Novel Mechanism for Endothelial Dysfunction
Dysregulation of Dimethylarginine Dimethylaminohydrolase

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Background—Asymmetric dimethylarginine (ADMA) is an endogenous inhibitor of nitric oxide synthase (NOS). Plasma levels of ADMA are elevated in individuals with hypercholesterolemia or atherosclerosis. We postulated that reduced degradation of ADMA may play a role in the accumulation of ADMA in these individuals. Accordingly, we studied the effects of oxidized LDL (oxLDL) or tumor necrosis factor-α (TNF-α) on the accumulation of ADMA by transformed human umbilical vein endothelial cells (ECV304) and on the enzyme dimethylarginine dimethylaminohydrolase (DDAH), which degrades ADMA.

Methods and Results—ECV304 were incubated with or without native LDL (100 μg/mL), oxLDL (100 μg/mL), or TNF-α (250 U/mL) for 48 hours. The concentration of ADMA in the conditioned medium was determined by high-performance liquid chromatography. Western blotting was performed to evaluate DDAH expression. We assayed DDAH activity by determining L-citrulline formation from ADMA. The addition of oxLDL or TNF-α to ECV304 significantly increased the level of ADMA in the conditioned medium. The effect of oxLDL or TNF-α was not due to a change in DDAH expression but rather to the reduction of DDAH activity. To determine whether dysregulation of DDAH also occurred in vivo, New Zealand White rabbits were fed normal chow or a high-cholesterol diet. Hypercholesterolemia significantly reduced aortic, renal, and hepatic DDAH activity.

Conclusions—These results suggest that the endothelial vasodilator dysfunction observed in hypercholesterolemia may be due to reduced degradation of ADMA, the endogenous inhibitor of NOS. (Circulation. 1999;99:3092-3095.)

Key Words: lipoproteins ■ endothelium ■ amino acids ■ asymmetric dimethylarginine ■ nitric oxide

Endothelium-derived nitric oxide (NO) is a potent vasodilator that plays a critical role in regulating vascular resistance and flow. In addition, NO inhibits key processes in atherogenesis, such as monocyte adhesion, platelet aggregation, and vascular smooth muscle proliferation. In metabolic disorders associated with atherosclerosis (eg, hypercholesterolemia, hypertension, and diabetes mellitus), a reduced endothelium-mediated, NO-dependent vasodilation is observed, which may contribute to the initiation and progression of atherosclerosis associated with these disorders. Although the mechanisms of endothelial vasodilator dysfunction are likely multifactorial, one contributing abnormality appears to be increased levels of asymmetric dimethylarginine (ADMA).

ADMA is an endogenous competitive inhibitor of NO synthase. It is thought to be derived from proteins that have been posttranslationally methylated and subsequently hydrolyzed to release ADMA. A number of cells elaborate ADMA, including human endothelial cells. ADMA may be excreted in the urine or metabolized by the enzyme dimethylarginine dimethylaminohydrolase (DDAH). This enzyme hydrolyzes ADMA to L-citrulline and dimethylamine. We hypothesized that lipid-induced dysregulation of DDAH may play an important role in the elevation of ADMA and the derangement of the NO synthase pathway in hypercholesterolemia.

Accordingly, we studied the effects of lipoprotein on the endothelial elaboration of ADMA and on the expression and activity of DDAH in cell culture and in vivo.

Methods

Cell Culture
Spontaneously transformed human umbilical vein endothelial cells (ECV304; ATCC) were cultured in medium M199 (Irvine Scientific) containing 10% FCS, 100 U/mL penicillin, and 100 μg/mL streptomycin (Gibco BRL). This cell line retains many of the characteristics of primary endothelial cells, including the synthesis of NO.

At 80% confluence, cells were washed and replaced with serum-free medium. After 2 hours, cells were incubated in the presence or absence (control) of native LDL (100 μg/mL), oxidized LDL (oxLDL; 30 or 100 μg/mL), or tumor necrosis factor-α (TNF-α; 250 U/mL) for 0, 24, or 48 hours. To assess cell viability, lactate
dehydrogenase activity in the conditioned medium was determined by a spectrophotometric assay (Sigma Diagnostics).

**Lipoprotein Preparation**

LDL was isolated by density-gradient ultracentrifugation of normal human plasma collected in EDTA (1 mg/mL) and quantified by Lowry assay. OxLDL was prepared by incubation of LDL (100 µg/mL) with CuSO4 (10 µg/mL) at 37°C for 24 hours. The extent of oxidation was monitored by measurement of thiobarbituric acid-reactive substances at 550 Å.

**Measurement of ADMA**

Concentrations of L-arginine and ADMA in the conditioned medium were measured by high-performance liquid chromatography and precolumn derivatization with o-phthalaldehyde, as previously described.

**Western Blotting**

Cells were resuspended in 500 µL of 0.1 mol/L sodium phosphate buffer (pH 7.0) and subsequently lysed by rapid freezing and thawing. Cell lysates were centrifuged at 15 000 rpm for 40 minutes, and supernatants were used for Western blotting and enzyme activity assay. Protein concentrations were measured by Lowry assay.

Membranes with transferred proteins were incubated with a mouse monoclonal antibody raised against purified rat DDAH 6 (1:1000) in Tris-buffered saline containing 4% nonfat milk and probed with a peroxidase-conjugated anti-mouse antibody (1:1000), with detection by ECL Western blotting reagents (Amersham).

**Enzyme Assay**

We assayed DDAH activity by determining l-citrulline formation in cell lysates, as previously described. As negative controls, cell lysates were boiled for 10 minutes to inactivate the enzyme, and l-citrulline formation was determined. Background values obtained were subtracted from the experimental data to provide genuine DDAH activity.

**Data Analysis**

Data are expressed as mean±SEM. Comparisons of multiple means were made by ANOVA followed by a Fisher’s protected least significant difference test. Comparisons between treatment groups in the rabbit study were made by Student’s unpaired t test. A value of *P*<0.05 was accepted as statistically significant.

**Results**

**ADMA Elaboration by Endothelial Cells**

The concentration of ADMA in the conditioned medium of control cells gradually increased with time to a level of 0.6±0.2 µmol/L at 48 hours (Figure 1). Incubation with native LDL (100 µg/mL) or a low dose of oxLDL (30 µg/mL) tended to increase the accumulation of ADMA (native LDL, 1.1±0.4 µmol/L; oxLDL, 1.2±0.3 µmol/L at 48 hours). Incubation with a high dose of oxLDL (100 µg/mL) or TNF-α (250 U/mL) for 48 hours significantly augmented ADMA accumulation (oxLDL, 3.9±1.3 µmol/L, *P*<0.05; TNF-α, 4.9±1.5 µmol/L, *P*<0.01) (Figure 1). L-Arginine concentrations in the medium did not change among 5 groups throughout the experimental period. Lactate dehydrogenase activity in the conditioned medium from endothelial cells incubated with native LDL, oxLDL, or TNF-α was not significantly higher than in medium from control cells (data not shown).

**DDAH Expression and Activity**

Incubation with oxLDL (100 µg/mL) or TNF-α caused no changes in the expression of protein levels of DDAH throughout the experimental period, as assessed by Western blot analysis (Figure 2A). DDAH activity was significantly decreased by 48-hour incubation with oxLDL (100 µg/mL) (63±8%; *P*<0.01) or TNF-α (63±8%; *P*<0.01), whereas control cells exhibited no significant change in DDAH activity (95±6%) (Figure 2B).

To determine whether this cytokine or lipid-induced dysregulation of DDAH occurred in vivo, New Zealand White rabbits were fed a normal chow (n=4) or 1% cholesterol diet (n=5). Bode-Boger et al previously showed that in this animal model, hypercholesterolemia increased plasma ADMA from 1.2 to 2.0 µmol/L. At 12 weeks, animals were killed, and the aortae, livers, and kidneys were removed for analysis of DDAH expression and activity. There were no differences between control and hypercholesterolemic animals in DDAH expression of the aorta, kidney, or liver (data not shown). However, DDAH activity in each of these tissues was significantly decreased in hypercholesterolemic animals (Table).

**Discussion**

The salient findings of this study are as follows: (1) OxLDL or TNF-α increases ADMA elaboration by endothelial cells. (2) This effect is associated with decreased DDAH activity, without a change in protein expression. (3) A similar dysregulation of DDAH activity is observed in vascular and extravascular tissues in rabbits fed a high-cholesterol diet. The data suggest that reduced activity of DDAH may be responsible for the elevated plasma ADMA and endothelial vasodilator dysfunction in hypercholesterolemia.

This study provides insight into a novel mechanism by which hyperlipidemia may disturb the NO synthase pathway in endothelial cells. Increased levels of ADMA, the endogenous NO synthase inhibitor, are observed in hypercholesterolemic animals and humans and may account for
the endothelial vasodilator dysfunction observed in this condition. ADMA plasma levels, which are 1.0 ± 0.1 μmol/L in healthy humans, are elevated 2-fold in hypercholesterolemic individuals and 3-fold in elderly patients with peripheral arterial occlusive disease and generalized atherosclerosis. Increased ADMA levels are associated with reduced NO elaboration in hypercholesterolemic subjects and in atherosclerotic patients, as judged by reduced nitrate excretion and impaired endothelium-dependent, NO-mediated forearm vasodilation. Previous studies have shown that exogenous ADMA concentrations between 1 and 10 μmol/L affect endothelium-dependent, NO-mediated vasodilation in rat mesenteric vessels and rat cerebral vessels and also regulate NO production by cultured macrophages. In the present study, the concentration of ADMA in the conditioned medium was increased 6- or 8-fold by incubation with oxLDL (100 μg/mL) or TNF-α, respectively, compared with control. These increases of ADMA may have pathophysiological significance, because they are in a range to inhibit the activity of NO synthase.

In humans, ADMA may be excreted by the kidney or metabolized by DDAH. We found that protein levels of DDAH were unaffected by oxLDL or TNF-α. By contrast, DDAH activity of endothelial cells was decreased to almost 60% of baseline values by incubation with oxLDL or TNF-α. Similarly, DDAH activity in several tissues was significantly decreased in rabbits fed a high-cholesterol diet, with no changes in protein expression. These results are similar to those obtained when endothelial cells are exposed to S-2-amino-4-(3-methylguanidino) butanoic acid (1 mmol/L), which inhibits DDAH activity and increases the accumulation of ADMA in the conditioned medium of endothelial cells.

ADMA and DDAH are widely distributed in tissues and may provide a mechanism for controlling NO synthesis in physiological or pathological states. Our results reveal that lipoproteins or cytokines may increase endothelial elaboration of ADMA by reducing DDAH activity. Decreased activity of DDAH may lead to local accumulation or release of intracellular ADMA and inhibition of NO synthase in disease states, including hypercholesterolemia. The increase in ADMA levels may explain the therapeutic benefit of supplemental L-arginine observed in patients with endothelial dysfunction.

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