Role of the Multidrug Resistance Protein-1 in Hypertension and Vascular Dysfunction Caused by Angiotensin II


Objective—Human endothelial cells use the multidrug resistance protein-1 (MRP1) to export glutathione disulfide (GSSG). This can promote thiol loss during states of increased glutathione oxidation. We investigated how MRP1 modulates blood pressure and vascular function during angiotensin II-induced hypertension.

Methods and Results—Angiotensin II–induced hypertension altered vascular glutathione flux by increasing GSSG export and decreasing vascular levels of glutathione in wild-type (FVB) but not in MRP1−/− mice. Aortic endothelium-dependent vasodilatation was reduced in FVB after angiotensin II infusion, but unchanged in MRP1−/− mice. Aortic superoxide (O2−) production and expression of several NADPH oxidase subunits were increased by angiotensin II in FVB. These effects were markedly blunted in MRP1−/− vessels. The increase in O2− production in FVB vessels caused by angiotensin II was largely inhibited by L-NAME, suggesting eNOS uncoupling. Accordingly, aortic tetrahydrobioppterin and levels of NO were decreased by angiotensin II in FVB but were unchanged in MRP1−/−. Finally, the hypertension caused by angiotensin II was markedly blunted in MRP1−/− mice (137±4 versus 158±6 mm Hg).

Conclusion—MRP1 plays a crucial role in the genesis of multiple vascular abnormalities that accompany hypertension and its presence is essential for the hypertensive response to angiotensin II. (Arterioscler Thromb Vasc Biol. 2007;27:762-768.)

Key Words: endothelial function ■ glutathione ■ hypertension ■ MRP1 ■ oxidative stress

Hypertension increases vascular production of reactive oxygen species (ROS).1 There is accumulating evidence that this contributes to the pathophysiologic consequences of hypertension.2 A major intracellular defense against ROS is the tripeptide glutathione (GSH).3 GSH serves as a cosubstrate for the glutathione peroxidases, which scavenge hydrogen peroxide and lipid peroxides. GSH also directly reacts with strong oxidants such as peroxyxinitrite. Depletion of the glutathione pool or inhibition of glutathione peroxidase alters vascular tone and leads to hypertension.4,5

The multidrug resistance proteins (MRPs) are members of the ATP-binding cassette (ABC) superfamily which use ATP for active transport of a variety of endogenously produced and exogenously administered molecules.6 MRP1 is a transporter of chemotherapeutic agents complexed to glutathione, leukotriene C4, glutathione disulfide (GSSG), and estrogens. Other MRPs have a myriad of cell functions including export of xenobiotics, cyclic nucleotides, lipids, and bilirubin. Recently, we found that MRP1 is the major transporter of GSSG in human endothelial cells.7 In this prior study, oxidative stress caused by oscillatory shear markedly enhanced the release of GSSG from cultured human aortic endothelial cells in an MRP1 dependent fashion. This led to a depletion of intracellular GSH and ultimately apoptosis. Blockade of MRP1 prevented these untoward effects of oxidative stress. We also showed that endothelial GSSG export was increased in an in vivo model of oxidative stress caused by desoxycorticosterone acetate-salt (DOCA-salt) hypertension in normal but not MRP1−/− mice. We selected DOCA-salt hypertension as a model of in vivo oxidative stress in this prior study because the increase in ROS is predominantly in the endothelium, mimicking our cell culture model. Moreover, in DOCA-salt hypertension, the degree of hypertension is relatively independent of vasomotor tone,8,9 and thus was identical between wild-type and MRP1−/− mice. These studies showed that the difference in GSSG extrusion caused by hypertension was independent of blood pressure levels, but markedly influenced by the presence of MRP1.

This prior study led to several important additional questions. First, it was not clear whether blood pressure could be influenced by MRP1 in a model less dependent on volume and sodium and more dependent on vasoconstriction. Second, because the increase in ROS is predominantly in the endothelium, it was difficult to examine the role of MRP1 in...
modulation of total vascular GSH. Third, it remained unclear how MRP1 could affect production of ROS and their various sources. In the present study, we examined the role of MRP1 in angiotensin II–induced hypertension. This model of hypertension is associated with an increase in production of ROS in both the endothelium and vascular smooth muscle, and is also dependent on vasomotor tone. This study provides new information about how angiotensin II–dependent hypertension reduces vascular intracellular glutathione and how this in turn affects ROS-producing enzymes. Moreover, our results strongly suggest that MRP1 plays myriad roles in regulation of blood pressure and vascular function and is a potential target for treatment of hypertension and vascular disease.

Materials and Methods

Animals
Animal experiments were approved by the Emory University Animal Care and Use Committee. Male MRP1−/− mice (FVB.129P2-Abccl1tm1Bor N12)10 and congenic wild-type animals (FVB/N) were purchased from Taconic. At 12 weeks of age the mice were anesthetized with Avertin 2.5% (0.3 mL per 25 g body weight, IP) and osmotic minipumps (Alzet Model 2002; Alzet Corp) were implanted to permit subcutaneous infusion of angiotensin II ([Val5]angiotensin II, infusion rate 400 ng/kg per min). Sham-operated animals underwent an identical surgical procedure, except that either no pump or an empty osmotic pump was implanted. Blood pressure was measured either noninvasively by a computerized tail-cuff system (Visitech Systems) or invasively by telemetry (Data Sciences International) as previously described.11,12 For in vitro studies the animals were killed with CO2 inhalation, and the aortas were removed and dissected free of adherent tissues.

Determination of Glutathione and GSSG Reductase Activity
Aortic GSSG release was determined as previously described.7 For measurement of vascular GSH and GSSG, aortas were homogenized in 150 µL ice-cold 5% perchloric acid containing 10 µmol/L γ-glutamylglutamic acid as an internal standard and were quantified by high-performance liquid chromatography (HPLC) as described previously.7 GSSG reductase activity was determined as described elsewhere, monitoring the disappearance of NADPH spectrophotometrically.13

Vascular Reactivity Studies
Two to 3-mm aortic ring segments were studied in organ chambers as previously described.11 Passive tension was adjusted to 1 g, and vessels were preconstricted to equal levels with PGE1. Relaxations to cumulative concentrations of acetylcholine and nitroglycerin were examined.

Measurements of Vascular O2− and NO Production and Bioprotein Content
Vascular O2− was determined by examining the oxidation of dihydroethidium (DHE) to 2-hydroxyethidium as described previously.14 NO production was measured by electron-spin resonance (ESR) using the specific colloidal probe Fe3+ diethyldithiocarbamate (Fe3+ (DETC)3) in the presence of 10µmol/L A23187 as described.15 Aortic bioprotein content was analyzed using HPLC and a differential oxidation method as performed previously.16

Western Blot
The MRP1 antibody was from Kamia Biomedical. The Nox4 antibody was a gift from D. Lambeth, Emory University, Atlanta, Ga. The monoclonal p22phox and the Nox1 antibodies have been described previously.12,17 eNOS, Nox2, p47phox, and p67phox antibodies were from BD Bioscience. The AT1, AT2, and CDK4 antibodies were from Santa Cruz.

Results

Angiotensin II–Induced Vascular GSSG Export
In preliminary studies, we found that vessels from FVB and MRP1−/− mice expressed similar amounts of the AT1 and AT2 angiotensin II receptors (supplemental Figure I, available online at http://atvb.ahajournals.org). To determine whether angiotensin II–induced hypertension leads to vascular extrusion of GSSG, aortas were incubated for 6 hours in media and the amount of released GSSG determined using HPLC. Angiotensin II infusion for 2 weeks caused a marked increase in GSSG release from the aorta compared with control conditions (Figure 1A). Endothelial removal increased GSSG export from FVB vessels both at baseline and after angiotensin II infusion, suggesting that the vascular smooth muscle largely contributes to this process in angiotensin II–induced hypertension. Vessels lacking MRP1 had significantly lower export of GSSG than wild-type FVB mice at baseline, and angiotensin II–induced hypertension had no effect on this (Figure 1A). These changes in aortic GSSG extrusion were accompanied by striking alterations in vascular glutathione. In normal mouse aorta, the level of total GSH and GSSG was 30 nmoles/mg protein. Angiotensin II–induced hypertension reduced this by 30% (Figure 1B, F(1,9)=12.59, P<0.05). This was predominantly attributable to a decrease in GSSG, although GSH was also reduced. In contrast, in MRP1−/− mice, the intracellular total GSH and GSSG at baseline was 40 nmoles/mg protein and was unaffected by angiotensin II infusion. These results are in keeping with a role of MRP1 in modulating GSSG export from vascular cells.

Because GSSG is rapidly converted to GSH by GSSG reductase, we also sought to determine whether the expression or function of this enzyme was altered by angiotensin II–induced hypertension or by the lack of MRP1. Protein levels of GSSG reductase were similar between FVB and MRP1−/− mice and were not changed by angiotensin II infusion (Figure 1C). The activity of GSSG reductase, however, was significantly increased in mice receiving angiotensin II infusion (Figure 1D). Levels of MRP1 protein were not altered by angiotensin II infusion (Figure 1E). Western analysis also confirmed a complete absence of MRP1 protein in the aorta of MRP1−/− mice.

Endothelium-Dependent Relaxation
Angiotensin II–induced hypertension is known to diminish endothelium-dependent relaxation. We sought to assess whether reducing thiol export could prevent this effect of angiotensin II. In FVB mice angiotensin II caused a reduction in the acetylcholine evoked endothelium-dependent relaxation as compared with sham-operated animals (Figure 2A).
In contrast, in mice lacking MRP1, angiotensin II infusion had absolutely no effect on endothelium-dependent relaxation to acetylcholine (Figure 2A). Endothelium-independent relaxation to nitroglycerin was not different between the groups (Figure 2B).

**Vascular O$_2^-$ Production**

One explanation for the preservation of endothelial function in the MRP1$^{-/-}$ mice could be a modulation of O$_2^-$ levels. As previously described, chronic angiotensin II infusion caused an increase in aortic O$_2^-$ production in wild-type FVB mice. In contrast, in mice lacking MRP1, angiotensin II had no effect on O$_2^-$ production (Figure 3A). To investigate the contribution of uncoupled nitric oxide synthase on aortic superoxide production, vessels were treated with L-N-arginine methyl ester (L-NAME, 100 μmol/L) for 30 minutes before exposure to DHE. L-NAME significantly reduced vascular O$_2^-$ in FVB mice previously treated with angiotensin II, while not significantly altering O$_2^-$ production in MRP1$^{-/-}$ mice treated with angiotensin II (Figure 3A). In vessels from angiotensin II–treated mice, O$_2^-$ production remained 2-fold higher in FVB as compared with MRP1$^{-/-}$ mice vessels after L-NAME treatment.

**NADPH-Oxidase Subunit Protein Expression**

It has previously been shown that angiotensin II–mediated O$_2^-$ production is in large part attributable to activation and increased expression of the NADPH-oxidase. Further, oxidative stress is known to increase expression of NADPH oxidase subunits. We therefore hypothesized that prevention of thiol export would blunt the increase in NADPH oxidase subunit expression caused by angiotensin II. At baseline, protein levels of Nox1, Nox2, Nox4, p22$^{phox}$, p47$^{phox}$, and p67$^{phox}$ were similar between FVB and MRP1$^{-/-}$ mice, as determined by Western blotting (Figure 3B and 3C). Nox4 expression was not altered by angiotensin II infusion in either group of mice. In contrast, chronic angiotensin II infusion increased Nox1, Nox2, p22$^{phox}$, p47$^{phox}$, and p67$^{phox}$ expression in FVB mice. These effects of angiotensin II were markedly blunted in the MRP1$^{-/-}$ mice (Figure 3B and 3C).
Effect of Angiotensin II on Vascular Tetrahydrobiopterin and NO Levels

As noted above, L-NAME blunted O$_2^-$ production in wild-type mice. NADPH oxidase-derived ROS can lead to H$_4$B oxidation and uncoupling of nitric oxide synthase. We therefore sought to determine whether the reduced levels of vascular O$_2^-$ in MRP1$^{-/-}$ mice might be associated with preservation of aortic H$_4$B. Even in the absence of angiotensin II, the amount of oxidized pterin was higher in FVB as compared with MRP1$^{-/-}$ vessels. In FVB mice chronic angiotensin II infusion caused a reduction in aortic H$_4$B and a slight increase in the ratio of oxidized to reduced H$_4$B, whereas in MRP1$^{-/-}$ mice angiotensin II had no effect on pterin levels or the ratio of reduced to oxidized H$_4$B (Figure 4A). These changes in H$_4$B levels were mirrored by the capacity of the vessels to produce NO (Figure 4B and 4C). Angiotensin II–induced hypertension caused a decline in vascular NO production in FVB mice, but had no effect in MRP1$^{-/-}$ vessels. The levels of eNOS protein were not significantly affected by angiotensin II or the presence of MRP1 (Figure 4D).

Role of MRP1 in Modulating Blood Pressure

To determine whether the preservation of endothelium-dependent relaxation or reduce vascular O$_2^-$ production blunted the blood pressure response to angiotensin II, we monitored arterial blood pressure using telemetry and noninvasively by the tail cuff method. While the baseline pressure...
vascular function. Our current results indicate that recycling pathways would be superior in terms of maintaining normal 
levels, preserved endothelium-dependent relaxation and pre-
sion caused by angiotensin II in MRP1+/− mice as compared with 
MRP1−/− mice (Figure 5 and supplemental Figure II).

Discussion
In this study we demonstrated that the multidrug resistance 
protein-1 is a major exporter of oxidized glutathione and 
contributes to angiotensin II–induced hypertension. Vascular 
levels of GSH were higher in MRP1+/− mice than in FVB 
mice and during angiotensin II–induced hypertension, the 
lack of MRP1 prevented the decline in vascular glutathione 
levels, preserved endothelium-dependent relaxation and pre-
vented the increase in O$_2^−$ caused by chronic angiotensin II 
infusion. This was associated with a blunted increase in 
Nox1, Nox2, p22phox, p47phox, and p67phox and a preservation of 
H$_2$B in MRP1−/−-vessels. The absence of MRP1 also pre-
vented the decrease in NO production caused by angiotensin II 
treatment. Finally, these favorable effects on vascular 
function were accomplished by a reduction in the hyper-
tension caused by angiotensin II in MRP1−/− mice. These data 
clearly demonstrate that MRP1 plays an important role in 
regulation of blood pressure in response to angiotensin II, 
likely via modulation of intracellular thiol levels.

When GSH is oxidized, mammalian cells have two options 
for removing the newly formed GSSG. One is recycling by 
the enzyme GSSG reductase. In the present study, we showed 
that the protein level of this enzyme wasn’t altered, but that 
its activity was modestly enhanced by angiotensin II–induced 
hypertension. This finding is in keeping with previous studies 
showing that GSSG reductase activity is modulated by 
substrate availability.$^{21}$ The second mechanism for GSSG 
removal is export. Our current results show that vascular cells 
predominantly use MRP1 for this process. Of note, MRP1 
protein expression wasn’t changed in vessels of wild-type 
mice during angiotensin II infusion. It is likely that the Km of 
MRP1 for GSSG is sufficiently high that the expression of 
this transporter does not need to be increased to handle higher 
levels of GSSG formed during persistent oxidative stress. 
Before the present study, it wasn’t clear which of these two 
pathways would be superior in terms of maintaining normal 
vascular function. Our current results indicate that recycling 
of GSSG to GSH by the reductase is clearly beneficial and 
that excessive thiol extrusion is deleterious. In addition to a 
reduction of total intracellular thiol and the attendant oxidant 
stress, export of GSSG by MRP1 requires that vascular cells 
synthesize new GSH de novo, which is metabolically de-
manding. The induction of GSSG reductase likely enhances 
GSSG recycling and thus preservation of higher levels of 
GSH. Of note, in MRP1−/− mice, the vascular levels of 
glutathione were substantially higher at baseline than in 
wild-type animals suggesting that MRP1 not only modulates 
intracellular thiol levels during pathological states but also 
under normal conditions. This is in keeping with data by 
Lorico et al in other tissues of MRP1−/− mice.$^{22}$

Our current findings also indicate that in angiotensin II–induced hypertension, MRP1 has a significant impact on 
vascular NO production. This is manifested as preserved 
endothelium-dependent vasodilatation and NO production as 
determined by ESR when MRP1 is absent. Unlike the case in 
wild-type vessels, angiotensin II failed to stimulate an in-
crease in O$_2^−$ in MRP1−/− vessels. Because of the rapid 
reaction between NO and O$_2^−$, this would likely preserve NO. 
We also found that the ratio of reduced to oxidized H$_2$B was 
higher at baseline and during angiotensin II infusion in 
MRP1−/− mice as compared with wild-type animals and that 
the depletion of H$_2$B observed in wild-type mice did not occur 
in MRP1−/− mice. This was not attributable to a change in 
eNOS expression; however, angiotensin II failed to stimulate 
an increase in O$_2^−$ in MRP1−/− vessels. A major source of 
H$_2$B oxidation is peroxynitrite (ONOO$^−$), which in turn is 
effectively scavenged by GSH.$^{23,24}$ Thus, preservation of H$_2$B 
could maintain eNOS function and NO production. Finally, 
as discussed below, the reduced induction of several NADPH 
oxidase components in MRP1−/− mice could reduce O$_2^−$ 
production and preserve NO.

Our results also indicate that the increase in expression of 
NADPH oxidase subunits caused by angiotensin II is modu-
lated by MRP1. Previously, it has been shown that angioten-
sin II–induced hypertension is associated with an 6- to 7-fold 
increase in Nox1 mRNA expression, but only a modest 
change in Nox4 message.$^{17,18}$ Desouki et al have shown that 
reactive oxygen species produced by the mitochondria can
stimulate Nox1 expression.25 Further, we have shown that treatment of rats with membrane targeted superoxide dismutase prevents upregulation of aortic p22<sub>phox</sub> mRNA during angiotensin II infusion.26 Recently, multiple NADPH oxidase subunits have been found to be increased in vessels of humans with coronary artery disease—a setting in which ROS production is increased.27 These studies suggest that expression of various components of the NADPH oxidase is redox sensitive and raise the possibility that the initial increase in ROS production caused by angiotensin II can lead to a further increase in NADPH oxidase subunit expression in a feed-forward fashion. It is interesting to speculate that in MRP1<sup>+/−</sup> mice, prevention of thiol export in response to angiotensin II favorably affected the endothelial intracellular redox status and suppressed the redox stimulus for Nox1 expression.

Several prior studies have supported the concept that various pathophysiological stimuli can activate multiple sources of ROS production and that there is an interaction between these systems.28 Our current findings are in keeping with this observation. In addition to the increase in NADPH oxidase subunits and activity caused by angiotensin II, we and others18 have found clear-cut evidence of eNOS uncoupling, as evidenced by partial inhibition of vascular O<sub>2</sub><sup>−</sup> production by L-NAME. Oxidation of H<sub>2</sub>B can lead to an accumulation of dihydrobiopterin or, when extensive, destruction of the pterin structure and loss of total cellular biopterin.23 It has been reported that GSH can serve to protect against H<sub>2</sub>B oxidation and that various oxidized species of glutathione can react with H<sub>2</sub>B.24,29 In keeping with these prior studies, we found that angiotensin II–induced hypertension is associated with a decrease in H<sub>2</sub>B levels. In contrast, H<sub>2</sub>B levels were not diminished in MRP1<sup>+/−</sup> mice, also likely contributing to the normal aortic O<sub>2</sub><sup>−</sup> production in these animals.

These favorable effects on vascular function in MRP1<sup>+/−</sup> mice were associated with a blunted increase in blood pressure caused by angiotensin II. Interestingly, this effect on blood pressure is similar to that we have previously observed in p47<sup>phox</sup><sup>−/−</sup> mice.19 Overexpression of either Nox1 or p22<sup>phox</sup> augments hypertension caused by angiotensin II, and knock-out of Nox1 has been shown in two independent studies to blunt hypertension.30–32 Moreover, treatment with H<sub>2</sub>B has proven effective in lowering blood pressure in animals.11,33 The blunting of Nox1 expression and preservation of H<sub>2</sub>B therefore likely contributed to the lower blood pressure response to angiotensin II in MRP1<sup>+/−</sup> mice. Other undefined mechanisms may also contribute.

In summary, our data indicate that the MRP1 plays a major role in modulating intracellular glutathione levels, NO production, O<sub>2</sub><sup>−</sup> flux, gene expression, vascular function, and blood pressure. The loss of thiols in response to angiotensin II is associated with myriad untoward vascular effects that are prevented when MRP1 is absent. These data strongly suggest that specific MRP1 blockade might prove effective in the treatment of hypertension and perhaps other vascular diseases.

Acknowledgments

We gratefully acknowledge excellent technical support by Louise McCann and Graciela Gamez.

Sources of Funding

This work was supported by NIH grants HL390006 and HL59248, NIH Program Project Grant HL58000 and a Department of Veterans Affairs Merit Grant, and the NH&MRC Australia. Dr Widder was supported by the Deutsche Akademie der Naturforscher Leopoldina (BMBF-LPD 9901/8-97). Dr Mueller was supported by the Deutsche Forschungsgemeinschaft (MU 1731/1-1).

Disclosures

None.

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Arterioscler Thromb Vasc Biol. 2007;27:762-768; originally published online February 1, 2007;
doi: 10.1161/01.ATV.0000259298.11129.a2

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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A: Western blots showing aortic angiotensin 1 and 2 receptor (AT1R and AT2R) expression in wild-type FVB and MRP1−/− mice.

B: Mean data for densitometry of AT1R/actin and AT2R/actin. Data are from 4 experiments.
Representative systolic (A) and diastolic (B) blood pressure over 24h with or without chronic angiotensin II infusion in FVB and MRP1-/- animals. Blood pressure was measured continuously every hour for 10 min in conscious mice using a telemetry system. Recordings are from typical animals in each group on the 7th day of angiotensin II infusion.