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Invited Review

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Dimethylarginine dimethylaminohydrolase (DDAH): expression, regulation, and function in the cardiovascular and renal systems

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Palm F, Onozato ML, Luo Z, Wilcox CS. Dimethylarginine dimethylaminohydrolase (DDAH): expression, regulation, and function in the cardiovascular and renal systems. *Am J Physiol Heart Circ Physiol* 293: H3227–H3245, 2007. First published October 12, 2007; doi:10.1152/ajpheart.00998.2007.—Asymmetric (N^G,N^G)-dimethylarginine (ADMA) inhibits nitric oxide (NO) synthases (NOS). ADMA is a risk factor for endothelial dysfunction, cardiovascular mortality, and progression of chronic kidney disease. Two isoforms of dimethylarginine dimethylaminohydrolase (DDAH) metabolize ADMA. DDAH-1 is the predominant isoform in the proximal tubules of the kidney and in the liver. These organs extract ADMA from the circulation. DDAH-2 is the predominant isoform in the vasculature, where it is found in endothelial cells adjacent to the cell membrane and in intracellular vesicles and in vascular smooth muscle cells among the myofibrils and the nuclear envelope. In vivo gene silencing of DDAH-1 in the rat and DDAH +/− mice both have increased circulating ADMA, whereas gene silencing of DDAH-2 reduces vascular NO generation and endothelium-derived relaxation factor responses. DDAH-2 also is expressed in the kidney in the macula densa and distal nephron. Angiotensin type 1 receptor activation in kidneys reduces the expression of DDAH-1 but increases the expression of DDAH-2. This rapidly evolving evidence of isoform-specific distribution and regulation of DDAH expression in the kidney and blood vessels provides potential mechanisms for nephron site-specific regulation of NO production. In this review, the recent advances in the regulation and function of DDAH enzymes, their roles in the regulation of NO generation, and their possible contribution to endothelial dysfunction in patients with cardiovascular and kidney diseases are discussed.

nitric oxide synthase; hypertension; diabetes mellitus; chronic kidney disease; asymmetric dimethylarginine

ASYMMETRIC (N^G,N^G) dimethylarginine (ADMA) is an endogenous methylated amino acid that inhibits the constitutive endothelial (e) or type III and neuronal (n) or type I isoforms of nitric oxide (NO) synthase (NOS) (49, 91, 103, 199). It is a less potent inhibitor of the inducible (i) or type II NOS isoform (41, 191, 213). Proteins are subject to methylation of arginine residues by protein arginine methyltransferase (PRMT). *S*-adenosylmethionine, which is synthesized from methionine and ATP, serves as the methyl donor and, in the process, is converted to *S*-adenosylhomocysteine, which itself can be hydrolyzed to homocysteine. Remethylation of homocysteine in the "remethylation pathway" regenerates methionine (14, 179). ADMA is released by protein hydrolysis and exported from the cell and taken up by other cells via system y^+ carriers of the cationic amino acid (CAT) family (14, 196, 212). ADMA is eliminated both by renal excretion and metabolic degradation. Its metabolism is facilitated by dimethylarginine dimethylaminohydrolases (DDAHs), which are expressed as type 1 and 2 isoforms. Recent studies have shown differential sites of expression of DDAH-1 and -2 in blood vessels and the kidneys and differential regulation of the renal expression of these isoforms by ANG II acting on type 1 receptors (AT_1-Rs) (127). DDAH also is subject to extensive posttranscriptional inhibition, for example by reactive oxygen species (ROS) generated by nicotinamide adenine dinucleotide phosphate (NADPH) oxidase or by homocysteine (65, 190). These concepts are described in greater details in the body of this review and are introduced in Fig. 1[1].

Since DDAH metabolizes ADMA (2) and regulates plasma levels of ADMA, it can determine bioavailable NO (36, 204). NO activity in blood vessels mediates an important component of the endothelium-dependent relaxation factor (EDRF) responses in blood vessels (131), prevents vascular remodeling (52, 184) maintains a stable blood pressure (BP) and renal

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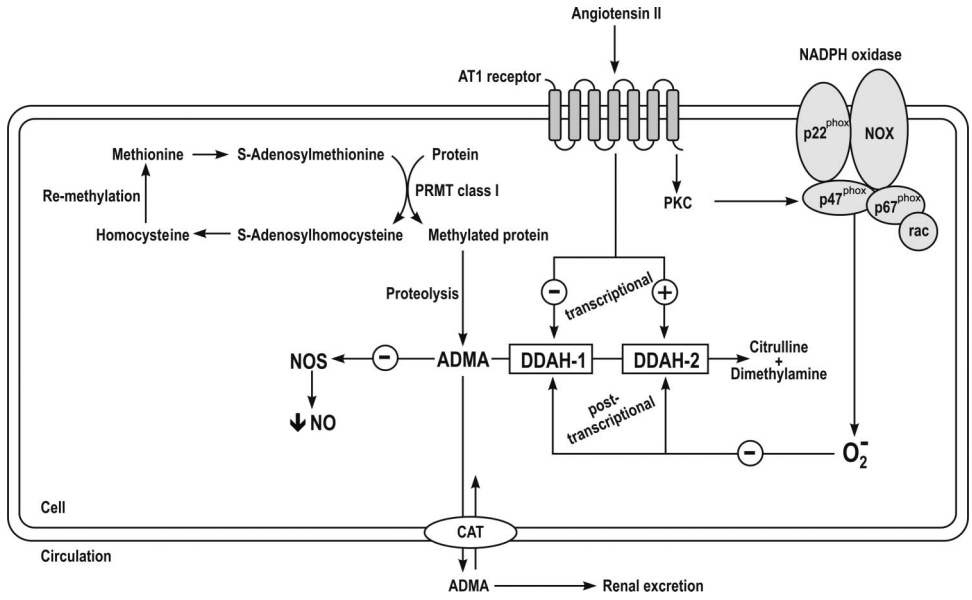

Fig. 1. Cell diagram of the formation of asymmetric methyl arginine (ADMA) involving protein arginine methyl transferase (PRMT) and the effect of ADMA to block nitric oxide (NO) formation by nitric oxide synthase (NOS), its metabolism by dimethylarginine dimethylaminohydrolase (DDAH) type 1 or 2, and its export from the cell via the cationic amino acid transporter (CAT), detailing some pathways of interaction with angiotensin II (ANG II) acting on type 1 receptors (AT_1-Rs) that activate protein kinase C (PKC) and the effect of superoxide anion ($O_2^{\bullet -}$) generated by nicotinamide adenine dinucleotide phosphate (NADPH) oxidase.

vascular resistance (RVR) (15, 38, 40), inhibits Na^+ entry into the thick ascending limb (TAL) of the loop of Henle (LH) (128) and the collecting ducts (CDs) (163), and prevents salt sensitivity (143). Consequently, DDAH could have a pivotal role in maintaining the homeostatic integrity of the cardiovascular and renal systems. This is the focus of this review. Readers are referred to recent reviews for further information on the cardiovascular and renal actions of NO (50, 86, 87, 151, 154, 195, 216, 217, 218) and ADMA (14, 18, 21, 34, 91, 99, 189, 192, 193, 196, 197, 234, 235). The chemistry of DDAH has been reviewed recently (78).

The type 1 isoform of DDAH in the rat is a 285-amino acid protein with a molecular mass of ~33 kDa (124). The K_m for metabolism of ADMA is 0.18 mM. The plasma levels of ADMA in humans and rats in most studies are in the range of 0.3 to 0.5 μmol/l (198). ADMA is accumulated in cells via CATs that are widely expressed (77), accounting for higher intracellular concentrations of ADMA. The intracellular ADMA concentrations in endothelial cells harvested from the carotid artery of the rabbit are 10-fold higher than plasma levels and increase further to ~25-fold higher in vessels from rabbits with streptozotocin-induced diabetes mellitus (105). Such high intracellular levels of ADMA should normally be well above the K_m for DDAH. Thus the intracellular concentrations of ADMA should be very sensitive to changes in the activity or expression of DDAH. This concept was tested recently in the rat in our laboratory (204), which reported that a 30–40% reduction in DDAH-1 expression in the liver, kidneys, and blood vessels of rats 72 h after intravenous injection of a small interference RNA (siRNA) targeted to DDAH-1 increases the serum ADMA concentrations by 20–30%. The authors report no changes in plasma ADMA levels in rats that had received a nontargeted control siRNA injection, or an injection of siRNA targeted to DDAH-2 (204). A limitation of this study is that tissue ADMA concentrations in the kidney, liver, and mesenteric artery were not evaluated. Therefore, this study could not relate intracellular ADMA levels to DDAH expression. Leiper et al. (90) reported that heterologous downregulation of DDAH in the mouse increases the concentration of ADMA in the plasma, brain, and lung by 20%. The modest increase in tissue ADMA levels in this model may relate to the modest decreases in DDAH expression in the brain and the fact that DDAH-2 is the principal isoform expressed in the lung. Moreover, possible confounding effects of changes in arginine uptake or PRMT function were not evaluated.

This review will consider the development of ideas on DDAH; the regulation of the transcription, the chromosomal location, the structure, and the regulation of the genes for DDAH; their sites of expression, posttranscriptional regulation, and protein-protein interactions; and their potential roles in disease states. The emphasis is on new findings in this rapidly developing field.

Brief Overview of Development of Knowledge on DDAH

The isolation of ADMA and its stereoisomer N^G,N'^G-dimethylarginine (symmetric dimethylarginine; SDMA) from human urine in 1970 by Kakimoto and Akazawa (70) established that they are normal products of metabolism and are subject to renal elimination. Ogawa et al. (122–124) in 1987 first described an enzyme that degrades ADMA, which was subsequently termed N^G,N^G-dimethylarginine dimethylaminohydrolase (EC 3.5.3.18). In 1999, Vallance, Leiper, and colleagues (93, 188) reported that DDAH has two isoforms (DDAH-1 and DDAH-2) that are expressed in bacteria, sheep, mice, rats, and humans. The finding by Tojo et al. that DDAH and NOS isoforms have distinct sites of expression in blood vessels and the kidney (182, 183) and that DDAH and NOS are co-expressed in cells (185) established that NO activity may be regulated by DDAH-induced changes in cellular ADMA concentration in a cell-specific manner (93, 204, 214).

DDAH catalyzes the metabolism of one molecule of ADMA to one molecule of L-citrulline and one molecule of dimethylamine. There is no evidence to date of specific cofactors required for the catalytic reaction. Purified DDAH has a maximum activity at pH 5.2 (124) to 6.5 (123). Its optimum temperature is 55°C (123). When incubated at 37°C and between pH 5.0 and 8.5, the enzyme is stable for at least 1 h

(93, 122, 123, 188). Knipp et al. report that DDAH-1 is a Zn(II)-containing protein (23, 80). Zn(II) is not involved in the catalytic process, but it is required to stabilize the enzyme in a fully active form (80). Most in vitro studies have been performed with DDAH-1. They report a wide variability of response to temperature and pH that may relate to the amount of Zn(II)-free enzyme that occurs under the condition of the buffer used to perform those experiments. In buffers that lack an appreciable metal-binding affinity, the effects of pH and temperature on enzyme activity reflect the presence of a small amount of Zn(II)-free DDAH-1. The Zn(II)-free enzyme has maximal activity at physiological pH (80).

Studies by Birdsey et al. (17) and Murray-Rust et al. (114) established that DDAH metabolizes ADMA intracellularly, whereas SDMA is not a substrate for DDAH. The specific affinity of DDAH for ADMA has been explained by its molecular structure. Murray-Rust et al. (114) describe that DDAH-1 has a substrate binding pocket that forms a small pore allowing the nonmethylated nitrogen side chain of ADMA to enter. On substrate binding, a loop closes the pore, and hydrophobic interactions of Phe75 with its $(CH_2)_3$-chain anchors the substrate ADMA in the active site pocket. On the other hand, SDMA has methyl groups on both nitrogens that render it sterically and electrostatically unable to enter this acidic pocket of DDAH. This is further reviewed by Knipp et al. (78). Vallance et al. (198) demonstrated further that ADMA and SDMA accumulate in the plasma of patients with end-stage renal disease (ESRD). This was postulated to be due to diminished ADMA metabolism by DDAH and diminished SDMA excretion by the functionally ineffective kidneys (189). Thus plasma ADMA will be dependent primarily on factors that affect DDAH expression and activity, whereas plasma SDMA will depend on the rate of renal excretion. Presently, there is little information on the handling of ADMA or SDMA by the nephron.

The observation that DDAH activity and protein expression do not always correlate led to the discovery by Leiper et al. (93) of a second human DDAH isoform, subsequently named DDAH-2. Their initial report was that DDAH-2 expression contributed only a minor fraction to total tissue DDAH activity. However, later studies established a marked cellular disparity in the expression of the two isoforms, with DDAH-2 making a predominant contribution in endothelial cells (188) where it determines NO bioactivity (204).

The crystal structure of DDAH-1 was published in 2007 by Leiper et al. (90). DDAH-2, whose human clone displays 62% homology at the amino acid level and 63% at the nucleotide level with human DDAH-1 (189), is considered to be the original form of the DDAH gene (188). DDAH-1 in rats has its highest expression in the kidney (122, 123). Human DDAH-1 has 93% identity with the rat enzyme at the protein level and displays similar enzymatic properties (93). It is now recognized that there are two major pathways for the clearance of ADMA, whose relative importance has distinct species variation (118).

ADMA is formed ubiquitously in all cells. It can be metabolized intracellularly by DDAH to citrulline and dimethylamine or be exported from the cell to the plasma by CAT, which is involved in both cellular release and cellular uptake of ADMA (179). ADMA is metabolized extensively by the kidneys and liver, which are principal sites of DDAH-1 expression. Some ADMA is excreted by the kidney (179). The sites of gene expression for DDAH correspond generally to those for NOS. DDAH-1 is expressed at sites of nNOS expression, such as the brain, and DDAH-2 at sites of eNOS expression such as endothelial cells, but there are exceptions to this rule (93). For example, the colocalization of DDAH and NOS isoforms in the kidney is more complex, as will be described below.

Clearly, DDAH activity must be considered in the context of factors that regulate L-arginine availability and NOS activity. In 1992, Vallance et al. (198) reported that ADMA accumulates in the plasma of patients with ESRD in sufficient concentration to inhibit NOS. This has led to the hypothesis that ADMA may contribute to the endothelial dysfunction, hypertension, atherosclerosis, and immune dysfunction of patients with ESRD (198). Moncada, Vallance, and colleagues reported further that ADMA causes dose-dependent vasoconstriction in rats (49) and humans (198). A growing body of epidemiological evidence links endothelial dysfunction with cardiovascular and renal disease risk factors or overt disease (56, 120, 121, 178). In parallel, intense physiological and biochemical investigation has thrown light on the mechanisms of ADMA accumulation and has highlighted the importance of DDAH activity, which is the focus of this review.

The Genes and Proteins for DDAH

Gene and protein expression studies. The expression of the DDAH-2 gene is increased by all-trans-retinoic acid (3), pioglitazone (a peroxisome proliferator-activated receptor-gamma ligand with antioxidant action) (201), and estradiol, which counters the effects of oxidized low-density lipoprotein (LDL) to reduce DDAH-2 expression in human endothelial cells (112). The expression of the gene for DDAH-2 is decreased by coupling factor 6 (CF6). CF6 is an essential component of the energy-transducing stalk of mitochondrial ATP synthase that inhibits phospholipase A_2, enhances ROS generation, and induces vasoconstriction (175). The expression of the protein for DDAH-2 is upregulated by all-*trans*-retinoic acid (3), pioglitazone (201), and estradiol (112), and is downregulated by CF6 (175), lipopolysaccharide (225), and high concentrations of glucose that generate ROS (160). The protein expression for DDAH-1 is increased by IL-1β (191) but is decreased by oxidized low-density lipoprotein (oxLDL) or TNF-α (64).

Chromosomal location of DDAH genes. Tran et al. (188) combined radiation hybrid mapping with fluorescence in situ hybridization to locate the human DDAH-1 gene on chromosome 1p22 and the DDAH-2 gene at the major histocompatibility complex (MHC) III region of chromosome 6p21.3. These chromosomal regions contain clusters of homologous genes. This has led to the proposal that a duplication in the gene for DDAH occurred when these clusters appeared in the hominid genome ~450 million years ago (188).

Structure of the genes and proteins for DDAH. The DDAHs have little homology to other mammalian arginine-modifying enzymes (93). However, Stone et al. (162a) report that bacteria and primitive eukaryotes express protein arginine deiminase (PAD), which also metabolizes arginine to citrulline and bears several similarities to DDAH. PAD may represent an

earlier evolutionary stage in the development of the DDAH gene (114, 144).

Human DDAH contains an 858-base pair (bp) open reading frame encoding a single 285-amino acid protein. Studies of the coding region of both DDAH-1 and DDAH-2 by Leiper, Vallance, and colleagues (93, 188) have shown that it is divided into six exons with highly conserved exon boundaries. In contrast, the length of the introns is distinctly different between DDAH-1 and -2 (93, 188).

The crystal structure of DDAH isolated from *Pseudomonas aureus* (PaDDAH) by Murray-Rust et al. (114) depicts a five-blade β/α-propeller topology consisting of five ββαβ-modules. The propeller-like arrangement of these modules forms a narrow channel with a central negatively charged core as the recognition site of the guanindinopropyl side chain of the methyl arginine substrate. It contains a conserved triad of His, Asp, and Cys amino acids, which catalyze the hydrolysis on the methyl arginine side chain.

A search of databases for expressed sequence tags from a number of organisms suggested to Tran et al. (188) that all DDAH-1 transcripts initiate from a single start site in the 5′-region of the open reading frame, but DDAH-2 transcripts have three transcription start sites in the 5′-region. These authors located a core promoter region of the DDAH-2 gene that contains a cytosine-phosphate diester-guanine (CpG) island in the 2 kilobases (kb) of DNA surrounding the transcription start sites. The presence of this island, and the lack of a TATA box in the promoter, may explain the widespread expression of the DDAH-2 in the fetus (188). The promoter region of DDAH-2 contains candidate transcription factor binding sites for early gene-2 factor, nerve growth factor-induced C/early growth response protein-2 gene, specificity protein-1, and interferon regulatory factor-1 (68).

The crystal structure of bacterial DDAH (PaDDAH) indicates that the active site contains a Cys-His-Glu catalytic triad (114), which are features shared by a new superfamily of arginine-modifying enzymes (48, 89, 114, 203). Despite this insight, studies have concluded that the active site varies among different species. The PaDDAH has His162 and Glu114 colocalized with Cys 249 on the coincident side of the site. The proposed catalytic mechanism for ADMA by PaDDAH is that ADMA is held within the active site of DDAH by a network of hydrogen bonds. The catalytic reaction occurs between the sulfur atom of Cys249 of DDAH and the carbon atom of the guanidine group of ADMA since replacement of the cystine residue of PaDDAH by serine inactivates the enzyme despite its retaining a normal solubility and folding. His162 and Glu114 maintain the reactive cystine in the active site in DDAH (114).

Further studies of the high-resolution crystal structure of bovine DDAH-1 and the alignment of DDAH protein sequences from mouse, sheep, and human sources, led to the conclusion that the catalytic site is composed of Cys273, Asp126, and His172 in DDAH-1; and Cys276, Asp125, and His171 in DDAH-2. These amino acids are conserved in all the mammalian species so far examined (47, 89, 114).

DDAH has a distinctive dimer interface, an apparently flexible loop that may close down the active site, and a unique substrate binding mode. The recent report by Frey et al. (47) of the crystal structure of bovine DDAH-1 confirms that it also contains a propeller-like fold similar to other arginine-modifying enzymes and a flexible loop that can adopt distinct conformations. This loop apparently may act as a lid to open or close a channel providing access of substrates to the active site.

The mouse, bovine, and human protein sequences show a homology of ~92% for DDAH-1 and 95% for DDAH-2. Species-specific variations in the amino acid sequences are found both in the substrate binding site and in the lid region. However, the amino acids that are proposed to be directly involved in substrate binding are conserved within each isoform among different species. The architecture of DDAH-2 is similar to that of DDAH-1, except in the substrate binding region.

Epigenetic regulation of DDAH gene expression. The methylation of mammalian genomic DNA at CpG islands and core histones are posttranslational modifications that are essential for normal development and gene regulation (16, 147). Studies in endothelial cells have not shown any effect of incubation with ADMA on methylation of the heavily methylated RNA binding protein Src-associated in mitosis, 68 kDa protein (Sam68) (137). Tomikawa et al. (187) analyzed the dynamics of epigenetic regulation of the upstream region of the mouse DDAH-2 gene. They reported that the expression of this gene in a stem cell population of trophoblast cell lineage is suppressed by a tissue-dependent, differentially methylated region in the regulatory site. This DNA region is hypermethylated in undifferentiated stem cells, which have a reduced DDAH-2 gene expression, but becomes hypomethylated when the DDAH-2 gene is expressed. Chromatin immunoprecipitation assays with antibodies to acetylated histone H3 and acetylated histone H4 reveal an increase in acetylation of these histones as cells differentiate. Inhibition of DNA methylation with 5′-Aza-2′-deoxycytidine (5-aza-dC) or inhibition of histone deacetylation with trichostatin A enhances DDAH-2 gene expression in undifferentiated cells. This important study identifies DNA methylation and histone acetylation in the promoter region of DDAH-2 as mechanisms that can control DDAH-2 gene transcription, at least in mice.

Single-nucleotide polymorphisms (SNIPs) of the DDAH genes. Using polymerase chain reaction (PCR) and single-strand conformational analysis, Jones et al. (68) have identified six common polymorphisms within the promoter region of the human gene for DDAH-2. A 6G/7G insertion/deletion polymorphism variant at position −871 in the core promoter region of DDAH-2 is associated with increased basal DDAH-2 protein expression.

Valkonen et al. (192) studied 1,609 middle-aged Finnish men who were participants in the Kuopio Ischemic Heart Disease Risk Factor Study. They identified another variant in the DDAH-2 gene and six variants in the DDAH-1 gene. A remarkable finding was an occurrence of coronary heart disease that was 50-fold higher among the carriers for a DDAH-1 mutation. About one-half of the relatives (spouses and siblings) of the 13 subjects identified as possessing the DDAH-1 mutation were carriers of this mutation. The family members identified in this study with this DDAH gene mutation had an increased prevalence of hypertension. Recently, Ryan et al. (142) reported a study of 236 patients undergoing elective cardiac surgery in which 107 were found to be homozygous carriers of a DDAH-2 −449G allele. The presence of this gene mutation was associated with a doubled probability that vasopressor infusions would be required after cardiac surgery.

Presumably, this mutation affects vascular function under the stress of trauma.

Sites of DDAH Expression and Function

Organ distribution of DDAH. DDAH-1 and -2 are predominantly cytoplasmic enzymes although some DDAH-1 is recovered in the membrane fraction of endothelial cell lysates (17).

DDAH-1 is widely expressed, especially in liver and kidney at sites of NOS expression (116, 117, 127, 185). The human kidney and liver are the major sites for metabolism of ADMA, leading to negative venoarterial gradients for ADMA across these organs (116, 117). This suggests that DDAH-1 in the liver and kidneys may be the guardian of circulating ADMA. DDAH-1 also is expressed strongly in the pancreas, forebrain, aorta, and peritoneal neutrophils and macrophages (76, 188). It is expressed at equivalent levels in fetal and adult tissues (188). A homozygous gene deletion for DDAH-1 is lethal in utero in the mouse. Although the precise mechanism for this lethality is not described in this work, this gene must fulfill some essential roles during embryogenesis. DDAH-1 +/− mice have decreased DDAH-1 protein expression in skeletal muscle, lung, brain, and heart with unaltered DDAH-2 expression. They show abnormalities in pulmonary vasculature that lead to pulmonary hypertension (90).

DDAH-2 is expressed at relatively high levels in all fetal tissues, while concentrations fall, and sites of expression become more selective, in adults (188). DDAH-2 predominates in the vascular endothelium, which is the site of eNOS expression (3, 127, 188, 204). It is widely expressed in the heart and placenta and is expressed heavily, but selectively, within the kidneys. DDAH-2 is also expressed in immune tissues that express iNOS. These include the spleen, thymus, peripheral leukocytes, lymph nodes, and bone marrow (188). The expression of DDAH-2 in cells of the immune system, coupled with the observation that the gene for DDAH-2 is located on chromosome 6p21.3 in the region of MHC III which predisposes to autoimmune diseases such as rheumatoid arthritis, raises the speculation that DDAH-2 may have a role in the modulation of host defense or immune tolerance in concert with iNOS. Perhaps relevant to this is the report of an increased risk of cardiovascular events in patients with systemic lupus erythematosus who have elevated plasma levels of ADMA (28).

Expression and function of DDAH within blood vessels. DDAH-1 has been described in the endothelium (88). However, the mRNA expression in the mesenteric resistance vessels of the rat for DDAH-2 is 5.1-fold greater than for DDAH-1 (204). A human endothelioma cell line expresses >10-fold more mRNA for DDAH-2 than DDAH-1 (204). Immunohistochemical staining of resistance vessels in the rat for DDAH-2 is strongly positive but is negative for DDAH-1 (204). Immunohistochemical studies locate DDAH-2 in the endothelial cells, vascular smooth muscle cells, and adventitia of these vessels. Overexpression of DDAH-2, but not DDAH-1, reverses the impaired NO production of endothelial cells exposed to glycated protein (96). These observations indicate DDAH-2 is the predominant isoform expressed in blood vessels and endothelium.

Recently, we have used an in vivo strategy of RNA interference in the rat by rapid intravenous bolus injection of a large volume of fluid containing small interference (si) RNAs targeted to DDAH-1 or -2 or nontargeted control constructs (204). There is a 30–60% reduction in the mRNA and protein expression corresponding to the target gene without detectable changes in the expression of the other isoform 3 days after injection of the specific targeted siRNA. Functional studies of mesenteric blood vessels dissected from rats after silencing of the DDAH-2 gene show almost complete inhibition of the EDRF/NO response to acetylcholine and an equivalent reduction in 4,5-diaminofluorescein acetoxymethyl ester-detectable NO activity, whereas these functions are minimally perturbed in vessels from rats after silencing of the DDAH-1 gene. Responses to the endothelium-independent vasodilator sodium nitroprusside and contractile responses to phenylephrine are not perturbed in vessels from mice after silencing of the DDAH-2 gene. DDAH-2 emerges from this study as the predominant isoform regulating bioactive NO in rat resistance vessels. However, primary cell cultures of pulmonary arterial endothelial cells from DDAH-1+/− mice produce significantly more ADMA, and less NO, than cells from DDAH-1 +/+ mice (90). The vessels from these DDAH-1 +/− mice have increased contractions to phenylephrine, reduced relaxations to acetycholine or the calcium ionophore A23187, and increased relaxations to sodium nitroprusside (an NO donor). These functional changes suggest a reduced endogenous capacity to generate and respond to endothelial NO in vessels from DDAH +/− mice (90). Presently, it is not clear whether these different conclusions concerning the primary role of DDAH-1 or -2 in endothelial cells represent species differences or differences in compensatory mechanisms to lifelong gene knockdown with the gene deletion method compared with relatively brief knockdown with the RNA interference method.

DDAH-2 is located with eNOS in the cytosol of endothelial cells. Recent studies of the ultrastructural distribution of DDAH-2 in the endothelial cells of the rat mesenteric resistance vessel in our laboratory (204) using preembedding immunoelectron microscopy locate DDAH-2 in the apical membrane and confirms its expression in cytoplasmic vesicles. The immunogold method reveals DDAH-2 in the smooth muscle fibrils and nuclei of vascular smooth muscle cells (204). Studies in vitro show that DDAH modulates iNOS expression in vascular smooth muscle cells by regulating metabolism of ADMA. This involves changes in IL-1β activation. Thus IL-1β increases the protein expression of DDAH and the DDAH enzyme activity. Inhibition of DDAH with 4124W abolishes changes in ADMA and attenuates NO synthesis after IL-1β stimulation. This suggests that DDAH may regulate cytokine-induced NO production and participate in the vascular injury accompanying atherosclerosis via effects on iNOS (191).

Studies by Achan et al. (3) relate beneficial effects of the vitamin A derivative all-*trans*-retinoic acid to its ability to enhance DDAH expression. It promotes angiogenic responses of vascular smooth muscle cells and endothelial cells, inhibits cellular proliferation, promotes vascular differentiation, and retards the development of atherosclerosis (3). All-*trans*-retinoic acid administration to endothelial cells increases the promoter activity and gene and protein expression of DDAH-2, reduces ADMA, and increases NO synthesis, despite unchanged eNOS expression. The finding that inhibition of DDAH blunts the increase in NO production induced by all-*trans*-retinoic acid implicates DDAH in these effects (3).

DDAH is implicated further in endothelial repair and angiogenesis (4). Pulmonary artery endothelial cells from DDAH-1 +/− mice have impaired motility that likely contributes to the associated defect in angiogenesis (221). These effects are mediated via activation of RhoA and Rho kinase and can be prevented by overexpression of DDAH-1 or -2, or administration of NO donors or 8-bromo-cGMP (191). Upregulation of DDAH increases the mRNA expression for the potent angiogenesis promoting agent, vascular endothelial growth factor (VEGF), and enhances vessel tube formation (159).

A recent study by Patschan et al. (134) reports that ADMA uncouples NOS, leading simultaneously to reduced NO synthesis and increased superoxide generation. Collectively, these reports establish the importance of DDAH in blood vessels for endothelial cell NO generation, EDRF/NO responses, and angiogenesis.

Expression and function of DDAH within the kidneys. The rat kidney expresses eNOS in microvascular endothelial cells and cells of the thick ascending limb of the loop of Henle (TAL). nNOS is expressed in tubular epithelial cells of the macula densa segment, Bowman's capsule of the glomerulus, and the collecting ducts (CDs). Although nNOS is not located immunohistochemically in the proximal tubule (PT), other studies have shown that it regulates proximal tubule cell function (216). iNOS immunoreactivity is widely expressed in the tubular epithelium, including the PT, TAL, distal convoluted tubule (DCT), and intercalated cells of the CDs (182, 185). DDAH is colocalized with eNOS, nNOS, and iNOS in these cells (127, 182, 185).

Immunohistochemical studies in the rat have located DDAH-1 in the PT, especially the pars recta (127). This is confirmed by a positive reaction in micro-Western analysis of individual PT segments dissected from the kidney, whereas glomeruli and more distal segments are negative. Immunoreactive DDAH-2 is located in the TAL, macula densa segment, DCT, and cortical and medullary CDs. These findings also are confirmed by a positive micro-Western analysis in the distal nephron segments (127). Electronmicroscopy has shown a subcellular localization of DDAH-2 in intracytoplasmic vesicles in macula densa cells (185). Thus DDAH expression within the cells of the nephron is highly isoform specific. This is reminiscent of the highly localized, cell-specific sites of expression of the NOS isoforms. Such discreet cellular localization patterns, coupled with evolving evidence for differential regulation of the individual isoforms of DDAH and NOS, for example by ANG II (127, 182, 183), provides for nephron site-specific regulation of NO generation. This may endow the kidney with its capacity to respond homeostatically to factors such as angiotensin or salt that may require discreet changes in segmental nephron reabsorption or segmental vascular resistance. This concept is developed later in the discussion of the pathophysiology of diabetes mellitus.

Microperfusion of individual loops of Henle in the rat with ADMA, N^G-monomethyl-L-arginine (L-NMMA), or SDMA inhibit the uptake of coperfused L-[^{14}C]arginine via system y^+ transport (212). Similar microperfusions of ADMA or L-NMMA also enhance the maximal tubuloglomerular feedback (TGF) response, whereas SDMA is ineffective. This demonstrates that all three methyl arginines inhibit the uptake of arginine from the lumen of the loop of Henle, whereas only ADMA and L-NMMA inhibit NOS in the macula densa, thereby enhancing TGF responses. Therefore, metabolism of ADMA by DDAH could enhance NOS substrate uptake into intracellular sites of NOS expression in the nephron as well as protect tubular NOS from competitive inhibition by ADMA, at least in the loop of Henle and macula densa segment (185).

DDAH Activity

DDAH activity measurements. DDAH hydrolyses ADMA or L-NMMA to L-citrulline and dimethylamine or monomethylamine, respectively. Determination of DDAH activity is based on the degradation of the substrate or on the formation of the reaction products (81, 94, 101). Tissue can be incubated with [^{14}C]ADMA or L-[^{14}C]NMMA and the strongly cationic ADMA separated from citrulline by an anionic exchange resin, yielding [^{14}C]citrulline in the reaction products to quantitate the degree of [^{14}C]ADMA that has been metabolized (102, 123). Some investigators have relied on unlabeled ADMA as substrate, with measurement of ADMA and citrulline, for example by high-pressure liquid chromatography (HPLC). Nonaka et al. (119) report a fast, sensitive column-switching HPLC-fluorescence detection method for determination of ADMA in plasma and renal tissue. A simpler colorimetric method has been developed for L-citrulline production based on its reaction with oximes, such as diacetyl monoxime, in which the color of the product is measured by spectrophotometric analysis (81). This assay requires attention to nonspecific color formation by urea and protein-bound L-citrulline (81, 173) and DDAH-independent production of L-citrulline by other enzymes, such as ornithine carbamoyltransferase and NOS (81, 101). Tain and Baylis (173) report the performance of their modified time-saving and inexpensive colorimetric assay of L-citrulline accumulation, which correlates closely with the direct measurement of renal ADMA consumption by HPLC.

DDAH activity assays based on the metabolism of [^{14}C]ADMA or L-[^{14}C] NMMA have been considered as the "gold standard" with high sensitivity and specificity (101), but there are concerns whether nonradioactive ADMA in the sample could compete with the tracer quantities of [^{14}C]ADMA in the assay. Recently, Maas et al. (101) have proposed using deuterium-labeled ADMA ([$^2H_6$]ADMA) as the substrate and double stable-isotope-labeled ADMA ([$^{13}C_5$-$^2H_6$]ADMA) as an internal standard in a stable-isotope-based assay suitable for 96-well plates to determine simultaneously the formation and the metabolism of ADMA. They applied this to renal and liver tissues of mice by using liquid chromatography-tandem mass spectrometry. This assay has the advantage of simultaneous determination of DDAH activity and endogenous formation of ADMA, SDMA, and L-arginine in tissue.

Nitrosylation. Activation of iNOS, for example during sepsis, can generate sufficient NO to nitrosate and inactivate adjacent constitutive NOS isoforms (11). More recently, Leiper et al. (89) have reported that both purified recombinant bacterial DDAH and mammalian DDAH extracted from the cytosol of rat kidneys are inhibited reversely by NO donors. Inhibition of DDAH by excessive NO occurs both in vitro and in vivo. It is difficult to be certain of the concentrations of NO that are required to inactivate DDAH. However, after cytokine treatment of a murine endothelial cell line, there is induction of iNOS, which produces sixfold greater amounts of NO, which

are sufficient to inhibit DDAH activity. Thus, after activation of iNOS, inactivation of DDAH by high ambient levels of NO may put a brake on excessive NO production both by inhibition of NOS by NO and by *S*-nitrosylation of DDAH expressed in adjacent cells. Inhibition of DDAH should limit ADMA metabolism and allow its accumulation to inhibit NOS. These concepts require further study.

Leiper et al. (89) report that a specific antibody that recognizes *S*-nitrosocysteine residues does not react with native DDAH but recognizes DDAH after treatment with the NO donor 2-(*N*,*N*-dimethylamino)-diazenolate-2-oxide·Na (DEANONOate). Thus NO can *S*-nitrosylate cysteines on DDAH. *S*-nitrosylation of PaDDAH involves a covalent attachment of a nitrogen monoxide group to the thiol side chain of the cysteine (Cys) 249 residue in the active site. Cys 221 and 273 residues are the corresponding targets for NO in the mammalian DDAH-1 (79, 89). Under basal conditions, DDAH is not detectably *S*-nitrosylated by the amount of NO synthesized by NOS. However, under some conditions where higher levels of NO are generated, for example, in cells in which iNOS is induced by cytokines, DDAH is *S*-nitrosylated and inactivated. Thus the amount of NO generated within cells after cytokine activation is sufficient to *S*-nitrosylate DDAH. Gow et al. (51) report that NO synthesized by each of the NOS isoforms *S*-nitrosylates a distinct subset of cellular proteins. This isoform-specific nitrosylation is proposed to result in part from the different localizations of the NOS isoforms within cell. Specifically, DDAH is *S*-nitrosylated after induction of the iNOS enzyme, which localizes to the cytosol. Although the nitrosylation reaction is covalent, the blockade is reversible because incubation in the presence of the reducing agent dithiothreitol (DTT), which restores sulfhydryl groups on Cys, reverses the DEANONOate-induced inhibition of DDAH activity (89, 167). DTT alone has no effect on DDAH. Knipp et al. (79) investigated the effect of NO on the structure and activity of DDAH-1. Using DEANONOate as an NO source, they found that whereas the native DDAH-1, which contains one tightly bound Zn^{2+} (holo-form), is resistant to *S*-nitrosylation, the zinc-depleted form of DDAH-1 (apo-form) is susceptible. Analysis by absorption spectroscopy, mass spectrometry, and fluorometric *S*-NO quantification reveals two of five cysteine residues of the zinc-depleted DDAH-1 (Cys 221 and 273) that react with NO, yielding cysteine-*S*-NO. These findings suggest that an increase in NO generation leads to S-nitrosylation of zinc-depleted forms of DDAH, which attenuates its activity, thereby permitting accumulation of ADMA, and inhibiting NOS activity.

Thiol oxidation. As described earlier, many factors have been identified that regulate the transcription or translation of the DDAH genes. In addition, DDAH activity is positively regulated by probucol (a potent antioxidant) (67), taurine (a semiessential amino acid with antioxidant properties) (174), insulin plus adiponectin (42), pravastatin (a cholesterol-lowering agent) (230), estradiol (59, 112), IL-1β (191), and fenofibrate or pyrrolidine dithiocarbamate (an antagonist of nuclear factor-κB) (228). DDAH activity is negatively regulated by CF6 (175), lipopolysaccharide (225), glycosylated bovine serum albumin (229), the erythropoietin analogs, epoetin-β and darbepoetin-α (146), high concentrations of glucose (160), oxLDL or TNF-α (64), cholesterol or homocysteine (167), and by cytomegalovirus infection (211). Almost all of these observations are performed in cultured endothelial cells or smooth muscle cells. Whether the effects of these factors on DDAH activity occurs in vivo needs to be elucidated.

Many of the inhibiting stimuli are accompanied by oxidative stress, whereas many of the activating stimuli are antioxidants. Redox modification of a sulfhydryl group in the catalytic region of the active site of DDAH could confer reversible sensitivity of the enzyme to oxidative stress (78, 167). For example, homocysteine oxidizes a sulfhydryl group in DDAH to form a mixed disulfide, which inactivates the enzyme (167). Tain and Baylis (173) report that a superoxide donor (2,3-dimethoxy-1,4 naphthoquinone) or NO donors (diethylamine NONOate or sodium nitrate) inhibit rat renal cortical DDAH activity in vitro. However, the detailed molecular mechanisms whereby many of these factors regulate DDAH activity and/or expression remain to be fully elucidated.

The flow of electrons through eNOS (223) or nNOS (138) is interrupted after the first reduction step in the presence of suboptimal concentrations of the substrate, L-arginine, or the cofactor tetrahydrobiopterin (BH_4). This directs NOS to generate superoxide ($O_2^{\cdot -}$) rather than NO. Under similar conditions, iNOS in murine macrophages (224) and in lipopolysaccharide- and cytokine-stimulated mouse epithelial cells (213) also generates superoxide anion ($O_2^{\bullet -}$). Peroxynitrate ($ONOO^-$) formed by the interaction of $O_2^{\bullet -}$ and NO can oxidize BH_4 to BH_2 and perpetuate this uncoupling of NOS that yields $O_2^{\bullet -}$ (222). Coincident oxidation of key reactive thiols in DDAH could impair its activity and permit ADMA accumulation. Inhibition of NOS by ADMA also can uncouple the enzyme and dictate $O_2^{\bullet -}$ generation by eNOS in endothelial cells (20) and by iNOS induced by lipopolysaccharides in mouse pulmonary epithelial cells (213), although high concentrations of ADMA are required. These findings demonstrate the importance of redox interruption of DDAH activity, with consequent accumulation of cellular ADMA, which could be a starting point for further elevation of ADMA, inhibition of NOS, and increase in $O_2^{\bullet -}$ generation, thereby initiating a feed-forward reaction.

Effects of arginine and citrulline. L-Arginine stimulates NO production in models of kidney disease (14), hypercholesterolemia, oxidative stress, inflammation, ischemia, and dietary salt restriction (31, 66, 77). However, a recent study in hepatic cells in vitro reports a dose-dependent inhibition of DDAH activity by L-arginine, which competes with ADMA for binding to DDAH (209), which should limit NO production. Nevertheless, the importance of this finding in vivo is not yet clear since supplementation with L- but not D-arginine reverses many of the vascular defects in DDAH-1 +/− mice (90) in which NO generation is diminished because of the accumulation of ADMA. Citrulline also competes with ADMA for metabolism by DDAH, but at concentrations likely above those found in vivo (102).

The finding that the bioactivity of NOS is limited by the availability of L-arginine, despite the fact that the plasma levels of L-arginine are many fold above the K_m for NO generation by NOS, has been referred to as the arginine paradox (5, 39). One explanation for this paradox is that NOS is subject to competitive inhibition by ADMA and therefore requires a higher substrate concentration for maximal activity. Consequently, the L-arginine:ADMA ratio should give greater insight into NOS activity than either component individually. A second expla-

nation derives from the recent study of Joshi et al. (69) that the guanidinium group of the arginine molecule binds to α-2-adrenoreceptors on human umbilical vein endothelial cells (HUVECs). This is followed by an increase in intracellular calcium via a pertussis toxin-sensitive G protein that activates PKC, which stimulates NO formation by eNOS (69).

Pharmacological inhibition. Three classes of DDAH inhibitors have been identified: pentafluorophenyl sulfonates (194); 2-chloroacetamidine (162); and a class of compounds derived the reversible inhibitor S-2-amino-4 (3-methylguanidino) butanoic acid (4124W) (141).

4124W is a chain-shortened analog of L-NMMA that acts as a somewhat weak competitive inhibitor of DDAH (3, 102, 191). MacAllister et al. (102) reported that 4124W inhibits the metabolism of L-[^{14}C]NMMA to [^{14}C]citrulline in rat liver homogenates (IC_{50}: 416 ± 66 μM) and cultured human endothelial cells (IC_{50}: 250 ± 34 μM). The addition of 4124W increased the accumulation of endogenously generated ADMA in the supernatant of cultured human endothelial cells from 3.1 ± 0.3 to 5 ± 0.7 μM. 4124W, in concentrations up to 1 mM, had no direct effect on eNOS activity but caused endothelium-dependent contractions of rat aortic rings, and reversed bradykinin-induced, endothelium-dependent relaxations of human saphenous veins. Moreover, 4124W dose dependently impairs the NO generation by all-*trans*-retinoic acid, inhibits IL-1β-induced upregulation of DDAH activity in rat VSMCs, and abolishes the reduction in ADMA by IL-1β in these cells (3, 191). Rossiter et al. (141) proposed that changing the charge around the critical guanidine moiety could disrupt ADMA hydrolysis by DDAH. They tested this hypothesis using a series of analogs of 4124W with varying guanidine substituents and amino acid moieties some of which inhibited DDAH activity as selective, substrate-based inhibitors that were effective in vivo.

Hartzoulakis et al. (53) combined physicochemical filtering, virtual screening, and hit analysis to identify inhibitors of PaDDAH. They isolated SR445, which had a much better activity than 4124W. They proposed that it inserts into the active site of DDAH with the indole moiety of SR445 taking the place of the arginine side chain of ADMA. SR445 is the most potent DDAH inhibitor identified to date with an IC_{50} of 2 μM. It is structurally distinct from previous inhibitors of both PaDDAH and mammalian DDAH.

A review of principal factors that regulate DDAH expression and activity is presented in Fig. 2.

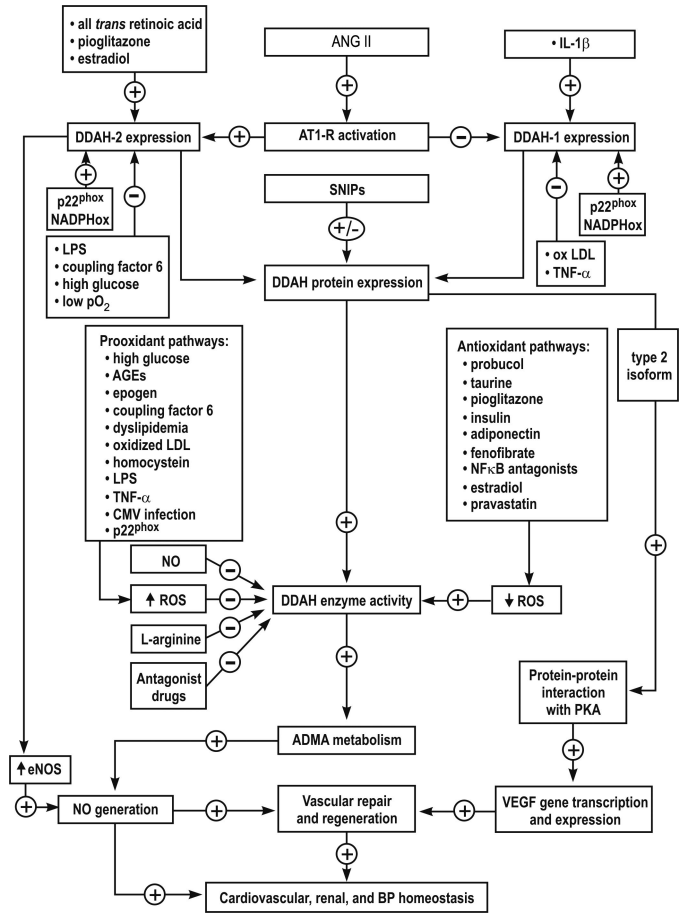

Fig. 2. Flow diagram of the action of ANG II acting on AT_1-Rs and other factors that can increase or decrease the activity of DDAH, and major consequences for cardiovascular function. oxLDL, oxidized low-density lipoprotein; CMV, cytomegalovirus; AGEs, advanced glycation end products; ROS, reactive oxygen species; PKA, protein kinase A; VEGF, vascular endothelial growth factor; eNOS, endothelial NOS; BP, blood pressure; NADPHox, NADPH oxidase. SNIPs, single nucleotide insertion polymorphisms.

Regulation of Gene Expression or Activity by DDAH or ADMA

DDAH can modulate the expression of a wide range of genes secondary to changes in ADMA concentrations and NO generation (158). For example, ADMA enhances lipopolysaccharide (LPS)-induced tissue factor (TF) expression in HUVECs (225). Smith et al. (158) report results from a DNA microarray assay that 142 genes in human coronary artery endothelial cells (HCAEC) are up- or downregulated by more than 1.7 fold when incubated with 2 μM ADMA (56 genes) or 100 μM ADMA (86 genes). Among these ADMA-stimulated genes are bone morphogenetic protein 2 inducible kinase (BMP2K), bone morphogenetic protein receptor 1A (BMPR1A), small family decapentaplegic-5 (Smad5), smooth muscle action (SMA)-related protein 5 (Smad5), and protein arginine methyltransferase 3 (PRMT3). The authors report an increased expression of BMP2K and PRMT3 in the brain, heart, and kidneys of DDAH-1 +/− mice that have increased plasma levels of ADMA. Bone morphogenetic proteins (BMPs) are multifunctional growth factors that belong to the transforming growth factor-β (TGF-β) superfamily (61). The BMP signaling pathway is important in the skeleton, kidney, and vasculature. Activation of this pathway may underlie the increased vascular calcification and progressive fibrosis of the kidney in chronic kidney disease (158). Interaction between the BMP and other major signaling pathways such as TGF-β/activin and p38 mitogen-activated protein kinase is an important cell signaling pathway (57). BMP2K is a putative serine/threonine protein kinase that attenuates osteoblast differentiation (71). Since ADMA reduces osteoblast differentiation and decreases osteocalcin expression, it is possible that the effects of ADMA on osteocalcin may be mediated by ADMA-induced changes in BMP2K (158). PRMT3 methylates protein arginine residues. Thus transcription of BMPR1A by increased cellular ADMA could relate chronic inflammation

and fibrosis to oxidative stress or DDAH expression, while transcription of PRMT-3 by ADMA could be a positive-feedback amplification loop whereby ADMA enhances its own production. These concepts await experimental analysis. Presently, the signaling pathways and transcription factors that mediate these effects of ADMA on gene regulation remain to be established.

DDAH regulates the expression of genes required for angiogenesis. In a preliminary report, Wang et al. (207) demonstrate significant downregulation of the mRNA and protein expression for eNOS in mesenteric resistance vessels of rats in which the gene for DDAH-2 has been silenced using in vivo siRNA. This is specific for DDAH-2 since there are no effects of silencing of the gene for DDAH-1 or of injection of a nontargeted siRNA. Smith et al. (159) reported that the mRNA for vascular endothelial growth factor (VEGF) is twice as high in ECV304 and murine endothelial cells that overexpress DDAH. Recently, Wojciak-Stothard et al. (221) report that ADMA induces stress fiber formation and inhibits the motility of mouse cultured pulmonary microvascular endothelial cells (PMVECs). These defects are prevented by overexpressing DDAH. They report further that PMVECs cultured from DDAH-1 +/− mice have reduced nitrite production and increased activity of the ras homolog gene family member A (RhoA), and defective angiogenic responses that are mimicked in DDAH-1 +/+ mice by exogenous ADMA (158). They propose that ADMA activates RhoA and Rho kinase in endothelial cells, which impairs endothelial cell motility and angiogenesis.

There is growing evidence that an increase in NO mediated by growth factors such as VEGF, TGF-β, and basic fibroblast growth factor (bFGF), plays a key role in angiogenesis (33, 58). The capillary tube formation induced by these growth factors is abolished by NOS antagonist (12, 133). Similarly, inhibition of NOS ablates the angiogenic effects of substance P or TGF-β in vitro (132, 233). VEGF-induced angiogenesis in the rabbit cornea model is blocked by N^{ω}-nitro-L-arginine methyl ester (L-NAME) (232). Cooke (33) provides evidence that ADMA acts as an endogenous inhibitor of angiogenesis by impairing the NOS/NO pathway. Using a disc angiogenesis system subcutaneously placed in normal or apo E-deficient mice, he reported that ADMA abrogates the effect of fibroblast growth factor on angiogenesis (33). This effect is reversed by L-arginine.

Hasegawa et al. (54) report that transfection of the gene for DDAH-2, but not DDAH-1, in primary cultures of bovine aortic endothelial cells transcriptionally activates VEGF. The remarkable finding of these authors is that this is accomplished in the absence of a change in NO generation by the direct binding of DDAH-2 to protein kinase A (PKA), leading to the phosphorylation of the transcription factor specificity protein 1 (Sp1), which translocates to the nucleus where it binds to the promoter region of the VEGF gene, leading to its transcription. This sequence of events is confirmed by the finding that an siRNA targeted to Sp1 blocks the upregulation of VEGF by DDAH-2. Tokuo et al. (186) report an analogous pathway whereby DDAH-1 binds directly to the COOH-terminal domain and to the cysteine/serine-rich domain of the small GTPase rat sarcoma (Ras)-pathway regulator neurofibromin 1 (NF-1), coinciding with the region containing specific PKA phosphorylation sites. Binding of DDAH-1 to NF-1 increases NF-1 phosphorylation by PKA. These observations are of special importance since they demonstrate that DDAH exerts its functions by two distinct mechanisms: hydrolysis of ADMA and protein-protein interaction. These protein-protein interactions that regulate gene transcription may also explain the unexpected finding in endothelial cell of rat mesenteric resistance vessels that DDAH-2 is located in the nucleus, which is not a site of NOS expression (204). The significance of DDAH expression in the nucleus is not yet clear. We recently reported that DDAH-2 is located in the nuclei of rat vascular smooth muscle cells (207). The dual effects of DDAH-2 that together both elicit VEGF gene transcription may contribute to its potent effects on angiogenesis and endothelial cell motility (4, 92). Angiogenesis begins with the proliferation and migration of endothelial cells as new capillaries sprout from preexisting blood vessels. Angiogenic growth factors are being used to induce "biological bypasses" to relieve ischemia and to preserve end-organ function (33).

Expression and Function of DDAH in Disease

Zoccali et al. have shown in a series of epidemiological studies that ADMA is a strong independent risk factor for cardiovascular disease (238), progression of chronic kidney disease (CKD) (237), and for the development of cardiovascular disease in patients with CKD (234, 236–238). The role of DDAH in NO deficiency, which can cause hypertension, cardiovascular disease, or kidney disease, is reviewed below.

Hypertension and angiotensin. Achan et al. (2) reported that infusion of ADMA into normal human subject increases their blood pressure only modestly because an increase in peripheral and pulmonary vascular resistances are offset by a decrease in cardiac output and cardiac dysfunction. Leiper et al. (90) reported that DDAH-1 +/− mice accumulate ADMA, which may account for reduced vascular NO signaling, endothelial dysfunction, increased systemic vascular resistance, and elevated systemic and pulmonary artery pressures. Arrigoni et al. (8) showed that newborn piglets with pulmonary hypertension due to 3 days of hypobaric hypoxia have reduced DDAH-2 protein expression and activity, which these authors propose as the cause of the hypoxic pulmonary hypertension. Mice transgenically overexpressing DDAH-1 have a reduced systolic blood pressure, systemic vascular resistance, and cardiac stroke volume (36). These findings suggest a particular role for DDAH in preventing pulmonary and systemic vasoconstriction.

On the other hand, there are no consistent changes in blood pressure of conscious, unrestrained rats measured by telemetry over 3 days after intravenous injections of siRNAs targeted to DDAH-1 or -2, which downregulate the respective proteins by 30–65% (204). The stable BP occurs despite a 30% increase in serum ADMA concentrations after silencing of the gene for DDAH-1 and almost complete abrogation of EDRF/NO signaling in mesenteric blood vessels after silencing of the gene for DDAH-2 (204). Thus moderate defects in DDAH over 3 days are not sufficient to elevate BP in conscious rats.

Endothelial dysfunction and reduced constitutive NOS activity are apparent in resistance vessels dissected and studied ex vivo from patients with essential hypertension (205). Some studies of human subjects with essential hypertension report elevated plasma levels of ADMA (35, 62, 135, 170). However,

others have failed to detect a relationship between blood pressure and ADMA in human subjects (108, 129, 148, 152).

The effect of renin system intervention on DDAH activity in human subjects has not been studied, but the effects on circulating ADMA in hypertension are variable. Some studies report that angiotensin-converting enzyme inhibitors (ACEIs) or angiotensin receptor blockers (ARB) reduce plasma levels of ADMA in patients with hypertension, CKD, metabolic syndrome, or diabetes mellitus (DM) (10, 30, 37, 62, 63, 115), whereas others report no changes in patients with hypertension or coronary artery disease (37, 210). Some of these controversies may derive from problems in measuring ADMA.

Incubation of human umbilical vein endothelial cells with ANG II over 24 h enhances their expression of PRMT, reduces DDAH activity, and doubles the release of ADMA into the medium (31). These effects are blocked by an AT_1-R antagonist. A subcutaneous infusion of ANG II into conscious mice for 4 wk at 60 $mg \cdot kg^{-1} \cdot 24\ h^{-1}$ increases their circulating ADMA fourfold (55). This ADMA response is blunted in transgenic mice overexpressing DDAH-2. These data relate an increase in ADMA with ANG II to a reduction in DDAH expression or activity. On the other hand, Tojo et al. (182) reported that increasing ANG II generation in rats endogenously by feeding a low-salt diet or exogenously by infusing ANG II for 2 wk increases DDAH protein expression in the renal cortex and glomeruli. However, this study predated the discovery of the two DDAH isoforms. A recent study by Onozato et al. (127) reports that DDAH-1 is expressed in proximal tubular cells and DDAH-2 in the afferent arteriole, the macula densa, and the collecting duct. The same study also reports that incubation of slices of rat kidney cortex with ANG II causes an AT_1-R dependent downregulation of DDAH-1 but a corresponding AT_1-R dependent upregulation of DDAH-2 (127). Thus ANG II can exert isoform-specific effects on DDAH expression, at least in the rat kidney, both of which entail AT_1-R activation. Furthermore, serum levels of ADMA in the study by Onozato et al. (127) appeared to relate to changes in DDAH-2 expression, which provides an explanation for the seemingly contradictory results from the previous study by Tojo et al. (182).

Recent reports suggest a possible role for DDAH in ANG II-induced inflammation. Tojo et al. (182) report that AT_1-R activation enhances the expression of DDAH and eNOS in the rat kidney. However, Chen et al. (31) report that incubation of endothelial cells with ANG II increases the production of ROS and the release of ADMA and decreases the levels of nitrite/nitrate. This suggests that ANG II may generate sufficient ROS to uncouple eNOS (thereby reducing NO and increasing $O_2^{\bullet -}$ generation) and inactivate DDAH, thereby impairing NO generation despite increased eNOS expression. Moreover, ADMA itself can contribute to uncoupling of NOS in vitro, which provides for amplification of ROS generation after inhibition of DDAH activity (20, 213). The generation of ROS, especially if coincident with activation of NOS, is an important initial event in inflammation. ADMA increases the generation of the downstream proinflammatory mediators TNF-α and IL-8, and activates the NF-κβ pathway and the binding of monocytes to endothelial cells (31). The finding that ANG II stimulates TNF-α, which itself inhibits DDAH, identifies a potential feed-forward cycle of vascular inflammation and dysfunction following an initial reduction in DDAH activity (64).

The physiological regulation of DDAH activity in *Pseudomonas aeruginosa* depends on the presence of an active-site cysteine residue Cys249 (161). Stone et al. (161) showed that DDAH activity varies according to the protonation state of Cys249 and demonstrated that metal ions inhibit binding of ADMA to Cys249. These results provide evidence for modulation of the reactivity of the active-site cysteine and thus for modulation of DDAH activity.

Pregnancy and preeclampsia. NO synthesis is increased in pregnancy and contributes to the reduced BP and peripheral vascular resistance (27). Plasma levels of ADMA normally decline during pregnancy (46). However, pregnant women with a high-resistance placental circulation who are at risk for preeclampsia and/or intrauterine growth restriction of the fetus (145) and those developing preeclampsia or the "hemolysis elevated liver enzymes and low platelet (HELLP)" syndrome have increased circulatory levels of ADMA (43, 46, 60, 136, 145, 156). The effects of any elevation of ADMA may be especially important in pregnancy since plasma levels of L-arginine are reduced by ~50% in women with normal pregnancy or preeclampsia (156).

One report concludes that the placenta is a source of ADMA since the authors found that placental venous blood contains a threefold higher level of ADMA than maternal systemic plasma (104). In contrast, a recent report detected no venoarterial gradient for ADMA, SDMA, or arginine across the placenta of women with normal pregnancy or preeclampsia, and no significant effect of preeclampsia on placental DDAH activity, which was 20-fold lower than in human liver tissue (156). Moreover, three studies have failed to detect an increase in plasma ADMA in women with preeclampsia (95, 100, 156), except for those with the HELLP syndrome, who have a 50% increase (156). Thus a defect in ADMA metabolism by DDAH in the placenta does not appear to be an adequate explanation for preeclampsia, but further studies are required. In fact, the plasma levels of ADMA during pregnancy have been found to correlate closely with blood pressure and with the functions of the liver and kidney. Accordingly, elevated ADMA levels may rather be a consequence of the preeclamptic and HELPP syndromes rather than a cause (156).

Cardiovascular disease and atherosclerosis. Schnabel et al. (148) report that plasma ADMA is an independent predictor of future cardiovascular events in patients with coronary artery disease. However, a subsequent study reported elevated plasma ADMA concentrations in patients with incident coronary artery disease only in smokers, those with elevated levels of plasma homocysteine, or those with impaired renal function (208). Circulating levels of ADMA are increased in patients with established atherosclerosis (111), peripheral vascular disease (19), coronary artery disease (208), or left ventricular hypertrophy (238). They are also elevated in subjects with many of the categories of risk for cardiovascular disease, including hypercholesterolemia (18, 22), raised circulating level of low density lipoproteins (64) or triglycerides (98), hyperhomocysteinemia (165, 166, 167), raised C reactive protein (236), smokers (208), older age (97), postmenopausal women (153), and patients with carotid artery remodeling (236). In a study of 363 patients with acute cerebrovascular disease, compared with 48 controls, ADMA was characterized as a weak, but independent, marker for acute stroke, but as a stronger marker for transient ischemic events (202).

The elevated plasma levels of ADMA in some conditions of increased cardiovascular risk have been linked experimentally to changes in DDAH expression or activity. Thus hindlimb ischemia in mice decreases the protein expression for DDAH and NOS and increases tissue ADMA concentrations (4). High levels of homocysteine, which are correlated with coronary and peripheral vascular disease, reduce DDAH enzyme activity in both cultured endothelial and nonvascular cells, leading to accumulation of ADMA and inhibition of NOS (167). This could underlie an impaired endothelium-dependent NO-mediated vasodilatation induced by homocysteine in the pig aorta (167). However, more recent data has shown that ADMA accumulates in patients with hyperhomocysteinemia only with the development of renal dysfunction (215).

Extracts from cigarette smoke reduce NO production by eNOS in cultured pulmonary artery endothelial cells (168). Smokers have elevated plasma concentrations of ADMA and reduced eNOS expression (13, 66, 208), which correlate with elevated concentrations of homocysteine (208). Nicotine downregulates the mRNA and the protein expression of DDAH-2 and reduces DDAH activity in endothelial cells (66). A recent study reports that endothelial cell dysfunction induced by nicotine is attenuated by the addition of L-arginine or by overexpression of DDAH-2 (66). Thus cigarette smoke, and/or the nicotine that it contains, could contribute to the endothelial dysfunction and cardiovascular disease of smokers by reducing DDAH-2 expression or function, thereby permitting the accumulation of ADMA that inhibits eNOS function and angiogenesis (66).

CKD. Patients with CKD have elevated plasma levels of ADMA (198), impaired EDRF/NO responses in isolated resistance vessels (205), and a marked increase in the frequency of cardiovascular events that are predicated by plasma levels of ADMA (237). An inverse relationship between plasma ADMA and glomerular filtration rate (GFR) is apparent in patients with coronary artery disease, which has been implicated in their increased cardiovascular risk (208). Remarkably, these processes can start very early in the natural history of CKD and can even predate any measurable reduction in GFR. Thus, among patients with autosomal dominant polycystic kidney disease (ADPKD), endothelial dysfunction and reduced constitutive NOS activity of subcutaneous dermal resistance vessels (205) and elevated plasma levels of ADMA (74, 206) and lipid peroxidation products (206) are reported even in those with normal values for GFR. At this very early stage of CKD-1, patients with ADPKD have cysts in their kidneys and a modest increase in albumin excretion, but normal clearance of ^{51}Cr-ethylenediamine tetraacetic acid (GFR marker) and normal ambulatory 24 h blood pressure values (198). These defects in ADMA metabolism in patients with early CKD are independent of other factors that elevate plasma ADMA (198). They could contribute to the subsequent decline in renal function and the development of cardiovascular disease since Zoccali et al. (139, 237) have shown that plasma levels of ADMA are independent predictors of these events in epidemiological studies of high-risk populations. Moreover, coronary endothelial function is impaired in the early stage of CKD in a dog model and has been related to downregulation the mRNAs for eNOS and DDAH-2 (177). However, it has been questioned whether elevated circulating levels of ADMA are merely a marker of kidney disease, akin to serum creatinine, or part of the pathophysiological process (197). Indeed, plasma levels of SDMA correlate more closely with parameters of renal function than do plasma levels of ADMA (75). Thus it is important to understand the regulation of DDAH in CKD.

Plasma concentrations of ADMA are elevated in subtotally nephrectomized rats in proportion to the degree of nephrectomy, despite increases in the renal clearance of ADMA, and correlate with increased blood pressure (106). Whereas hydralazine treatment prevents the hypertension in this model, it does not prevent progressive reduction in GFR (106). Transfection of the gene for DDAH-1 also prevents hypertension in this model (106). These findings demonstrate a pathogenic role for defective DDAH in the elevation of blood pressure in a model of CKD.

Elevated circulating levels of ADMA in CKD have been related to a combination of a reduced renal ADMA excretion and a reduced catabolism of ADMA by DDAH (14). DDAH-2 expression is downregulated in the kidneys of reduced renal mass models in the dog (125) or rat (106). A decreased activity of DDAH in CKD could also entail functional inhibition by oxidative stress (14).

There are other effects in addition to a rise in blood pressure that may be a consequence of decreased DDAH activity in the remnant kidney model. Thus overexpression of DDAH-1 in rats with surgical reduction of renal mass prevents the progressive reduction in GFR and loss of peritubular capillaries, suppresses tubulointerstitial fibrosis and proteinuria, and reduces the renal gene and protein expression of TGF-β (107). Overexpression of TGF-β by ADMA may contribute to the progression of CKD since TGF is upregulated in many models of CKD and infusion of TGF causes hypertension, reduces GFR and renal medullary blood flow, and leads to fibrosis of the kidneys (72). Thus strategies to maintain DDAH activity might have beneficial effects in preventing irreversible kidney failure and hypertension.

Liver disease. The healthy liver extracts and metabolizes substantial quantities of L-arginine and ADMA from the circulation (214). Patients with liver failure have increased circulating levels of both L-arginine and ADMA (116, 157) that are reversed by successful liver transplantation (157). This identifies the liver as an important organ for ADMA elimination in humans (157). An increase in plasma ADMA in patients with alcoholic hepatitis is accompanied by decreased hepatic DDAH protein expression and increased hepatic protein expression of PRMT-1 (113).

DM and insulin resistance. Several studies have reported elevated concentrations of circulating ADMA in patients with DM Type I (6, 176) and II (1, 9), prior gestational diabetes (110), and in some rat models of insulinopenia (127, 226, 227) and insulin resistance (29, 94, 164, 172). The concentration of ADMA in endothelial cells stripped from the carotid artery of the rabbit are 10-fold higher than in plasma and increase a further two- to threefold after induction of streptozotocin-induced DM (105). Plasma ADMA predicts progression to ESRD in diabetic patients with CKD (139) and predicts macrovascular complications in a rat model of Type 1 DM (226). Treatment of diabetic patients with metformin lowers their blood glucose concentration and their circulating levels of ADMA (9). The combination of streptozotocin administration and high-fat diet elevates plasma ADMA levels consistently (94). There are some conflicting findings. Thus one study in

Wistar rats in a model of streptozotocin-induced diabetes reports unaltered plasma ADMA after 4 wk of diabetes (130), whereas another apparently similar study in Sprague-Dawley rats with a similar duration of streptozotocin-induced DM reports increased plasma levels of ADMA and SDMA (127).

The mechanism of elevated ADMA in models of DM has been the focus of several studies (94, 127, 130, 171, 226, 227). Hyperglycemia can activate proinflammatory cytokines such as TNF-α that could impair endothelial function in diabetes (45, 167). Eid et al. (42) report that TNF-α decreases DDAH activity in human endothelial cells in culture and promotes the accumulation of ADMA. The increase in ADMA in human cultured endothelial cells induced by TNF-α can be reversed by insulin or adiponectin, which increase DDAH activity. Endothelial cells exposed to glycosylated serum albumin have decreased DDAH-2 gene expression, reduced DDAH activity, and consequently increased ADMA accumulation and decreased NOS activity (96). These effects are prevented by overexpression of the DDAH-2 gene (96).

We reported recently that early insulinopenic diabetic rats have a 50% increase in renal cortical concentrations of ANG II, an increase in plasma levels of ADMA, a decrease in renal cortical expression of DDAH-1, but an increase in expression of DDAH-2 (127). All of these changes are prevented by 2 wk of administration of the ARB telmisartan, which also reduces renal cortical PRMT-1 expression. Consequently, a reduction in circulating levels of ADMA by an ARB in this model of DM Type 1 may relate both to a decrease in ADMA synthesis by PRMT-1 and to an increase in renal ADMA metabolism by DDAH-1. The direct effects of AT_1-R stimulation in the kidney were studied further in kidney slices from normal rats (127). Incubation with ANG II led to an AT_1-R-dependent downregulation of DDAH-1 expression and upregulation of DDAH-2 expression, consistent with the effects of telmisartan on the expression of these isoforms of DDAH in the diabetic rat kidney.

These findings may throw light on some paradoxical findings in models of insulinopenic DM that are characterized, on the one hand, by severe endothelial dysfunction and oxidative stress of microvessel (149, 150), yet, on the other, by nNOS-dependent glomerular hyperfiltration (82, 83). This is not easily attributed to expression of nNOS since its expression in the kidney of diabetic rats is variously reported to be reduced (44), unchanged (126), or increased modestly (32, 73, 84, 155, 200), whereas the renal expression of eNOS is more regularly reported to be increased (32, 126, 169, 200) after the early initiation phase (73). An upregulation of DDAH-2 in the juxtaglomerular apparatus may contribute to the upregulation of eNOS in models of DM (207). It is not very likely that an upregulated nNOS could account for the dramatic nNOS-dependent glomerular hyperfiltration in the diabetic model. A reduction in DDAH-1 in the proximal tubules of the kidneys of rats with diabetes may impair renal ADMA extraction, thereby increasing the circulating and renal parenchymal levels of ADMA that could contribute to the later development of salt sensitivity and hypertension (143, 181). Indeed, NOS plays a critical role in animal models of insulinopenic DM in offsetting the effects of oxidative stress to cause a rapid increase in blood pressure (24). However, an increase in DDAH-2 in the juxtaglomerular apparatus could reduce local levels of ADMA, including within the macula densa cells. nNOS is expressed heavily in macula densa cells where it blunts vasoconstriction of the adjacent renal afferent arteriole, for example by the TGF process, thereby increasing the nephron GFR (212, 220). NO generated by macula densa nNOS is implicated in the regulation of renal hemodynamics, for example the renal vasodilation accompanying high salt intake (219). Microperfusion of ADMA into the macula densa segment of the rat nephron enhances TGF and reduces glomerular capillary pressure by a mechanism that apparently entails inhibition of NOS since it can be prevented by microperfusion of L-arginine (212). Thus upregulation of DDAH-2 in the macula densa cells of the

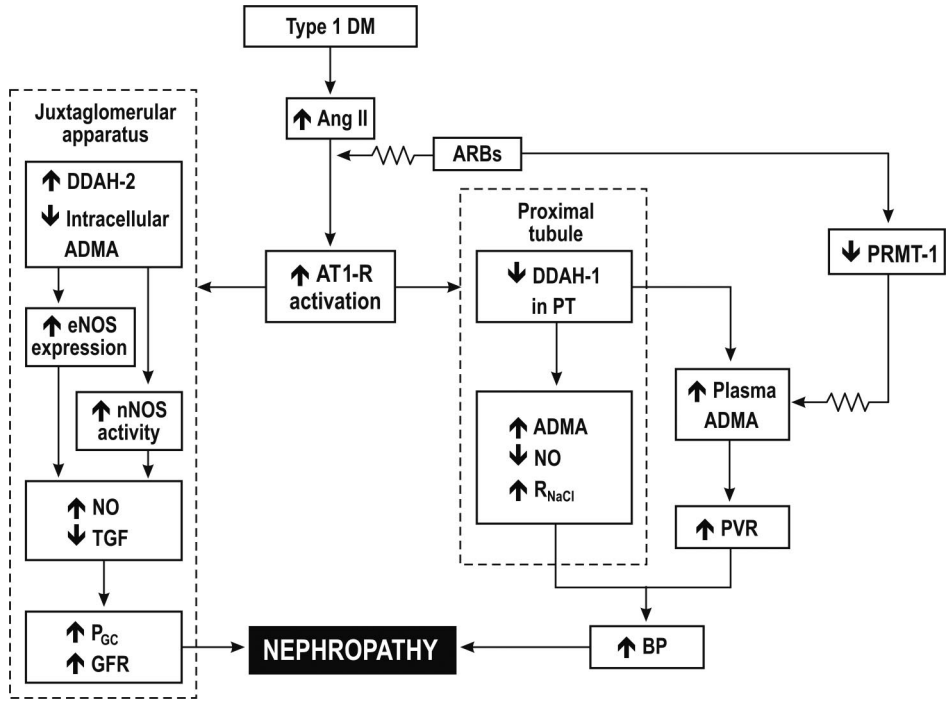

Fig. 3. Flow diagram outlining a hypothesis for distinct pathophysiological roles of intrarenal ANG II acting on type 1 receptors on DDAH-1 or -2 in the glomerular hyperfiltration and hypertension of diabetes mellitus (DM) Type 1 and their roles in the progression of nephropathy. ARBs, angiotensin receptor blockers; MD, macula densa; PT, proximal tubule; TGF, tubuloglomerular feedback; P_{GC}, glomerular capillary hydraulic pressure; GFR, glomerular filtration rate; R_{NaCl}, reabsorption of salt; PVR, peripheral vascular resistance; nNOS, neuronal NOS.

diabetic rat kidney may limit local ADMA accumulation, thereby enhancing nNOS-dependent afferent arteriolar vasodilation and glomerular hyperfiltration. Indeed, NO derived from macula densa nNOS in rat models of early DM increases the single-nephron GFR even in the absence of activation of TGF (85, 180). These findings might help to explain the apparently paradoxical role of nNOS-dependent glomerular hyperfiltration despite increased circulating levels of ADMA. These concepts are outlined in Fig. 3 but require further experimental investigation.

A second finding of potential clinical relevance to diabetic nephropathy is that the increase in ADMA and the decrease in renal protein expression for DDAH-1 in the kidneys of diabetic, insulinopenic rats are prevented by an ARB that also reduces the renal expression of PRMT-1 (127). Clinical trials of patients with DM and nephropathy have reported that patients randomized to receive an ARB (26) or an ACEI (140), compared with equally antihypertensive treatments with drugs that do not perturb the renin-angiotensin system, have a reduced rate of loss of renal function. Studies by Anderson and Brenner (7), Brenner (25), and Zatz et al. (231) in a rat model of insulinopenic DM have shown that ACEIs reduce the elevated levels of single-nephron glomerular filtration, glomerular capillary pressure, and BP. These actions of ACEIs or ARBs on glomerular hemodynamics are not seen in normal rats and depend on nNOS (82, 83). One potential explanation is that a reduction in ANG II generation or action on AT_1-Rs reduces the expression of DDAH-2 in the juxtaglomerular apparatus, leading to an increased intracellular ADMA that could inhibit nNOS activation specifically in those cells expressing DDAH-2. A reduced DDAH-2 expression within the juxtaglomerular apparatus may allow sufficient accumulation of ADMA to inhibit nNOS-dependent glomerular hyperfiltration (Fig. 3). This hypothesis requires further study.

Several other mechanisms directly or indirectly related to hyperglycemia have been described that could contribute to impaired DDAH activity in DM. ANG II stimulates oxidative stress (24) that could lead to posttranscriptional inhibition of DDAH activity. ANG II induces the expression of proinflammatory cytokines, growth factors, and adhesion molecules and the transcription factor NF-κB that can further promote ROS generation (45, 167). High glucose concentration itself can inhibit the expression of DDAH in cultured endothelial cells (160). Rats exposed to glycosylated proteins have endothelial dysfunction and impaired DDAH activity that can be restored by insulin (42, 229). Thus dysfunction of DDAH could underlie endothelial dysfunction in diabetes.

Conclusions and Clinical Perspectives

As research progresses, DDAH emerges as an important regulator of NO bioavailability and the integrity of renal and vascular function. The finding that the gene for DDAH is evolutionarily well conserved suggests that the enzyme confers an important survival advantage. A decrease in DDAH expression or activity is emerging as a potential mediator of cardiovascular and kidney diseases that accompany ANG II action, oxidative stress, CKD, hypertension, inflammation, and insulin resistance. Several recent findings have clarified the regulation and action of DDAH. Immunohistochemical studies in the rat show nephron site-specific expression of DDAH-1 and -2. DDAH-2 is expressed not only at sites of NOS expression in endothelial cells but also in vascular smooth muscle cells and in the cell nucleus. DDAH-2 can undergo protein-protein interaction, which leads to enhanced generation of VEGF that contributes to its role in promoting angiogenesis (54). Studies of gene silencing or deletion in rodents lead to the conclusion that plasma levels of ADMA are regulated by DDAH-1, whereas DDAH-2 can be of predominant importance for preserving endothelial function in resistance vessels. The recent finding that gene transfer of DDAH prevents hypertension and progressive loss of kidney function in a model of reduced renal mass implies that it plays a key role in the pathophysiology of CKD.

NO contributes substantially to the regulation of BP, endothelial function, and host defense. It is therefore not surprising that circulating ADMA is a strong predictor of cardiovascular morbidity and mortality in those with vascular disease and of the development of ESRD in those with CKD. Thus lowering of ADMA plasma concentrations in patients at risk could be a major therapeutic goal. However, the findings that silencing of the DDAH-2 gene in the rat blocks EDRF/NO responses without a change in circulating ADMA demonstrates that this goal is inadequate. Apparently, ADMA can accumulate intracellular in endothelial cells sufficiently to block their production of NO without a spillover into the plasma that elevates circulating ADMA levels.

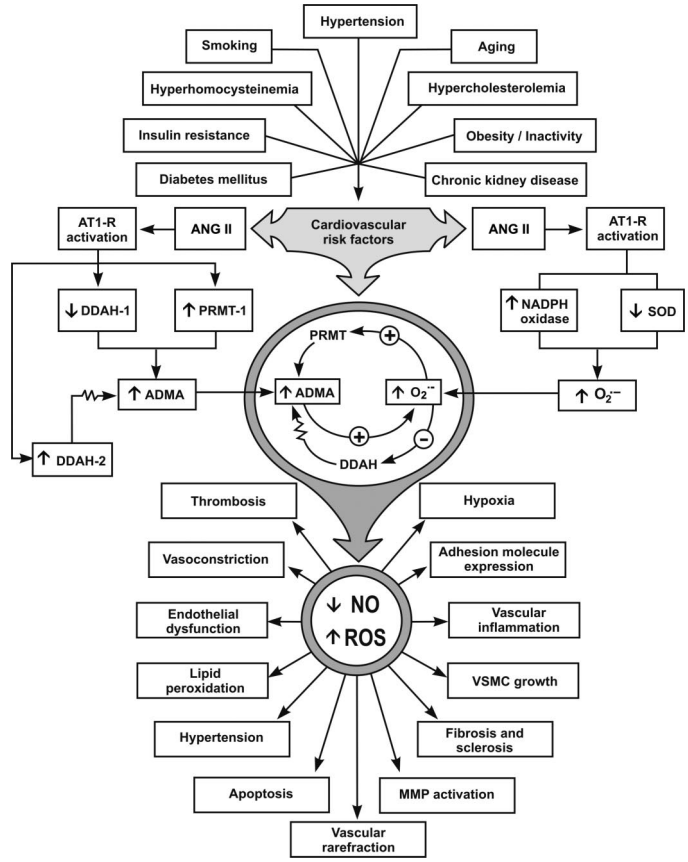

Fig. 4. Flow diagram outlining the interactive roles of ROS and ADMA in relating cardiovascular risk factors to pathophysiological changes in tissues that may underlie cardiovascular and kidney disease. For abbreviations, see legends to Figs. 1, 2 and 3. SOD, superoxide dismutase; MMP, matrix metalloproteinase; VSMC, vascular smooth muscle cell.

Reversible inhibitors of DDAH may have a role in the management of diseases with excess NO production, such as septic shock. However, interventions that enhance DDAH activity, decrease ADMA production, and facilitate NO signaling are of far greater potential importance. Enhancing the activity of DDAH or decreasing the activity of class 1 PRMT may become novel therapeutic strategies for preventing progressive cardiovascular and kidney diseases but currently lack a clear experimental basis.

Finally, the close interaction between the NOS/DDAH pathways and those that generate or metabolize ROS may underlie the finding that both an elevation of ADMA and an elevation of ROS are implicated in the ensuing vascular and organ dysfunction. These heuristic concepts are illustrated in Fig. 4.

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