Asymmetric Dimethylarginine and Reactive Oxygen Species
Unwelcome Twin Visitors to the Cardiovascular and Kidney Disease Tables

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Abstract—Plasma levels of asymmetric dimethylarginine or markers of reactive oxygen species are increased in subjects with risk factors for cardiovascular disease or chronic kidney disease. We tested the hypothesis that reactive oxygen species generate cellular asymmetric dimethylarginine that together cause endothelial dysfunction that underlies the risk of subsequent disease. Rat preglomerular vascular smooth muscle cells transfected with p22phox had increased NADPH oxidase activity, enhanced activity and expression of protein arginine methyltransferase, and reduced activity and protein expression of dimethylarginine dimethylaminotransferase and of cationic amino acid transferase 1 resulting in increased cellular levels of asymmetric dimethylarginine. Rats infused with angiotensin II had oxidative stress. The endothelial function of their mesenteric arterioles was changed from vasodilation to vasoconstriction, accompanied by increased vascular asymmetric dimethylarginine. All of these changes were prevented by Tempol. In vivo silencing of dimethylarginine dimethylaminotransferase 1 increased plasma levels of asymmetric dimethylarginine, whereas silencing of dimethylarginine dimethylaminotransferase 2 impaired endothelial function. We suggest that initiation factors, such as angiotensin II, expressed in blood vessels or tissues of subjects with cardiovascular and kidney disease risk factors generate reactive oxygen species from NADPH oxidase that enhances cellular asymmetric dimethylarginine in an amplification loop. This leads to adverse changes in vascular and organ functions, as a consequence of reduced tissue levels of NO and increased reactive oxygen species. Thus, we conclude that reactive oxygen species and asymmetric dimethylarginine form a tightly coupled amplification system that translates cardiovascular/kidney risk into overt disease. (Hypertension. 2012;59:00-00.)

Key Words: hypertension ■ endothelial function ■ NO ■ angiotensin II ■ NADPH oxidase

Symmetric (N\text{G}-, N\text{G}-) dimethylarginine (ADMA) and its stereoisomer, symmetric (N\text{G}-, N\text{G}-) dimethylarginine (SDMA) were isolated from human urine in 1970.\textsuperscript{1} Ogawa et al\textsuperscript{2,3} purified an enzyme from rats, termed dimethylarginine dimethylaminohydrolase (DDAH). He demonstrated its expression in the kidney\textsuperscript{4} and its ability to metabolize ADMA to citrulline and dimethylamine. Valence et al\textsuperscript{5} reported a large increase in plasma concentrations of ADMA in patients with chronic kidney disease (CKD) and end-stage renal disease and showed that ADMA inhibited NO synthase (NOS) and that ADMA and symmetrical dimethylarginine inhibited cationic amino acid transferase (CAT). ADMA was shown to impair endothelial function and to raise peripheral and pulmonary vascular resistance.\textsuperscript{6} Durban et al\textsuperscript{7} isolated protein arginine methyltransferase (PRMT) enzymes from brain and showed that they asymmetrically (class 1) or symmetrically (class 2) methylated arginine moieties on proteins, which, after hydrolysis, liberated ADMA or SDMA within the cell.\textsuperscript{8} The major pathway for arginine uptake and for ADMA or SDMA exit from cells was shown to be via the family of CATs.\textsuperscript{9} Extensive epidemiological studies by others\textsuperscript{10–17} established close relationships between plasma levels of ADMA and most of the CKD and cardiovascular disease (CVD) risk factors. In prospective studies, plasma ADMA predicted the subsequent development of CKD or CVD or the rate of progression of CKD.\textsuperscript{12,16} These aspects of ADMA and DDAH have been reviewed recently.\textsuperscript{18–20} Collectively, they identify ADMA as an important endogenous inhibitor of NO generation, thereby predisposing to endothelial dysfunction, vasoconstriction, salt sensitivity, and vascular remodeling, which are precursors of hypertension, CKD, and CVD.

Reactive oxygen species (ROS) represent a family of unstable, reactive, intermediate metabolites of oxygen, notably superoxide anion (O\textsubscript{2}\textsuperscript{−}) and hydroxyl radical (•OH). ROS generally also refer to more stable compounds, such as hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}). ROS can have diverse effects. Thus, the isolated, perfused afferent arteriole is contracted by O\textsubscript{2}\textsuperscript{−}\textsuperscript{21,22} and by H\textsubscript{2}O\textsubscript{2} at relatively high concentrations,\textsuperscript{23} whereas lower concentrations of H\textsubscript{2}O\textsubscript{2} impair myogenic contractions.\textsuperscript{23} Moreover, H\textsubscript{2}O\textsubscript{2} generated by an uncoupled endothelial NOS (eNOS) in small vessels from angiotensin II (Ang II)-infused rats caused vasorelaxation.\textsuperscript{23} Clearly, ROS are a diverse species with agent- and...
concentration-dependent effects on microvascular function. However, during states of oxidative stress, the predominant microvascular effects of ROS have usually been vasoconstriction. Much work initiated by Harrison and colleagues established important roles for $O_2^{-}$ and $H_2O_2$ generated by NADPH oxidase and superoxide dismutase in the development of hypertension and associated microvascular and organ dysfunction. NADPH oxidase was shown to be activated by Ang II, endothelin 1, thromboxane A$_2$, and aldosterone or by vascular stretch. Although difficult to quantify in vivo, several studies reported that markers of ROS accompanied or predicted the subsequent development of hypertension, CKD, and CVD. ROS and antioxidants have been extensively reviewed recently.

NO has potent vasodilator actions on blood vessels and protective effects on the kidneys, heart, and brain in hypertensive models. Rubanyi and Vanhoutte demonstrated that the endothelium-derived relaxing factor (EDRF) was destroyed by $O_2^{-}$ in the blood vessel even before the development of NO. In fact, the major route for bioactivation of NO in the tissues is by reaction with $O_2^{-}$ to form the oxidative molecule, peroxynitrite (ONOO$^-$). Long-term provision of excess NO by oral nitrite therapy can reduce blood pressure and moderate angina pectoris. However, tolerance develops to its effects because of oxidative stress, and long-term therapy can be toxic, perhaps because of ONOO$^-$ formation.

Clearly, both ADMA and ROS have considerable potential to mediate hypertension and its adverse effects on CVD and CKD. They share similar clinical epidemiology where evidence of increased levels of both circulating ADMA and circulating markers of ROS accompany CVD and CKD risk factors and can predict subsequent adverse events. They share a similar pathophysiological end point where both can reduce bioactive NO albeit via different mechanisms. Thus, ADMA reduces NO primarily by inhibition of NOS, whereas ROS inhibit NO by its bioactivation to peroxynitrite and by oxidation and inactivation of the NOS cofactor tetrahydrobiopterin, which uncouples the enzyme and directs it to generate ROS rather than NO. Accordingly, our studies have concluded that p22phox-induced generation of NADPH oxidase reduces NO primarily by inhibition of NOS, whereas ROS increases ADMA. These studies encompass cellular, vascular, and whole animal projects, which are the subject of this review.

**Generation of ADMA by ROS**

Evidence that ROS can increase ADMA has been quite sparse. Bo¨ger et al reported that incubation of human endothelial cells with low-density lipoprotein cholesterol, as an oxidizing agent, upregulated the synthesis of ADMA. They demonstrated that ROS could enhance the generation of S-adenosylmethionine that increased the methylation of arginine epitopes by PRMT. Activation of NADPH oxidase by lysophosphatidylcholine increased ADMA. In contrast, increasing bioactive NO by oral administration of nitrate prevented ROS and restored normal levels of ADMA in a rat model of salt-sensitive hypertension and CKD.

We developed a cellular model of enhanced NADPH oxidase to explore its role in ADMA generation, metabolism, and transport. Microvessels express the neutrophil oxidase (NOX)-2$^3$ and NOX-4$^{50,51}$ isoforms of NADPH oxidase. Both require p22phox for membrane localization and for tissue expression. Ang II infusion increased the expression of p22phox in the kidneys$^{52}$ and the renal afferent arterioles.$^{21,53}$ Moreover, in vivo knockdown of p22phox in rats with targeted small interference RNAs (siRNAs) prevented the progressive increase in blood pressure and oxidative stress (indexed by isoprostane excretion) in rats infused with Ang II.$^{52}$ Incubation of rat preglomerular vascular smooth muscle cells with Ang II increased their expression of p22phox and led to concentration-dependent increases in $O_2^{-}$ and $H_2O_2$ generation at physiologically relevant levels of Ang II of $10^{-10}$ m. These responses were prevented by the flavoprotein antagonist diphenyleneiodonium or the redox-cycling nitroxy Tempol.$^{54}$

We developed a cell model of oxidative stress without the complicating effects of Ang II or other agonists to generate ROS. Stable transfection of preglomerular vascular smooth muscle cells with a vector containing p22phox (S-p22phox) compared with control cells transfected with an empty vector (wild type) led to 2.5- to 4.0-fold increases in mRNA and protein expression for p22phox and equivalent increases in $O_2^{-}$ and $H_2O_2$. PRMT-3 (a class 1 enzyme) was increased in wild-type cells incubated with 100 μmol/L of H$_2$O$_2$ and in S-p22phox versus wild-type cells, accompanied by a marked increase in asymmetrical methylated arginine moieties on proteins (an indirect measure of PRMT activity) detected by a specific antibody. Unexpectedly, the mRNAs for DDAH-1 and -2 and the activity of the DDAH-2 gene promoter were increased in S-p22phox, yet the protein expressions for DDAH-1 and -2 and the DDAH enzyme activity were reduced by 50%. Recently, Martin-Garrido et al demonstrated that the inositol 1,4,5-triphosphate receptor in vascular smooth muscle cells was oxidatively modified by H$_2$O$_2$ and that this marked the protein for proteasomal degradation. Consistent with this, S-p22phox cells had a marked increase in oxidatively modified proteins (detected by using an Oxy Blot analysis) that was prevented by coinubcation with Tempol or catalase. Moreover, pretreatment of S-p22phox cells with epoxomicin to inhibit proteasomal degradation prevented the reduction in DDAH-1 or -2 protein expression in S-p22phox cells. S-p22phox cells had a 30% reduction in CAT activity, assessed by lysine-inhibitable [C$^{14}$]-ADMA uptake, accompanied by an equivalent reduction in the mRNA expression of CAT-1. However, unlike this study, CAT-1 mRNA did not correlate with CAT activity in the kidneys of rats with CKD. Finally, levels of ADMA in S-p22phox cells were increased 3-fold, but this was reduced by epoxomicin. We concluded that p22phox-induced generation of NADPH oxidase increased the synthesis and reduced the metabolism and cellular export of ADMA.

ADMA added to the medium of tetrahydrobiopterin-depleted endothelial cells (to uncouple NOS) caused dose-dependent increases in $O_2^{-}$ generation from eNOS, although this required 1 μmol/L of ADMA, which is 2 or 3 times higher than plasma levels. Whether eNOS-derived $O_2^{-}$ contributed to ADMA generation in endothelial cells is not yet known but seems probable. Collectively, these results demonstrate a potential positive feed-forward mechanism whereby cellular $O_2^{-}$ generation via NADPH oxidase can...
increase ADMA that, in endothelial cells, can further increase $O_2^-$ generation from eNOS.

**Relationship of ADMA and ROS to Endothelial Dysfunction in Vessels From Ang II–Infused Rats**

Studies of the effects of Ang II on ADMA have produced conflicting results. Incubation of endothelial cells with Ang II enhanced the expression of PRMT, reduced the DDAH activity, and doubled the release of ADMA. These effects were dependent on Ang II type 1 receptors. Mice infused with Ang II for 2 weeks had a 4-fold increase in circulating ADMA, which was related to reduced DDAH activity, because this rise in ADMA was blocked in DDAH-2 transgenic mice. Indeed, Ang II decreased DDAH-1 expression in the rat kidney and stimulated tumor necrosis factor-$
\alpha$ generation in blood vessels that itself inhibited DDAH and could thereby provide another pathway to increase levels of ADMA. Onozato et al confirmed that incubation of rat kidney cortex sections with Ang II downregulated DDAH-1 expression. Moreover, some studies have reported a reduction in plasma ADMA with angiotensin-converting enzyme inhibitors or angiotensin receptor blockers. On the other hand, the anticipated increase in plasma ADMA with Ang II infusion has been inconsistent. Thus, prolonged infusions of Ang II did not change ADMA in 2 studies except at high rates of Ang II infusion into rats over 6 weeks that caused renal damage.

To resolve these controversies, we studied the effects of Ang II on ADMA synthesis, metabolism, and membrane transport in isolated glomerular vascular smooth muscle cells. These studies were followed by measurement of plasma and tissue levels of ADMA in mesenteric resistance arterioles (MRAs) of rats and related these to endothelial function in these vessels.

Incubation of rat pregglomerular vascular smooth muscle cells with Ang II increased the activity of NADPH oxidase but decreased the activities of DDAH and CAT. This was accompanied by dose-dependent increases in $O_2^-$ generation and in cellular levels of ADMA. This is consistent with decreased metabolism of ADMA by DDAH. However, there were no parallel short-term increases in medium ADMA, consistent with decreased cellular export of ADMA by CAT. The cellular pathways for ADMA synthesis, metabolism, and transport and the sites of interaction with ROS identified in these studies are shown in Figure 1.

To test whether the conclusions derived from these cellular studies could be translated into in vivo changes in endothelial function, rats were infused for 2 weeks with Ang II at a rate that raised their MAP slowly after 2 to 3 days (slow pressor response) or with vehicle. Alternate rats in each group received the redox cycling nitroxide Tempol or vehicle added to the drinking water to prevent the development of oxidative stress.

MRAs dissected from rats after 2 weeks of Ang II infusion and studied in an isometric myograph had a diminished expression of DDAH-2, a diminished acetylcholine-induced EDRF response and NO activity, a diminished EDHF response, and the appearance of a new endothelium-dependent contracting factor response accompanied by acetylcholine-induced generation of microvascular ROS. All of these effects were prevented in rats given Tempol throughout. Ang II did not change the plasma levels of ADMA yet led to a ROS-dependent (Tempol-inhibitable) increase of 40% to 100% in the tissue levels of ADMA in the MRA, the aorta, and the kidney cortex. These studies demonstrated that ROS enhanced cellular levels of ADMA in resistance arterioles and transformed endothelial function from a beneficial mode of vasodilatation to a detrimental mode of vasoconstriction.

**Figure 1.** Cellular mechanisms involved in the generation, action, metabolism, and transport of asymmetric dimethylarginine (ADMA) and the major sites of interaction with reactive oxygen species (ROS).
An increase in ADMA in endothelial cells likely contributed to the endothelial dysfunction by impairing NOS activity, NO generation, and EDRF. However, the relative importance of increased ADMA and increased NO bioinactivation by O$_2$•• in the blood vessels was not established in this study.

Coincubation of endothelial cells with ADMA led to a dose-dependent increase in their production of O$_2$••.57,70 This demonstrated the potential for ADMA to increase endothelial ROS, but further work is required to determine the importance of this effect in vivo.

Selective Expression and Function of DDAH Isoforms

The finding that DDAH and NOS were expressed at similar sites in the kidneys suggested functional interactions.60 Using isoform-specific antibodies, DDAH-1 was found to be expressed weakly in blood vessels but strongly in the renal proximal tubules.62 Because the kidney is the major site for plasma ADMA clearance from the circulation in rats and humans,71,72 these findings predicted that plasma ADMA would depend on DDAH-1. In contrast, DDAH-2 was expressed strongly in the endothelium, vascular smooth muscle cells and adventitia of blood vessels, and in the macula densa cells, the distal tubules, and the collecting ducts of the kidney.60 This finding predicted that endothelial function would depend on DDAH-2.

To test these predictions, rats were administered specific siRNAs targeted to either DDAH-1 or -2 and compared with nontargeted control siRNAs. The targeted siRNAs led to isoform-specific reductions of 35% to 70% of the mRNA and protein expressions for DDAH in the kidney cortex, liver, and MRAs after 1 and 3 days.73 Only knockdown of DDAH-1 increased plasma ADMA, whereas only knockdown of DDAH-2 blunted the EDRF and NO responses of MRAs to acetylcholine. This was of interest because previous studies had not found a significant role for DDAH-2 in metabolizing ADMA.74,75 To test whether the reduced EDRF response after knockdown of DDAH-2 could be ascribed to competitive inhibition of eNOS by accumulated ADMA, vessels were incubated with a 5-fold molar excess of the NOS substrate, l-arginine. This improved, but did not correct, the EDRF responses, thereby pointing to an additional defect after DDAH-2 knockdown other than competitive inhibition of NOS by ADMA. Indeed, the MRAs from rats given siRNA to DDAH-2 were found to have a reduced expression of the mRNA and protein for eNOS. We concluded that DDAH-2 preserved endothelial function by a combination of metabolizing endothelial ADMA and by enhancing the expression of eNOS.

Conclusions

These studies have disclosed 3 major sites of cellular interaction between ROS and ADMA (Figure 1). How might this fit into the pathophysiology of cardiovascular and kidney disease?

Our hypothesis is shown in Figure 2. Clinical CVD and CKD risk leads to the elaboration of initiator mechanisms that can activate NADPH oxidase to enhance ROS generation. ROS, in turn, can stimulate the expression of the initiating factors, thereby setting up the first ROS-dependent feed-forward enhancing mechanism. For example, ROS increase renal or vascular levels of Ang II,76 thromboxane A$_2$,21 and endothelin177,78 and can activate the sympathetic nervous system79 and aldosterone receptor signaling.80 ROS also can increase PRMT class I activity, decrease DDAH activity, and decrease CAT activity, thereby increasing cellular ADMA. Over time, this can be accompanied by increased circulating ADMA levels.19 Because ADMA itself also can increase ROS, this establishes a second potential ROS-dependent, amplifying, feed-forward cellular loop, whereby an initial increase in cellular ROS can stimulate the generation of ADMA that further stimulates ROS production. These effects of ROS to increase ADMA would impair cellular NO generation in addition to the established effects of ROS to bioinactivate NO and to uncouple eNOS by oxidation of its tetrahydrobiopterin cofactor.46 NO deficiency and oxidative stress dictate a series of pathophysiological events that culminate in overt vascular, cardiovascular, or CKD (Figure 2).

The proposed sequence outlined in Figure 2 may explain the durability of major risk factors, such as hypertension, and the frequency with which effective antioxidant, such as Tempol, reduce hypertension and its consequences in animal models. Thus, of 63 reports of the long-term (days or months) effects of the redox-cycling antioxidant Tempol in animal models of hypertension, the blood pressure was reduced significantly in 87%.25 Tempol was effective in high- and low-renin, salt-sensitive and salt-resistance, genetic and environmental models in rabbits, rats, and mice. For example, hypertension initiated in rats by the acute placement of a renal artery clip is mediated initially by Ang II. However, over time, the importance of angiotensin diminishes and is replaced by oxidative stress,81 which becomes the principal determinant of the hypertension and renal vasconstriction.82 Thus, during established 2-kidney, 1-clip hypertension, Tempol restored blood pressure, renal hemodynamics, and oxygenation, whereas an angiotensin receptor blocker was not effective.83 ROS generated in a clipped or ischemic kidney or during prolonged Ang II infusion can recruit other mediators that include thromboxane A$_2$,21 endothelin1,77,87 and the sympathetic nervous system87 and can enhance aldosterone signaling.80 In turn, these can activate ROS further to establish a ROS-dependent positive feedback loop (Figure 2).

What can be done to break this chain of events? Production of ROS could be a preferred target, because this is at the apex of 2 positive feedback loops linking cardiovascular/kidney risk to disease. Unfortunately, vitamin E is the only widely available antioxidant for use in human clinical trials. Vitamin E was almost ineffective in blocking cellular superoxide production with Ang II.86 Moreover, the dose of vitamin E required to suppress oxidative stress in humans was 4- to 8-fold higher than that used in clinical trials.89 Thus, the oxidative stress hypothesis has yet to be tested rigorously in human subjects. In contrast, Tempol is as effective as superoxide dismutase itself in preventing cellular superoxide generation by Ang II.88 Tempol is a general purpose antioxidant that reduces the level of many ROS, including hydroxyl radical, peroxynitrite, and hydrogen peroxide, in addition to superoxide.25 Tempol has been reported effective in many
animal models in protecting organs from the effects of hypertension and other cardiovascular risk factors. Tempol could be a model for future drug development to break the positive amplification loops that reinforce the tight relationship between cardiovascular and kidney disease risk and established disease.

I thank Wing Kam Emily Chan and Glenda Baker for preparing the article.

I gratefully acknowledge the widespread support and collaboration from many colleagues in the conduct of these studies. This review formed the basis for my presentation at the American Heart Association Council on High Blood Pressure Research Excellence in Hypertension 2011 award presentation.

None.
References


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_Hypertension_. published online January 3, 2012;
_Hypertension_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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