Pathogenic Cycle Between the Endogenous Nitric Oxide Synthase Inhibitor Asymmetrical Dimethylarginine and the Leukocyte-Derived Hemoprotein Myeloperoxidase

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Background—The nitric oxide synthase inhibitor asymmetrical dimethylarginine (ADMA) and the leukocyte-derived hemoprotein myeloperoxidase (MPO) are associated with cardiovascular diseases. Activation of monocytes and polymorphonuclear neutrophils (PMNs) with concomitant release of MPO is regulated in a nitric oxide–dependent fashion. The aim of the study was to investigate a potential 2-way interaction between ADMA and MPO.

Methods and Results—Ex vivo, ADMA uptake by isolated human PMNs, the principal source of MPO in humans, significantly impaired nitric oxide synthase activity determined by gas chromatography–mass spectrometry. In humans, short-term ADMA infusion (0.0125 mg · kg⁻¹ · min⁻¹) significantly increased MPO plasma concentrations. Functionally, PMN exposure to ADMA enhanced leukocyte adhesion to endothelial cells, augmented NADPH oxidase activity, and stimulated PMN degranulation, resulting in release of MPO. In vivo, a 28-day ADMA infusion (250 μmol · kg⁻¹ · d⁻¹) in C57Bl/6 mice significantly increased plasma MPO concentrations, whereas this ADMA effect on MPO was attenuated by human dimethylarginine dimethylaminohydrolase1 (hDDAH1) overexpression. Moreover, the MPO-derived reactive molecule hypochlorous acid impaired recombinant hDDAH1 activity in vitro. In MPO⁻/⁻ mice, the lipopolysaccharide-induced increase in systemic ADMA concentrations was abrogated.

Conclusions—ADMA profoundly impairs nitric oxide synthesis of PMNs, resulting in increased PMN adhesion to endothelial cells, superoxide generation, and release of MPO. In addition, MPO impairs DDAH1 activity. Our data reveal an ADMA-induced cycle of PMN activation, enhanced MPO release, and subsequent impairment of DDAH1 activity. These findings not only highlight so far unrecognized cytokine-like properties of ADMA but also identify MPO as a regulatory switch for ADMA bioavailability under inflammatory conditions.

Key Words: arteriosclerosis ■ endothelium ■ inflammation ■ leukocytes ■ nitric oxide synthase

Over the past decade, the endogenous nitric oxide (NO) synthase (NOS) inhibitor asymmetrical dimethylarginine (ADMA) has emerged as a novel cardiovascular risk factor. Elevated plasma ADMA concentrations have been demonstrated in a variety of patient cohorts with cardiovascular risk factors and overt cardiovascular disease. Moreover, ADMA appears to be an independent predictor of cardiovascular and all-cause mortality. Leukocyte-derived NO has emerged as a critical determinant of the activation state of a cell. Accordingly, elevated ADMA concentrations are associated with increased leukocyte activation. The major pathway for elimination of ADMA is its metabolism by
the enzyme dimethylarginine dimethylaminohydrolase (DDAH), which exists in 2 isoforms (DDAH1 and DDAH2), yielding l-citrulline and dimethylamine. A minor portion of circulating ADMA is excreted via the kidneys. Importantly, the activity of the DDAH enzyme has been shown to be regulated in a redox-sensitive fashion. Thus, DDAH inactivation under conditions of increased production of vascular-derived reactive oxygen and nitrogen species may result in increased ADMA concentrations.

Clinical Perspective on p ●●●

Endothelium-derived NO plays a crucial role in the regulation of vascular tone, platelet activity, leukocyte adhesion, and development of arteriosclerosis. A decreased NO bioavailability by impaired synthesis resulting from the presence of endogenous NOS inhibitors and/or increased NO inactivation by elevated oxidative stress leads to the development of endothelial dysfunction. Important initial steps in the course of endothelial dysfunction are an impaired release of NO from the endothelium, an increased expression of adhesion molecules, and the consecutive enhanced attachment of polymorphonuclear neutrophils (PMNs) to endothelial cells.

On activation, PMNs release myeloperoxidase (MPO), an NO-oxidizing hemoprotein with various proinflammatory properties. The MPO enzyme is stored within primary granules of neutrophils and, to a lesser extent, in monocytes and macrophages, and is released in the course of degranulation. MPO catalyzes the generation of oxygen- and nitrogen-derived reactive species and promotes oxidative damage of host tissue at sites of inflammation, including arteriosclerotic lesions. The enhanced NO consumption by MPO may explain, in part, the importance of MPO in the context of endothelial dysfunction and cardiovascular diseases. Elevated concentrations of circulating MPO are associated with the presence of coronary artery disease and powerfully predict cardiovascular events in patients with acute coronary syndrome.

We hypothesized that increased ADMA concentrations lead to enhanced PMN activation, subsequent degranulation, and MPO release. In addition, we hypothesized that MPO—by impairing DDAH activity, which leads to ADMA accumulation and decreased NO production—may contribute to endothelial dysfunction and cardiovascular diseases via an additive pathway. Therefore, the aim of the present study was to investigate a potential 2-way interaction between the ADMA/DDAH pathway and MPO.

Methods

Isolation of Human PMNs

Experiments focusing on the ADMA effect on intracellular NOS enzyme activity and activation of neutrophils were performed in isolated human PMNs. Blood was taken from healthy volunteers, and PMNs were isolated as described previously. Neutrophils were isolated from peripheral blood of healthy volunteers, using a gradient of polymyxin B sulfate and dextran. Isolated human PMNs were preincubated with saline, ADMA (100 μmol/L), or SDMA (100 μmol/L) for 30 minutes at 37°C. Cells were centrifuged, washed in Hank balanced salt solution buffer, and sonicated, and the lysate was centrifuged again. ADMA and SDMA concentrations in the supernatant were measured by liquid chromatography–tandem mass spectrometry (LC-MS/MS) as recently described. Isotope-labeled [2H6]-ADMA (in-house synthesis) was used as internal standard for quantification of ADMA and SDMA.

Determination of ADMA Uptake by HL-60 Cells

HL-60 cells (ATCC, Wesel, Germany) were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum. For differentiation, the cells were incubated with 1% dimethyl sulfoxide for 96 hours and characterized by flow cytometric quantification of CD11b integrin expression and by determination of reactive oxygen species production. After differentiation, 1×10⁶ cells/mL were incubated with increasing concentrations of ADMA (0, 0.5, 1, 10, and 100 μmol/L) for 2 hours in Hank balanced salt solution supplemented with L-arginine (400 μmol/L). After repeated washing, HL-60 cells were resuspended in ice-cold phosphate buffer, containing PBS and homogenized, and nuclei and membrane fractions were separated. The proteins from cytosolic fraction were removed by centrifugation through 3-kDa filters (Millipore, USA). ADMA concentrations in the cytosolic fraction were determined by LC-MS/MS and related to total cytosolic protein content determined before filtration of proteins.

Determination of Intracellular Dimethylarginine Accumulation in Human PMNs

Isolated human PMNs were preincubated with saline, ADMA (100 μmol/L), or SDMA (100 μmol/L) for 30 minutes at 37°C. Cells were centrifuged, washed in Hank balanced salt solution buffer, and sonicated, and the lysate was centrifuged again. ADMA and SDMA concentrations in the supernatant were measured by liquid chromatography–mass spectrometry as described previously. The lysed cells were treated with protease inhibitors, and a sterile solution of N-[guanidine-15N2]-arginine hydrochloride (100 μmol/L; 15N isotope purity >98%; Eurisotop, Saarbrücken, Germany) dissolved in 0.45% sodium chloride was added and incubated for additional 30 minutes. Supernatant samples were collected before and 30 minutes after administration of labeled L-arginine. Reaction products were derivatized with pentafluorobenzyl bromide (Sigma, Hamburg, Germany) and extracted into toluene (Merck, Darmstadt, Germany). The conversion of L-[guanidine-15N2]-arginine to 15N-labeled nitrite was detected as the 15N over 14N isotope ratio with gas chromatography–mass spectrometry. 14N-nitrite concentration was determined subsequently by gas chromatography–mass spectrometry, and 15N-nitrite concentration was calculated from the isotopic ratio. Concentration was normalized to cell number.

Determination of the High-Affinity Cationic Amino Acid Transporter-1 in Human PMNs

For detection of the transmembrane cationic amino acid transporter-1 (CAT-1) transport system in isolated human PMNs by Western blot analysis, PMNs were lysed (100 mmol/L sodium dihydrogen phosphate; 0.1% Triton X-100) and sonicated on ice, and polyacrylamide gel electrophoresis and immunoblotting with an anti-CAT-1 antibody (1:500; Abnova, Heidelberg, Germany) were performed according to the manufacturer’s protocol.

Effect of ADMA on Human PMN Degranulation and Release of Oxygen Species

MPO and elastase concentrations in the supernatant of human PMNs were determined by ELISA according to the manufacturer’s recommendations (Calbiotech and IBL, Hamburg, Germany, respectively). MPO activity in isolated human PMNs was determined by photometrically assessing the oxidation of 3,3’-5,5’-tetramethylbenzidine as described. The generation of superoxide anion by isolated human PMNs was determined as linear rate of superoxide dismutase–inhibitable reduction of cytochrome c as...
-described. The measurement was initiated by addition of cytochrome c (5 μL, 100 μmol/L) and simultaneous application of superoxide dismutase (50 μL, 300 U/mL) to the samples.

Effect of ADMA on Adhesion of Human PMNs to Human Umbilical Vein Endothelial Cells

Human PMNs were isolated and preincubated with the fluorescent dye acetylmethoxy-calcein. The labeled cells were added to cultured human umbilical vein endothelial cells and cocultivated with Hank balanced salt solution buffer, SDMA (100 μmol/L), or ADMA (100 μmol/L) for 30 minutes. The cells were washed and light emission was measured in a fluorometer (Berthold Twinkle LB 970; excitation/emission wavelength, 495/515 nm).

Animals

The investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (publication No. 85–23, revised 1996). The study protocol was approved by the Administrative Panel on Laboratory Animal Care, Hamburg University (protocol No. 70/07). The in vivo effect of MPO on methylarginine concentrations on lipopolysaccharide (LPS) stimulation was assessed with MPO-deficient (MPO−/−) mice. For the experiments, female MPO−/− and control mice (at 12–16 weeks of age; both groups were on a C57Bl/6 background) were generated as previously described. Male human DDAH1 (hDDAH1) transgenic mice were mated with C57Bl/6 female mice (Charles River Laboratories Germany, Sulzfeld, Germany). Offsprings were screened for transgene expression by polymerase chain reaction analysis as described earlier. All experiments investigating the impact of hDDAH1 overexpression on the effects of MPO were conducted in female, 6- to 8-week-old, heterozygous hDDAH1 transgenic mice and age-, sex-, and weight-matched wild-type (WT) littermates housed in a temperature-controlled animal facility with a 12-hour light/dark cycle and free access to tap water and rodent chow.

Assessing the In Vivo Effect of ADMA on MPO Plasma Concentrations in C57Bl/6 Mice

To study the in vivo effect of exogenous ADMA on MPO plasma concentrations, osmotic minipumps (ALZET, model 2002) were implanted in female C57Bl/6 (C57) mice (age, 8–12 weeks; weight, 23–28 g) for 28 days. The minipumps were loaded with either ADMA (250 μmol · kg⁻¹ · d⁻¹) or vehicle (saline and KCl; 136±5 mmol/L). All animals were anesthetized by intraperitoneal injection of 0.12 mg ketamine and 0.016 mg xylazine/10 g body weight. An osmotic minipump was implanted into the abdomen of each animal after dissection and midline incision. Finally, the wound was closed with nylon sutures. To assess plasma MPO concentrations, blood samples were taken at day 28 and determined by ELISA according to the manufacturer’s recommendations (Hycult Biotech, Uden, the Netherlands). ADMA and SDMA plasma concentrations were measured by LC-MS/MS.

Effect of MPO on Recombinant hDDAH1 Activity In Vitro

The effect of MPO and its enzymatic product hypochlorous acid (HOCl) on the activity of recombinant hDDAH1 enzyme was measured by a modified version of the method developed by Knipp and Vysok. Briefly, 1 μg hDDAH1 was incubated for 15 minutes at 37°C with active MPO (0.001, 0.005, 0.01, 0.05, 0.1, and 0.5 μg/100 μL; Planta Natural Products, Vienna, Austria) or heat-inactivated MPO (boiled at 95°C for 30 minutes) and substrate (10 μmol/L hydrogen peroxide [H₂O₂]; Sigma, Germany) or, in a different setting, with HOCl (0.1, 1, 10, and 100 μmol/L; Sigma, Germany). After the addition of 1 mmol/L of the DDAH substrate ADMA in a final reaction volume of 100 μL, the samples were incubated for 1 hour at 37°C. After incubation, 200 μL of fresh prepared color developing reagent solution was added to each sample. After 15 minutes of incubation at 95°C in an oven, the plate was cooled to room temperature, and citrulline formation was measured at 540 nm.

In Vivo Effect of MPO−/− on Methylarginine Concentrations

To study the in vivo effect of MPO on plasma ADMA and SDMA concentrations, MPO−/− and control C57 mice received an intraperitoneal injection of 12.5 mg/kg LPS. Four hours after LPS administration, the mice were anesthetized, and blood samples for determination of methylarginine concentrations were obtained by puncturing the inferior vena cava and drawing the blood to EDTA-rinsed syringes. The blood was immediately centrifuged at 4°C (10 minutes at 4000 rpm), and the supernatant was stored at −80°C. ADMA, SDMA, and N⁵-monomethyl-L-arginine (L-NMMA) plasma concentrations were measured by LC-MS/MS with isotope-labeled [¹³C₆]-ADMA and [¹⁵N₁]-L-NMMA used as internal standards for quantification of ADMA and SDMA and of L-NMMA, respectively.

In Vivo Impact of hDDAH1 Overexpression on the Effects of MPO

To assess the potency of DDAH1 on opposing the effects of MPO, we investigated the impact of hDDAH1 overexpression on MPO plasma concentrations after 28 days of ADMA treatment via osmotic minipumps. HDDAH1 transgenic mice and their WT littermates were treated with saline- or ADMA-loaded osmotic minipumps (250 μmol · kg⁻¹ · d⁻¹) over a period of 28 days. MPO plasma concentrations were determined at day 28 as described earlier. In a second approach, we challenged hDDAH1 and WT littermate mice with an intraperitoneal LPS injection (12.5 mg/kg) and determined ADMA and SDMA plasma concentrations by LC-MS/MS 4 hours after injection.

In Vivo Effect of Short-Term ADMA Infusion on MPO Plasma Concentrations in Humans

To assess the effect of short-term ADMA infusion on MPO plasma concentrations in humans, 10 healthy, normotensive men and women were studied before, during, and after a short-term ADMA infusion after written, informed consent. This study was approved by the University of Michigan Institutional Review Board. ADMA was manufactured for human administration by Bachem AG (Bubendorf, Switzerland) and approved by the Federal Drug Administration (investigational new drug 77,330). After a baseline period, ADMA was administered intravenously at 0.0125 mg · kg⁻¹ · min⁻¹. Plasma MPO, ADMA, SDMA, and L-NMMA concentrations were measured at baseline, at 60 minutes of ADMA infusion, and 20 minutes after the ADMA infusion was discontinued by LC-MS/MS. MPO plasma concentrations were determined by ELISA technique as described earlier.

Statistical Analysis

The data are expressed as mean±SEM. All data were tested with the Kolmogorov-Smirnov test and revealed normal distributions. To test for differences between groups, the Student paired or unpaired t test as appropriate or 1-way ANOVA followed by Bonferroni post hoc test was used. A value of P<0.05 was considered statistically significant.

Results

ADMA Incubation Leads to Intracellular Accumulation and Impairs NOS Activity in PMNs

In line with previous findings in macrophages, the CAT-1 transport system was detected in human PMNs (Figure 1A). Incubation with ADMA or SDMA (100 μmol/L) resulted in an increase in ADMA and SDMA in the cell lysate, respectively (Figure 1B). Additional experiments in HL-60 cells revealed a significant 6-fold increase in intracellular ADMA.
on incubation with 10 μmol/L ADMA (control versus 10 μmol/L ADMA, 0.14±0.02 versus 0.86±0.09 μmol ADMA/mg protein; P=0.042; Figure 1C). In accordance with the enhanced intracellular ADMA or SDMA accumulation, NOS enzyme activity in PMNs was significantly reduced after treatment with either ADMA or SDMA (3.52±0.18, 0.53±0.07, and 1.08±0.04 nmol 15N-nitrite/1×10⁶ PMNs for control, ADMA, and SDMA, respectively; each P<0.001; ADMA versus SDMA, P=0.028; Figure 1D).

ADMA Enhances Adhesion of Isolated Human PMNs to Human Umbilical Vein Endothelial Cells

As a further marker of PMN activation, adhesion to endothelial cells was tested. Without prior stimulation, only a few PMNs tended to adhere to endothelial cells (Figure 3A). Coincubation with 100 μmol/L SDMA led to a slight increase in PMN adhesion (43.0±2.6 relative fluorescence units, values of control subtracted). In contrast, coincubation with 100 μmol/L ADMA caused a significant, 2-fold increase in PMN adhesion (85.4±17.5 relative fluorescence units, values of control subtracted; P=0.021; Figure 3B) compared with the SDMA effect.

Long-Term In Vivo ADMA Treatment Increases MPO Plasma Concentration in C57 Mice

Long-term treatment with exogenous ADMA via osmotic minipumps resulted in significantly increased MPO plasma concentrations in C57 mice after 28 days of treatment (saline versus ADMA treated, 26.3±1.2 versus 42.6±5.9 ng/mL; P=0.018; Figure 4A and Figure 1A in the online-only Data Supplement). The ADMA plasma concentrations significantly increased on ADMA treatment (saline versus ADMA treated, 0.72±0.03 versus 2.00±0.25 μmol/L; P<0.001; Figure 4B and Figure 1B in the online-only Data Supplement), whereas SDMA plasma concentrations did not change significantly (saline versus ADMA treated, 0.11±0.01 versus 0.13±0.01 μmol/L; P=0.244; Figure 4C and Figure 1C in the online-only Data Supplement).

In Vitro Effect of MPO on Recombinant hDDAH1 Activity

H₂O₂ plus MPO treatment resulted in a significant reduction of recombinant hDDAH1 activity by 78% (100% versus 22%; P<0.001; Figure 5A). To investigate a direct protein-protein interaction between hDDAH1 and MPO, heat-inacti-
vated MPO and H2O2 were added, which did not affect hDDAH1 activity (100% versus 99%; P=1.00; Figure 5A). Both MPO (plus 10 μmol/L H2O2) and the MPO product HOCl itself dose-dependently impaired hDDAH1 activity, starting with an MPO concentration of 0.005 μg/100 μL (P<0.001; Figure 5B) and an HOCl concentration of 0.1 μmol/L (P=0.003; Figure 5C), respectively.

**In Vivo Effect of MPO on ADMA and SDMA Concentrations After Inflammatory Stimulation**

Under baseline conditions, no significant differences in ADMA plasma concentrations were observed between MPO−/− and C57 mice (MPO−/− versus C57, 0.82±0.04 versus 0.84±0.04 μmol/L; P=1.000; Figure 6A and Figure IIA in the online-only Data Supplement). However, after stimulation with LPS, ADMA plasma concentrations in C57 mice increased significantly (C57+LPS, 1.08±0.08 μmol/L; P=0.023 versus baseline), whereas the LPS-induced increase in ADMA in MPO−/− mice was not significant (MPO−/−+LPS, 0.95±0.07 μmol/L; P=0.790 versus baseline). SDMA plasma concentrations did not show a significant difference between the groups under baseline conditions (MPO−/− versus C57, 0.16±0.01 versus 0.16±0.01 μmol/L; P=1.000; Figure 6B and Figure IIB in the online-only Data Supplement). Under LPS treatment, SDMA plasma concentrations increased significantly in both groups without any significant difference between the 2 groups (C57+LPS, 0.25±0.02 μmol/L; MPO−/−+LPS, 0.25±0.02 μmol/L; each P<0.001 versus baseline). Interestingly, LPS treatment decreased L-NMMA plasma concentrations in both groups of animals (MPO−/− versus MPO−/−+LPS; 0.23±0.01 versus 0.10±0.01 μmol/L, P<0.001; C57 versus C57+LPS; 0.20±0.03 versus 0.13±0.02 μmol/L, P=0.080), whereas the L-NMMA plasma concentrations did not significantly differ between MPO−/− and C57 mice. Creatinine concentrations in the blood samples of all animal groups treated with saline/LPS revealed no significant increase after LPS challenge and no significant difference between the 2 groups.

**hDDAH1 Overexpression in Mice Attenuates the Effect of Long-Term ADMA Infusion and Short-Term Inflammatory Stimulation In Vivo**

ADMA-treated WT mice showed a significant increase in MPO concentrations at day 28 compared with saline-treated WT mice (WT+saline versus WT+ADMA, 95.3±6.3 versus 179.7±17.8 ng/mL; P=0.005; Figure 7A and Figure IIIA in the online-only Data Supplement). Interestingly, overexpression of the hDDAH1 transgene protected against the ADMA-induced increase in MPO (hDDAH1+salamine versus hDDAH1+ADMA, 125.8±21.6 versus 132.5±16.8 ng/mL;
$P=0.851$), underlining the potency of DDAH overexpression in opposing the MPO effect. In terms of the MPO plasma concentrations in WT and hDDAH1 mice treated with a saline-loaded minipump, we did not see significant differences after 28 days of treatment.

Before LPS application, hDDAH1 mice revealed significantly lower ADMA plasma concentrations compared with WT mice (hDDAH1 versus WT at baseline, 0.48 ± 0.02 versus 0.67 ± 0.03 μmol/L; $P=0.041$; Figure 7B and Figure IIB in the online-only Data Supplement). Four hours after LPS stimulation, ADMA plasma concentrations in WT mice were significantly elevated (WT + LPS, 0.95 ± 0.08 μmol/L; $P=0.019$), whereas hDDAH1 mice showed no significant increase in ADMA plasma concentrations (hDDAH1 + LPS, 0.52 ± 0.02 μmol/L; $P=0.308$). In addition, we assessed the SDMA plasma concentrations, showing no significant differences between the hDDAH1 and WT mice either at baseline (hDDAH1 versus WT, 0.17 ± 0.01 versus 0.15 ± 0.01 μmol/L; $P=1.000$) or after 4 hours of LPS treatment (hDDAH1 + LPS versus WT + LPS, 0.25 ± 0.02 versus 0.19 ± 0.02 μmol/L).

Figure 4. In vivo effect of long-term asymmetrical dimethylarginine (ADMA) infusion on myeloperoxidase (MPO) plasma concentrations. MPO plasma concentrations significantly increased in C57Bl/6 (C57) mice on long-term ADMA treatment (250 μmol · kg$^{-1}$ · d$^{-1}$; 28 days; A). Long-term ADMA infusion significantly increased ADMA (B), whereas SDMA plasma concentrations (C) were not significantly affected by ADMA treatment. C57 + saline, n = 14; C57 + ADMA, n = 13.

Figure 5. In vitro effect of myeloperoxidase (MPO) on recombinant human dimethylarginine dimethylaminohydrolase1 (DDAH1) enzyme activity. Addition of MPO (10 μg) and its substrate hydrogen peroxide ($H_2O_2$; 10 μmol/L) resulted in a significant decrease in recombinant human DDAH1 (hDDAH1) activity, whereas heat-inactivated MPO (10 μg) and $H_2O_2$ did not (A). In addition, MPO and $H_2O_2$ (B) and its product, hypochlorous acid (HOCl; C), dose dependently impaired hDDAH1 activity. n = 3 for controls, heat-inactivated MPO, and each individual concentration; † $P<0.001$. 

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$P=0.095$) but with a significant increase after LPS application in both groups. To exclude an artificial finding resulting from the LPS effect in the moribund animal, we determined the creatinine concentrations in all 4 groups, which revealed no significant increase after LPS challenge and no significant difference between the 2 groups.

**Short-Term ADMA Infusion Increases MPO Plasma Concentrations in Humans**

With ADMA infusions, there was a significant increase in mean plasma MPO concentrations ($189.7 \pm 10.5$ versus $207.7 \pm 16.0$ pmol/L; $P=0.006$; the Table) associated with the short-term rise in plasma ADMA concentrations ($0.74 \pm 0.06$ pmol/L).

**Figure 6.** Dimethylarginine concentrations in myeloperoxidase-deficient (MPO$^{-/-}$) mice after lipopolysaccharide (LPS) stimulation. Under baseline conditions, asymmetrical dimethylarginine (ADMA) plasma concentrations showed no significant differences between MPO$^{-/-}$ and C57 mice (A). After LPS stimulation, ADMA concentrations in C57 mice increased significantly, whereas the LPS-induced increase in ADMA in MPO$^{-/-}$ mice was attenuated. Symmetrical dimethylarginine (SDMA) plasma concentrations at baseline did not differ significantly between the 2 groups (B) and increased significantly in both groups after LPS treatment but without any significant difference between the 2 groups. ADMA and SDMA, respectively: C57, $n=20$; MPO$^{-/-}$, $n=17$; C57 + LPS, $n=19$; MPO$^{-/-}$ + LPS, $n=17$.

**Figure 7.** Effect of human dimethylarginine dimethylaminohydrolase1 (hDDAH1) overexpression on long-term asymmetrical dimethylarginine (ADMA) infusion and short-term lipopolysaccharide (LPS) challenge in vivo. ADMA-treated wild-type (WT) mice showed a significant increase in myeloperoxidase (MPO) concentrations at day 28 compared with saline-treated WT mice, whereas hDDAH1 overexpression was able to attenuate the ADMA-induced increase in MPO (A). MPO: WT + saline, $n=4$; WT + ADMA, $n=5$; hDDAH1 + saline, $n=2$; hDDAH1 + ADMA, $n=7$. Four hours after LPS stimulation, ADMA plasma concentrations in WT mice were significantly elevated, whereas hDDAH1 mice showed no significant increase in ADMA concentrations (B). ADMA: WT, $n=9$; hDDAH1, $n=9$; WT + LPS, $n=9$; hDDAH1 + LPS, $n=9$. 

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versus 5.82±0.51; *P<0.001, baseline versus 60 minutes of ADMA infusion). Conversely, discontinuation of ADMA infusions was associated with normalization of plasma MPO (207.7±16.0 versus 189.7±10.5 pmol/L; †P<0.001 versus baseline), although ADMA concentrations (5.82±0.51 versus 3.28±0.22 mol/L; †P<0.001 versus baseline) remained significantly above preinfusion levels at 20 minutes. SDMA and L-NMMA plasma concentrations were unaffected by ADMA infusions, as demonstrated in the Table.

Table. Concentrations of Myeloperoxidase, Asymmetrical Dimethylarginine, Symmetrical Dimethylarginine, and N\textsuperscript{\textgreek{n}}-Monomethyl-L-Arginine

<table>
<thead>
<tr>
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<th>Baseline</th>
<th>ADMA Infusion (60 min)</th>
<th>ADMA Discontinued (20 min)</th>
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<tbody>
<tr>
<td>MPO, pmol/L</td>
<td>189.7±10.5</td>
<td>207.7±16.0</td>
<td>192.6±13.0</td>
</tr>
<tr>
<td>ADMA, μmol/L</td>
<td>0.74±0.06</td>
<td>5.82±0.51†</td>
<td>3.28±0.22†</td>
</tr>
<tr>
<td>SDMA, μmol/L</td>
<td>0.41±0.03</td>
<td>0.43±0.03</td>
<td>0.46±0.03</td>
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<tr>
<td>L-NMMA, μmol/L</td>
<td>0.07±0.01</td>
<td>0.09±0.01</td>
<td>0.06±0.01</td>
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ADMA indicates asymmetrical dimethylarginine; SDMA, symmetrical dimethylarginine; MPO, myeloperoxidase; and L-NMMA, N\textsuperscript{\textgreek{n}}-monomethyl-L-arginine. n=10 healthy, normotensive individuals.

*P<0.006, †P<0.001 for baseline vs ADMA infusion and ADMA infusion vs ADMA discontinued, respectively.

Discussion

Leukocyte activation and concomitant release of MPO play a pivotal role in the development of endothelial dysfunction. Because both steps are regulated in an NO-dependent fashion, we investigated the interaction between the endogenous NOS inhibitor ADMA and the leukocyte-derived hemoprotein MPO. The present study provides evidence of the impact of an ADMA-induced pathogenic cycle on increased PMN activation, enhanced MPO release, and subsequent impairment of DDAH activity, which in turn accounts for an increase in ADMA concentrations (Figure 8). Briefly, the salient findings are the following. First, ADMA accumulates in human PMNs and impairs intracellular NOS activity. Second, ADMA accumulation in PMNs leads to PMN degranulation and increases superoxide release, resulting in enhanced PMN adhesion to human umbilical vein endothelial cells ex vivo. Third, the hypothesis of an ADMA/MPO interaction is confirmed in human individuals challenged with a short-term ADMA infusion. Fourth, in vitro, MPO via its product HOCl decreases recombinant hDDAH1 activity. Fifth, in vivo, the LPS-induced increase in plasma ADMA concentrations is attenuated in MPO−/− mice, and overexpression of the hDDAH1 gene is able to oppose the effects of MPO.

NO inhibits PMN activation and thereby exerts antiinflammatory effects. Treatment of mice with the NOS inhibitor N\textsuperscript{\textgreek{n}}-nitro-L-arginine or a soluble guanylyl cyclase inhibitor enhances neutrophil migration, rolling, and adhesion to the endothelium. The NOS substrate L-arginine and methylated arginines, eg, ADMA, are transported into the cells via the high-affinity CAT-1 transporter. In humans, this transporter is encoded by the SLC7A1 gene and has been demonstrated in neurons, endothelium, and macrophages. In the present study, we were able to detect the expression of the CAT-1 transporter in isolated human PMNs. We hypothesize that increased concentrations of circulating ADMA and subsequent enhanced cellular uptake by CAT-1 cause an intracellular ADMA accumulation in PMNs. This in turn may affect the activation state of PMNs. Indeed, NOS activity of PMNs was significantly impaired by ADMA; subsequently, characteristics of PMN degranulation, ie, MPO and elastase release, increased on ADMA stimulation in our present study. PMN

Figure 8. Pathogenic cycle between the nitric oxide (NO) synthase (NOS) inhibitor asymmetrical dimethylarginine (ADMA) and leukocyte-derived myeloperoxidase (MPO). ADMA plasma concentrations are elevated under conditions of increased cardiovascular risk factors (1). Elevated ADMA concentrations accumulate in polymorphonuclear neutrophils (PMNs) and impair intracellular NOS activity (2), resulting in activation, degranulation, and adhesion of PMNs (3). As a consequence, MPO plasma concentrations and local MPO activity are enhanced (4). Subsequently, the MPO product HOCl impairs dimethylarginine dimethylaminohydrolase1 (DDAH) activity (5), resulting in a further increase in ADMA concentrations (6).
Degranulation is a crucial process that occurs through tightly controlled receptor-coupled mechanisms, leading to regulated exocytosis. Phosphorylation of p38 mitogen-activated protein kinase (MAPK) is considered a key event affecting the activation state of PMNs. Activation of p38 MAPK induces activation of the NADPH oxidase-dependent respiratory burst. We have previously shown that the addition of MPO to PMNs increased phosphorylation of p38 MAPK. Interestingly, ADMA enhances phosphorylation of p38 MAPK in endothelial cells. In our present study, exposure to ADMA led to degranulation of PMNs; therefore, ADMA acted as a mediator of the activation state of PMNs (ie, enhanced MPO activity, superoxide release). Another possible mechanism by which altered NO bioavailability may influence PMN degranulation has been described by Fortenberry et al. These investigators showed that NO impairs oxidative cell function of PMNs and increases neutrophil cell death in part by enhancing DNA fragmentation, resulting in a markedly impaired release of toxic granula content (ie, MPO). As a result of the ADMA-induced PMN activation in our present study, the isolated human PMNs showed a significantly increased adhesion to human umbilical vein endothelial cells ex vivo. To assess the impact of ADMA on PMN and MPO release in vivo, we investigated the effect of long-term ADMA treatment in C57 mice. In this model, long-term ADMA infusion significantly increased MPO plasma concentrations in mice. Intriguingly, our hypothesis of an ADMA/MPO interaction was confirmed in humans challenged with a short-term ADMA infusion, resulting in significantly increased MPO plasma concentrations.

Studies using hDDAH1 transgenic mice underlined the impact of lowering ADMA plasma concentrations in inflammatory responses within the vessel and/or myocardium. Overexpression of the hDDAH1 gene diminished the development of transplant vasculopathy after heterotopic heart transplantation. DDAH1 overexpression in the recipient reduced ADMA plasma concentrations, myocardial oxidative stress, cytokine elaboration, and inflammation in the allograft. These effects were associated with less graft coronary artery disease and improved function of the allograft. In another study, hDDAH1 overexpression markedly reduced myocardial reperfusion injury after occlusion and reopening of the left coronary artery. Decreased NO bioavailability in ischemia reperfusion injury triggers a cascade of pathophysiological events, ie, upregulation of adhesion molecules, leukocyte adhesion to endothelial cells, transmigration of PMNs, and subsequent tissue damage of the reperfused myocardium.

Stuhlinger et al demonstrated that ADMA concentrations were elevated and DDAH activity was impaired in the early phase of reperfusion, resulting in reduced NO bioavailability. Interestingly, enhanced leukocyte activity after ischemia reperfusion (determined by enzyme activity of MPO) was markedly reduced in hearts of hDDAH1 transgenic mice. In our present study, hDDAH1-overexpressing mice were protected against an ADMA-induced increase in plasma MPO and an LPS-induced increase in plasma ADMA concentrations. Therefore, the findings of our present study may offer an explanation for the potency of DDAH overexpression in opposing the MPO effect, eg, in myocardial reperfusion injury.

NO serves as a physiological substrate for MPO, and MPO attenuates NO-dependent smooth muscle cell relaxation. Thus, it was discussed that MPO may serve as a catalytic sink for NO, limiting its bioavailability. Indeed, our data demonstrate that MPO impairs the production of NO by impairing DDAH activity. Redox-sensitive inactivation of DDAH by superoxide and peroxynitrite has been described by Leiper et al. In our present study, hDDAH1 incubation with MPO and its substrate H2O2 or with the MPO product HOCl alone resulted in hDDAH1 inactivation. The possibility that, besides a mechanism-based effect of MPO on DDAH function, MPO may exert its effect on DDAH by direct protein-protein binding was excluded by showing that heat-inactivated MPO and H2O2 protein did not affect hDDAH1 activity.

In vivo ADMA plasma concentrations did not differ between MPO−/− and C57 mice at baseline. However, after stimulation with LPS, ADMA concentrations increased significantly in control mice, whereas in MPO−/− mice, this increase was blunted. SDMA plasma concentrations did not differ at baseline and increased significantly to the same extent in both mouse groups after LPS stimulation, suggesting that changes in renal function caused by systemic inflammation cannot explain the demonstrated differences in ADMA concentrations exclusively. More probably, the lack of MPO-induced inactivation of DDAH may have contributed to this effect in MPO−/− mice.

Recently, Wang et al performed an elegant study showing that patients with significant coronary artery disease revealed higher ADMA and SDMA plasma concentrations. Furthermore, dose-dependent increases in systemic ADMA and SDMA concentrations within the individuals were strongly associated with an increased risk for experiencing a major cardiovascular event over a 3-year follow-up period. Intriguingly, these investigators observed an increased arginine methylation index, a ratio indicating an increased posttranslational arginine methylation. These investigators concluded that the relationship between arginine methylation pathways and the development of coronary artery disease extends beyond direct NOS inhibition. Considering our recent results, this might be due to the competition of SDMA and ADMA with L-arginine at the CAT system in leukocytes, leading to a reduction of intracellular L-arginine concentrations in PMNs. In previous studies, impaired systemic NO production was not consistently associated with SDMA concentrations. However, the effect of SDMA on the intracellular L-arginine/NO homeostasis of PMNs may be more fragile, contributing to a decreased NO production, subsequent degranulation, and initiation of cardiovascular disease, additive to the intracellular ADMA accumulation.

Surprisingly, lower L-NMMA plasma concentrations were associated with significant coronary artery disease in the study by Wang et al. The authors argue that this association may reflect a heightened posttranslational modification of proteins through dimethylation reactions and proteolysis, producing ADMA and SDMA, in these patients. Indeed, inflammatory and redox-sensitive pathways (ie, activation
and degranulation of PMNs), which are known to enhance protein arginine residue posttranslational modification by methylation and subsequent proteolysis, may be a key mechanism accounting for the strong association of ADMA and cardiovascular disease. Our experiments in which we challenged C57 mice with LPS further support the hypothesis by Wang et al. In these studies, L-NMMA plasma concentrations decreased whereas ADMA concentrations increased after inflammatory stimulation.

Certainly, apart from the mechanisms described here, alternative mediators may influence the ADMA-MPO pathway. Besides the inactivation of DDAH by HOCl, DDAH is described to be inactivated by the oxidized low-density lipoprotein, which can be generated from low-density lipoprotein in an MPO-dependent fashion. Moreover, MPO may affect the accumulation of ADMA by modulating ADMA has been shown to activate the transcription factor with C57 mice (preliminary data not shown). Apart from that, production of chemokines. Therefore, it is conceivable that our data not only highlight so far unrecognized cytokine-like properties of ADMA but also identify MPO as a regulatory switch for ADMA bioavailability under inflammatory conditions. We believe that understanding the underlying mechanisms of the ADMA/MPO interaction may open a new avenue for the treatment of cardiovascular disease.

Conclusions

Our data reveal an ADMA-induced cycle of PMN activation, MPO release, and subsequent impairment of hDDAH1 activity. In addition, our data not only highlight so far unrecognized cytokine-like properties of ADMA but also identify MPO as a regulatory switch for ADMA bioavailability under inflammatory conditions. We believe that understanding the underlying mechanisms of the ADMA/MPO interaction may open a new avenue for the treatment of cardiovascular disease.

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Disclosures

Drs Maas, Schwedhelm, and Böger are named inventors on patents relating analytical assays for methylarginines and receive royalties from them. The other authors report no conflicts.

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The nitric oxide synthase inhibitor asymmetrical dimethylarginine (ADMA) and the leukocyte-derived hemoprotein myeloperoxidase (MPO) are associated with cardiovascular diseases. Activation of monocytes and polymorphonuclear neutrophils with concomitant release of MPO is regulated in a nitric oxide–dependent fashion. The present article describes an ADMA-induced cycle of polymorphonuclear neutrophil activation, MPO release, and subsequent impairment of human dimethylarginine dimethylaminohydrolase1 (hDDAH1) activity. In addition, the data not only highlight so far unrecognized cytokine-like properties of ADMA, but also identify MPO as a regulatory switch for ADMA bioavailability under inflammatory conditions. This finding suggests that understanding the underlying mechanisms of the ADMA/MPO interaction may open a new avenue for treatment of cardiovascular disease.
Pathogenic Cycle Between the Endogenous Nitric Oxide Synthase Inhibitor Asymmetrical Dimethylarginine and the Leukocyte-Derived Hemoprotein Myeloperoxidase


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Supplemental Material

Supplemental Figure Legends

Supplemental figure 1:

In vivo effect of chronic ADMA infusion on MPO plasma concentrations

MPO plasma concentrations significantly increased in C57Bl/6 mice upon chronic ADMA treatment (250 µmol/kg/d; 28 days; median at baseline vs. day 28, 26.0 vs. 35.3 ng/ml; 1A). Chronic ADMA infusion significantly increased ADMA (median at baseline vs. day 28, 0.70 vs. 1.64 µmol/l; 1B), whereas SDMA plasma concentrations (median at baseline vs. day 28, 0.13 vs. 0.12 µmol/l; 1C) were not significantly affected by ADMA treatment. C57+saline: N=14, C57+ADMA: N=13.

Supplemental figure 2:

Dimethylarginine concentrations in MPO \(^{-/-}\) mice after LPS stimulation

Under baseline conditions, ADMA plasma concentrations showed no significant differences between MPO \(^{-/-}\) and C57 mice (MPO \(^{-/-}\) vs. C57: median at baseline, 0.79 vs. 0.86 µmol/l; 2A). After lipopolysaccharides (LPS) stimulation, ADMA concentrations in C57 mice increased significantly (C57: median at baseline vs. LPS, 0.86 vs. 0.95 µmol/l), whereas the LPS-induced increase of ADMA in MPO \(^{-/-}\) mice was attenuated (MPO \(^{-/-}\): median at baseline vs. LPS, 0.79 vs. 0.91 µmol/l). SDMA plasma concentrations at baseline did not differ significantly between the two groups (MPO \(^{-/-}\) vs. C57: median at baseline, 0.16 vs. 0.16 µmol/l; 2B), increased significantly in both groups after LPS-treatment (C57: median at baseline vs. LPS; 0.16 vs. 0.25 µmol/l and MPO \(^{-/-}\): median at baseline vs. LPS, 0.16 vs. 0.25 µmol/l), however, without any significant difference between the two groups. ADMA: C57: N=20, MPO \(^{-/-}\): N=17, C57+LPS: N=19, MPO \(^{-/-}\)+LPS: N=17; SDMA: C57: N=20, MPO \(^{-/-}\): N=17, C57+LPS: N=19, MPO \(^{-/-}\)+LPS: N=17.
Supplemental figure 3:

*Effect of hDDAH1 overexpression on chronic ADMA infusion and acute LPS challenge in vivo*

ADMA-treated WT mice showed a significant increase in MPO concentrations at day 28 when compared with saline-treated WT mice (WT: median at baseline vs. day 28, 99.4 vs. 176.5 ng/ml; 3A), whereas hDDAH1 overexpression was able to attenuate the ADMA-induced increase of MPO (hDDAH1: median at baseline vs. day 28, 125.8 vs. 113.9 ng/ml; 3B). *MPO*: WT+saline: N=4, WT+ADMA: N=5, hDDAH1+saline: N=2, hDDAH1+ADMA: N=7.

Four hours after LPS stimulation, ADMA plasma concentrations in WT mice (WT: median at baseline vs. LPS, 0.67 vs. 1.00 µmol/l; 3B) were significantly elevated, whereas hDDAH1 mice showed no significant increase in ADMA concentrations (hDDAH1: median at baseline vs. LPS, 0.47 vs. 0.52 µmol/l). Creatinine concentrations revealed no significant increase after LPS challenge in all 4 groups and no significant difference between the two groups. *ADMA*: WT: N=9, hDDAH1: N=9, WT+LPS: N=9, hDDAH1+LPS: N=9.
Supplemental figure 1: von Leitner/Klinke et al.

(A) MPO [ng/ml]  
MPO [ng/ml] over time (day 28) for C57+saline and C57+ADMA groups. The p-value is 0.018.

(B) ADMA [µmol/l]  
ADMA [µmol/l] over time (day 28) for C57+saline and C57+ADMA groups. The p-value is <0.001.

(C) SDMA [µmol/l]  
SDMA [µmol/l] over time (day 28) for C57+saline and C57+ADMA groups. The p-value is 0.244.
Supplemental figure 2: von Leitner/Klinke et al.

(A) ADMA [µmol/l]

(B) SDMA [µmol/l]

C57 MPO- C57+LPS MPO-+LPS

p=0.790

p=0.023

p<0.001

p<0.001
Supplemental figure 3: von Leitner/Klinke et al.

(A) MPO [ng/ml]

- WT + saline
- WT + ADMA
- hDDAH1 + saline
- hDDAH1 + ADMA

(B) ADMA [µmol/l]

- WT + saline
- WT + LPS
- hDDAH1 + saline
- hDDAH1 + LPS

p-values:
- p=0.088
- p=0.134
- p=0.005
- p=0.851
- p=0.005
- p=0.019
- p<0.001
- p=0.041