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Research Paper

Synergistic Effect of Amlodipine and Atorvastatin in Reversing LDL-Induced Endothelial Dysfunction

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Purpose. Statins and certain calcium channel blockers may improve nitric oxide (NO) release and endothelial function through various mechanisms, but their combined effects are not well understood.
Methods. The separate versus combined effects of amlodipine (AML) and atorvastatin (AT) on NO and peroxynitrite ($ONOO^-$) were measured in human umbilical vein endothelial cells (HUVEC) in the presence and absence of low-density lipoprotein (LDL) using electrochemical nanosensors.
Results. The combination of AML (5 μmol/l) and AT (3-6 μmol/l) directly stimulated NO release that was about twofold greater than the sum of their separate effects ($p<0.05$). This synergistic activity is attributed to enhanced endothelial NO synthase (eNOS) function and decreased cytotoxic $ONOO^-$. LDL (100 mg/dl) caused a dysfunction of HUVEC manifested by a 60% reduction in NO and an almost twofold increase in $ONOO^-$. Treatment with AML/AT partially reversed the effects of LDL on endothelial function, including a 90% increase in NO and 50% reduction in $ONOO^-$. Small-angle X-ray diffraction analysis indicates that AML and AT are lipophilic and share an overlapping molecular location in the cell membrane that could facilitate electron transfer for antioxidant mechanisms.
Conclusion. These findings indicate a synergistic effect of AML and AT on an increase in NO concentration, reduction of nitroxidative stress. Also, AML/AT partially restored the NO level of LDL-induced dysfunctional endothelium. Their combined effects may be enhanced by antioxidant properties related to their intermolecular actions in the cell membrane and an increase in the expression and coupling of endothelial nitric oxide synthase.

KEY WORDS: endothelium; LDL; nitric oxide; oxidative stress.

INTRODUCTION

Endothelial dysfunction contributes to mechanisms of atherogenesis and its clinical manifestations, including coronary artery disease (1–3). Cardiovascular risk factors have been linked to a loss of nitric oxide (NO) in the dysfunctional endothelium (4,5), resulting in an abnormal vasodilatation in response to various stimuli (6–8). There is an evidence that multiple risk factors, including hypertension and hyperlipidemia, lead to a synergistic effect on endothelial dysfunction, likely through oxidative stress mechanisms (9,10). Epidemiologic studies have demonstrated an increase in cardiovascular risk with hypertension and hyperlipidemia, as compared to their separate effects (11,12).

A reduction in nitric oxide (NO) bioavailability with hypertension has been linked to endothelial nitric oxide synthase (eNOS) uncoupling and NAD(P)H oxidase activity, resulting in increased superoxide anion (O_2^-) formation (13–15). Hyperlipidemia is also associated with eNOS uncoupling due to changes in plasma membrane caveolae levels and L-arginine availability (16–18). With a loss in NO bioavailability, there is enhanced susceptibility of the vessel to atherosclerotic processes, including smooth muscle cell proliferation and migration, expression of adhesion molecules, and platelet aggregation (19).

Cardiovascular agents, including angiotensin-converting enzyme (ACE) inhibitors, certain calcium channel blockers (CCBs) and HMG-CoA reductase inhibitors (statins), have been shown to improve endothelial-dependent NO release, albeit through different mechanisms (20–26). In this study, we selected a lipophilic CCB (amlodipine) with evidence of direct endothelial actions and antioxidant properties enhanced in the presence of atorvastatin (27,28). Both amlodipine and atorvastatin interact strongly with the cell membrane and have been shown to reduce the risk of cardiovascular disease and slow plaque progression in randomized clinical trials (29–31). A recent prospective study in over 10,000 hypertensive subjects showed a threefold reduction in cardiovascular events when atorvastatin was combined with amlodipine as compared to another anti-hypertensive agent, atenolol, despite similar reduction in LDL levels and reduction of blood pressure (32). In animal models of atherosclerosis, this drug combination improves certain aspects of plaque progression, beyond their separate effects (33).

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The hypothesis of this study was that the combination of a lipophilic CCB and statin may produce an enhanced effect on endothelial-dependent NO release, as compared to either agent separately. This hypothesis was tested in primary cultures of human endothelial cells (HUVEC) in the absence or in the presence of low-density lipoprotein (LDL). Treatment with amlodipine and atorvastatin was correlated with eNOS expression and coupling efficiency, including measurement of NO bioavailability and peroxynitrite ($ONOO^-$) induced nitroxidative stress. The molecular cell membrane interactions of these agents were directly ascertained by small-angle X-ray diffraction approaches. The results of this analysis provide insight into pharmacologic approaches to the treatment of endothelial dysfunction using combination approaches.

MATERIALS AND METHODS

Materials

The phospholipid 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) and cholesterol powder purchased from Avanti Polar Lipids Inc. (Alabaster, AL) were dissolved in HPLC-grade chloroform and stored at −80°C. Amlodipine and atorvastatin powder were obtained from Pfizer Central Research (Groton, CT) and solubilized in redistilled ethanol.

Cell Culture Preparations

We tested the separate versus combined effects of amlodipine and atorvastatin on HUVEC obtained from American Type Culture Collection. The HUVEC culture was incubated in 95% air/5% carbon dioxide at 37°C and passaged by an enzymatic (trypsin) procedure (34). Briefly, cells were seeded in collagen coated flasks and monitored until 75% of the cell clumps adhered (0.5–1 h). Non-adhering cells were poured off and the adhering cells were incubated in minimum essential medium (MEM) containing 10% fetal bovine serum (FBS) at 37°C under 5% CO_2 and 95% air. The medium was changed every 2 days. After 4–6 days, the primary cultures formed a confluent monolayer. The cell monolayer from the stock flask was dissociated by 2–3 min of exposure to tryspin in 0.15 mol/l NaCl, 0.01 mol/l sodium phosphate and 0.02% EDTA at 24°C. When the cells rounded up, they were resuspended in MEM supplemented with 10% FBS and seeded at a final concentration of 2×10^4 cells 35-mm dish.

Confluent cells ($4–5\times10^5$ cells/35-mm dish) were used for electrochemical measurements of NO and $ONOO^-$. Cells were directly stimulated with different concentrations of AML and/or AT. In a set of separate experiments cells were co-incubated with LDL (100 mg/dl), LDL and AML, AT, or a combination of AML and AT. NO and $ONOO^-$ release in these cells was measured after stimulation of eNOS with calcium ionophore (A23187, 1 μmol/l).

Preparation of the Tandem Sensor for NO and ONOO Detection

Concurrent measurements of NO and $ONOO^-$ were performed with electrochemical nanosensors (400–500 nm diameter) (35–37) combined into one working unit with a total diameter of 1.0–1.5 μm. The design was based on previously developed and well-characterized chemically modified carbon-fiber technology. Each of the sensors was made by depositing a sensing material on the tip of carbon fiber (length, 4 to 5 μm; diameter, 5 μm). The fibers were sealed with nonconductive epoxy and electrically connected to copper wires with conductive silver epoxy. A microburner was used to reduce the tip of carbon fiber to 400–500 nm diameter. We used a conductive film of polymeric nickel (II) tetrakis (3-methoxy-4-hydroxyphenyl) porphyrin for the NO sensor (37,38), and a polymeric film of Mn(III)- [2,2] paracyclophenylporphyrin for the $ONOO^-$ sensor (35,39).

Measurement of NO and $ONOO^-$

The concurrent measurement of NO and $ONOO^-$ levels were conducted with an electrochemical system consisting of a three-electrode system: tandem electrochemical nanosensors (working electrodes), a platinum wire (0.1 mm) counterelectrode and a saturated calomel reference electrode. Amperometry was performed with a computer-based Gamry VFP600 multichannel potentiostat. Amperometry was used to measure changes in NO and $ONOO^-$ concentrations from its basal level with time (detection limit of 1 nmol/l and resolution time <50 ms for each sensor). Amperometric-measured current at the peak potential characteristic for NO (0.65 V) oxidation and $ONOO^-$ (−0.40 V) reduction was directly proportional to the local concentrations of these compounds in the immediate vicinity of the sensor. Linear calibration curves were constructed for each sensor from 5 nmol/l to 3 μmol/l before and after measurements with aliquots of NO and $ONOO^-$ standard solutions, respectively. The tandem system of NO/$ONOO^-$ nanosensors was lowered with the help of a computer-controlled micromanipulator until it reached the surface of the cell membrane (a small piezoelectric signal, 6 to 8 pA, of 1 to 3 ms duration was observed at this point). The sensors were slowly raised 4±1 μm from the surface and moved horizontally to another single cell. The eNOS agonist CaI A23187 or AML, AT or combination of both ML/AT was then injected with a microinjector that was also positioned by a computer-controlled micromanipulator.

Preparation of Lipid Bilayers for Diffraction Analysis

Multilamellar lipid vesicles (MLVs) consisting of POPC and cholesterol were prepared in a buffer (0.5 mmol/l HEPES and 154.0 mmol/l sodium chloride, pH, 7.2) by the method of Bangham (40). The final phospholipid concentration was 2.5 mg/ml and the mole ratio of cholesterol to phospholipid was 0.2:1. The mole ratio of drug to phospholipid was 1:10, resulting in a final concentration of <5%, by mass. Membrane samples were oriented for diffraction analysis by centrifugation in a Sorvall AH-629 swinging bucket ultracentrifuge rotor at 35,000×*g* for 50 min at 5°C. Samples were then placed in hermetically sealed canisters that controlled temperature and relative humidity (74%) using a saturated salt solution consisting of tartaric acid, as previously described in detail (41). The ultrastructure of the lipid vesicles was analyzed by transmission electron microscopy (TEM) and shown to form stable lipid bilayers.

Small-angle X-ray Diffraction Analysis of Drug/Lipid Structure

Small-angle X-ray diffraction approaches were used to examine the molecular membrane location of amlodipine and atorvastatin in membranes reconstituted from bovine cardiac phosphatidylcholine and cholesterol. A detailed explanation of membrane diffraction analysis has been described previously (42). In brief, X-ray diffraction experiments were conducted by aligning the samples at grazing incidence with respect to a collimated X-ray source. In addition to direct calibration of the detector system, cholesterol monohydrate crystals were used to verify the calibration, as previously described. The unit cell periodicity (*d*-space) of the membrane lipid bilayer was calculated using Bragg's Law. Corrected diffraction orders obtained from samples in this study were analyzed using Fourier summation to yield one-dimensional electron density profiles (Å versus electrons/Å^3) of the membrane lipid bilayer.

Western Immunoblotting Analysis

Samples of endothelial cell homogenate, equalized for protein content, were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE; 5% gels) and transferred to polyvinylidene difluoride (PVDF) membranes. Levels of the enzyme eNOS were detected with polyclonal anti-eNOS antibody (Santa Cruz Biotechnology) (25). Bands were detected by horseradish peroxidase–conjugated secondary antibodies and visualized by chemiluminescence.

Calculations and Statistical Analysis

When applicable (comparison between 2 values), statistical analysis was done with Student's *t* test. For multiple comparisons, results were analyzed by analysis of variance (ANOVA) followed by Bonferroni's and Dunn's correction. Data are presented as mean ± SEM. Means were considered significantly different at $P<0.05$.

RESULTS

Synergistic Release of NO from HUVEC with Acute Amlodipine and Atorvastatin Treatment

Endothelial-dependent release of NO was measured using electrochemical nanosensors placed near the HUVEC surface. The separate and combined effects of amlodipine (AML) and atorvastatin (AT) on the release of NO are demonstrated in Fig. 1A. Separately, acute AT treatment had little effect on release of NO over the range of concentrations that were evaluated (1.0 to 10.0 μmol/l). By contrast, AML caused a significant increase in NO release, 30±2 nmol/l, at 1.0 μmol/l and at 10.0 μmol/l, NO increased to 252±22 nmol/l. Remarkably, when AML and AT were combined at constant concentration of 5.0 μmol/l of AML and variable concentrations (from 1.0–10.0 μmol/l) of AT, the amount of NO was more than additive, but actually synergistic. The drug combination caused an increase in levels of bioavailable NO from 135±11 nmol/l (at micromolar ratio 5/1 of AML/AT) to 281±26 nmol/l (at 5/10 of AML/AT ratio; Fig. 1A). Figure 1B shows

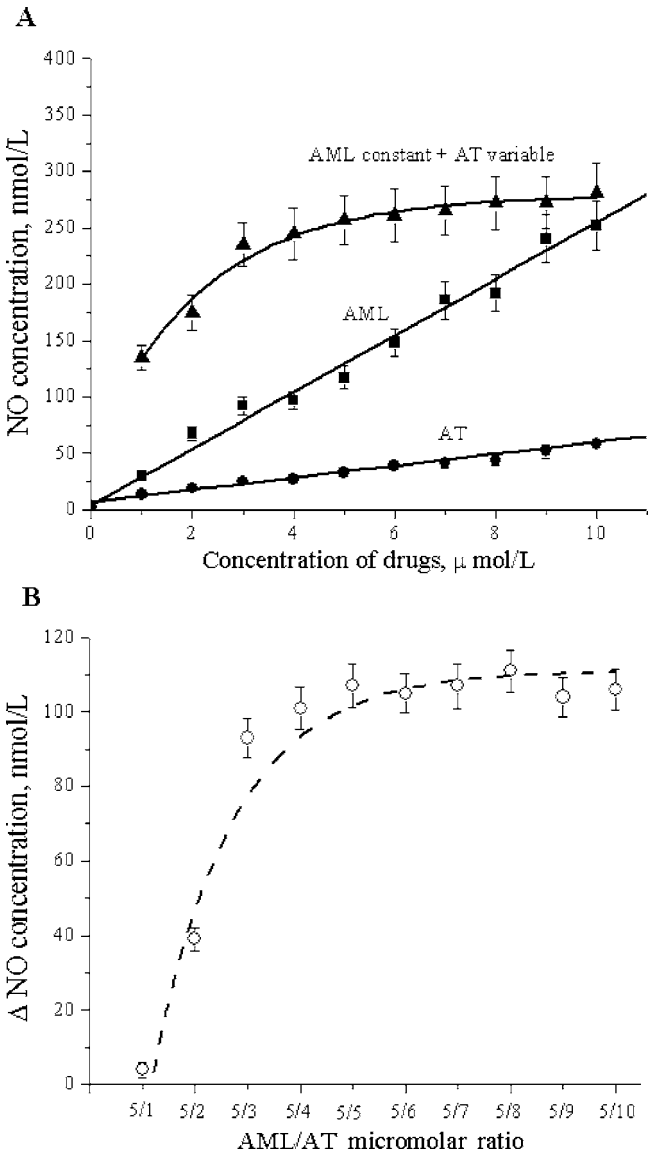

Fig. 1. **A** Maximal nitric oxide concentration released from a single HUVEC after direct stimulation with different concentrations of atorvastatin (AT, *filled circle*), amlodipine (AML, *filled square*), and combination of 5 μmol/l AML and variable concentrations (from 1-10 μmol/l) of AT (*filled triangle*). Nitric oxide concentration was monitored continuously for 20 s and maximal concentration was observed 1.0±0.2 s from an injection of the drugs. **B** A synergistic effect of the combination of AML (at 5 μmol/l) and different concentrations of AT (from 1–10 μmol/l) on NO release from a single HUVEC. Δ[NO] represents a net maximal increase in NO concentration, and was calculated by subtracting the sum of NO concentrations measured by each of the drugs from the maximal concentration of NO measured by the combination of the two drugs ($\Delta[NO] = [NO]_{AML/AT\ combination} - [NO]_{AML} + [NO]_{AT}$).

a synergistic effect of the combination of AML (at 5.0 μmol/l) and different concentrations of AT (1.0–10.0 μmol/l). Δ[NO] represents a net increase in NO concentration and was calculated by subtracting the sum of NO concentrations measured for each of the drugs from the maximal concentration of NO measured for the combination of the two drugs

($\Delta[NO] = [NO]_{AML/AT\ combination} - [NO]_{AML} + [NO]_{AT}$). The synergistic effect of the combination of AML and AT on NO release increased from 4.0±0.2 nmol/l (at 5/1 AML/AT ratio) to 95±5 nmol/l (at 5/3 AML/AT ratio) and reached a plateau of 105±5 nmol/l (at 5/5 AML/AT ratio). The increase of the synergistic effect was minimal above 5/3 micromolar ratio. Therefore, the combination of 5/3 AML/AT ratio was selected to perform the experiments involving LDL-induced endothelial dysfunction.

Effect of Amlodipine and Atorvastatin Combination on Calcium Ionophore-induced Nitric Oxide Release

We evaluated the effects of the combination of AML and AT (5/3 micromolar ratio) on endothelial-dependent NO release from HUVEC as a function of incubation time (Fig. 2). We tested the receptor–independent release of NO from these cells using a CaI (1.0 μmol/l). Unlike a receptor-dependent agonist (e.g., acetylcholine), the CaI can produce a large increase in intracellular calcium levels, leading to full activation of available NO synthase in the endothelial cells through increased levels of the co-factor, calcium–calmodulin. The release of NO increased markedly from 410±17 nmol/l (control) to 516±15 nmol/l after 1 h incubation with AML/AT combination. With exposure of the cells to the drug combination for 24 h, the amount of bioavailable NO increased to 640±20 nmol/l.

Effect of Amlodipine and Atorvastatin Combination on eNOS Expression

To understand the basis for enhanced NO bioavailability with treatment of the combination, we measured eNOS levels in these HUVEC preparations using Western blot techniques. In Fig. 3, the time- and dose-dependent effects of the drug combination on eNOS expression were reviewed. At increasing ratios of AT to AML, there was a 40% increase in eNOS levels after 1-h of treatment at equimolar concentrations of the two agents. Consistent with measurements of NO release, a longer incubation period of 24 h produced a much greater increase in eNOS protein levels (>80%).

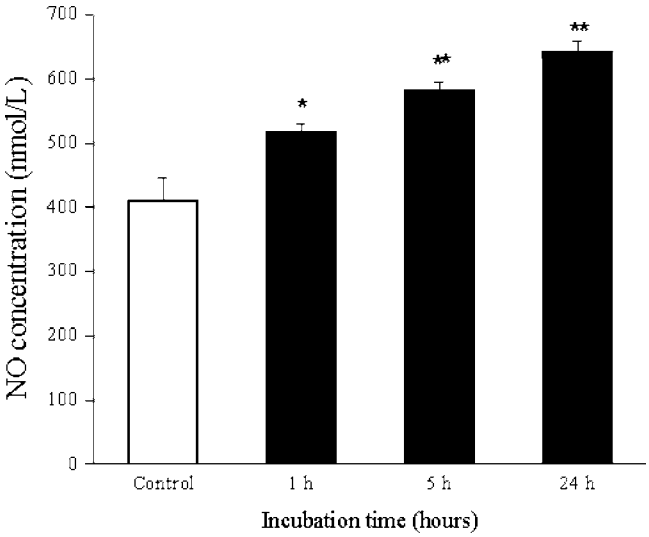

Fig. 2. CaI (A23187, 1.0 μmol/l)-stimulated maximal NO concentration in non-treated HUVEC (control, *open bar*) or after incubation with AML/AT (5/3 micromolar ratio) for various time periods up to 24 h (*solid bars*). *Asterisk*, $p<0.01$ vs control; *double asterisk*, $p<0.001$ vs control.

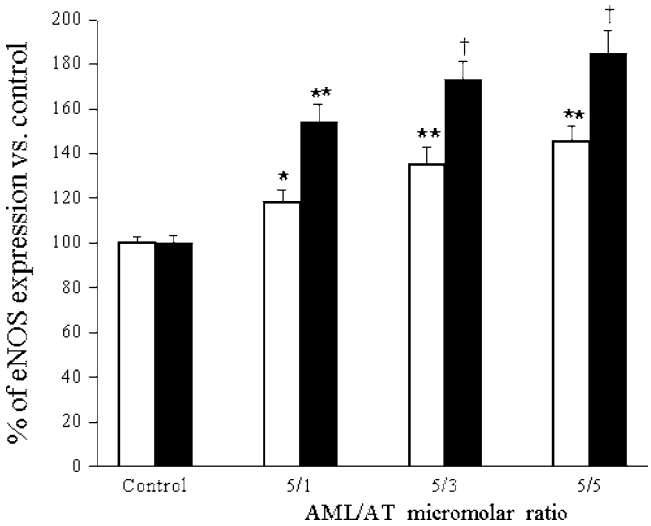

Fig. 3. Percentage change in cellular levels of eNOS protein expression from control HUVEC as a function of increasing the ratio of AT to AML. The amount of eNOS expression was measured at 1 h (open bars) vs. 24 h (*solid bars*) after co-incubation with increasing ratios of AT to AML (5/1, 5/3 and 5/5 μmol/l). *Asterisk*, $p<0.01$ vs control; *double asterisk*, $p<0.001$ vs control; *dagger*, $p<0.0001$ vs control.

Effect of Amlodipine and Atorvastatin Combination on Nitroxidative Stress

A result of eNOS uncoupling is the transfer of one electron to molecular oxygen instead of five electrons oxidation of L-arginine, resulting in superoxide anion generation. At higher levels, superoxide will react with NO to form a cytotoxic peroxynitrite ($ONOO^-$), a main component of nitroxidative stress. To assess the effect of the drug combination on nitroxidative stress levels, the amounts of both $ONOO^-$ and NO were simultaneously measured in these cells using tandem nanosensors. After incubation with AML/AT (5/3 micromolar ratio), there was a 40% reduction in levels of $ONOO^-$ following stimulation with calcium ionophore at 1.0 μmol/l (Fig. 4A). As a result, the ratio of NO to $ONOO^-$ maximal concentrations increased by more than twofold from 2.1 to 5.4 (Fig. 4B).

Effect of Amlodipine and Atorvastatin on NO/$ONOO^-$ Release in HUVEC following Co-incubation with LDL

Figure 5 summarizes the effects of CaI (1.0 μmol/l)-stimulated NO production from HUVEC incubated with different concentrations of LDL. The amount of NO released was inversely related to the level of LDL added to the HUVEC, especially over the range from 50 mg/dl to 150 mg/dl. The addition of LDL (100 mg/dl) caused a decrease in NO levels by approximately 60% (150±12 nmol/l). When the HUVECs were pre-incubated simultaneously with LDL (100 mg/dl), and AML or AT or a combination of AML/AT a marked increase in NO levels was observed (Fig. 6A).

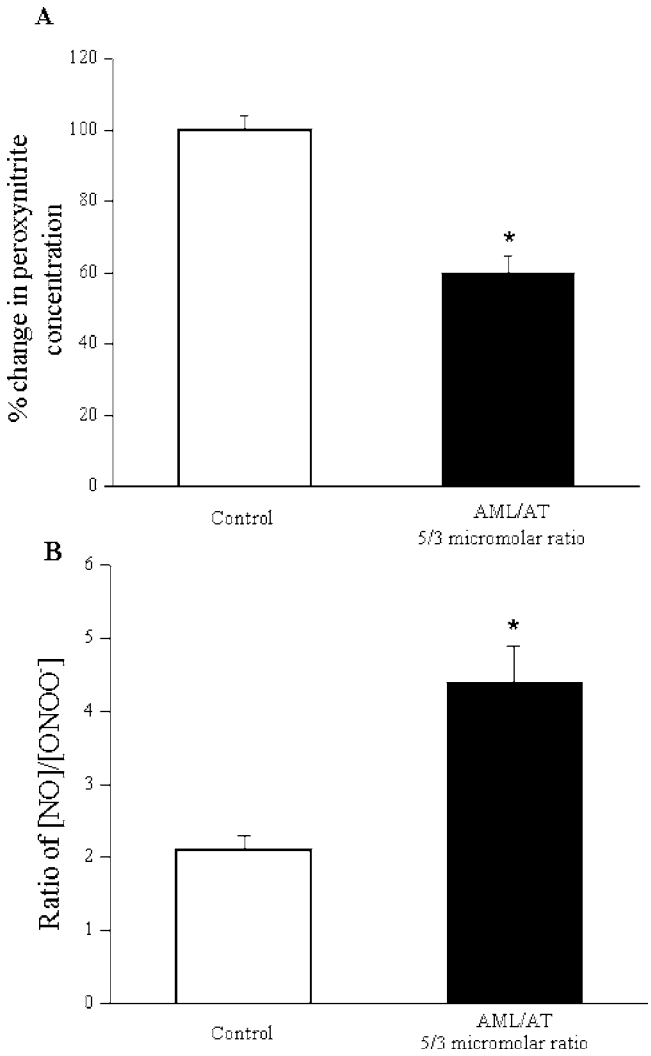

Fig. 4. A Percentage change (vs control) in CaI (1.0 μmol/l)-stimulated peroxynitrite ($ONOO^-$) release and **B** ratio of NO/$ONOO^-$ concentration in non-treated HUVEC and AML/AT (5/3 μmol/l)-treated cells for 1 h (*solid bars*). *Asterisk*, $p<0.0001$ vs non-treated, control cells.

However, the increase in the NO level was much more significant after preincubation with AML/AT combination (from control 150±12 nmol/l to 330±15nmol/l) than after preincubation with either ML or AT. Treatment of HUVEC with LDL also caused a dramatic increase (about twofold) in the $ONOO^-$ concentration following stimulation with CaI (Fig. 6B). The $ONOO^-$ levels increased from 167±12 control to 340±15 nmol/l. After pre-incubation of HUVEC with either AML, AT or AML/AT $ONOO^-$ concentration decreased. Again, the most significant reduction in $ONOO^-$ was observed after one incubation with the combination of AML/AT.

Effect of Amlodipine and Atorvastatin Combination on eNOS Expression in HUVEC following LDL Treatment

To study the effect of AML, AT or AML/AT combination treatment on the expression of the eNOS enzyme following LDL enrichment, Western blot assays were performed. As shown in Fig. 7, pre-incubation of HUVEC with 100 mg/dl LDL for 24 h reduced the expression of eNOS protein level by 40% from control. However, pre-incubation of HUVEC with 100 mg/dl LDL and AML, AT or AML/AT partially restored this expression. The highest increase (about 25%) in the expression of eNOS was observed after incubation with AML/AT.

Molecular Interactions of Amlodipine and Atorvastatin with Membrane Lipids

Small-angle X-ray diffraction approaches were used to directly determine the molecular distribution of AML/AT in the membrane lipid bilayer, as previously described (43). At 20°C, the *d*-space value for the membrane, including surface hydration, was 54.2 Å, which was consistent with previous X-ray diffraction studies of both intact and reconstituted vascular smooth muscle cell plasma membrane and cardiac sarcolemma (44,45).

Membrane samples, prepared in the absence and presence of drug, produced reproducible diffraction orders. Fourier analysis of the data produced a one-dimensional electron density profile (Å versus electrons/$Å^3$), which depicts the electron density of the lipid molecules. The time-averaged length of the phospholipid molecules was 27 Å. The addition of the AML and AT at a low concentration (<1% by mass) produced distinct changes in the molecular structure of phospholipid molecules that indicate their locations in the membrane, as summarized in Table I. Relative to the terminal methylene segments in the membrane bilayer center, the addition of AML separately produced a pronounced increase in electron density 12–20 Å corresponding to a well defined location associated with the glycerol backbone and upper acyl chain region of the phospholipid molecules. An overlapping but broader distribution was observed for AT that included the upper acyl chain region as well as the phospholipid headgroups, 12–27 Å. When the two molecules were added together to the membrane, the equilibrium location was 11–27 Å and consistent with a strongly overlapping location.

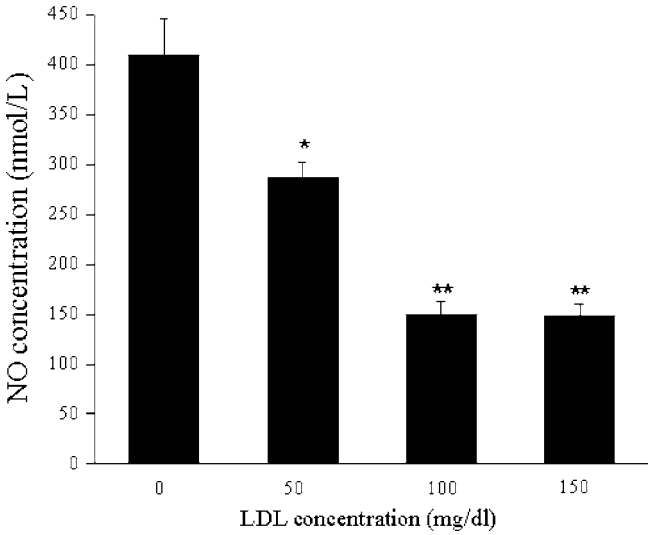

Fig. 5. CaI (1.0 μmol/l)-stimulated maximal NO release in HUVEC after incubation with LDL at various concentrations for 24 h. *Asterisk*, $p<0.01$ and *double asterisk*, $p<0.001$ vs control.

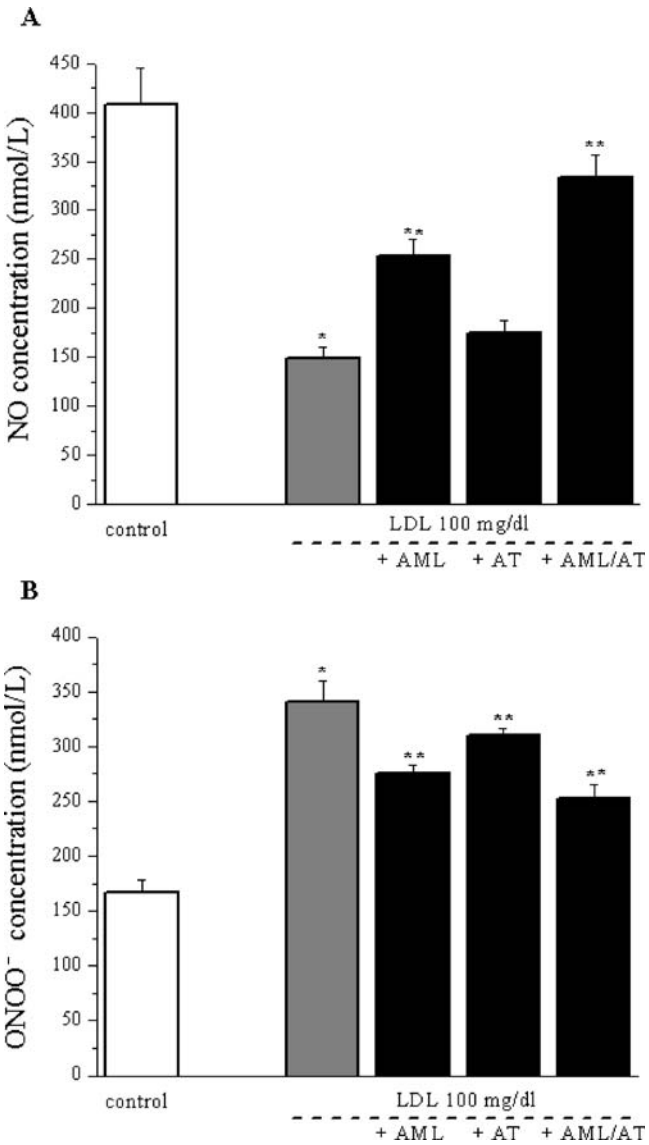

Fig. 6. CaI (1.0 μmol/l)-stimulated maximal **A** NO and **B** $ONOO^-$ release in non-treated HUVEC (control, *open bars*) cells and HUVEC cells incubated with 100 mg/dl LDL (*grey bars*) or incubated with 100 mg/dl LDL and AML (5 μmol/l), AT (1 μmol/l) or AML/AT (5/3 micromolar ratio; *solid bars*). *Asterisk*, $p<0.0001$ vs control; *double asterisk*, $p<0.01$ vs nontreated.

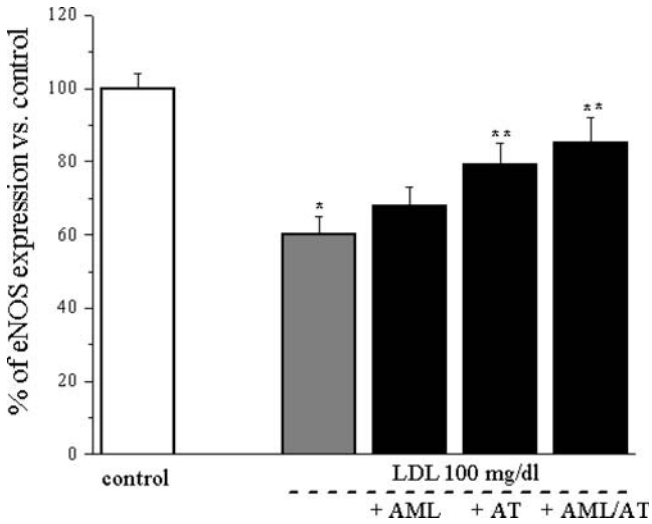

Fig. 7. Percentage change in cellular levels of eNOS protein expression from non-treated HUVEC (control, *open bars*) cells and HUVEC cells incubated with 100 mg/dl LDL (*grey bars*) or are incubated with 100 mg/dl LDL and AML (5 μmol/l). AT (1 μmol/l) or AML/AT (5/3 micromolar ratio; *solid bars*) for 24 h. *Asterisk*, $p<0.0001$ vs control; *double asterisk*, $p<0.01$ vs nontreated.

DISCUSSION

Risk factors for cardiovascular disease are associated with endothelial dysfunction, resulting in increased susceptibility to atherogenic processes (3,19). Loss in endothelial function is associated with eNOS uncoupling, as evidenced by reduced NO bioavailability and enhanced superoxide anion (O_2^-) generation. Agents that effectively treat risk factors such as hypertension and hyperlipidemia have been shown to improve endothelial function and responsiveness of vessels to stimuli of vasodilatation by various mechanisms, including a study that used a combination approach (20–25,46). The results from this study clearly indicate that a treatment of HUVEC with AML and AT combination produced a synergistic increase in endothelial-dependent NO release at the cellular level, beyond their known effects on risk factors. The basis for this effect is attributed to both enhanced eNOS expression and improved eNOS function, as measured in these studies at the cellular level.

Both, AML and AT, improved endothelial NO production. However, this effect was much more significant after treatment with AML/AT combination. An improvement in endothelial NO with the combination of AML/AT treatment was especially apparent in endothelial cells following LDL enrichment, a condition characterized by a marked imbalance between NO and $ONOO^-$ levels due to eNOS uncoupling. The causal relationship between elevations in LDL and endothelial dysfunction has been previously described at the cellular level (47–49). It has been shown that elevated LDL causes downregulation of eNOS expression (50,51). LDL can also enhance the generation of O_2^- which scavenges NO, resulting in cytotoxic peroxynitrite formation (15,17,48,49,52). Thus, hyperlipidemia is causally related to endothelial dysfunction which is accompanied by eNOS uncoupling. The eNOS uncoupling may depend on the severity and duration of

Table I. Molecular Membrane Locations of Amlodipine, Atorvastatin and the Combination

Drug	Membrane Location	Molecular Distribution (27 Å Total Phospholipid Length)
Amlodipine	Glycerol backbone/ phospholipid acyl chains (upper)	12–20 Å
Atorvastatin	Phospholipid headgroup/ phospholipid acyl chains (upper)	12–27 Å
Amlodipine/ atorvastatin	Phospholipid headgroup/ phospholipid acyl chains (upper)	11–27 Å

hyperlipidemia and availability of substrates and co-factors for eNOS.

Statins have been shown to improve NO synthesis by lowering serum LDL as well as by mechanisms unrelated to HMG–CoA reductase inhibition, including upregulation of eNOS expression (53) and reduced O_2^- formation (54,55). Additionally, statins stimulate endothelial NO production by causing a reduction in plasma membrane caveolin levels (56). By interfering with cholesterol biosynthesis and lowering plasma membrane cholesterol levels, AT was shown to attenuate the expression of caveolin-1, thereby allowing for the activation of eNOS by co-factors. These effects on NO metabolism were observed in the absence of changes in cytosolic eNOS levels and were reversed with mevalonate. By modulating plasma membrane microdomains and the expression of associated proteins, statins have an important effect on endothelial function (24).

The results of this study indicate that both AML and AT have a beneficial effect on endothelial NO production and reduction of cytotoxic $ONOO^-$. However, the combination of AML and AT caused a synergistic increase in the endothelial-dependent NO release. Both of these drugs can contribute to the synergistic effect in different but complimentary pathways. In a separate treatment of the endothelial cells, AT increased expression of eNOS more significantly than AML. However AML was much more effective antioxidant than AT as manifested by a significant reduction in $ONOO^-$. Therefore, the basis for the unexpected synergistic effect of AML/AT combination may be through their ability to (1) interfere with sources of reactive oxygen species (eNOS and NAD(P)H), (2) an increase in eNOS content, (3) an enhanced eNOS coupling. In support of this concept, previous studies have demonstrated that AML has chain-breaking antioxidant properties as a result of physico-chemical interactions with phospholipid polyunsaturated fatty acids (PUFAs), thereby interfering with propagation of free radicals (57). More recently, Franzoni *et al.* have shown that hydroxyl radicals generated by the Fenton reaction were rapidly and efficiently scavenged by AML in a manner that was superior to Trolox or glutathione (58). The presence of AT may further enhance the antioxidant activity of AML by facilitating electron transfer and further proton stabilization mechanisms with its multiple conjugated rings. The common location of these molecules in the membrane is consistent with such a model, as directly determined by small-angle X-ray diffraction approaches (Table I). Amlodipine has a formal positive charge due to its amino ethoxy function while atorvastatin has negative polarity mediated by its heptenoic acid side chain. Thus, the combination of AT with AML may potentiate their separate antioxidant actions through complementary mechanisms mediated by their physio-chemical properties.

These direct endothelial effects of AML/AT combination are separate from their benefits in risk factor management. It is well known that risk factors such as hypertension and hyperlipidemia have adverse effects of endothelial-dependent vasodilation that can be attributed to eNOS uncoupling mechanisms. During hypertension, in particular, there are marked reductions in functional NO, despite increased eNOS enzyme levels (59). Similar changes in eNOS coupling mechanisms and loss in substrate availability have been reported in experimental models of hyperlipidemia (16,17) and are consistent with findings from this study. Thus, pharmacologic agents that manage risk factors while also improving eNOS coupling and decreasing an nitroxidative stress may provide important benefits in the treatment of heart diseases.

CONCLUSION

The results of this study indicate that amlodipine and atorvastatin produced a synergistic effect on endothelial-dependent mechanisms of NO biosynthesis. The basis for this cellular activity is attributed to changes in eNOS expression, increased coupling efficiency of eNOS and a decrease in cytotoxic $ONOO^-$. The fact that amlodipine and atorvastatin are lipophilic and share a common membrane location may also facilitate antioxidant mechanisms and reduce oxidative/nitroxidative stress.

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