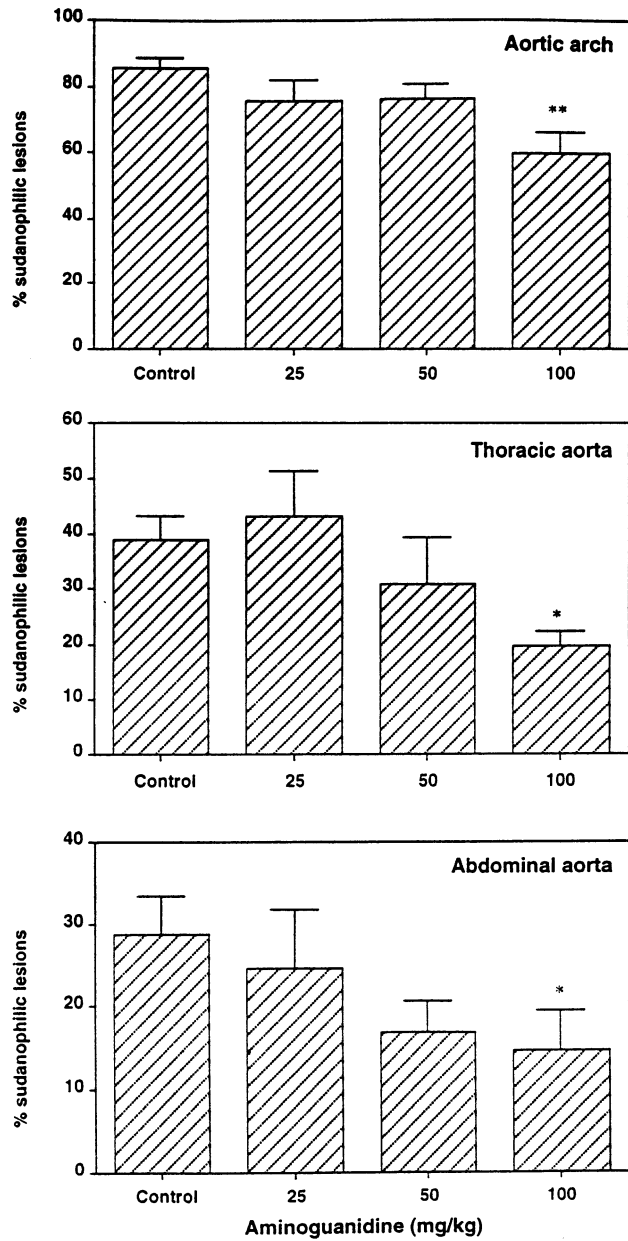


Fig. 1. Data are shown as mean ± S.E.M. for percentage sudanophilic lesions in the aortic arch (upper panel), thoracic aorta (middle panel) and abdominal aorta (lower panel). \*  $P < 0.05$ , \*\*  $P < 0.01$  versus control.

on AGE levels in thoracic or abdominal aorta (thoracic aorta 25 mg/kg A,  $0.51 \pm 0.12$ ; 50 mg/kg A,  $0.60 \pm 0.18$ ; abdominal aorta 25 mg/kg A,  $0.21 \pm 0.03$ ; 50 mg/kg A,  $0.18 \pm 0.01$  arbitrary U/mg protein).

In addition, the regional variation was similar to that observed for sites of atherosclerotic plaque formation, with the level of tissue AGEs in the aortic arch being three fold greater than in the abdominal aorta (Fig. 2). A positive correlation between tissue AGE levels and atherosclerotic plaque formation in both the control ( $r = 0.75$ ,  $P < 0.01$ ) and the high dose aminoguanidine group ( $r = 0.59$ ,  $P < 0.02$ ) was observed.

#### 4. Discussion

This study has demonstrated that administration of aminoguanidine, an inhibitor of AGE formation, reduces atherosclerotic plaques in the cholesterol-fed rabbit. The reduction in plaque formation by aminoguanidine observed in our study is comparable to previous papers which have used agents including lovastatin [28], perindopril [29] and nifedipine [30] to achieve reductions in plaque formation between 58–82% in the cholesterol-fed rabbit model.

Since the anti-atherogenic effect of aminoguanidine appears to be independent of hypolipidemic or antioxidant effects, the data raise the possibility that AGEs may play a role in atherogenesis.

Previous studies have examined the effect of aminoguanidine in experimental diabetes. In alloxan diabetic rats, treatment with aminoguanidine over 16 weeks prevented the diabetes-related increase in AGE formation and cross-linking of arterial wall connective tissue [3]. Functional and structural abnormalities associated with diabetic nephropathy in the streptozotocin rat, including albuminuria and mesangial expansion, were retarded by the administration of aminoguanidine

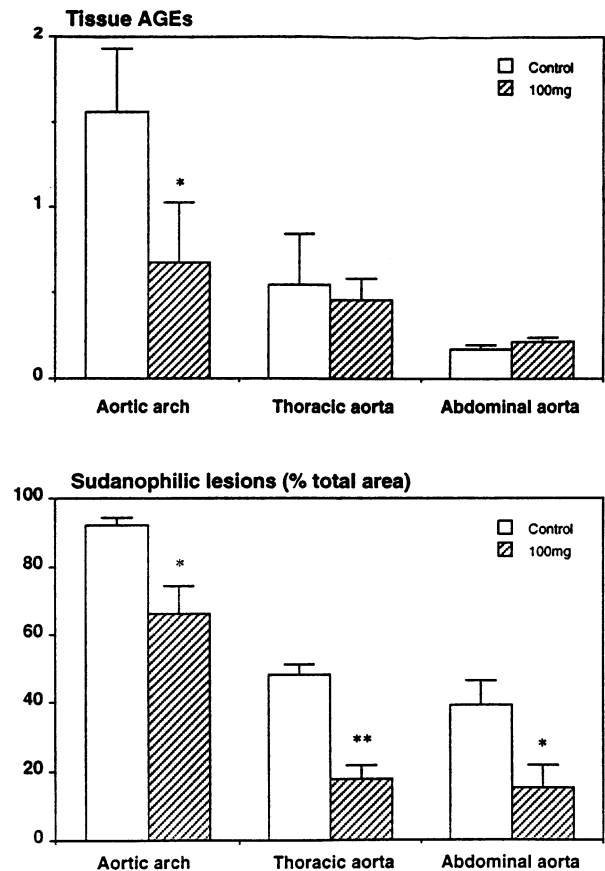


Fig. 2. Data are shown as mean ± S.E.M. for tissue AGEs (upper panel) and percentage sudanophilic lesions (lower panel). \*  $P < 0.05$ , \*\*  $P < 0.01$  versus control.

over 32 weeks [20]. Furthermore, AGEs injected into non-diabetic rats produced a significant increase in vascular permeability [31], glomerulosclerosis and albuminuria [32], which were prevented by aminoguanidine. In experimental diabetic retinopathy, aminoguanidine prevented the diabetes-induced increase in albumin vascular clearance [33], acellular capillaries and microaneurysm formation in both normotensive [19] and hypertensive rats [34]. These data are consistent with the concept that the ameliorating effects of aminoguanidine in diabetic angiopathy are mediated by prevention of AGE formation [1].

In non-diabetic animals, several authors have suggested an atherogenic role for AGEs and/or their receptors. In a recent study, AGEs were administered to non-diabetic Watanabe heritable hyperlipemic (WHHL) rabbits for 4 months which resulted in focal intimal proliferation and oil-red-O positive lipid deposits in the ascending aorta [11]. The intravenous administration of AGEs for 16 weeks in Sprague Dawley rats produced a marked mononuclear infiltration of the aortic wall [35]. AGEs have been extracted from non-diabetic human atherosclerotic plaques [36] and the levels increase in proportion to the severity of superficial plaques [37]. This is in contrast to levels of early glycation products which are similar in normal and atherosclerotic plaques in non-diabetic patients [15]. Immunohistochemically, AGEs have been localized in atherosclerotic plaques from euglycemic WHHL rabbits [38], diabetic [39] and non-diabetic subjects [40].

The precise mechanisms linking AGEs with atherogenesis may involve receptor dependent and independent pathways at both intra- and extravascular sites. Minor injury to the blood vessel wall may expose sub-endothelial AGEs, thereby promoting monocyte infiltration and initiating the development of an atherosclerotic lesion [35]. Alternatively, AGEs may initiate or exacerbate plaque formation by increasing vascular permeability [7], releasing growth factors and cytokines [10] or by increasing the expression of vascular adhesion molecules [11]. In the vasculature, the atherogenic effects of AGEs may be mediated via the integral membrane protein, RAGE [4]. Atherosclerotic plaques from diabetic and non-diabetic subjects [41] demonstrate increased expression of RAGE, as assessed by immunohistochemical techniques. Diabetes is associated with increasing non-enzymatic glycation of circulating apolipoproteins [42] and lipids [43]. This provides increased substrate for subsequent advanced glycation of lipoproteins raising the possibility that aminoguanidine may act not only on tissue AGEs but also on circulating AGEs. Therefore, inhibiting the formation of AGEs has the potential to prevent some of the above processes.

In this study, the anti-atherogenic effect of aminoguanidine cannot be accounted for in terms of a

lipid-lowering effect. The diet-induced hyperlipidaemia persisted throughout the treatment period, and was unaltered by aminoguanidine. By contrast, in a small group of diabetic subjects treated with aminoguanidine for 4 weeks, plasma total- and LDL-cholesterol were decreased by 19 and 28%, respectively [44]. This discrepancy may be due to variation in the degree of hypercholesterolemia and species differences in the two studies. It does not appear to be due to the dose of aminoguanidine used, since comparable plasma aminoguanidine levels were obtained in both studies.

Aminoguanidine did not appear to have direct anti-oxidant properties in this study since the level of plasma TBARS was similar in treated and untreated animals in the pilot study. One cannot exclude the possibility that the effects of higher doses of aminoguanidine may be partly related to inhibition of lipid peroxidation. This may be due to the lack of specificity or sensitivity of the assay used, since aminoguanidine has been shown to prevent the lipid oxidation of human LDL induced by AGEs *in vitro* [14]. Furthermore, glycated proteins generate free radicals [45] which can potentially oxidize lipids. Positive tissue staining for malondialdehyde following the infusion of AGE-albumin in non-diabetic Sprague Dawley rats supports the pro-oxidative effects of AGEs [46]. It has been suggested that lipid peroxidation may be involved in atherogenesis, since oxidized-LDL is cytotoxic to the endothelium and vascular smooth muscle cells [47] and stimulates the formation of foam cells [48]. It is therefore possible that the inhibition of AGE formation by aminoguanidine may have reduced lipid peroxidation in the sub-endothelial space, even though this was not reflected in plasma levels of TBARS. To further exclude that aminoguanidine is not acting as an inhibitor of oxidation, a more detailed biochemical assessment of lipid peroxidation including measurement of tissue oxidation products is required. Although aminoguanidine may act as an inhibitor of oxidation, in a recent study of experimental diabetic nephropathy, the antioxidants probucol and butylated hydroxytoluene failed to reproduce the effect of aminoguanidine [49].

Finally, it is possible that the antiatherosclerotic action of aminoguanidine is due to an entirely different mechanism. A recent study in cholesterol-fed WHHL rabbits showed that incubation of atherosclerotic blood vessels with aminoguanidine *in vitro* restored the impaired contractile responsiveness to phenylephrine [50]. This beneficial effect of aminoguanidine was attributed to the inhibitory action of aminoguanidine on nitric oxide synthase (NOS) activity [51] rather than advanced glycation. Although NOS activity was not measured in our study, previous papers have documented an increase in the activity of the inducible form of NOS in aortas from atherosclerotic compared to normal rabbits

[50,52]. It is therefore possible that aminoguanidine prevents plaque formation by decreasing NOS activity *in vivo*. However, two lines of evidence argue against this being a significant role. Firstly, chronic administration of L-arginine, the precursor of nitric oxide, prevents plaque formation [53] whereas neointimal plaque formation is accelerated by the NOS inhibitor N<sup>G</sup>-nitro-L-arginine when administered to cholesterol-fed rabbits [54]. Secondly, AGEs quench nitric oxide *in vitro* and *in vivo* [8,31,55]. The significance of nitric oxide in the mechanism of action of aminoguanidine therefore awaits further studies.

Although glucose is necessary for glycation, the formation of AGEs is not confined to the diabetic state. Biochemical and morphometric studies have identified increased levels of AGEs in human atherosclerotic plaques from diabetic [39] and non-diabetic [40] blood vessels. Our study has shown that aminoguanidine decreases the formation of atherosclerotic plaques in a dose-dependent manner, in the non-diabetic cholesterol-fed rabbit model. There was a correlation between increased AGE levels and sites of atheroma formation, suggesting that glycation may contribute to the pathogenetic process of atherogenesis. However, in the aortic arch, a high dose of aminoguanidine was associated not only with a reduction in plaque formation but also with a decrease in AGE formation, suggesting a link between inhibition of AGE products and the development of atherosclerosis. However, this association was not evident at other sites within the aorta.

Since it has previously been shown that diabetes is a state of increased AGE production, as assessed by collagen-related fluorescence in the rat aorta [3,20], it is possible that the anti-atherosclerotic effect of aminoguanidine might be enhanced in the diabetic-hypercholesterolemic model. The clinical relevance of our results is not known but additional studies are warranted in diabetic and non-diabetic models to assess the effect of aminoguanidine on the development of atherosclerosis and to further elucidate its anti-atherogenic mechanism of action.

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