The Role of the Multidrug Resistance Protein-1 in Modulation of Endothelial Cell Oxidative Stress

Cornelius F.H. Mueller,* Julian D. Widder,* Joseph S. McNally, Louise McCann, Dean P. Jones, David G. Harrison

Abstract—Glutathione (GSH) is the major source of intracellular sulfhydryl groups. Oxidized GSH (GSSG) can be recycled to GSH by the GSH reductase or exported from the cell. The mechanism by which GSSG is exported and the consequence of its export from endothelial cells has not been defined previously. We found that human endothelial cells express the multidrug resistance protein-1 (MRP1) and use this as their major exporter of GSSG. Oscillatory shear stress, which is known to stimulate endothelial cell production of reactive oxygen species, decreased intracellular GSH. In contrast, laminar shear significantly increased intracellular GSH. Oscillatory shear also caused a robust export of GSSG that was prevented by the MRP1 inhibitor MK571 and by MRP1 small interfering RNA. MRP1 inhibition prevented the decline in intracellular GSH, preserved the intracellular GSH Nernst potential, and reduced apoptosis caused by oscillatory shear. In aortas of hypertensive mice, endothelial disulfide export was doubled, and this was prevented by MK571 and was not observed in aortas of hypertensive MRP1−/− mice. Further, the altered endothelium-dependent vasodilatation caused by hypertension was ameliorated in MRP1−/− mice. GSSG export by MRP1 leads to a perturbation of endothelial redox state and ultimately endothelial cell apoptosis. Endothelial MRP1 may provide a novel therapeutic target for prevention of vascular disease. (Circ Res. 2005;97:637-644.)

Key Words: endothelial cells ■ glutathione ■ MRP1 ■ oscillatory shear stress ■ oxidative stress

A major cellular defense against oxidative stress is reduced glutathione (GSH), a tripeptide consisting of cysteine, glutamate, and glycine.1,2 The antioxidant properties of GSH relate to its ability to directly reduce strong oxidants such as peroxynitrite and to its role as a cosubstrate for the enzyme GSH peroxidase.3 Of the total GSH pool, oxidized GSH (GSSG) normally represents <2%. Perturbations of the GSH/GSSG ratio can affect the ratio of other redox couples, such as NADH/NAD+ and NADPH/NADP+, and can induce cellular apoptosis.4,5 This ratio can be quantified as a Nernst potential for the GSH/GSSG couple, and this is generally regulated tightly to maintain cellular homeostasis.6

To maintain the GSH/GSSG Nernst potential in the setting of oxidative stress, cells may either synthesize GSH de novo or modulate GSSG levels. Cells use two competing mechanisms to maintain GSSG at low levels. The first is reduction by the enzyme GSH reductase, and the second involves GSSG export from the cell. For example, organs perfused with peroxide release GSSG.7 To date, it is unclear whether these competing mechanisms have similar effects on cellular function.

Within the endothelium, oxidative stress has been implicated in the pathogenesis of atherosclerosis, hypertension, altered vasomotion, and apoptosis.8 Endothelial production of reactive oxygen species (ROS) is increased by a variety of pathophysiological stimuli, including cytokines, hypertension, and altered mechanical forces. In particular, oscillatory shear stress, which occurs at points in the circulation predisposed to atherosclerosis, is a potent stimulus for ROS production.9 For example, on the outer wall of the carotid bulb, oscillations of +15 to −13 dyne have been observed. Similar degrees of oscillation occur in the proximal coronary arteries and the distal aorta. ROS produced in response to oscillatory shear have been implicated in enhanced monocyte adhesion as well as increased tissue factor and adhesion molecule expression.10,11

Despite the interest in endothelial oxidative stress and the importance of GSH in modulating the intracellular oxidative environment, virtually nothing is known about factors that regulate export of GSH from the endothelial cell, what the consequences of GSH depletion are, and how GSH transport can be modulated in the endothelium. Recent studies in other cells have shown that GSSG export can be mediated by a family of nine multidrug resistance proteins (MRPs).12 Although MRPs mediate drug resistance in cancer cells via export of drugs coupled to GSH, it has become apparent that some MRP proteins are also capable of transporting GSSG, and that GSSG may, in some cases, be their major endoge-
nous substrate.13 MRP1 has been identified in brain microvascular endothelial cells, but its role in endothelial GSH homeostasis has not been defined.14 In the present study, we demonstrate that MRP1 plays a major role in endothelial export of GSSG caused by oxidative stress in cell culture and in vivo. We further demonstrate that inhibition of GSSG export by MRP1 blockade prevents endothelial cell apoptosis and improves endothelial function.

Materials and Methods

Materials Used
Iodoacetic acid, dansylchloride, γ-glutamylglutamate (γ-glu-glu), boric acid, chloroform, and acetone were obtained from Sigma. Antibodies for Western blotting were purchased from Kamiya-Biomedical. All other materials for Western blotting were acquired from Bio-Rad. MK571, the caspase 3 colorimetric assay kit, and the annexin V ELISA kit were purchased from Alexis Biochemicals. Oligofectamine and Optimem were obtained from Invitrogen. Ovarian carcinoma-4 (OVCAR4) cell protein was provided by Dr Jonathan Simons, Winship Cancer Center, Emory University.

Cell Culture
Postconfluent human aortic endothelial cells (HAECs; Cambrex) between passages 4 and 10 were used for the experiments. Cells were maintained at 5% CO2 at 37°C and passaged every 6 to 10 days when confluent. Cells were exposed to either unidirectional laminar shear (15 dyne) or oscillatory shear (±15 dyne) using a cone and plate viscometer as described previously.15 Media was changed 1 hour before shear exposure.

High-Performance Liquid Chromatography Detection of GSH, GSSG, and Mixed Disulfides
Endothelial cells were harvested in 500 μL of 5% perchorlic acid containing 10 μM/L γ-glu-glu as an internal standard. The precipitated proteins were separated by centrifugation for 2 minutes at 14,000 rpm. GSH, GSSG, and the disulfide cys-GSH were quantified by high-performance liquid chromatography (HPLC) from the acid-soluble supernatant as N,N-bis-dansyl or S-carboxy-methyl-N-dansyl derivatives as described previously.16 To determine media levels of disulfide, 150 μL of media was centrifuged and transferred to a fresh tube before perchorlic acid (10%) was added.

The Eh values for the GSH/GSSG pool were calculated using the Nernst equation with combined constants for a two-electron transfer: \( E_v = E_0 + \frac{30 \log([\text{disulfide}]/[\text{thiol}])}{n} \), using \( E_0 = -264 \text{ mV} \) for GSSG/GSH.4,6

Detection of MRP1 mRNA and Protein
MRP1 protein was detected by Western blotting and its mRNA quantified using real-time polymerase chain reaction (PCR). Details of these are provided in the supplement, available online at http://circres.ahajournals.org.

Suppression of MRP1 by Small Interfering RNA
Specific siRNAs were produced using the Silencer small interfering RNA (siRNA) construction kit (Ambion) for human MRPI and GAPDH (AS-MRP1, AAT TCT CAA TGG GAT CAA AGT). A negative control siRNA sequence (Ambion) was also used. Details of transfection are provided in the online supplement.

GSH Reductase Activity
GSH Reductase activity was determined as described previously, monitoring the disappearance of NADPH spectrophotometrically.17

Deoxycorticosterone Acetate–Salt Hypertension
Deoxycorticosterone acetate (DOCA)–salt hypertension was created as described previously.18 Sham-operated mice underwent anesthesia and surgical incisions in the flank and midscapular regions but did not have nephrectomy or insertion of the DOCA pellet. Male MRP1−/− mice19 and the corresponding wild-type FVB mice (Taconic) underwent similar treatment. On the days of experiments, mice were killed using carbon dioxide inhalation. The aortas were excised and adherent tissue removed. For measurements of disulfide release, eight 2-mm sections were incubated in 150 μL of EBM2 media for 6 hours in an incubator at 37°C, pH 7.4, CO2 5%.

Studies of Vascular Reactivity
Ring segments (3 mm) of mouse aortas were studied in organ chambers and isometric tension recorded as described previously.18 Passive tension was set to 1 g, and vascular relaxation to cumulative concentrations of acetylcholine or sodium nitroprusside was determined after preconstriction with phenylephrine.

Statistical Analysis
Values are expressed as mean±SE and were compared between groups using ANOVA. The Student-Newman–Keuls post hoc test was performed when significance was indicated. For comparisons between two groups, Student’s t test was used. Differences were considered significant when \( P<0.05 \).

Results
Oxidative Stress Decreases Intracellular GSH and Stimulates GSSG Release From Human Endothelial Cells
Areas of the circulation exposed to oscillatory shear are predisposed to atherosclerotic lesion formation, and oscillatory shear is known to stimulate endothelial cell O2− and hydrogen peroxide production.9,20 We therefore examined the effect of oscillatory shear on intracellular GSH levels and the export of GSH into the media. Both 4 and 12 hours of oscillatory shear decreased intracellular GSH compared with static conditions (Figure 2A and 1B). In contrast, laminar shear had no effect on intracellular GSH after 4 hours and significantly increased intracellular levels of GSH after 12 hours. The media levels of the mixed disulfide cys-GSH, which forms rapidly in presence of GSSG and cysteine, were increased 3-fold by oscillatory shear. Laminar shear also increased media disulfide levels but only by about half that of oscillatory shear (Figure 2A and 2B). These data indicate that oscillatory shear is a potent stimulus for the oxidation of GSH and ultimately for its release from endothelial cells.

Role of MRP1 in Endothelial Thiol Export
In other cells, members of the MRP family have been implicated in transport of GSSG.13 One of the best characterized transporters of GSSG is MRPI, and we therefore sought to determine whether HAECs expressed MRPI. Western analysis for MRPI in HAECs demonstrated a protein of identical molecular weight to that observed in ovarian carcinoma cell line OVCAR4, which is known to express MRPI at high levels (supplemental Figure IA, available online at http://circres.ahajournals.org). The levels of MRPI protein were not affected by 12 hours of oscillatory shear (supplemental Figure IB). To determine whether MRPI contributes to endothelial cell export of disulfide, cells were exposed to the specific MRPI inhibitor MK571 (50 μmol/L) before shear.21 MK571 completely prevented the increase in extracellular disulfide export caused by oscillatory and laminar shear while having only a small effect on disulfide export in static cells. MK571 also blocked the accumulation of extra-
cellular disulfide in the setting of laminar shear (supplemental Figure IC).

To avoid nonspecific pharmacological effects that might accompany use of MK571, we also used siRNA to suppress MRP1 expression. The siRNA against MRP1 reduced its mRNA levels, as determined by real-time PCR, by \( \approx 70\% \) (supplemental Figure IIA). The specific MPR1 siRNA also reduced endothelial cell MRP1 protein levels by \( 64\% \pm 4\%; n=8 \) (supplemental Figure IIB). In contrast, neither mock transfection with oligofectamine alone, nor transfection with the GAPDH siRNA affected MRP1 mRNA or protein levels, although GAPDH siRNA reduced GAPDH protein (supplemental Figure IIB). Downregulation of MRP1 with the MRP1 siRNA significantly reduced the export of disulfide into the medium and preserved the intracellular levels of GSH compared with GAPDH siRNA controls (Table and supplemental Table I). Importantly, downregulation of MRP1 with the MRP1 siRNA did not increase intracellular GSSG levels during either oscillatory or laminar shear.

The above changes in intracellular GSH caused by oscillatory shear and the MRP1 siRNA were reflected by changes in the intracellular GSH/GSSG Nernst potential. In cells transfected with the GAPDH siRNA control, oscillatory shear caused an increase in the GSH/GSSG Nernst potential to \( -271 \text{ mV} \) (supplemental Figure IIC and Table). In contrast, oscillatory shear had no effect on the intracellular GSH/GSSG Nernst potential in cells transfected with the MRP1 siRNA. Laminar shear had no effect on the intracellular GSH/GSSG Nernst potential (supplemental Figure IIC and Table).

The Role of MRP1 in Endothelial Cell Survival During Oxidative Stress Induced by Oscillatory Shear

The above data show that HAECs express MRP1 and that MRP1 is the major source for GSSG export when these cells are exposed to oscillatory shear. Removal of GSSG by MRP1 could be expected to have beneficial effects via elimination of this oxidizing molecule or could have deleterious effects by decreasing intracellular thiol pools. Because intracellular GSH depletion is an important trigger for apoptosis,\(^{22}\) we examined the role of MRP1 in this process. Exposure of cells transfected with either GAPDH siRNA or negative control siRNA to oscillatory shear for 16 hours increased caspase-3

Figure 1. Effect of 4 and 12 hours shear on intracellular GSH in HAECs. A, Mean data for HAECs exposed to static conditions or 15 dyne/cm\(^2\) of oscillatory (osc) or laminar (lam) shear. GSH levels were determined by HPLC and normalized to cellular protein levels (\( n=6 \) to 8; \( *P<0.05 \)). B, Representative HPLC tracings illustrating intracellular GSH resulting from laminar and oscillatory shear. The retention time for the GSH peak is shown at 27 minutes.

Figure 2. Effect of 4 and 12 hours of shear on extracellular disulfide in HAECs. A, Mean data for HAECs exposed to static conditions or 15 dyne/cm\(^2\) of oscillatory (osc) or laminar (lam) shear. Media disulfide levels were determined by HPLC and normalized to cellular protein values (\( n=6 ; *P<0.05 \)). B, Representative HPLC tracings showing extracellular disulfide. The retention time for the disulfide peak is 26 minutes.
activity and annexin V by 2-fold compared with static conditions (Figure 3A and 3B). This was completely prevented by downregulation of MRP1 using siRNA. Laminar shear had no significant effect on either caspase 3 activity or annexin V levels (Figure 3A and 3B).

**Effect of Shear and MRP1 siRNA on GSH Reductase Activity**

Because we observed an increase in reduction of GSSG to GSH during MRP1 inhibition and oxidative, we examined activity of GSH reductase. Inhibition of MRP1 expression with siRNA had minimal effect on GSH reductase activity; however, in the presence of oscillatory shear, siRNA inhibition of MRP1 expression increased GSH reductase activity by 40% (Figure 4). Neither the negative control nor GAPDH siRNA altered GSH reductase activity during oscillatory shear. Unidirectional laminar shear markedly stimulated GSH reductase activity in cells transfected with negative-control siRNA and siRNA against either GAPDH or MRP1 (Figure 4).

**MRP1 Modulates Endothelial GSSG Export and Endothelial Function in Hypertension**

To determine the role of MRP1 in vascular oxidative stress in vivo, we studied mice with DOCA-salt hypertension. This model of hypertension is associated with a large increase in ROS production by the endothelium.18 Interestingly, MRP1 expression was increased in vessels of hypertensive C57BLk/6 mice (Figure 5A). Segments of aortas from control and hypertensive C57BLk/6 mice were incubated in media for 6 hours, and disulfide release was measured by HPLC. Hypertension increased disulfide release 2-fold, and this was abolished completely by MK571 (Figure 5B). Denudation of the endothelium also eliminated disulfide release, in keeping with our previous observation that DOCA-salt hypertension predominantly increases ROS production by the endothelium (Figure 5B).18

To further examine the role of MRP1 in modulation of vascular disulfide release, we studied MRP1−/− mice and their respective FVB controls under control (sham surgery) conditions and after induction of DOCA-salt hypertension. The increase in blood pressure caused by DOCA-salt hypertension in MRP1−/− mice was identical to that we observed in C57BLk/6 mice. Systolic pressures averaged 123±4 mm Hg in FVB shams, 146±4 mm Hg in FVB hypertensive mice, 115±3 mm Hg in MRP1−/− shams, and 144±3 mm Hg in MRP1−/− DOCA-salt hypertensive mice, respectively (n=8 to 11). The aortas of MRP1−/− mice released only one third as much disulfide as FVB controls, and DOCA-salt hypertension did not increase this further (Figure 5C). As a measure of endothelial cell function, we examined endothelium-dependent vasodilatation evoked by acetylcholine, which reflects endothelial NO availability. This was impaired in FVB mice with DOCA-salt hypertension (Figure 6A). In contrast, endothelium-dependent vasodilatation was only mildly impaired in the MRP1−/− mice with hypertension. Endothelium-independent vasodilatation to sodium nitroprusside was not altered by DOCA-salt hypertension and similar between FVB and MRP1−/− mice (Figure 6B).

**Discussion**

In the present study, we defined a novel role for the membrane transporter MRP1 in regulation of endothelial cell oxidative stress in response to mechanical stimuli in cell culture and hypertension in vivo. In human endothelial cells, MRP1 was found to be critical for export of GSSG during the
oxidative stress caused by oscillatory shear. In intact vessels, endothelial MRP1 played a similar role in thiol transport, and this was ameliorated in the setting of DOCA-salt hypertension. DOCA-salt hypertension was accompanied by diminished endothelium-dependent relaxation, and this was ameliorated in MRP1\textsuperscript{-/-} mice. Our studies indicate that the extracellular thiol transport mediated by MRP1 in response to oxidant stress may predispose to endothelial cell apoptosis, and that inhibition of MRP1 allows endothelial cells to maintain their intracellular redox potential and prove beneficial in promoting endothelial cell survival and function.

ROS exposure increases cellular GSSG content within a short time and rapidly decreases the GSH/GSSG ratio.\textsuperscript{23} This can induce three major cellular defense strategies. First, exposure to ROS induces enzymatic synthesis of GSH via upregulation of GSH synthetase.\textsuperscript{24} A second mechanism to restore the GSH/GSSG ratio is via the action of GSH reductase, which rapidly converts GSSG to GSH using NADPH as a substrate.\textsuperscript{2} The third strategy involves cellular export of GSSG.\textsuperscript{25} Our current data indicate that although export of GSSG by MRP1 may serve as an immediate mechanism for the cell to rid itself of an oxidized species, this process is in competition with GSH reductase (Figure 7), and if stimulated for a prolonged period, it may reduce the total intracellular thiol pool and paradoxically have an undesirable effect on the intracellular ratio of GSH/GSSG. We found that inhibition of MRP1 increases GSH reductase activity during oscillatory shear. The combined effects of inhibiting export of GSSG and increasing GSH reductase activity would allow recycling of GSSG to GSH and ultimately maintenance of the intracellular redox state.

To date, the major interest in MRP1 has been in its role in transport of the anticancer drugs when they are coupled to GSH.\textsuperscript{19} A less appreciated role of MRP1 is transport of GSSG. As an example, MRP1 exports GSSG in astrocytes exposed to oxidative stress.\textsuperscript{26} MRP1 also participates in GSSG transport and drug resistance in brain choroidal cells.\textsuperscript{27} It is of interest that many chemotherapeutic agents also induce cellular oxidant stress, and that oxidant stress may be a common mechanism for modulation of MRP1 induction not only in hypertension but also in chemoresistance.
In the circulation, regions exposed to oscillatory shear are predisposed to develop atherosclerotic lesions. Our in vitro model approximates oscillations that have been documented to occur at these sites. Oscillatory shear induces a variety of proatherosclerotic events, including adhesion molecule expression, enhanced tissue factor expression and monocyte adhesion. As in the present study, these effects of oscillatory shear are often completely opposite of the effects of laminar shear, which seems to inhibit atherosclerosis. In cultured endothelial cells, we found that oscillatory shear stimulated endothelial cell apoptosis, and that this was prevented by preserving intracellular GSH via downregulation of MRP1. These findings are in keeping with several previous studies showing that depletion of GSH and alterations in the GSH/GSSG Nernst potential can stimulate apoptosis in a variety of cells. GSH depletion has been shown to decrease protein levels of antiapoptotic protein Bcl-2, and to cause apoptosis. Moreover, verapamil, which activates GSH export via MRP1, induces apoptosis in baby hamster kidney cells.

Of note, the increase in disulfide released into the medium in response to oscillatory shear was striking, averaging 50 nmol/mg protein. This represents ~50% of the intracellular content of GSH (Table and Figures 1 and 2). Thus, during oscillatory shear, there is not only loss of the existing intracellular GSH but a requirement for the endothelial cell to synthesize large amounts of additional GSH de novo. This process requires cysteine, glycine, and glutamate, and two molecules of ATP. This metabolic demand may contribute to some of the untoward cellular events caused by oxidative stress. Prevention of GSSG export by MRP1 inhibition would prevent the need for additional GSH de novo synthesis and reduce this metabolic demand.

In the present study, we induced endogenous oxidative stress using two different stimuli. In HAECs, we used oscillatory shear, whereas we induced oxidative stress in mouse aortas via induction of DOCA-salt hypertension. On first inspection, these two stimuli seemed unrelated. However, there are several strengths of this approach. First, we demonstrated the importance of MRP1 in endothelial cell GSH homeostasis in human cells, and our findings thus have implications for human pathophysiology. Second, we extended these findings to an in vivo setting and demonstrated that MRP1 mediated GSSG release from intact aortic rings in response to hypertension. By using MRP1−/− mice, we were able to precisely identify this transporter as the source of GSSG export. Third, both of the stimuli we used evoke endogenous production of ROS rather than requiring addition of exogenous ROS. This is important because the exogenous addition of ROS may not reflect those generated by intracellular enzymes. Fourth, our studies indicate that endothelial export of GSSG by MRP1 is not unique to one stimulus of oxidative stress but occurs in response to at least two stimuli. In preliminary studies, we also found that addition of hydrogen peroxide to HAECs also stimulates GSSG export, and that this can be inhibited by MRP1 siRNA treatment. It is therefore likely that multiple causes of oxidant stress would activate GSSG transport by MRP1 in the endothelium. Finally, in systolic hypertension associated with a wide pulse pressure, the degrees of shear oscillation would be expected to increase at sites prone to this flow perturbation. Thus,
hypertension may worsen oscillatory shear under some circumstances.

In the DOCA-salt hypertensive aortas, we observed an increase in MRP1 expression; however, we do not believe this is necessary to explain the increase in GSSG export from these vessels. In cultured endothelial cells, oscillatory shear stimulated increased GSSG export without increasing MRP1 protein. This suggests that the ambient levels of MRP1 are sufficient to extrude increased GSSG formed in response to an oxidative stimulus. Furthermore, Balcerczyk et al demonstrated that overexpression of MRP1 failed to alter responses to oxidative stress imposed on Chinese hamster cells.34

Because GSH export was mediated by the endothelium in DOCA-salt hypertensive mice, we also examined endothelium-dependent vasodilatation in these vessels. In conduit vessels, endothelium-dependent vasodilatation is dependent on NO and reflects overall integrity of the endothelium. Our experiments indicate that this function of the endothelium was preserved in MRP1−/− mice with hypertension. The precise mechanisms whereby endothelial NO production may be altered by MRP1 remain undefined; however, alterations of the GSH/GSSG ratio may alter levels of other ROS, which could, in turn, affect NO production or bioavailability. It is important to note that in the DOCA-salt model of hypertension, enhanced vasodilation is not generally associated with a reduction in blood pressure. As an example, Huang et al have shown that minoxidil has no effect blood pressure in this model while reducing systemic vascular resistance by 30%.35 We have shown previously that treatment of rats with a membrane-targeted superoxide dismutase improved endothelium-dependent vasodilation but had no effect on blood pressure in rats with DOCA-salt hypertension.36

In summary, the current studies elucidated a new mechanism whereby endothelial cells modulate intracellular levels of GSH and GSSG via the action of the MRP1. This newly recognized function of MRP1 plays a role in cell survival and endothelial cell function. Vascular oxidative stress is increased in numerous conditions other than hypertension, including diabetes, atherosclerosis, and aging. Attention has focused on sources of ROS such as the NADPH oxidase, xanthine oxidase, the mitochondria, and antioxidant enzymes such as the superoxide dismutases, GSH peroxidase, and catalase. Our current study indicates that MRP1 also has an important role in modulation of endothelial cell oxidative stress and that modulation of its activity might represent an important therapeutic target for treatment of vascular disease.

Acknowledgments
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References
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On-line supplement

Methods

Western analysis: For detection of MRP1 in human cells, we used the clone MRPm6 as primary antibody (Kamiya Biomedical) at a dilution of 1:300. To detect MRP1 in mouse aortas, we used the monoclonal antibody MRPr1 at a dilution of 1:50. Actin immunoblotting was used to normalize for loading variations.

Quantitative Real-time PCR: Isolation of total RNA from HAEC’s was performed using TRI reagent (Molecular Research Center, Inc). Total RNA (3 µg) was reverse transcribed using random primers and a SuperScript III kit (Invitrogen). Endothelial cDNA was amplified using a LightCycler real-time thermocycler (Roche Diagnostics, Indianapolis, IN) with following primers for MRP1 (forward 5’-CAGAGCAGGAGCAGGATGCAGAGGA-3’ and reverse 5’-GGAACCAGCGCGACACATGCTG-3’). The mRNA copy numbers were calculated from standard curves generated using MRP1 and 18S templates. The pSRα-MRP plasmid for these standards were generously provided by Dr. Gary Kruh (Fox Chase Center, Philadelphia). The resulting mRNA levels were expressed as a ratio to the level of 18S mRNA.

Suppression of MRP1 by siRNA: siRNA was preincubated in Eppendorf-tubes containing 0.591 ml Optimem and 9µl Oligofectamine per sample. siRNA (25 nmol/L) was added at a final volume of 3 ml. After 4 hours of incubation at 37°C and 5% CO₂, 6 ml EBM2 media was added. This procedure was repeated after 48 hours.
**Online Table 1:** Intracellular GSH, GSSG and Nernst Potential. Effect of oscillatory and laminar shear stress and MRP1 downregulation.

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<th>GSSG (nMoles/mg protein)</th>
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*p < 0.05 vs static GAPDH. n=6-9

On-line supplement figure legends:

**Online Figure 1:** Expression of MRP1 and its role in HAEC’s: A: Fifteen µg protein from HAEC’s and OVCAR 4 cells were separated on a 7.5% SDS Polyacrylamide gel and Western analysis performed using the MRPM6 clone antibody against human MRP1. B: HAEC’s were exposed to oscillatory shear over 12 hours and MRP1 protein expression assessed by Western blot (n=3-4) C: HAEC’s were exposed to shear over 12 hours with or without the MRP1 inhibitor MK571 (50 µmol/L). Inhibition of MRP1 by treatment with MK571 decreased accumulation of extracellular disulfide compared to untreated cells. (n=3-4, *p<0.05 for oscillatory shear vs. static, **p<0.05 for MK571 vs. nontreated)

**Online Figure 2:** MRP1 inhibition using siRNA – effect on intracellular GSH/GSSG Nernst potential. Cells were treated with 25 nmol/L siRNA against either GAPDH,
MRP1 or were mock transfected (exposed to oligofectamine without siRNA). A: MRP1 mRNA expression in HAEC’s determined by real time PCR and normalized to 18S. (n=3, *p<0.05. vs. no transfection (-), GAPDH-siRNA and mock transfection). Panel B: Representative Western Blot showing MRP1 and GAPDH expression in cells treated with the respective siRNAs or mock transfection. C: Intracellular GSH/GSSG Nernst potentials (Eh GSH/GSSG) in MRP1 siRNA transfected cells compared to cells transfected with siRNA for GAPDH (n=7-9, *p<0.05). Cells were exposed to either static conditions, laminar or oscillatory shear stress as in figure 1 in manuscript.
Figure 1: On-line supplement

A

![Image of HAECs and OVCAR-4 proteins](image)

190 kDa

HAECs  OVCAR-4

B

![Image of MRP1 and Actin](image)

MRP1  Actin

C

![Bar graph of nmoles disulfide/mg Protein](image)

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<th>Condition</th>
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Statistical significance indicated by * (p < 0.05), ** (p < 0.01)
Figure 2: On-line supplement

(A) Bar graph showing MRPI mRNA/18 S levels with error bars. The treatments include: - (mock), GAPDH siRNA, MRP1 siRNA, and mock.

(B) Western blot images with bands labeled as MRP1, actin, mock, GAPDH, and MRP1.

(C) Bar graph showing Intracellular $E_h$ (mV) with error bars. The treatments are Static GAPDH, Static MRP1, Osc GAPDH, Osc MRP1, Lam GAPDH, Lam MRP1.