Multidrug Resistance Protein-1 Affects Oxidative Stress, Endothelial Dysfunction, and Atherogenesis via Leukotriene C₄ Export

Cornelius F.H. Mueller, MD; Kerstin Wassmann, MD; Julian D. Widder, MD; Sven Wassmann, MD; Chia Hui Chen, MD; Barbara Keuler, MS; Alexey Kudin, PhD; Wolfram S. Kunz, PhD; Georg Nickenig, MD

Background—We recently showed that the multidrug resistance related protein-1 (MRP1) is important for the management of oxidative stress in vascular cells. However, the underlying mechanism and the in vivo relevance of these findings remain elusive. We hypothesize that inside-outside transport of leukotriene C₄ (LTC₄) via MRP1 is a substantial proatherogenic mechanism in the vasculature. To test this hypothesis, we investigated the effects of MRP1 inhibition and LTC₄ receptor blockade (Cys-LT1 receptor) in vitro and in vivo.

Methods and Results—MRP1 is expressed abundantly in vascular smooth muscle cells (VSMCs). Pharmacological inhibition of MRP1 via MK571 reduces angiotensin II–induced reactive oxygen species release by 59% (L012 fluorescence) in VSMCs. The release of reactive oxygen species after angiotensin II stimulation also is inhibited by blockade of the Cys-LT1 receptor with montelukast. Incubation of VSMCs with recombined LTC₄ causes enhanced rates of reactive oxygen species and proliferation in wild-type and MRP1⁻/⁻ VSMCs. Accordingly, the LTC₄ release in the cell culture supernatant of MRP1⁻/⁻ VSMCs is significantly decreased compared with wild-type cells. To extend our observations to the in vivo situation, atherosclerosis-prone apolipoprotein E–deficient mice on a high-cholesterol diet were treated with placebo, the MRP1 inhibitor MK571, or the Cys-LT1 receptor inhibitor montelukast for 6 weeks. Treatment with MK571 or montelukast reduced vascular reactive oxygen species production, significantly improved endothelial function, and ameliorated atherosclerotic plaque generation by 52% and 61%, respectively.

Conclusions—These findings indicate that MRP1 and LTC₄ exert proatherosclerotic effects and that both MRP1 and LTC₄ are potentially promising targets for atheroprotective therapy. (Circulation. 2008;117:2912-2918.)

Key Words: angiotensin ■ atherosclerosis ■ interleukins ■ muscle, smooth ■ multidrug resistance-associated proteins

Multidrug resistance proteins (MRPs) are members of the ATP-binding cassette family and originally were identified as mediators of chemoresistance in cancer cells. MRPs are active transporters for a variety of endogenously produced and exogenously administered molecules, including chemotherapeutic agents. Presently, MRP1 is the best-investigated member of this protein family. MRP1 acts as transporter for substances such as glutathione, oxidized glutathione, estrogen, and leukotriene C₄ (LTC₄), which are all potentially important for the regulation of reactive oxygen species (ROS) production in vascular cells. We have recently shown that MRP1 is expressed in human aortic endothelial cells and that modulation of MRP1 expression affects vascular function. MRP1 blockade or depletion stabilized the intracellular redox potential, decreased ROS-induced endothelial cell apoptosis, and improved vascular function in mice with desoxycorticosterone acetate (DOCA) salt–induced hypertension. Furthermore, it has been shown that hypertension caused by angiotensin (Ang) II is markedly reduced in MRP1⁻/⁻ mice compared with wild-type mice.

Clinical Perspective p 2918

So far, the underlying intracellular mechanisms of the reported effects remain unclear. An increased turnover rate of the glutathione redox system has been discussed as the most important effect of MRP1 depletion/blockade. Glutathione is one of the most abundant thiol antioxidants in cells. It is involved in cellular division, protein and DNA synthesis, maintenance of cellular redox homeostasis, and regulation of apoptosis. Catalyzed by the enzyme glutathione peroxidase,
glutathione can be easily converted to its oxidized form (GSSG). GSSG in turn can be either exported out of the cytoplasm via MRP1 or regenerated to glutathione by the enzyme glutathione reductase. Either accumulation or increased export of GSSG leads to cellular apoptosis. We have recently demonstrated that MRP1 blockade reduces oxidative stress by increasing the activity of the glutathione reductase in human aortic endothelial cells. However, this moderate upregulation may only partially explain the observed vaso-protective effects in an in vivo situation. Therefore, we hypothesized that additional intracellular mechanisms contribute to the atheroprotective effects of MRP1 blockade or depletion.

LTC₄, the conjugation product of glutathione and leukotriene A₄, is a mediator of inflammation, increases postcapillary permeability, causes vasoconstriction, and induces oxidative stress. LTC₄ is metabolized in vascular smooth muscle cells (VSMCs), macrophages, and leukocytes. In VSMCs, LTC₄ release can be triggered by Ang II.

LTC₄ mediates its biological effects by binding to the Cys-LT1 receptor. Because the Cys-LT1 receptor is located on the cellular membrane of VSMCs, transport of LTC₄ from the intracellular to the extracellular compartment is necessary to promote LTC₄ effects via its receptor. It has been shown that LTC₄ displays a high affinity to MRP1. Thus, we hypothesize that MRP1 could be important for atherogenesis and that inside-outside transport of LTC₄ could resemble a relevant mechanism.

Methods

Materials

Antibodies for Western blotting were purchased from Kamiya Biomedical (Seattle, Wash). All other materials necessary for Western blotting were acquired from Bio-Rad Laboratories (Hercules, Calif). MK571 and the caspase 3 colorimetric assay were obtained from Alexis Biochemicals (San Diego, Calif). DCF (2',7'-dichlorofluorescin), Ang II, and LTC₄ were purchased from Sigma.

Polymerase Chain Reaction

Total RNA (3 μg) was reverse transcribed using random primers and a SuperScript III kit (Invitrogen). DNA was amplified with the following primers for MRP1 (forward, 5'-CAGAGGAGGAGGATGCAGAGGA-3'; reverse, 5'-GGA-ACACGGCGCAGCAGACATGT-3').

Determination of ROS production

Intracellular ROS production in VSMCs was measured by L012 fluorescence as described previously. To perform these measurements, VSMCs were cultivated in 96-well plates until they reached confluence. The cells were then stimulated for up to 12 hours with the different substances indicated in the Results section. Cells were then harvested (trypsin) and resuspended in equal volumes of Krebs-HEPES-buffer with 100 μmol/L L012. Chemiluminescence was assessed after an incubation for 5 minutes over a time course of 15 minutes in a scintillation counter (Lumat LB 9501, Berthold, Bad Wildbad, Germany) in 1-minute intervals.

Determination of LTC₄ Release of VSMCs

For determination of LTC₄ release in cell culture supernatant of VSMCs, the leukotriene C/D/E Biotrak assay (Amersham) was used. VSMCs were cultured in a 96-well plate until they reached confluence. The cells were then incubated with the different substances (eg, MK571/montelukast) for 12 hours as indicated in Results. Afterward, 200 μL cell culture supernatant was collected and added to the 96-well plate provided with the kit for 2 hours. The following steps were performed according to the manufacturer's protocol.

Animal Experiments

Twelve-week-old apolipoprotein E–deficient (ApoE−/−) mice were fed a Western diet for 6 weeks. The animals were treated with either MK571, a pharmacological antagonist of MRP1 (5 mg · kg⁻¹ · d⁻¹), or montelukast (1 mg · kg⁻¹ · d⁻¹). Placebo-treated animals served as the control group. The mice were killed, and the aortas were excised. The vessel was then immersed in chilled buffer containing (in mmol/L) NaCl 118.0, CaCl₂ 2.5, KCl 4.73, MgCl₂ 1.2, KH₂PO₄ 1.2, NaHCO₃ 25.0, NaEDTA 0.026, and D(-)-glucose 5.5, pH 7.4. Perivascular adipose tissue was carefully removed. Rings (3 mm) were mounted in organ chamber baths filled with the described buffer (37°C, continuously aerated with 95% O₂ and 5% CO₂) and attached to a force transducer, and isometric tension was recorded. The vessels were gradually stretched over 60 minutes to a resting tension of 10 mN, which was maintained throughout the experiment, and were allowed to equilibrate for another 30 minutes. Drugs were added in increasing concentrations to obtain cumulative concentration-response curves: KCl 20 and 40 mmol/L, phenylephrine 1 mmol/L to 10 μmol/L, carbachol 10 mmol/L to 100 μmol/L, and nitroglycerin 1 mmol/L to 10 μmol/L. The drug concentration was increased when vasorelaxation or resistance was completed. Drugs were washed out before the next substance was added. Atherosclerotic lesion development was determined as published previously. Sections of the aortic root were embedded in Tissue Tek optical coherence tomography embedding medium, snap-frozen, and stored at −80°C. Samples were sectioned on a Leica cryostat and placed on poly-L-lysine–coated slides for analysis. Sections of the aortic root were then stained with Oil Red O and analyzed with software measuring the atherosclerotic lesion area in relation to the lumen of the vessel.

Statistical Analysis

Values are expressed as mean±SEM and were compared between groups by use of ANOVA. The Student-Newman-Keuls post hoc test was performed when significance is indicated. For comparisons between 2 groups, Student's t test was used. All authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.
Results
Pharmacological MRP1 Inhibition Decreases Ang II–Induced ROS Release
We assessed the expression of MRP1 in cultured VSMCs by Western blotting and reverse-transcription polymerase chain reaction and found a strong expression of MRP1. This expression was not altered by Ang II stimulation for up to 16 hours (data not shown). In a following experiment, we investigated whether pharmacological inhibition of MRP1 affected the Ang II–induced ROS generation. VSMCs were grown to confluence and incubated with either Ang II (10^{-6} mol/L) or the pharmacological MRP1 inhibitor MK571 (5 μmol/L) or coincubated with both substances. After 12 hours of incubation, the generation of ROS was determined by L012 fluorescence (Figure 1A). MK571 significantly diminished the ROS generation in VSMCs after coincubation with Ang II by 59±5%. To rule out side effects of the pharmacological inhibitor, we performed a similar experiment using MRPI^{+/−} cells (Figure 1B). Stimulation with Ang II resulted in a significantly diminished generation of ROS in MRPI^{−/−} VSMCs by 79±9% compared with wild-type VSMCs. These findings indicate an important role of MRP1 in Ang II–induced ROS production in VSMCs.

Ang II Causes LTC4 Release in VSMCs
We wondered whether MRP1–associated ROS generation was connected to LTC4 levels in the cell culture supernatant. LTC4 was detected in the cell culture media (Figure 2A). Ang II stimulation resulted in an increase in LTC4 of 75±15% in wild-type cells and had no effect in MRPI^{−/−} cells. This finding indicates that Ang II induces inside-outside transport of LTC4.

LTC4 Leads to ROS Generation in VSMCs: Blockade of the LTC4 Receptor and MRP1 Inhibits ROS Generation
VSMCs were grown to confluence and then incubated with LTC4, Ang II, MK571, and montelukast, a pharmacological Cys-LT1 receptor inhibitor. After a 12-hour stimulation, ROS release was determined by L012 chemiluminescence (Figure 3A). Ang II (10^{-6} mol/L) and LTC4 (10^{-6} mol/L) stimulation resulted in enhanced ROS generation by 134±30.4% and 107±36.2%, respectively, above the level of the control cells. Incubation with MK571 (5 μmol/L) and montelukast (10^{-6} μmol/L) alone did not affect ROS release. MK571 did not prevent the LTC4–induced oxidative stress; montelukast inhibited the LTC4–caused release of ROS. These findings demonstrate that MRP1–mediated LTC4 export caused ROS generation in VSMCs after stimulation with Ang II. We performed a similar experiment in MRPI^{−/−} cells to exclude nonspecific effects of MK571 (Figure 3B). Stimulation with MK571 and Ang II lead to a significant reduction in LTC4 release compared with wild-type cells. These findings indicate that Ang II induces inside-outside transport of LTC4.
LTC₄ increased ROS production, whereas stimulation with Ang II showed no effect. Incubation with Montelukast and coincubation with Montelukast and LTC₄ did not alter the ROS level in MRP1⁺⁻ VSMCs.

**Pharmacological MRP1 Inhibition and Cys-LT1 Receptor Blockade Affect ROS Production, Endothelial Function, and Atherogenesis in ApoE⁻⁻/⁻ Mice In Vivo**

To extend our findings to the in vivo setting, 3-month-old atherosclerosis-prone ApoE⁻⁻/⁻ mice receiving a cholesterol-enriched diet for 6 weeks were treated with MK571 (5 mg · kg⁻¹ · d⁻¹), montelukast (1 mg · kg⁻¹ · d⁻¹), or placebo. All mice were fed a high-fat, cholesterol-rich diet containing 21% fat, 19.5% casein, and 1.25% cholesterol.

The animals were then killed, aortas were excised, and endothelial function was analyzed in organ chamber experiments. Pharmacological inhibition of MRP1 and Cys-LT1 receptor inhibition reduced ROS production (L012 chemiluminescence) by 55% and resulted in a significantly lower percentage of atherosclerotic plaques (Oil Red O staining) by 52% ± 11%. Application of MK571 or montelukast improved endothelial function compared with placebo-treated controls (Figure 4). Endothelium-independent relaxation (nitroglycerin) remained unchanged. Application of MK571 or montelukast improved endothelial function to almost identical extents. Furthermore, we analyzed the development of atherosclerotic lesions in the aortic sinus (Figure 5). Treatment with MK571 diminished the size of atherosclerotic plaques (Oil Red O staining) by 52% ± 11%. Treatment with montelukast showed a similar effect, with a reduction of 61% ± 10%.

**Discussion**

Here, we show that MRP1 is expressed in VSMCs and acts as a transport protein for LTC₄. Inhibition of MRP1 reduces inside-outside transport of LTC₄, causing reduced ROS production. Furthermore, both inhibition and blockade of the Cys-LT1 receptor decrease ROS production, improve endothelial function, and ameliorate atherogenesis in vivo. These findings support prior publications showing a beneficial role of MRP1 inhibition in DOCA salt–hypertensive mice and in mice with Ang II-induced hypertension.

Although the major scientific interest in MRP1 was focused on its role in mediating resistance to anticancer drugs in tumor cells, there is now growing evidence that MRP1 also plays an important role in vascular function and in the setting of oxidative stress. Most of the chemotherapeutic agents transported via MRP1 require coupling to glutathione to be oxidized. Glutathione is one of the substrates with the highest affinity to the MRP1 protein. The idea that MRP1 might affect the management of oxidative stress was groundbreaking when Hirrlinger et al. discovered that oxidative stress causes the release of glutathione via MRP1 in astrocytes. Later, it was reported that in addition to the reduced form of glutathione, its oxidized form (GSSG) can be transported via MRP1. The glutathione redox system belongs to the basic antioxidative systems in almost every cell type.

It enables cells to cope with oxidative stress and almost exclusively determines the intracellular redox potential in human aortic endothelial cells. Given the importance of this system, we focused in prior studies mainly on the effects of MRP1 inhibition on the glutathione redox system. Oxidative stress causes the release of glutathione via MRP1 in astrocytes. Later, it was reported that in addition to the reduced form of glutathione, its oxidized form (GSSG) can be transported via MRP1. The glutathione redox system belongs to the basic antioxidative systems in almost every cell type.
stress results in human aortic endothelial cells in extrusion of GSSG and finally in glutathione depletion, which is one of the strongest triggers of apoptosis and cellular death. On the other hand, accumulation of GSSG also can trigger apoptosis. It was therefore surprising that MRP1 inhibition led in human aortic endothelial cells to improved cellular survival. Obviously, uncontrolled GSSG accumulation did not occur. We discovered that MRP1 inhibition upregulated the activity of the enzyme glutathione reductase, one of the key enzymes of the glutathione redox system, catalyzing the reduction of GSSG back to glutathione. However, this increase was only moderate (40%) and only partially explained the beneficial effects of MRP1 inhibition. In the same study, we analyzed the expression of MRP1 in the vascular wall of DOCA salt–hypertensive mice and found it expressed at a remarkable level. VSMCs present the majority of cells within the vascular wall. Therefore, a relevant expression of MRP1 in VSMCs was a likely finding. It was also likely that the glutathione redox system would be less important in VSMCs because these cells are less exposed to sudden oxidative stress and show different coping mechanisms and physiological reactions like the induction of cellular growth when exposed to ROS. One major trigger of ROS generation in VSMCs is Ang II. Widder et al investigated the effect of Ang II infusion in MRP1−/− mice and reported a variety of effects connected with MRP1 in respect to vascular function. Among them are a lower blood pressure, reduced production of ROS, and better endothelial function in MRP1−/− animals compared with control mice. We hypothesized that besides the already reported effects of MRP1 inhibition on the glutathione/GSSG system, alternative mechanisms should contribute to the observed effects in VSMCs.

Among the substrates with high affinity for MRP1 is LTC4. LTC4 mediates inflammatory reactions in respiratory tissue, and it is instrumental in the pathogenesis and treatment of asthma. In respiratory tissue, LTC4 induces ROS production via EGF receptor transactivation and ERK1/2 phosphorylation. A potential connection between MRP1 and LTC4 was supported by observations in MRP1−/− mice showing an insusceptibility of MRP1−/− mice to infection with pneumococci combined with dramatically improved survival. The major source of LTC4 in human tissue is leukocytes, but LTC4 also can be generated and released by VSMCs. It was therefore reasonable to assume that at least part of the observed antiatherosclerotic effects could be based on reduced generation and release of LTC4 by VSMCs. Especially in early stages of atherosclerotic lesion development, this VSMC-driven LTC4 release might be of importance. In advanced stages, the major source of LTC4 is most likely macrophages. In our experimental setting, Ang II stimulation of VSMCs resulted in an increased LTC4 release. This finding is in accordance with the results of Luchtefeld et al, who demonstrated that Ang II enhances leukotriene formation in VSMCs via induction of the lipoxigenase 5. Leukotriene formation resulted in an increased ROS production. The study presented here demonstrates that Ang II induces intracellular ROS formation via induction of LTC4, which is transported to

**Figure 4.** Endothelium-dependent vasorelaxation in ApoE−/− mice. Three groups of 5 mice each received proatherogenic diet for 6 weeks. One group also received MK571 in their drinking water (5 mg · kg−1 · d−1). Another group received montelukast in their diet (1 mg · kg−1 · d−1). Afterward, the animals were killed, the aortas were excised, and endothelial function was determined. Pharmacological inhibition of MRP1 and the Cys-LT1 receptor resulted in significantly improved endothelial function compared with placebo-treated controls. Endothelium-independent relaxation (nitrroglycerin) remained unchanged (n=5; P<0.05).
its extracellular lying receptor via MRPI. In MRPI<sup>−/−</sup>-VSMCs, Ang II was unable to induce either LTC<sub>4</sub> or ROS generation, indicating that inside-outside transport of LTC<sub>4</sub> via MRPI is necessary for Ang II–induced ROS production. In this study, we measured ROS mainly by determining L012 chemiluminescence, which represents the intracellular ROS generation. We did not determine extracellular sources of ROS, e.g., extracellular superoxide dismutase, which might also be important. The effects of MRPI inhibition and reduced leukotriene formation on extracellular ROS production need to be determined in future studies.

Our in vivo data support the thesis that LTC<sub>4</sub>-mediated production of ROS is an essential part of the physiological reactions in VSMCs after Ang II stimulation. Inhibition of MRPI or blocking of the Cys-LT1 receptor showed antiatherogenic effects in ApoE<sup>−/−</sup> mice. Our findings imply that the proinflammatory reactions caused by Ang II within the vascular wall are substantially connected to Ang II–mediated cytokine release. As shown in the online Data Supplement, Ang II stimulation may initiate an intrinsic vicious cycle, starting with the generation of LTC<sub>4</sub> in VSMCs. In a second step, LTC<sub>4</sub> is transported to the extracellular compartment via export by MRPI. The Cys-LT1 receptor, located on the extracellular membrane of VSMCs, can be stimulated by released LTC<sub>4</sub>. This in turn triggers the production of ROS and the further generation of leukotrienes, promoting finally a self-sustaining process. There are 2 potential ways to interfere with this cycle. The first way is to block MRPI and therefore interrupt the release of LTC<sub>4</sub>. The second way to interrupt LTC<sub>4</sub>-mediated effects is the blockade of the Cys-LT1 receptor. Montelukast (Singulair) is a Cys-LT1 receptor antagonist that is used for asthma treatment. So far, the effect of montelukast on vascular function has remained undetermined. We now demonstrate that pharmacological inhibition of MRPI and Cys-LT1 receptor prevents Ang II–induced ROS production, improves endothelial function, and reduces atherosclerotic plaque formation in ApoE<sup>−/−</sup> knockout mice. This mechanism also might be of importance in humans.

Taken together, our findings indicate a decisive role of MRPI in mediating LTC<sub>4</sub> transport, intracellular redox homeostasis, and finally vascular function and atherogenesis. Moreover, MRPI blockade and Cys-LT1 receptor blockade may provide new and interesting targets for the treatment of endothelial dysfunction and atherosclerosis.

**Acknowledgments**

The excellent technical support by Thomas Czech and Verena Becker is greatly appreciated.

**Sources of Funding**

Dr Mueller was supported by the Deutsche Forschungsgemeinschaft (MU 1731/1-1) and the BONFOR program of the Universitätsklinikum Bonn (O-109.0020). Dr Widder was supported by the Deutsche Akademie der Naturforscher Leopoldina (BMBF-LPD 9901/8-97).

**Disclosures**

None.
References
11. Leslie RM, Deeley RG, Cole SP. Toxicological relevance of the multidrug resistance protein 1, MRP1 (ABCC1) and related transporters. Toxicology. 2001;167:3–23.

CLINICAL PERSPECTIVE
Multidrug resistance proteins (MRPs) are active transporters for a variety of endogenously produced and exogenously administered molecules, including glutathione, oxidized glutathione, estrogen, and leukotriene C4 (LTC4), all of which are of potential importance for the regulation of reactive oxygen species production in vascular cells. In endothelial cells, MRP1 blockade or depletion stabilized the intracellular redox potential and decreased reactive oxygen species–induced endothelial cell apoptosis. LTC4 is a mediator of inflammation, increases postcapillary permeability, causes vasoconstriction, and induces oxidative stress. LTC4 is metabolized in VSMCs, macrophages, and leukocytes. The release of LTC4 can be triggered by angiotensin II. The biological effects of LTC4 are mediated via the Cys-LT1 receptor. In this study, we show that release of LTC4 via MRPI is important for atherogenesis and endothelial function in apolipoprotein E–deficient mice and that inside-outside transport of LTC4 resembles a relevant pro-oxidant mechanism. There are 2 potential ways to interfere with this mechanism. The first way is to block MRPI and therefore interrupt the release of LTC4. The second way to interrupt LTC4-mediated effects is the blockade of the Cys-LT1 receptor. Montelukast is a Cys-LT1 receptor antagonist used for asthma treatment. So far, the effect of montelukast on vascular functions has remained undetermined. We now demonstrate that pharmacological inhibition of MRPI, as well as Cys-LT1 receptors, prevents angiotensin II–induced reactive oxygen species production, improves endothelial function, and reduces atherosclerotic plaque formation in apolipoprotein E–deficient knockout mice. This mechanism might also be of importance in humans.
Multidrug Resistance Protein-1 Affects Oxidative Stress, Endothelial Dysfunction, and Atherogenesis via Leukotriene C4 Export

_Circulation_. 2008;117:2912-2918; originally published online May 27, 2008; doi: 10.1161/CIRCULATIONAHA.107.747667
_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2008 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/117/22/2912

Data Supplement (unedited) at:
http://circ.ahajournals.org/content/suppl/2008/05/27/CIRCULATIONAHA.107.747667.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Circulation_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to _Circulation_ is online at:
http://circ.ahajournals.org/subscriptions/