Plasma Concentration of Asymmetric Dimethylarginine, an Endogenous Inhibitor of Nitric Oxide Synthase, Is Elevated in Monkeys With Hyperhomocyst(e)inemia or Hypercholesterolemia

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Abstract—Hyperhomocyst(e)inemia is associated with endothelial dysfunction. Mechanisms responsible for endothelial dysfunction in hyperhomocyst(e)inemia may involve impaired bioavailability of endothelium-dependent nitric oxide. We tested the hypothesis that hyperhomocyst(e)inemia is associated with an elevated plasma concentration of asymmetric dimethylarginine (ADMA), an endogenous inhibitor of nitric oxide synthase. One group of adult cynomolgus monkeys was fed either a control or hyperhomocyst(e)inemic diet for 4 weeks in a randomized crossover design. The second group was fed an atherogenic diet that produces both hyperhomocyst(e)inemia and hypercholesterolemia for 17 months, followed by an atherogenic diet supplemented with B vitamins for 6 months to decrease plasma homocyst(e)ine concentration. Human endothelial cells were used to study the effects of methionine and homocysteine in the presence or absence of B vitamins or the methylation inhibitor S-adenosylhomocysteine on the formation of ADMA and its inactive stereoisomer, symmetric dimethylarginine. The hyperhomocyst(e)inemic diet produced 2- to 3-fold increases in plasma levels of homocyst(e)ine and ADMA (both \( P<0.05 \)). The atherogenic diet also produced elevated plasma levels of homocyst(e)ine and ADMA (both \( P<0.05 \)). Supplementation of the atherogenic diet with B vitamins decreased the plasma levels of homocyst(e)ine but did not affect the plasma levels of ADMA or endothelial function. There was a strong correlation between plasma ADMA and homocyst(e)ine and a strong inverse correlation between ADMA and carotid artery relaxation to acetylcholine. ADMA release by cultured endothelial cells was significantly increased in the presence of methionine or homocysteine. This effect was blocked by S-adenosylhomocysteine but not by B vitamins. We conclude that plasma levels of ADMA are elevated in hyperhomocyst(e)inemia. Because ADMA acts as a competitive inhibitor of endothelial nitric oxide synthase, these findings suggest a novel mechanism for impaired endothelial function in hyperhomocyst(e)inemia. (Arterioscler Thromb Vasc Biol. 2000;20:1557-1564.)

Key Words: nitric oxide ■ endothelium ■ asymmetric dimethylarginine ■ homocysteine

Hyperhomocyst(e)inemia, which is a risk factor for atherosclerotic vascular disease, is associated with endothelial dysfunction in monkeys and humans. (The term “hyperhomocyst(e)inemia” is used in this article to indicate that plasma homocyst(e)ine assays measure the total concentration of thiol, disulfide, and mixed disulfide adducts of homocysteine.) Mechanisms that produce endothelial dysfunction in hyperhomocyst(e)inemia are not clear but may involve a direct toxic effect of homocyst(e)ine on endothelial cells and oxidative inactivation of nitric oxide (NO) by homocyst(e)ine. Another mechanism that may contribute to endothelial dysfunction in atherosclerosis is the generation of asymmetric dimethylarginine (ADMA), an endogenous competitive inhibitor of NO synthase.

Plasma levels of ADMA and its biologically inactive stereoisomer, symmetric dimethylarginine (SDMA), are elevated in hypercholesterolemic rabbits and humans. Elevation of ADMA is associated with reduced activity of NO synthase in this animal model. Similar observations have been made in patients with peripheral arterial disease and generalized atherosclerosis and in hypercholesterolemic humans. Dimethylarginines are probably formed from the degradation of methylated proteins. A major source of methyl groups used for various methylating reactions is the demethylation of methionine to homocysteine. This methyl group may then be transferred, directly or indirectly, to L-arginine to yield \( \text{N}^2,\text{N}^6 \)-dimethyl-L-arginine (ADMA) and/or \( \text{N}^6,\text{N}^{\text{D}} \)-dimethyl-L-arginine (SDMA).
In the present study, we tested the hypothesis that moderate diet-induced hyperhomocyst(e)inemia is associated with increased plasma concentration of ADMA in cynomolgus monkeys. We measured plasma concentrations of ADMA and determined whether there is an association between elevated ADMA concentration and endothelial dysfunction in monkeys fed a hyperhomocyst(e)inemic diet, an atherogenetic diet [which induces hypercholesterolemia and moderate hyperhomocyst(e)inemia], an atherogenetic diet supplemented with B vitamins [to decrease homocyst(e)ine levels], or a control diet.1,13

Endothelial cells are capable of synthesizing ADMA and, in minor amounts, SDMA.14,15 Therefore, we sought to determine whether the formation of ADMA and SDMA by cultured human endothelial cells is increased in the presence of high concentrations of methionine or homocysteine and whether inhibition of S-adenosylmethionine–dependent methylases reduces this effect. We also investigated the effect of B vitamins on ADMA formation in the presence of elevated homocysteine concentrations in cell cultures.

Our results indicate that plasma concentrations of ADMA are elevated in monkeys with hyperhomocyst(e)inemia and/or hypercholesterolemia and that plasma levels of ADMA correlate strongly with endothelial dysfunction. We have also demonstrated in the present study that human endothelial cells are a source of ADMA and that ADMA formation by endothelial cells is increased in the presence of high methionine or homocysteine levels in a manner reversible by the methylation inhibitor S-adenosylhomocysteine.

Methods

Animals and Experimental Protocol

Sixteen adult cynomolgus monkeys (Macaca fascicularis) were studied in 2 separate groups. Details of the study protocols have been published previously.1,13 In group 1, 7 monkeys were assigned to receive, in a randomized crossover design, either a control diet (Purina Monkey Chow,Ralston-Purina) or a hyperhomocyst(e)inemic diet enriched in methionine or homocysteine and free of choline, for 4 weeks, followed by the other diet for 4 weeks. In group 2, 9 monkeys were assigned to receive an atherogenetic diet for 17 months, followed by an other diet for 4 weeks. In group 2, animals were anesthetized again, another venous blood sample was drawn, and measurement of the vasomotor response to collagen infusion in vivo was repeated. The remaining carotid artery was removed and placed in oxygenated Krebs’ solution. The animals were killed by administration of sodium pentobarbital (200 mg/kg IV) and exsanguinated. The study protocol was approved by the University of Iowa Animal Care and Use Committee.

Cell Culture

The spontaneously transformed human umbilical vein endothelial cell line ECV304 (American Type Culture Collection)17 was cultured in medium 199 (GIBCO-BRL) 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 synthesis of angiogenin-converting enzyme, NO, and prostacyclin, and the expression of endothelial adhesion molecules, including intercellular adhesion molecule-1 and lymphocyte function–associated antigen-3. ECV304 cells were maintained in medium 199 until they reached confluence. The cells were washed, and fresh medium was added. Cells were then maintained in culture for an additional 24 hours in the absence or presence of methionine (1×10^{-4} to 4×10^{-4} mol/L), DL-homocysteine (10^{-3} mol/L), DL-homocysteine plus S-adenosylhomocysteine (10^{-4} mol/L), or DL-homocysteine plus B vitamins (10^{-3} mol/L pyridoxine, 10^{-4} mol/L cobalamin, and 10^{-4} mol/L folic acid) before the cells and media were harvested for measurement of ADMA and SDMA levels.

Biochemical Analyses

Concentrations of L-arginine and dimethylarginines in plasma and in cell supernatants were determined by high-performance liquid chromatography (HPLC) with precolumn derivatization with o-phthalaldehyde by a modification of a previously described method.7 Samples were spiked with 10 μmol/L homocitrulline as an internal standard and extracted on CBA solid-phase extraction cartridges (Varian). The eluates were dried under nitrogen, and residues were dissolved in bidistilled water for HPLC analysis. HPLC was carried out on a liquid chromatography system (Gynkotek) consisting of 2 HPLC pumps with a gradient controller (model M 480 HDG), a spectral fluorescence detector RF 1002, and an automatic injector (model GINA 160). Samples and standards were incubated for exactly 30 seconds with the o-phthalaldehyde reagent (5.5 mg/mL o-phthalaldehyde in borate buffer, pH 8.5, containing 0.4% 2-mercaptoethanol) before automatic injection into the HPLC system. Chromatographic separation was performed on a C18 column (Macherey and Nagel) with the fluorescence monitor set at excitation and emission wavelengths of 340 and 455 nm, respectively. Samples were eluted from the column isocratically with 0.96% citric acid/methanol (2:1 [vol/vol], pH 6.8) at a flow rate of 1 mL/min. The coefficients of variation of this method were 5.2% for within-assay determination and 5.5% for between-assay determination; the detection limit of the assay was 0.1 μmol/L.

Fasting plasma homocyst(e)ine concentrations were measured by HPLC and electrochemical detection, according to the method of Smolin and Schneider,18 as previously described.15 Plasma total cholesterol levels were determined by using methods established by the Lipid Research Centers and standardized by the Centers of Disease Control as described previously.20
Endothelium-Dependent Vascular Function Ex Vivo

After removal of loose connective tissue, the common carotid artery was cut into multiple rings, each 5 mm wide. Carotid artery rings were suspended in organ chambers containing oxygenated Krebs’ buffer maintained at 37°C and connected to force transducers to measure changes in isometric tension. Rings were precontracted to a tension of 1.0 g by stepwise addition of prostaglandin F2\textsubscript{a} (1 to 3 \textmu mol/L), and relaxation concentration response curves were generated by cumulative addition of acetylcholine or sodium nitroprusside (each 1 nmol/L to 10 \textmu mol/L).

Calculations and Statistical Analyses

Data are given as mean±SEM. Statistical significance was tested by ANOVA, followed by the Fisher protected least significant difference test. Linear regression curves and correlation coefficients were calculated according to the least squares method. Multiple regression analysis was performed for endothelium-dependent vasodilation ex vivo, with cholesterol, ADMA, and homocyst(e)ine concentrations used as independent variables. A linear ADMA-homocyst(e)ine interaction term was also included as an independent variable to assess the potential interaction between both molecules on endothelial function. Statistical significance was accepted at \( P < 0.05 \).

Results

Effects of Hyperhomocyst(e)inemic Diet on Plasma Homocyst(e)ine, Total Cholesterol, l-Arginine, and ADMA Levels

The hyperhomocyst(e)inemic diet increased plasma homocyst(e)ine levels 2.7-fold compared with the control diet (\( P < 0.05 \), Table). Plasma total cholesterol concentrations were not significantly different between these groups (Table).

Plasma ADMA levels were elevated 3-fold in animals fed the hyperhomocyst(e)inemic diet (\( P < 0.05 \), Figure 1A). Plasma SDMA levels did not differ significantly between the 2 groups of animals (Table). Plasma concentrations of l-arginine were elevated during the hyperhomocyst(e)inemic diet (\( P < 0.05 \), Table). The l-arginine/ADMA ratio was significantly lower in the group fed the hyperhomocyst(e)inemic diet (\( P < 0.05 \), Figure 1B).

Effects of Atherogenic Diet and B Vitamin Supplementation on Plasma Homocyst(e)ine, Total Cholesterol, l-Arginine, and ADMA Levels

The atherogenic diet increased plasma total cholesterol and homocyst(e)ine concentrations (Table). B vitamin supplementation decreased homocyst(e)ine to control levels but did not significantly affect total cholesterol levels (Table). ADMA levels were elevated in monkeys fed the atherogenic diet and remained unchanged after B vitamins were added to the diet (Figure 1A). Plasma concentrations of l-arginine and SDMA were not significantly changed by the atherogenic diet with or without supplemental vitamins (Table). The l-arginine/ADMA ratio was significantly decreased in these 2 groups compared with monkeys fed the control diet; vitamin supplementation did not affect this ratio (Figure 1B).

Regression Analysis of Plasma ADMA and Homocyst(e)ine Concentrations

There was a significant correlation between plasma ADMA concentration and plasma homocyst(e)ine concentration in monkeys fed the hyperhomocyst(e)inemic or control diet.
Regression Analysis of Plasma ADMA Concentration and Endothelium-Dependent Vasodilator Function Ex Vivo

Isolated carotid arteries from monkeys showed a concentration-dependent relaxation response to acetylcholine, and maximum acetylcholine-induced relaxation was significantly impaired in carotid arteries from hyperhomocyst(e)inemic animals compared with control animals. Maximum relaxation was 80.7±7.7% in monkeys fed the control diet, 48.7±12.2% in monkeys fed the hyperhomocyst(e)inemic diet (P=0.05 versus the control diet), and 53.3±9.4% in monkeys fed the atherogenic diet without supplemental B vitamins. Supplementation of the atherogenic diet with B vitamins did not improve maximum relaxation to acetylcholine (54.6±9.4%), although sensitivity to the lowest concentrations of acetylcholine was modestly increased.

In simple regression analysis, a strong inverse correlation was found between ADMA plasma concentration and maximum acetylcholine-induced vasodilation of carotid artery ex vivo for monkeys fed control or hyperhomocyst(e)inemic diets (R=0.65, P=0.01; n=14). A significant inverse correlation was also observed when data from all animals were included in the analysis (R=0.50, P=0.004; n=32). Multiple regression analysis indicated that plasma ADMA concentration was the only independent predictor of endothelium-dependent relaxation ex vivo (R=0.553, P=0.02), whereas plasma homocyst(e)ine or total cholesterol levels were not independent predictors of endothelium-dependent relaxation. Inclusion of an ADMA-homocyst(e)ine interaction term did not increase the correlation coefficient (R=0.554, P=0.03), indicating the absence of an additive effect of ADMA and homocyst(e)ine on endothelial dysfunction of the carotid artery (P=0.82 for interaction).

Regression Analysis of Plasma ADMA and Blood Flow Response to Collagen In Vivo

Intra-arterial infusion of collagen decreased hindlimb blood flow by 42±9% in monkeys fed the hyperhomocyst(e)inemic diet and by 14±11% in monkeys fed the control diet.
Effects of Methionine and Homocysteine on ADMA Release by Endothelial Cells

Under control conditions (medium free of methionine and homocysteine), ECV304 human endothelial cells produced 13.7 ± 1.3 pmol of ADMA per micromgram of protein over 24 hours and 10.3 ± 3.6 pmol of SDMA per micromgram of protein over 24 hours. ADMA formation was concentration-dependently increased by increasing the concentrations of methionine (P < 0.05 versus control for 200 and 400 μmol/L, Figure 4). Incubation with 1.0 mmol/L homocysteine also significantly elevated ADMA production (27.8 ± 2.9 pmol/μg protein over 24 hours, P < 0.05 versus control). SDMA formation was not significantly affected under the same conditions. The increased production rate of ADMA in the presence of homocysteine was completely reversed in the presence of the methylation inhibitor S-adenosylhomocysteine (13.6 ± 1.8 pmol/μg protein over 24 hours, P < 0.05 versus homocysteine; P = NS versus control), but it was not affected by the addition of B vitamins (34.2 ± 4.4 pmol/μg protein over 24 hours, P = NS versus homocysteine).

Discussion

The major findings of the present study are that (1) plasma levels of ADMA, an endogenous NO synthase inhibitor, are elevated in monkeys with diet-induced hyperhomocyst(e)inemia and/or hypercholesterolemia; (2) there is a significant linear correlation between plasma levels of ADMA and homocyst(e)ine in hyperhomocyst(e)inemic and hypercholesterolemic monkeys; (3) plasma levels of ADMA are inversely correlated with endothelium-dependent relaxation of carotid artery rings in vivo and with the blood flow response to collagen in vivo; (4) dietary supplementation with B vitamins, which normalizes plasma homocyst(e)ine levels, does not decrease plasma ADMA concentration or restore normal endothelium-dependent vasomotor function in hypercholesterolemic monkeys; and (5) release of ADMA by endothelial cells is increased in the presence of high methionine or homocysteine levels in a manner reversible by the methylating inhibitor S-adenosylhomocysteine.

We observed that plasma concentration of ADMA was elevated in monkeys fed an atherogenic diet that produces hyperhomocyst(e)inemia and hypercholesterolemia. Supplementation of the atherogenic diet with B vitamins decreased plasma homocyst(e)ine to control levels but did not affect the plasma concentration of ADMA or improve endothelium-dependent vasodilatation in these monkeys.13 B vitamins are essential cofactors for the transsulfuration of homocyst(e)ine to cystathionine by cystathionine β synthase (vitamin B6) and the remethylation of homocyst(e)ine to methionine by methionine synthase (folic acid, vitamin B12), as seen in Figure 5.12,21 Thus, dietary supplementation with B vitamins promotes the transsulfuration and remethylation of homocysteine, resulting in decreased plasma levels of homocyst(e)ine.21,22 Despite decreasing plasma homocyst(e)ine, however, supplementation with B vitamins may not decrease protein methylation or the generation of ADMA, because intracellular levels of S-adenosylmethionine may actually increase as a consequence of increased turnover of the homocyst(e)ine-methionine pathway (Figure 5).23

In cultured endothelial cells, incubation with methionine concentration-dependently increased ADMA levels. This effect was also induced by homocysteine; it was reversed by the methylation inhibitor S-adenosylhomocysteine, but it was not changed by the addition of B vitamins. Thus, these data in vitro corroborate our findings in monkeys in vivo and further support the proposed model (Figure 5) that N-methylation of L-arginine to ADMA may occur concomitantly with the demethylation of methionine to homocysteine. Wang et al24 recently reported that the addition of homocysteine to endo-
thelial cells in the presence of adenosine and an adenosine deaminase inhibitor can inhibit carboxy methylation of p21<sup>CIP1</sup> by increasing the levels of S-adenosylhomocysteine. These findings are in contrast to our present results, which were obtained in the absence of exogenous adenosine. The differences may be due to different culture conditions and differential regulation of specific methyltransferases. It is very likely that the effects of homocysteine on methylation reactions are dependent on intracellular concentrations of adenosine, methionine, and B vitamins as well as other factors that influence the levels of S-adenosylmethionine and S-adenosylhomocysteine.

Factors unrelated to homocysteine metabolism may have affected ADMA levels in our in vivo study. We have previously reported that ADMA levels increase in the presence of hypercholesterolemia in animals<sup>7,8</sup> and humans.<sup>10</sup> Dimethylarginines are excreted by the kidneys and accumulate in chronic renal failure.<sup>25</sup> However, accumulation of ADMA has been shown to occur in atherosclerotic humans<sup>9</sup> and in cholesterol-fed rabbits<sup>8</sup> in spite of normal renal function. ADMA is metabolized to citrulline by the enzyme dimethylarginine dimethylaminohydrolase (DDAH).<sup>26</sup> Inhibition of DDAH produces gradual constriction of vascular segments, which is reversed by L-arginine, further supporting the view that the ratio between endogenous ADMA and L-arginine regulates endothelial NO synthase activity.<sup>27</sup> Reduced DDAH activity has been proposed to account for the elevation of ADMA in hypercholesterolemia<sup>28</sup> and hyperglycemia.<sup>29</sup> We cannot exclude the possibility that modulation of DDAH activity contributed to elevated ADMA concentration in the present study, although preliminary data suggest that homocysteine does not affect DDAH activity in vitro (R.H.B. et al, unpublished data, 2000).

Our finding that monkeys fed hyperhomocysteinemic diets had significantly elevated plasma ADMA levels is consistent with the hypothesis that methyl groups incorporated into dimethylarginines may be supplied during the demethylation of methionine to homocysteine. This reaction results in the cleavage from S-adenosylmethionine of 1 methyl group that may be inserted directly or indirectly into L-arginine, thereby forming methylated arginine analogues, such as ADMA and SDMA (Figure 5). Indeed, it has recently been shown that S-adenosylmethionine, an intermediate in the conversion of methionine to homocysteine, is the source of methyl groups for the methylation of arginine residues within proteins by the enzyme protein-arginine methyltransferase-1 of yeast.<sup>30</sup> At least 3 different isoforms of protein-arginine methyltransferases, which have different tissue distribution and different product specificities for ADMA or SDMA, have been characterized.<sup>31,32</sup> We report in the present study that cultured human endothelial cells produce more ADMA than SDMA under control conditions. This finding, which is in accordance with previous studies,<sup>14,15</sup> suggests that the methyltransferase present in endothelial cells preferentially methylates arginine in a manner yielding asymmetric dimethylarginine. Selective elevation of ADMA levels, but not of SDMA levels, in hyperhomocysteinemic monkeys may therefore be explained by the altered endothelial metabolism of methylarginines.

Hyperhomocysteinemia is associated with impaired endothelial function in animals<sup>1</sup> and humans.<sup>2,3,33</sup> However, mechanisms responsible for endothelial dysfunction in hyperhomocysteinemia have remained unclear.<sup>34</sup> In high concentrations, homocysteine is directly toxic to cultured endothelial cells,<sup>4</sup> and it may decrease endothelial production of NO through oxidative mechanisms.<sup>5</sup> Our present findings suggest that increased generation of ADMA may be an alternative mechanism of endothelial dysfunction in hyperhomocysteinemia. We observed that the plasma concentration of ADMA was inversely correlated with the acetylcholine-induced relaxation of carotid artery rings ex vivo in hyperhomocysteinemic monkeys. In multiple regression analysis, plasma ADMA was a better predictor of endothelial dysfunction than either plasma homocysteine or total cholesterol concentrations. No interaction was found between ADMA and homocysteine in relation to endothelial function, which further supports the hypothesis that homocysteine may impair endothelial function via ADMA instead of potentiating the detrimental effects of both molecules on endothelial NO formation.

There also was an inverse correlation between plasma ADMA concentration and changes in blood flow in response to collagen, but there was no correlation with changes in hindlimb blood flow in response to acetylcholine in vivo ($R = 0.014, P = NS$). The explanation is not clear for the finding that plasma ADMA correlated well with vascular responses to collagen, but not acetylcholine, but may be related to different mediators for responses to collagen and acetylcholine. It is likely that vasodilatation in response to the activation of platelets by collagen is mediated by NO and thus is inhibited by ADMA. In contrast, the response of resistance vessels to acetylcholine may not be mediated by NO<sup>35</sup> and thus may not be inhibited by ADMA.

Despite the 2- to 3-fold elevation of plasma ADMA concentrations in hyperhomocysteinemic monkeys, ADMA levels were still far below those of L-arginine in plasma (2 to 3 μmol/L versus 60 to 100 μmol/L). These levels may seem unlikely to antagonize L-arginine as a substrate for NO synthase.<sup>36</sup> Studies in vitro, however, have shown that intracellular concentrations of ADMA are higher in cultured endothelial cells than in the extracellular fluid, which suggests an accumulation of ADMA within cells.<sup>14</sup> We have recently found that ADMA significantly and concentration-dependently inhibits conversion of L-[guanidino-<sup>15</sup>N]<sub>2</sub>arginine to [<sup>15</sup>N]<sub>2</sub>arginine to [<sup>15</sup>N]nitrate in primary human endothelial cells within the concentration range between 0.5 μmol/L and 10 μmol/L (R.H.B., S.M.B.-B., unpublished data, 2000). This finding indicates that ADMA concentrations like those reported in the present study are within the steep part of the concentration-effect curve and may well contribute to the modulation of NO synthase activity. Extracellular concentrations of ADMA between 1 and 10 μmol/L inhibit endothelium-dependent vasodilation in isolated blood vessels,<sup>37,38</sup> and ADMA inhibits the release of NO by cultured endothelial cells<sup>14</sup> and macrophages<sup>15</sup> within the same concentration range. Moreover, in young asymptomatic adults with hypercholesterolemia, elevated plasma concentrations of ADMA are significantly related to the degree of impaired endothelium-dependent forearm vasodilation.<sup>10</sup> Taken together, these studies suggest that ADMA, within the concentration range found in the present study, contributes to the regulation of endothelial NO synthase activity.
From a therapeutic point of view, the observation that lowering homocyst(e)ine plasma concentrations with B vitamins does not improve endothelial-dependent vasodilation may have far-reaching implications. Because a relationship between elevated homocyst(e)ine concentration and cardiovascular disease has been established in epidemiological studies, the implicit notion is that lowering homocyst(e)ine levels might reduce cardiovascular risk. Preliminary data indicate that these patients also have elevated ADMA plasma concentration (R.H.B., K.S., unpublished data, 2000). Therefore, if methylation of L-arginine is the link between homocyst(e)ine and vascular dysfunction in humans, a beneficial effect would not be expected during supplementation with B vitamins. Prospective interventional clinical trials are necessary to clarify this issue.

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