Aminoguanidine and the Effects of Modified LDL on Cultured Retinal Capillary Cells

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PURPOSE. Compared with normal low density lipoprotein (N–LDL), LDL minimally modified in vitro by glycation, minimal oxidation, or glycoxidation (G–, MO–, GO–LDL) decreases survival of cultured retinal capillary endothelial cells and pericytes. Similar modifications occurring in vivo in diabetes may contribute to retinopathy. The goal of this study was to determine whether low concentrations of aminoguanidine might prevent cytotoxic modification of LDL and/or protect retinal capillary cells from previously modified LDL.

METHODS. Minimal in vitro modification of LDL (3 days, 37°C) was achieved with glucose (0, 50 mM), under antioxidant conditions (for N–LDL, G–LDL), or under mild oxidant conditions (for MO–, GO–LDL) in the presence/absence of aminoguanidine (0, 1, 10, 100 μM). Glucose and aminoguanidine were then removed by dialysis. Confluent bovine retinal capillary endothelial cells (n = 13) and pericytes (n = 14) were exposed to LDL (100 mg/l) for 3 days, with and without aminoguanidine (100 μM) in media. Cell counts were determined by hemocytometer.

RESULTS. A decrease in cell counts after exposure to modified compared with N–LDL was confirmed (P < 0.001) but was significantly mitigated if LDL had been modified in the presence of aminoguanidine (P < 0.001). Aminoguanidine was as effective at 1 μM as at the higher concentrations. Aminoguanidine (100 μM) present in culture media conferred no additional protection, and showed slight evidence of toxicity. Aminoguanidine present during LDL modification had no effect on measured glycation or oxidation products, or on LDL oxidizability.

CONCLUSIONS. Very low concentrations of aminoguanidine mitigate toxicity of LDL exposed to stresses that simulate the diabetic environment. This action may contribute to the beneficial effects of aminoguanidine observed in experimental diabetic retinopathy. (Invest Ophthalmol Vis Sci. 2000;41:1176–1180)

In a previous study,1 we demonstrated that compared with unmodified low density lipoprotein (normal LDL, N–LDL) in vitro glycated, minimally oxidized, and glycoxidized LDL (G–LDL, MO–LDL, GO–LDL) reduce cell counts and protein in cultured retinal capillary endothelial cells and pericytes. Slight increases in glycation and/or oxidation of LDL are present from the onset of diabetes and, thus, could contribute to early retinopathy. We now investigate whether micromolar concentrations of aminoguanidine can mitigate these effects, if present either during LDL modification or subsequently during exposure of cells to modified LDL.

Aminoguanidine inhibits the development2 and progression3 of experimental diabetic retinopathy in streptozocin-diabetic rats and also the development of atherosclerosis,4 diabetic nephropathy,5 neuropathy,6 and cataracts.7 Its effects are attributed to inhibition of advanced glycation reactions8 and/or inhibition of nitric oxide synthase (NOS)9 or other enzymes.

In its effects on advanced glycation, aminoguanidine scavenges reactive carbonyl intermediates formed by free radical oxidation of carbohydrates, fructoselysine (FL, the early protein glycation product), and lipids (reviewed in Reference 10). Reactive carbonyls damage proteins, phospholipids, and other macromolecules.10 In diabetes, carbonyl formation is enhanced by increased availability of substrate (glucose, FL, lipoproteins), perhaps increased susceptibility of lipoproteins to oxidation, perhaps increased “oxidative stress.”10,11 These considerations led to a “carbonyl stress hypothesis” for the development of diabetic complications, proposing that glycemic and oxidative stresses combine to determine complication risk.10,11 One carbonyl-derived product in proteins is Nε-carboxymethyllysine (CML),12 whose concentration is measured in this study in apoB, the apolipoprotein of LDL.

The other established action of aminoguanidine is enzyme inhibition. In intact cells, both inducible and constitutive NOS (in neurons) are 50% inhibited by 6 to 10 μM and 100 μM aminoguanidine, respectively.9 In retinal capillaries, inducible NOS is present in both endothelial cells and pericytes; constitutive NOS is found in endothelial cells only.13 It is also possible that aminoguanidine could inhibit an LDL-associated enzyme, or protect it from inactivation by glycoxidative stress.

In this study, to maintain pathophysiological and pharmacological relevance, we used very mildly modified LDL and low...
(micromolar) concentrations of aminoguanidine (i.e., levels at or below those in plasma and tissues of aminoguanidine-treated animals and humans).

**METHODS**

Aminoguanidine hydrochloride was a gift from Alteon (Northvale, NJ). All other reagents were obtained from Sigma Chemical (St. Louis, MO) unless otherwise specified.

**LDL Isolation**

Seven separate preparations of pooled normal human LDL were used. For each, 180 ml venous blood was drawn into EDTA (4.2 mM final concentration) from each of a group (5 or 6 volunteers) of normolipemic, nondiabetic, healthy humans (none taking medications or vitamin supplements). The study was approved by our Institutional Review Board at the Medical University of South Carolina, and informed consent was obtained from all volunteers. Plasma was pooled and LDL (1.019 < d < 1.063 g/ml) isolated by sequential ultracentrifugation as described previously. To maintain antioxidant conditions, all salt solutions used to adjust solvent densities and dialysis buffer solutions contained 270 mM (0.01% wt/vol) EDTA, and dissolved oxygen content was minimized in these solutions by purging with nitrogen before use. Pooled LDL samples were divided into 15 aliquots for in vitro modification.

**In Vitro Modification of LDL**

The 13 aliquots of LDL were incubated concurrently (3 days, 37°C, pH 7.4, in phosphate-buffered saline with EDTA, 270 μM) to prepare normal LDL (N-LDL, control incubation) and 12 different modified preparations: G-LDL, MO-LDL, and GO-LDL each prepared in the presence of 0, 1, 10, and 100 μM aminoguanidine (logistics precluded incubation of N–LDL with aminoguanidine as above) were incubated as described for MO–LDL (aminoguandine as above) were incubated as for N–LDL except under air rather than nitrogen and without added DTPA. To obtain only minimal oxidation, EDTA was not removed. To obtain only minimal oxidation, EDTA was not removed.

**Control Incubation (N-LDL).** One aliquot of LDL was incubated under nitrogen with added diethylenetriaminepentacetic acid (DTPA; 1 mM) to enhance antioxidant conditions.

**In Vitro Glycation (G-LDL).** Four aliquots of LDL (aminoguanidine concentrations of 0, 1, 10, and 100 μM) were incubated for N-LDL, but with added α-glucose (final concentration 50 mM).

**In Vitro Minimal Oxidation (MO-LDL).** Four aliquots of LDL (aminoguanidine as above) were incubated as for N-LDL except under air rather than nitrogen and without added DTPA. To obtain only minimal oxidation, EDTA was not removed.

**In Vitro Glycoxidation (Glycation and Minimal Oxidation Combined; GO-LDL).** Four aliquots of LDL (aminoguanidine as above) were incubated as for MO-LDL, but with added α-glucose (50 mM final concentration).

After incubation, samples were dialyzed against NaCl (150 mM) containing EDTA (270 μM) under nitrogen at 4°C to remove glucose, aminoguanidine, and DTPA if present. Protein content was determined by the method of Lowry, as previously described: it was typically approximately 5 g/l.

**LDL Characterization**

Fructoseamine and CML in LDL were determined by gas chromatography/mass spectrometry as described previously. Thiobarbituric acid–reacting substances (TBARS) were measured spectrophotometrically, as described previously. Susceptibility of LDL to in vitro oxidation was measured by a modified Esterbauer technique, as described previously. Briefly, after removal of EDTA by chromatography (Sephadex G25, medium, column; Pharmacia, Piscataway, NJ) and filtration to remove aggregates, LDL was diluted to 100 mg/l cholesterol (Sigma) with phosphate-buffered saline, which had been purged with oxygen for 10 minutes immediately beforehand. Oxidation was initiated by 5 μM CuCl₂ (37°C), and conjugated diene formation was monitored by measuring absorbance (234 nm; model DU 650 spectrophotometer; Beckman, Fullerton, CA), as described previously.

**Culture of Bovine Retinal Capillary Endothelial Cells and Pericytes**

Bovine globes were obtained from a slaughterhouse, and retinal capillary endothelial cells and pericytes were cultured as described previously. Endothelial cells exhibited typical morphology, positive staining for factor VIII (Atlantic Antibodies, Stillwater, MN), and ability to incorporate fluorescein-labeled acetylated LDL (DiI-Ac-LDL; Biomedical Technologies, Stoughton, MA). Pericytes stained positively for 3G5 ganglioside, negatively for factor VIII, and did not incorporate DiI-Ac-LDL.

**Protocol**

Thirteen experiments were performed with endothelial cells, using two different pooled LDL preparations and three different cell batches. Fourteen experiments were performed with pericytes, using seven different pooled LDL preparations and five different cell batches. Within each experiment, cells were exposed to 26 different conditions: the 13 LDL preparations described above, each studied with and without aminoguanidine (100 μM) in culture media.

For each experiment, cells were plated into four (12-well) plates (Costar, Cambridge, MA) and grown to confluency. One plate was used at day 0 for baseline determinations. Growth medium in the remaining three plates, among which 26 of the 36 wells were occupied, was replaced by serum-free medium containing 1% albumin and supplemented with the various LDL preparations (100 mg/l LDL protein ± aminoguanidine (0 or 100 μM)). After 3 days' incubation, medium was aspirated, and cell counts were determined. Using baseline values for each experiment (defined in figure legends), values at day 3 were expressed as a percentage of those at day 0.

**Cell Counting**

For counting, cells were trypsinized, resuspended in 100 μl growth medium to which 200 μl 1% trypsin blue solution was added. Using a hemocytometer, total and viable cells in four 0.1 μl volumes were counted. The total of these four cell counts was multiplied by 750 to estimate cells/well. Counting was performed by a single observer unaware of sample identity. Intra-assay coefficients of variation were obtained by assessing reproducibility of cell counts from replicate wells at day 0 and averaging 6.1% and 5.5% for endothelial cells and pericytes, respectively. Because 80% to 90% of cells were viable, and because the conclusions obtained from analyses of total and viable cell counts were essentially identical (except as detailed below), we present only “total cell count” data (termed “cell counts”).
Cell Counts, % Day 0 Values

A

N-LDL  G-LDL  MO-LDL  GO-LDL

0  20  40  60  80  100

B

[AG] (umol) in Modifying Incubation

0  10  100

Statistical Analysis

SAS (Cary, NC) and Sigma Stat (SPSS, Chicago, IL) statistical software were used. In individual experiments, cell counts on day 3 were expressed as percentages of day 0. Significant overall differences according to cell type were identified by MANOVA. Subsequently, for each cell type, significant overall differences were evaluated according to LDL modification, presence of aminoguanidine in LDL-modifying incubation, and presence of aminoguanidine in cell culture medium, using a three-way MANOVA. If the MANOVA yielded positive results, differences within individual modification groups were evaluated using the Tukey multiple comparison procedure. Hypotheses were tested at the 0.05 level of significance.

RESULTS

Cell Culture Studies

A global MANOVA demonstrated differing responses between the two cell types (P < 0.001), which were therefore analyzed separately. For both cell types, a subsequent three-way MANOVA identified significant differences in cell counts attributable to type of modification of LDL (e.g., normal, glycated) independent of aminoguanidine (P < 0.0001) and absence/presence of aminoguanidine in the LDL-modifying incubation (0, 1, 10, 100 μM; P < 0.001; Fig. 1). The presence of 100 μM aminoguanidine in cell culture media caused a slight but significant reduction in pericyte counts (P < 0.02), but the effect did not reach significance for endothelial cells (Fig. 1). Permissible comparisons were then performed using the Tukey method for multiple comparisons:

Modification of LDL. In both cell types, exposure to any of the modified LDL significantly reduced cell counts compared with N-LDL; also GO-LDL was more toxic than G-LDL (P < 0.05, multiple comparisons, Tukey method, Fig. 1). Generally, the toxicity of modified LDL increased as follows: G-LDL < MO-LDL < GO-LDL, as previously observed.1

Effect of Presence/Concentration of Aminoguanidine in LDL-Modifying Incubation. For endothelial cells, 1 or 10 μM aminoguanidine in the modifying incubation significantly increased cell counts compared with 0 aminoguanidine (P < 0.05, Fig. 1A). For pericytes, this effect was seen with 10 or 100 μM aminoguanidine (Fig. 1B). When the three levels of aminoguanidine were directly compared, no differences were observed among them, and each abolished the significance of reductions in cell numbers after exposure to modified LDL.

Aminoguanidine in the Cell Culture Medium. Aminoguanidine (100 μM) in cell culture media resulted in a small but significant overall reduction in pericyte counts (P < 0.02; Fig. 1B). A similar trend, not reaching significance, was observed with endothelial cells (Fig. 1A). Using “live cell counts” (trypan blue exclusion), a significant negative effect of aminoguanidine was observed for endothelial cells (P < 0.001) but not for pericytes. Therefore, at this relatively high concentration, aminoguanidine exhibits some direct cytotoxicity. However, the effect is small, and the net effect of aminoguanidine present during LDL modification and in cell culture media remained beneficial (Fig. 1).

Degree of Modification of LDL

Fructoselysine, CML, and TBARS content of five LDL pools are summarized in Table 1. Effects of aminoguanidine were analyzed within individual modification groups (e.g., G-LDL was...
comparing only with G-LDL prepared in the presence of aminoguanidine). LDL exposed to glucose (G-LDL, GO-LDL) showed significant (10-fold) increases in FL content compared with N-LDL and MO-LDL. As expected, aminoguanidine had no effect on FL formation.

Carboxymethyllysine was slightly elevated after 3 days' exposure to glucose or oxidative stress, and the increase became significant when both stresses were combined (GO-LDL). Aminoguanidine had no significant effect on CML formation.

TBARS in MO-LDL and GO-LDL were significantly higher than in N-LDL (P < 0.05) but nevertheless remained low in all the modified lipoproteins. Aminoguanidine had no significant effect on TBARS formation.

Modification of LDL (aminoguanidine) had no effect, compared with N-LDL, on electrophoretic mobility on agarose gels (data not shown). We have previously shown that modified LDL was determined as in experiments not recognized by the macrophage scavenger receptor.15

Oxidizability of each LDL preparation was unaffected by the prior presence of aminoguanidine during LDL modification. As expected, oxidizability increased (i.e., lag phase shortened) as the extent of modification increased: N-LDL < G-LDL < MO-LDL < GO-LDL. However, when oxidizability of each modified LDL was determined with aminoguanidine (0, 1, 10, 100 μM) present during the Cu²⁺-mediated oxidation of the Esterbauer technique, aminoguanidine had a pro-oxidant effect with increasing concentration (data not shown), as has been observed by others at these low concentrations.

### DISCUSSION

In this work, we used concentrations of aminoguanidine that simulated those attained in vivo in animal and human studies.3,14 In a study where aminoguanidine inhibited progression of retinopathy in rats,3 mean plasma levels were 18.64 μg/ml (260 μM), and tissue levels were presumably lower. In a short-term study in humans,14 trough plasma levels were 10 μg/ml (140 μM). In our study, even at 1 μM, aminoguanidine significantly inhibited the development of toxicity in modified LDL toward retinal capillary endothelial cells. This is remarkable because LDL apoprotein concentration in the modifying incubation was approximately 5 g/l (10 μM), so LDL particles outnumbered aminoguanidine molecules by as much as 10:1. For pericytes, 10 μM aminoguanidine was required for a significant effect; however, in contrast, aminoguanidine in cell culture media had no protective effect and, indeed, was slightly toxic by some measures.

The mechanism by which aminoguanidine inhibits complications in experimental diabetes is unknown. Its effects are usually attributed to inhibition of advanced glycation reactions (by scavenging reactive dicarbonyls) and/or to inhibition of NOS and perhaps other enzymes. In this study, aminoguanidine was effective at very low concentrations when present during LDL modification (i.e., in a cell-free environment) but ineffective in the presence of cells. This makes an action mediated by enzyme inhibition unlikely, although it is possible that an LDL-associated enzyme (e.g., platelet activating factor acetylhydrolase) might be implicated. An action involving reactive carbonyls seems more probable. In LDL, both the protein (apoB) and lipid components of the particle are targets for, and sources of, reactive carbonyls. As targets, lysines in apoB and amino groups in surface aminophospholipids may be modified. As sources, glycated residues (in apoB and surface phospholipids) and fatty acid residues (in phospholipids and cholesterol esters) may be oxidized, generating carbonyls.10 We measured several parameters of modification: FL, CML, TBARS, electrophoretic mobility, and susceptibility to oxidation. These reflected the consequences of mild glycoxidative stress but were not influenced by the presence of aminoguanidine. Nevertheless, aminoguanidine may act by inhibiting formation of unidentified products in LDL, which mediate important cellular responses. Such responses may involve amplification via signaling pathways, so that very low concentrations of aminoguanidine could inhibit a biological response dependent on a small input signal. An effect on signaling is consistent with our observation that aminoguanidine in culture media at submicromolar concentrations can abolish the toxic effects of high glucose levels (50 mM).22 In both scenarios, aminoguanidine is present in a high-glucose aqueous environment in contact with a lipid surface (i.e., phospholipids and free cholesterol on either LDL or cell membrane surfaces).

Aminoguanidine is polar and water-soluble, so the surface of LDL is its most likely site of action. Oxidation products of surface cholesterol and phospholipids have been implicated in the effects of modified LDL on cells23,24 and may be scavenged by aminoguanidine. A protective effect of aminoguanidine at the LDL surface is consistent with our recent studies using vitamin E.25 Vitamin E is an amphiphilic antioxidant that re-

### Table 1. Levels of FL, CML, and TBARS in Normal and Modified LDL Pools

<table>
<thead>
<tr>
<th>LDL Pools</th>
<th>FL (mm/ml Lys)</th>
<th>CML (mm/ml Lys)</th>
<th>TBARS (ml MDA/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-LDL</td>
<td>0.28 ± 0.02</td>
<td>0.015 ± 0.001</td>
<td>0.90 ± 0.13</td>
</tr>
<tr>
<td>G-LDL</td>
<td>2.87 ± 0.39*</td>
<td>0.027 ± 0.002</td>
<td>1.60 ± 0.21</td>
</tr>
<tr>
<td>+1 μM AG</td>
<td>3.41 ± 0.53</td>
<td>0.022 ± 0.003</td>
<td>1.48 ± 0.35</td>
</tr>
<tr>
<td>+10 μM AG</td>
<td>2.87 ± 0.44</td>
<td>0.023 ± 0.002</td>
<td>1.54 ± 0.09</td>
</tr>
<tr>
<td>+100 μM AG</td>
<td>3.37 ± 0.48</td>
<td>0.025 ± 0.008</td>
<td>1.44 ± 0.23</td>
</tr>
<tr>
<td>MO-LDL</td>
<td>0.33 ± 0.03</td>
<td>0.022 ± 0.003</td>
<td>2.04 ± 0.40**</td>
</tr>
<tr>
<td>+1 μM AG</td>
<td>0.42 ± 0.06</td>
<td>0.041</td>
<td>3.95 ± 1.22</td>
</tr>
<tr>
<td>+10 μM AG</td>
<td>0.31 ± 0.01</td>
<td>0.026 ± 0.008</td>
<td>3.24 ± 0.91</td>
</tr>
<tr>
<td>+100 μM AG</td>
<td>0.32 ± 0.03</td>
<td>0.042 ± 0.009</td>
<td>4.12 ± 1.05</td>
</tr>
<tr>
<td>GO-LDL</td>
<td>2.41 ± 0.32*</td>
<td>0.034 ± 0.008**</td>
<td>2.42 ± 0.56**</td>
</tr>
<tr>
<td>+1 μM AG</td>
<td>3.46 ± 0.53</td>
<td>0.051 ± 0.007</td>
<td>3.43 ± 0.93</td>
</tr>
<tr>
<td>+10 μM AG</td>
<td>2.67 ± 0.45</td>
<td>0.044 ± 0.009</td>
<td>5.00 ± 1.14</td>
</tr>
<tr>
<td>+100 μM AG</td>
<td>3.58 ± 0.19</td>
<td>0.061</td>
<td>3.64 ± 1.04</td>
</tr>
</tbody>
</table>

Small sample volumes precluded some assays in some pools. AG = aminoguanidine. Statistics (relating to LDL modified in the absence of aminoguanidine): *P < 0.05 versus N-LDL and MO-LDL; **P < 0.05 versus N-LDL (MANOVA). Modification in the presence (versus absence) of AG had no significant effect on any measured parameter.
sides on the surface of LDL, and presupplementation of LDL with vitamin E (before exposure to glycoxidative stress) abolishes the development of toxicity toward retinal capillary cells. We also found that β-isofrom-specific protein kinase C inhibitors abolish the toxic effects of modified LDL toward retinal cells.20 Overall, these data are consistent with a sequence in which lipid modification leads to activation of a membrane-dependent signaling pathway. Further studies on the effects of aminoguanidine on glycoxidative modification of the phospholipid and cholesterol components of the LDL surface (and also cell membranes) are needed.

Aminoguanidine present during LDL modification was removed by dialysis and was not present when cells were exposed to LDL. To test the effect of aminoguanidine in culture media, which contained physiological 5 mM glucose, we used a single concentration, 100 μM, similar to that found in vivo in plasma of treated animals3 and humans.14 We hypothesized that some toxicity might develop from cell-mediated oxidative modification of LDL or that previously modified LDL might be more susceptible to such damage. Also, inhibition of NOS2 or perhaps other enzymes might alter cell survival. We found no protective effect of aminoguanidine in culture media, and conclude that inhibition of cellular enzymes is not the mechanism for the beneficial effect of aminoguanidine in this model. Indeed, exposure of cells to 100 μM aminoguanidine resulted in a slight decrease in cell numbers. Nevertheless, the net effect of aminoguanidine present both during LDL modification and in culture media (the closest simulation of in vivo conditions) remained beneficial.

Several studies have addressed the effects of aminoguanidine on in vitro oxidizability of LDL, but are not directly comparable to our work: they used higher (by 10- to 50,000-fold) and often nonpharmacological aminoguanidine concentrations, and more severe copper (Cu²⁺)-mediated oxidative stress (Estebauer technique). In our work, aminoguanidine, present during mild LDL modification but then removed by dialysis, had no effect on subsequent susceptibility of LDL to Cu²⁺-oxidation. In contrast, we found that when present during Cu²⁺-mediated oxidation, the same concentrations of aminoguanidine had a progressive pro-oxidant effect (data not shown), consistent with the observations of others.21

In conclusion, although results obtained from cell culture systems must be interpreted with caution, our findings may explain in part why aminoguanidine inhibits the development of experimental diabetic retinopathy. The protective effect we observed is mediated through an action on LDL modification, not on the cells themselves. The existence of a pathway leading from specific toxic LDL modifications through cell signaling processes to altered cell responses may proffer multiple opportunities for intervention. Aminoguanidine was protective at remarkably low concentrations, suggesting that doses lower than those generally used may be effective, reducing the risk of drug toxicity.

References