Effect of Aminoguanidine on Lipid Peroxidation in Streptozotocin-Induced Diabetic Rats

Sung-Hee Ihm, Hyung Joon Yoo, Sung Woo Park, and Jahei Ihm

Diabetes mellitus is postulated to be associated with increased lipid peroxidation, which may contribute to vascular complications. One potential mechanism of the increased lipid peroxidation in diabetes is lipid-linked advanced glycosylation and oxidation. Aminoguanidine (AMGN), the prototype inhibitor of advanced glycosylation end product (AGE) formation, has been recently shown to prevent oxidative modification of low-density lipoprotein (LDL) in vitro at a moderate concentration. It is unknown whether AMGN may act as an antioxidant against lipid peroxidation under hyperglycemia in vivo. To investigate the in vivo effect of AMGN on lipid peroxidation in diabetes, we administered AMGN (1 g/L in drinking water) or vitamin E (400 mg/d for 5 d/wk) to streptozotocin (STZ)-induced diabetic rats for 9 weeks and measured plasma lipid hydroperoxides by ferrous oxidation with xylenol orange II (FOX method) and red blood cell (RBC) membrane malondialdehyde (MDA) and related aldehydes as thiobarbituric acid–reactive substances (TBARS). Plasma lipid hydroperoxide was higher in STZ-induced diabetic rats versus control rats (mean ± SD, 7.53 ± 2.03 *v* 5.62 ± 0.44 μmol/L, $P < .05$; n = 8 to 14). RBC membrane TBARS were also higher in STZ-induced diabetic rats than in control rats (2.67 ± 0.46 *v* 1.81 ± 0.19 nmol/mL, $P < .05$). Plasma lipid hydroperoxide was lower in AMGN-treated (6.23 ± 0.59 μmol/L, $P < .05$) and vitamin E–treated (5.29 ± 0.27 μmol/L, $P < .05$) diabetic rats than in untreated diabetic rats. RBC membrane TBARS were also lower in AMGN-treated (1.93 ± 0.12 nmol/mL, $P < .05$) diabetic rats than in untreated diabetic rats. There was no significant difference in plasma glucose, cholesterol, and triglyceride levels among diabetic groups. Although the mechanism(s) of action of AMGN on lipid peroxidation in vivo should be studied further, these results suggest that AMGN may have an additional beneficial effect as an antioxidant against lipid peroxidation in a prevention trial for diabetic vascular complications.

SINCE THE FIRST REPORT by Sato et al[1] of an increase in lipid peroxidation products in the plasma of diabetic patients, numerous studies have shown that lipid peroxidation is enhanced in the plasma and tissues of diabetic subjects.[2] Lipid peroxidation in plasma lipoproteins and in the cellular membrane, which may contribute to the development of vascular disease in diabetes, is thought to reflect the increased oxidative stress in diabetes.[3,4] Mechanisms that contribute to increased oxidative stress in diabetes include increased nonenzymatic glycosylation and autoxidative glycosylation.[5-8]

Hyperglycemia causes the formation of nonenzymatically derived glycation and dehydration-condensation complexes known as advanced glycosylation end products (AGEs). AGEs have been proposed to promote vascular disease by generating oxidizing intermediates and inducing oxidant stress in vascular cells.[9-11] AGE oxidation plays an important role in initiating lipid oxidation of low-density lipoprotein (LDL) in diabetes.[9] In vitro studies have shown that the incubation of lipids with glycosylated proteins resulted in an elevation of lipid peroxidation.[10] The interaction of AGEs with their cellular receptor leads to oxidant stress, manifested by the appearance of lipid peroxidation products.[11] Thus, increased glycation of tissue and plasma proteins in diabetes may stimulate the oxidation of lipids, which may in turn stimulate autoxidative reactions of sugars, enhancing damage to both lipids and proteins in the circulation and vascular wall.[4]

The importance of AGEs in the development of diabetic vascular complications has been supported by experiments with aminoguanidine (AMGN), the prototype inhibitor of AGE formation.[12-14] AMGN has been shown to inhibit both AGE formation and the development of vascular complications in diabetic animal models. The mechanism of action of AMGN is known to be the entrapment of reactive dicarbonyl intermediates formed from the oxidation of enediol rearrangements of Amadori products. Recently, AMGN has been shown to prevent oxidative modification of LDL directly in vivo at a moderate concentration.[15,16] It is unknown whether AMGN may act as an antioxidant against lipid peroxidation in vivo.

In this study, to investigate the in vivo effect of AMGN on lipid peroxidation in diabetes, we administered AMGN or vitamin E to streptozotocin (STZ)-induced diabetic rats for 9 weeks and measured plasma lipid hydroperoxides by ferrous oxidation with xylenol orange II (FOX method) and red blood cell (RBC) membrane malondialdehyde (MDA) and related aldehydes as thiobarbituric acid–reactive substances (TBARS).

MATERIALS AND METHODS

Induction of Experimental Diabetes

Male Sprague-Dawley rats aged 10 weeks and weighing 220 to 280 g were used. They had free access to water and standard laboratory diet pellets. Diabetes was induced by a single intraperitoneal dose of STZ 50 mg/kg body weight (Sigma, St Louis, MO) dissolved in citrate buffer (pH 4.5). Control rats (n = 13) were intraperitoneally injected with citrate buffer. Of 32 diabetic rats, 10 were selected at random to receive AMGN (Sigma) 1 week after STZ injection. AMGN was supplied in the drinking water at a concentration of 1 g/L for 9 weeks.[17] Eight diabetic rats were also selected at random to receive vitamin E (Sigma) 1 week after STZ injection. Vitamin E was administered orally at a dose of 400 mg/d for 5 d/wk for 9 weeks.[18] Ten weeks after STZ injection, heparinized blood samples were collected from the retro-orbital venous

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sinus under ether anesthesia. Plasma and RBCs were separated by centrifugation at 2,000 × *g* for 15 minutes at 4°C. The plasma glucose level was measured by a glucose oxidase method. Plasma cholesterol and triglyceride levels were measured using enzymatic procedures (Sigma).

Measurement of Plasma Lipid Hydroperoxides

Whole-plasma lipid hydroperoxides were determined using the FOX assay.[19,20] Briefly, 90-μL aliquots of plasma were transferred to six microcentrifuge vials; 10 μL 10-mmol/L triphenylphosphine (TPP) in methanol was added to three vials to remove lipid hydroperoxides, and 10 μL methanol was added to the other three vials. All vials were then vortexed and incubated at room temperature for 30 minutes prior to addition of 900 μL FOX reagent (250 μmol/L ammonium ferrous sulfate, 100 μmol/L xylenol orange, 25 mmol/L H_2SO_4, and 4 mmol/L butylated hydroxytoluene [BHT] in 90% vol/vol methanol in a final vol of 100 mL). After incubation at room temperature for a further 30 minutes, the vials were centrifuged at 12,000 × *g* for 10 minutes. Absorbance of the supernatant was then determined as a function of the mean absorbance difference for samples with and without elimination of lipid hydroperoxides by TPP. Ammonium ferrous sulfate, H_2SO_4, BHT, xylenol orange, and TPP of the highest purity available were purchased from Sigma. The coefficient of variation for individual samples with this method was 4.5%.

Measurement of TBARS in RBC Membrane

MDA and related aldehydes in RBC membrane were determined by the TBA method.[21] Aliquots of 0.2 mL from packed-RBC samples were mixed thoroughly with 0.8 mL phosphate-buffered saline and 0.025 mL BHT solution (88 mg BHT/10 mL absolute alcohol). After adding 0.5 mL 30% trichloroacetic acid, the samples were placed on ice for 2 hours and then centrifuged at 2,000 × *g* at 25°C for 15 minutes. One milliliter of supernatant was mixed with 0.075 mL 0.1-mol/L EDTA and 0.25 mL 1% TBA (Sigma)/0.05N NaOH and placed on boiling water for 15 minutes. Absorbance was determined at 532 and 600 nm (A_{532} and A_{600}). The concentration of lipid peroxidation products (nanomoles per milliliter of packed cells) was calculated as $A_{532} - A_{600}$ using the extinction coefficient for the MDA-TBA complex of 1.56×10^5 $mol^{-1} \cdot cm^{-1}$.

Statistical Analysis

Data are expressed as the mean ± SD. Selected comparisons were performed with Student's *t* test, ANOVA, and linear regression. A *P* values less than .05 was considered statistically significant.

RESULTS

Plasma Glucose, Cholesterol, and Triglycerides

Plasma glucose, cholesterol, and triglyceride levels were higher in STZ-induced diabetic rats versus control rats (Table 1). There was no significant difference in plasma glucose, cholesterol, and triglyceride levels among diabetic groups.

Table 1. Body Weight and Plasma Glucose, Cholesterol, and Triglyceride Levels at 10 Weeks

Group	No. of Rats	Weight (g)	Plasma Level (mmol/L)		
			Glucose	Cholesterol	Triglyceride
Control	13	450 ± 20	7.0 ± 0.2	2.1 ± 0.1	1 3 ± 0.3
STZ	14	295 ± 18*	20.3 ± 1.7*	4.0 ± 0.4*	5.0 ± 0.8*
STZ + AMGN	10	297 ± 27*	19.1 ± 2.1*	4.1 ± 0.5*	4.9 ± 1.1*
STZ + vitamin E	8	290 ± 37*	19.8 ± 3.0*	4.9 ± 1.0*	5.8 ± 1.1*

NOTE. Values are the mean ± SD.
*$P < .05$ *v* control.

Effects on Plasma Lipid Hydroperoxides

Plasma lipid hydroperoxide levels were higher in STZ-induced diabetic rats compared with control rats (7.53 ± 2.03 *v* 5.62 ± 0.44 μmol/L, $P < .05$). There was no significant correlation between plasma lipid hydroperoxides and plasma cholesterol in control ($r = .06$, $P = .42$) or STZ-induced diabetic ($r = .12$, $P = .31$) rats. There also was no significant correlation between plasma lipid hydroperoxides and plasma triglycerides in control ($r = .09$, $P = .33$) or STZ-induced diabetic ($r = .20$, $P = .15$) rats. Plasma lipid hydroperoxides were significantly lower in AMGN-treated (6.23 ± 0.59 μmol/L) and vitamin E–treated (5.29 ± 0.27 μmol/L) diabetic rats versus untreated diabetic rats (Fig 1).

Effects on TBARS in RBC Membrane

RBC membrane TBARS were also higher in STZ-induced diabetic rats compared with control rats (2.67 ± 0.46 *v* 1.81 ± 0.19 nmol/mL packed cells, $P < .05$). RBC membrane TBARS were significantly lower in AMGN-treated (1.93 ± 0.12 nmol/mL packed cells) and vitamin E–treated (1.85 ± 0.12 nmol/mL packed cells) diabetic rats versus untreated diabetic rats (Fig 2).

DISCUSSION

Diabetes is postulated to be associated with increased lipid peroxidation, which may contribute to the vascular complications of diabetes.[4] By-products of lipid peroxidation, such as TBARS and conjugated dienes, are increased in RBC membranes[21] and synaptosomal membranes in diabetic subjects.[22] Our results show that RBC membrane TBARS were higher in STZ-induced diabetic rats compared with control rats (Fig 2). It has been pointed out that the TBA assay unaccompanied by high-performance liquid chromatography lacks specificity when applied to plasma, because plasma contains many substances that can react in the TBA assay.[23] In the present study, plasma total lipid hydroperoxide levels were specifically measured using the FOX assay with TPP authentication of the signal. With this method, Nourooz-Zadeh et al[19,20] reported that plasma lipid hydroperoxide levels are substantially higher in diabetic patients compared with control subjects. Our results show that the plasma lipid hydroperoxide level was higher in STZ-induced diabetic rats versus control rats (Fig 1).

There are many possible explanations for the increased lipid peroxidation observed in diabetic rats, one of which may be an increase in peroxidizable lipids due to altered lipoprotein metabolism in the diabetic state. In this study, there was no significant correlation between plasma cholesterol or triglyceride and plasma lipid hydroperoxide levels in control or STZ-induced diabetic rats. This finding is consistent with the result obtained by Nourooz-Zadeh et al[19] in studying diabetic and nondiabetic humans. These results suggest that plasma lipid hydroperoxide levels may not be a simple function of total lipids, although we cannot exclude the possibility that hyperlipidemia might contribute to lipid peroxidation to some degree in diabetes via an increased availability of peroxidizable lipids. Also, since we did not measure any lipid parameter in RBCs, we could not standardize the RBC MDA level per lipid parameter.

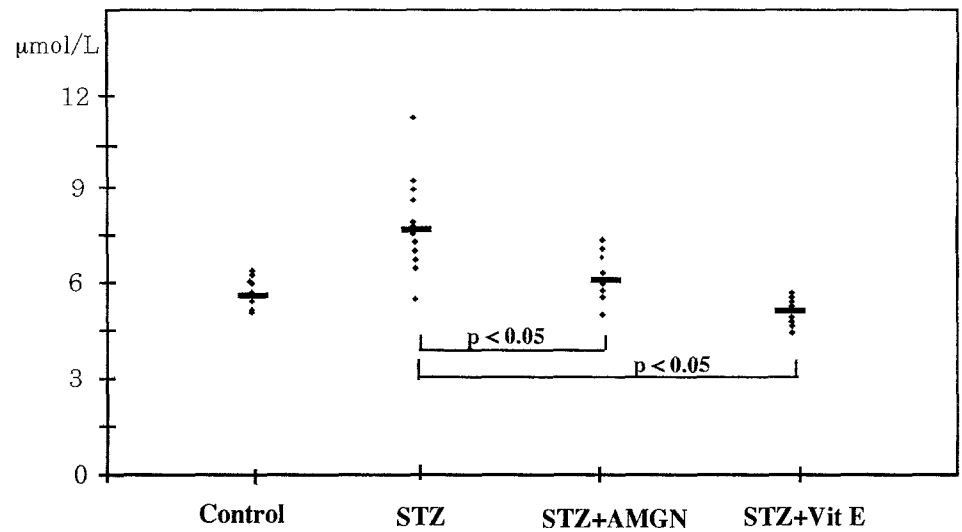

Fig 1. Plasma lipid hydroperoxides at 10 weeks. Vit, vitamin.

These are limitations of our study, although it has been reported that the RBC MDA level is higher in diabetic subjects versus control subjects regardless of whether the MDA level is calculated per milliliter of packed cells, per micromole of RBC phospholipid, or per number of cells.[21]

One of the factors contributing to the increased lipid peroxidation in diabetic subjects may be the higher rate of glycosylation. Nonenzymatic glycation of proteins is closely linked to oxidative processes.[5-7] AGEs have been shown to generate oxidizing intermediates and to induce oxidant stress in vascular cells in vitro.[9-11] However, it has not been studied as to whether AGEs can contribute to increased oxidative stress in diabetes in vivo. In our study, diabetic rats treated with AMGN for 9 weeks showed a significantly lower plasma lipid hydroperoxide level and RBC membrane TBARS than untreated diabetic rats (Figs 1 and 2). This result is in concordance with a recent study showing that the vitreous lipid peroxide level in diabetic rabbits is decreased by AMGN treatment.[24] In our study, there was no significant difference in plasma cholesterol and triglyceride levels between AMGN-treated and untreated diabetic rats. The results that the inhibitor of AGE formation reduced lipid peroxidation in vivo in diabetes without a change in plasma lipid levels supports the view that increased nonenzymatic glycosylation contributes to increased oxidative stress in diabetes.

The structure of AMGN does not resemble that of traditional antioxidants. Nevertheless, recent studies have shown that AMGN prevents the increase of oxidant-induced lipid peroxides in rat retinal cell cultures,[24] and that AMGN prevents oxidative modification of LDL directly in vitro at a moderate concentration.[15,16] These results suggest that AMGN is capable of inhibiting the oxidative modification of lipids by trapping reactive breakdown products of lipid peroxidation such as aldehydes and by acting as an antioxidant.[16,24] The actual concentration of AMGN in the various tissues in which oxidation may occur is unknown. Thus, whether AMGN may have localized antioxidant effects on lipid peroxidation in vivo is unknown. The AMGN-induced inhibition of lipid peroxida-

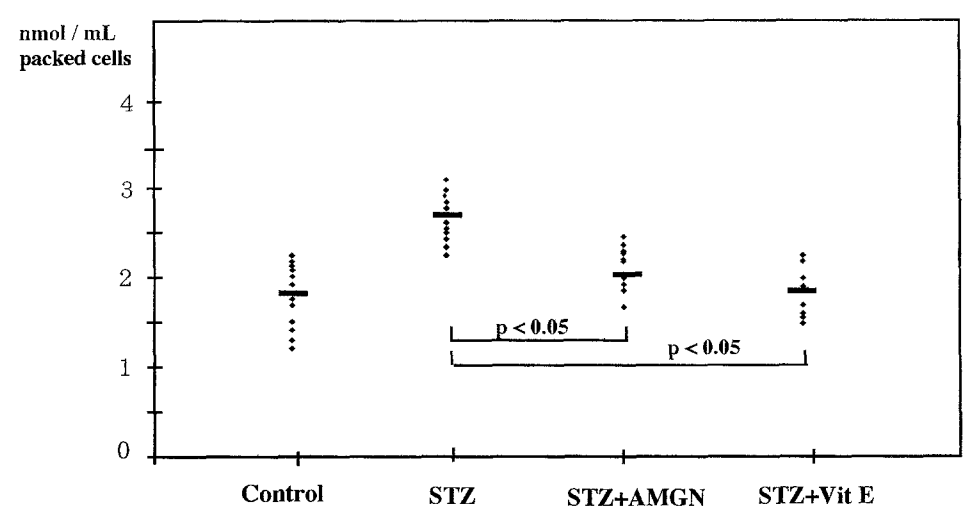

Fig 2. RBC membrane TBARS at 10 weeks. Vit, vitamin.

tion in plasma and RBC membranes observed in this study may have resulted from the inhibition of AGE formation and the resultant reduction of free-radical formation,[25] from the trapping of reactive breakdown products of lipid peroxidation, or from direct antioxidant action.[16] We could not determine which was the major mechanism in this study.

In addition, AMGN is a well-known selective inhibitor of inducible nitric oxide (NO) synthase.[26] The reaction between NO produced by endothelial cells and superoxide anion yields peroxynitrite, which is itself a poweful oxidizing agent capable of initiating LDL oxidation in vitro.[27] In diabetes, increased glucose and free fatty acid concentrations may stimulate endothelial cells and macrophages to secrete NO and superoxide, resulting in increased peroxynitrite formation and increased lipid peroxidation.[3,28] Thus, we cannot exclude the possibility that inhibition of NO production by AMGN may have contributed to the inhibition of lipid peroxidation observed in this study.

In our study, diabetic rats treated with vitamin E, a potent free-radical scavenger, for 9 weeks showed significantly lower plasma lipid hydroperoxides and RBC membrane TBARS than untreated diabetic rats (Figs 1 and 2). This result is in concordance with the findings from previous clinical studies that administration of vitamin E to diabetic patients results in decreased LDL oxidizability[29] or plasma MDA[30] without a significant change in blood glucose levels.[29-31] Although AMGN was shown to have antioxidant activity at a higher concentration, it was also demonstrated that a low concentration (0.01 mmol/L) of AMGN promoted lipid peroxidation.[16] The prooxidant activity of AMGN can be reduced by pretreatment of LDL with vitamin E in vitro.[16] These observations suggest that combination therapy with AMGN and vitamin E may provide potent antioxidant activity in vivo.

In conclusion, we observed that AMGN in vivo reduces lipid peroxidation in the plasma and RBC membrane, which is enhanced in diabetes. Although the finding that an inhibitor of AGE formation reduced lipid peroxidation in diabetic rats suggests that increased nonenzymatic glycosylation contributes to increased oxidative stress in diabetes, the mechanism(s) of action of AMGN on lipid peroxidation in vivo should be studied further. Whatever the mechanism, these results suggest that AMGN may have an additional beneficial effect as an inhibitor of lipid peroxidation in a prevention trial for diabetic vascular complications.

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