ORIGINAL RESEARCH COMMUNICATION

Protective Role of Methionine Sulfoxide Reductase A Against Ischemia/Reperfusion Injury in Mouse Kidney and Its Involvement in the Regulation of Trans-Sulfuration Pathway

Jee In Kim,¹ Seung Hee Choi,² Kyong-Jin Jung,¹ Eujin Lee,² Hwa-Young Kim,² and Kwon Moo Park¹

Abstract

Aims: Methionine sulfoxide reductase A (MsrA) and methionine metabolism are associated with oxidative stress, a principal cause of ischemia/reperfusion (I/R) injury. Herein, we investigated the protective role of MsrA against kidney I/R injury and the involvement of MsrA in methionine metabolism and the trans-sulfuration pathway during I/R. **Results:** We found that MsrA gene-deleted mice (*MsrA*^{-/-}) were more susceptible to kidney I/R injury than wild-type mice (*MsrA*^{+/+}). Deletion of MsrA enhanced renal functional and morphological impairments, congestion, inflammatory responses, and oxidative stress under I/R conditions. Concentrations of homocysteine and H₂S in the plasma of control *MsrA*^{-/-} mice were significantly lower than those in control *MsrA*^{+/+} mice. I/R reduced the levels of homocysteine and H₂S in both *MsrA*^{+/+} and *MsrA*^{-/-} mice, and these reductions were significantly more profound in *MsrA*^{-/-} than in *MsrA*^{+/+} mice. I/R reduced the expression and activities of cystathionine-β-synthase (CBS) and cystathionine-γ-lyase (CSE), both of which are H₂S-producing enzymes, in the kidneys. These reductions were more profound in the *MsrA*^{-/-} mice than in the *MsrA*^{+/+} mice. **Innovation:** The data provided herein constitute the first *in vivo* evidence for the involvement of MsrA in regulating methionine metabolism and the trans-sulfuration pathway under normal and I/R conditions. **Conclusion:** Our data demonstrate that MsrA protects the kidney against I/R injury, and that this protection is associated with reduced oxidative stress and inflammatory responses. The data indicate that MsrA regulates H₂S production during I/R by modulating the expression and activity of the CBS and CSE enzymes. *Antioxid. Redox Signal.* 18, 2241–2250.

Introduction

METHIONINE SULFOXIDE REDUCTASES (Msrs) catalyze the reduction of free and protein-based methionine sulfoxide to methionine. They consist of two families according to substrate stereospecificity. MsrA is specific for the *S*-form of methionine sulfoxide, whereas MsrB reduces the *R*-form (9, 28, 30). A single MsrA and three MsrBs (MsrB1, B2, and B3) are present in mammalian cells (16, 34). The principal function of Msrs is to repair oxidatively damaged proteins by reversing the oxidation of methionine residues. The cyclic oxidation/reduction of methionine is believed to be an important process in scavenging reactive oxygen species (ROS) (21, 44). A number of studies have demonstrated that MsrA and MsrB function as antioxidant enzymes, protecting cells against oxidative stress (22, 24, 25, 29).

Methionine, an essential amino acid in mammals, supports the methylation cycle involving *S*-adenosylmethionine and is

Innovation

The present study shows that MsrA deletion exacerbates the susceptibility of the kidneys to I/R injury, thereby indicating that MsrA is an important protein in protecting the kidney against I/R injury. Additionally, MsrA knockout reduces the levels of plasma homocysteine and H₂S, both of which are relevant to I/R injury. Moreover, MsrA regulates the activity and expression of both CBS and CSE enzymes in the trans-sulfuration pathway during I/R injury. A better understanding of the MsrA-homocysteine-H₂S axis will clearly be of benefit in regard to efforts to preserve kidney function under I/R injury conditions.

relevant to the biosynthesis of cysteine and other compounds (19, 45). Homocysteine is generated by methionine

¹Department of Anatomy, Kyungpook National University School of Medicine, Daegu, Republic of Korea.

²Department of Biochemistry and Molecular Biology, Yeungnam University College of Medicine, Daegu, Republic of Korea.

metabolism and is converted to cysteine by the consecutive actions of cystathionine- β -synthase (CBS) and cystathionine- γ -lyase (CSE) in the trans-sulfuration pathway. Methionine restriction leads to the downregulation of the trans-sulfuration pathway by reducing levels of CBS (39). Cysteine is a substrate of the two pyridoxal-5'-phosphate-dependent enzymes, CBS and CSE, generating hydrogen sulfide (H_2S) (42). H_2S , which has been known for decades as a toxic gas, is currently recognized as a gaseous physiological molecule (1, 11, 42, 48). Recent studies have demonstrated that H_2S performs a cytoprotective role in a variety of organs, including the heart, brain, intestine, and kidney, against ischemia/reperfusion (I/R) injury (5, 41, 49). Thus, it is possible that Msrs may be involved in the regulation of the trans-sulfuration pathway and the production of H_2S .

I/R in the kidney induces acute kidney injury, which is characterized by high mortality and morbidity. No effective therapeutic strategy for the treatment of I/R injury has yet been developed (3). I/R injury is associated with a number of factors, including ROS, cytokines, and chemokines, all of which are generated abundantly during I/R (3, 43). ROS are recognized as a major cause of kidney I/R injury, as they induce lipid peroxidation, protein dysfunction, cellular cytoskeletal disruption, cellular integrity degradation, DNA breakdown, and inflammatory responses (3, 7, 16, 33).

MsrA is known to be abundantly expressed in the kidney (26). Considering that ROS play a causal role in kidney I/R injury, we hypothesized that MsrA might protect the kidney against I/R injury. We also hypothesized that MsrA might affect homocysteine and H_2S production in methionine metabolism and the trans-sulfuration pathway. To test these hypotheses, we employed MsrA gene-deleted mice in a kidney I/R injury model (23). We found that MsrA knockout ($MsrA^{-/-}$) mice were much more susceptible to kidney I/R injury than wild-type mice ($MsrA^{+/+}$), and that MsrA could indeed regulate the production of homocysteine and H_2S under I/R conditions.

Results

MsrA gene deletion accelerates renal functional and morphological impairments after I/R injury

To investigate the functions of MsrA in the susceptibility of the kidney to I/R insult, $MsrA^{+/+}$ and $MsrA^{-/-}$ mice were subjected to bilateral renal ischemia for 30 min. 30 min of bilateral renal ischemia markedly increased the plasma creatinine (PCr) levels in both $MsrA^{+/+}$ and $MsrA^{-/-}$ mice at 4 and 24 h after ischemia, indicating that 30 min of renal ischemia resulted in kidney dysfunction (Fig. 1A). The increase of PCr levels in the $MsrA^{-/-}$ mice was more profound than in the $MsrA^{+/+}$ mice (Fig. 1A). Consistent with renal functional impairment, I/R resulted in severe loss of the brush borders of kidney tubular epithelial cells, luminal congestion, and atrophy and dilation of the tubules in the outer medulla of the kidney (Fig. 1B). These post-ischemic histological changes were more severe in the $MsrA^{-/-}$ mice than in the $MsrA^{+/+}$ mice (Fig. 1B). In accordance with PCr concentrations, histological damage scores were higher in the $MsrA^{-/-}$ mice than in the $MsrA^{+/+}$ mice (Fig. 1C). These results indicate that the deletion of MsrA gene accelerates renal injury after I/R insult.

MsrA gene deletion enhances post-ischemic kidney congestion and inflammatory responses

The severe congestion in the outer medulla was noted in the hemi-sect kidney at 24 h after ischemia (Fig. 2A). The congestion in the $MsrA^{-/-}$ mouse kidney was significantly greater than in the $MsrA^{+/+}$ mouse kidney (Fig. 2A). The infiltration of leukocytes has been implicated in the cell damage occurring pursuant to I/R insult. Expression levels of lymphocyte antigen 6 complex (Ly6G), an index leukocyte, were significantly higher in $MsrA^{-/-}$ than in $MsrA^{+/+}$ (Fig. 2B). Microscopic examination revealed much greater accumulation of leukocytes in the interstitium in the $MsrA^{-/-}$ mice than in the $MsrA^{+/+}$ mice (Fig. 1B). The data suggest that MsrA

FIG. 1. Concentration of plasma creatinine (A) and histological damage (B, C) after I/R. $MsrA^{+/+}$ and $MsrA^{-/-}$ male mice were subjected to either 30 min of bilateral renal ischemia (Ischemia) or sham-operation (Sham). (A) Concentrations of plasma creatinine were determined at 4 and 24 h after the surgeries ($n=6$). (B, C) Kidneys were harvested at 24 h after the surgeries. Kidney sections were prepared at a $2\ \mu m$ thickness and stained with periodic acid Schiff (B), and tubular damage was scored as described in the Materials and Methods section. Damaged and congested tubules are indicated by "*" and "c", respectively. Four kidneys in each experimental animal group were used ($n=4$). Results are expressed as means $\pm$ SE. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

FIG. 2. Congestion and Ly6G expression in kidneys. *MsrA*^{+/+} and *MsrA*^{-/-} male mice were subjected to either 30 min of bilateral renal ischemia (Ischemia) or sham-operation (Sham). Kidneys were harvested at 24 h after the surgeries. (A) Kidneys were hemi-sectioned and the image was taken. (B) Ly6G expression was determined by Western blot analysis using anti-Ly6G antibody; β -actin was used as a loading control. The densities of blots were quantified using the Lab Works program ($n=4$). Results are expressed as means $\pm$ SE. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

gene deletion induces enhanced post-ischemic congestion and inflammation in the kidney.

MsrA gene deletion augments kidney oxidative stress after I/R injury

Since MsrA functions as an antioxidant enzyme, we investigated its protective role against I/R-induced oxidative stress. 30 min of bilateral renal ischemia, followed by 24 h reperfusion, significantly increased kidney tissue H_2O_2 levels (Fig. 3A). The H_2O_2 levels were significantly higher in the I/R-subjected *MsrA*^{-/-} mouse kidney than in the I/R-subjected *MsrA*^{+/+} mouse kidney (Fig. 3A). The control levels of H_2O_2 , when sham operation was performed, were not significantly different between the *MsrA*^{+/+} and *MsrA*^{-/-} kidneys (Fig. 3A). The levels of protein-carbonyl content were significantly higher in the sham-operated *MsrA*^{-/-} kidney than in the *MsrA*^{+/+} kidney (Fig. 3B), thereby indicating that MsrA deletion resulted in increased basal levels of protein oxidation. I/R increased the protein-carbonyl content in the mouse kidneys (Fig. 3B). A significantly higher increase in the protein-carbonyl content was observed in the kidneys of *MsrA*^{-/-} mice after I/R injury, compared with the *MsrA*^{+/+} mice (Fig. 3B). The levels of lipid peroxidation were also significantly greater in the *MsrA*^{-/-} mouse kidney after I/R than in the *MsrA*^{+/+} mouse kidney (Fig. 3C), while no significant differences between the sham-operated *MsrA*^{+/+} and *MsrA*^{-/-} kidneys were detected. These results indicate that the increased susceptibility of *MsrA*^{-/-} mice to I/R injury is associated with enhanced oxidative stress.

I/R reduces the expression and activity of Msrs in the kidney

Next, we attempted to determine whether I/R affected the activity and expression of Msrs. We first determined the activity and expression levels of MsrA and MsrB1 in the kidneys. It should be noted that among the three Msrs, selenoenzyme MsrB1 is the main MsrB in the kidney (10). The MsrA protein level in the *MsrA*^{+/+} kidney declined significantly at 24 h after ischemia (Fig. 4A). Consistent with the reduced MsrA protein levels, its mRNA levels were reduced

by 30% (Fig. 4B). In accordance with the reduced MsrA expression in association with I/R injury, the MsrA activity was reduced by 30% after I/R (Fig. 4C). In the case of MsrB1, I/R did not alter both the protein and mRNA levels of MsrB1 in the *MsrA*^{+/+} kidney (Fig. 4A and B). However, MsrB activity was slightly but significantly reduced in the I/R-subjected *MsrA*^{+/+} mouse kidney (Fig. 4D). The expression levels of MsrB2 and MsrB3 were not altered after I/R in the *MsrA*^{+/+} kidney (data not shown). MsrB activity in the I/R-subjected *MsrA*^{-/-} mouse kidney decreased markedly when compared with that measured in the I/R-subjected *MsrA*^{+/+} kidney (Fig. 4D). Consistent with the data of MsrB enzyme activity, Western blot and RT-PCR analyses showed 60% and 20% reductions in MsrB1 protein and mRNA levels, respectively, in the *MsrA*^{-/-} kidney after I/R (Fig. 4A and B). Taken together, the data show that I/R reduces MsrA expression and activity in the *MsrA*^{+/+} kidney, and that it reduces MsrB1 expression and activity more profoundly in the *MsrA*^{-/-} kidney than in the *MsrA*^{+/+} kidney.

MsrA gene deletion accelerates the decrease of hydrogen sulfide after I/R injury

H_2S is generated during the metabolism of homocysteine, a metabolite of methionine. Since MsrA may affect methionine metabolism, we investigated H_2S production under sham and I/R conditions. Plasma H_2S levels were lower in the *MsrA*^{-/-} mice than in the *MsrA*^{+/+} mice at 24 h after either sham operation or I/R (Fig. 5A). Although no significant differences in the H_2S levels of kidney tissue were noted between the sham-operated *MsrA*^{-/-} and *MsrA*^{+/+} mice (Fig. 5B), I/R induced reductions in H_2S to a significantly greater degree in the kidneys of *MsrA*^{-/-} mice as compared to *MsrA*^{+/+} mice (Fig. 5B).

No significant difference in CBS expression was observed between the *MsrA*^{-/-} and *MsrA*^{+/+} mice with sham operation; however, interestingly, the post-I/R decrease in expression was significantly more profound in the *MsrA*^{-/-} mice than in the *MsrA*^{+/+} mice (Fig. 6A). Consistent with its similar expression levels after sham operation, the CBS activity of the *MsrA*^{+/+} and *MsrA*^{-/-} kidneys did not differ (Fig. 6B). However, 24 h after I/R, CBS activity declined

FIG. 3. Levels of H₂O₂, protein-carbonyl content, and lipid peroxidation in kidney. *MsrA*^{+/+} and *MsrA*^{-/-} male mice were subjected to either 30 min of bilateral renal ischemia (Ischemia) or sham-operation (Sham). Kidneys were harvested at 24 h after the surgeries. Levels of H₂O₂ (A), protein-carbonyl content (B), and lipid peroxidation (C) were determined as described in the Materials and Methods section. Results are expressed as means $\pm$ SE ($n=4$).

significantly in the kidneys of both *MsrA*^{+/+} and *MsrA*^{-/-} mice. As expected, the decline of CBS activity in the *MsrA*^{-/-} mouse was much greater than that in the *MsrA*^{+/+} mouse (Fig. 6B).

The levels of CSE expression did not differ between the kidneys of the *MsrA*^{-/-} and *MsrA*^{+/+} mice after sham operation (Fig. 6A). In contrast, 24 h after ischemia, the CSE expression levels were greatly reduced in both the *MsrA*^{-/-} and *MsrA*^{+/+} kidneys. Furthermore, the CSE expression after I/R was significantly lower in the *MsrA*^{-/-} mice than in the *MsrA*^{+/+} mice (Fig. 6A). Consistent with the reduced CSE expression, activity was also reduced in the kidneys of both the *MsrA*^{-/-} and *MsrA*^{+/+} mice after ischemia (Fig. 6C). As expected, the reduction in CSE activity was greater in the *MsrA*^{-/-} mice than in the *MsrA*^{+/+} mice (Fig. 6C). Taken together, the data indicate that MsrA regulates H₂S production during I/R by modulating the expression and activity of the CBS and CSE enzymes.

MsrA gene deletion reduces homocysteine levels

Finally, to determine whether MsrA gene deletion affects methionine metabolism, we measured the levels of homocysteine, a product of methionine metabolism and a substrate of CBS for H₂S generation. The basal level of homocysteine in the plasma of *MsrA*^{-/-} mice was significantly lower than in that of *MsrA*^{+/+} mice (Fig. 7A). After ischemia, plasma homocysteine level declined significantly in both types of mice. Much lower levels of plasma homocysteine were observed in the I/R-subjected *MsrA*^{-/-} mice than in the I/R-subjected *MsrA*^{+/+} mice (Fig. 7A). In the kidneys, a reduction in basal homocysteine level was observed in the *MsrA*^{-/-} mice relative to the *MsrA*^{+/+} mice, but this reduction was not significant (Fig. 7B). I/R reduced kidney homocysteine levels in both the *MsrA*^{+/+} and *MsrA*^{-/-} mice, but a significant reduction was observed only in the *MsrA*^{-/-} mice. Collectively, these data indicate that MsrA modulates homocysteine production under normal conditions, and this effect can be augmented by kidney I/R.

Discussion

Herein, we report the link between MsrA and kidney I/R injury for the first time. Additionally, this study provides the first evidence that MsrA regulates the production of H₂S, which is highly implicated in I/R injury, under *in vivo* conditions. The deletion of the *MsrA* gene exacerbated kidney I/R injury and inhibited H₂S production under I/R conditions by downregulating the expression and activity of CBS and CSE and the substrate homocysteine.

Considering that ROS play a causal role in I/R injury, it is expected that the antioxidant enzyme MsrA may protect against such injury. A recent study by Zhao *et al.* reported that MsrA overexpression confers resistance to I/R injury in the heart (51). Prentice *et al.* reported that MsrA overexpression protects cardiac myocytes against hypoxia/reoxygenation (38). The data reported herein clearly demonstrate the protective role of MsrA against kidney I/R injury.

Under normal conditions, H₂O₂ and lipid peroxidation levels in the *MsrA*^{-/-} kidney did not differ significantly compared to those observed in *MsrA*^{+/+} mice. Additionally, no significant functional and histological impairments were noted in sham-operated *MsrA*^{-/-} mice. In contrast, under

FIG. 4. Expression and activities of MsrA and MsrB1 in kidneys. *MsrA*^{+/+} and *MsrA*^{-/-} male mice were subjected to either 30 min of bilateral renal ischemia (Ischemia) or sham-operation (Sham). Kidneys were harvested 24 h after the surgeries. (A) The protein levels of MsrA and MsrB1 were analyzed via Western blotting (upper panels); β -actin expression was used as a loading control. Protein size is indicated on the left of the figure. The densities of blots were quantified using the ImageJ program (lower panels). (B) The mRNA levels of MsrA and MsrB1 were analyzed by RT-PCR. β -actin was used to normalize the amount of RNA in each sample. DNA size is indicated on the left of the figure. The densities of bands were quantified using the ImageJ program. (C, D) MsrA and MsrB activities were determined as described in the Materials and Methods section. Results are expressed as means $\pm$ SE ($n=4$). ND, not detectable.

pathological I/R conditions, the *MsrA*^{-/-} mouse kidney, with more severe functional and histological impairments, evidenced greater levels of H₂O₂ and lipid peroxidation than were seen in the *MsrA*^{+/+} mice kidney. Protein-carbonyl content is generally employed as a marker for protein oxidation and oxidative stress. The accumulation of protein-carbonyl is positively correlated with the methionine oxidation content (27). In this study, the basal content of carbonylated proteins of the normal kidneys was significantly greater in the *MsrA*^{-/-} mice than in the *MsrA*^{+/+} mice, which indicates that MsrA carries out an antioxidant defense function even under normal conditions. Moreover, after I/R, like H₂O₂ and lipid

peroxidation, the content of protein-carbonyl in the *MsrA*^{-/-} mice kidneys was also significantly higher than in the *MsrA*^{+/+} mice. Taken together, the data indicate that MsrA is highly important for defense against pathologically induced oxidative stresses such as I/R injury. Notably, the I/R-induced increase in protein carbonylation was similar between the *MsrA*^{+/+} and *MsrA*^{-/-} mice. Since proteins in the *MsrA*^{-/-} mice have already been oxidatively damaged to a significant degree in the resting state, we speculate that additional oxidative stress induced by I/R may be somewhat less compelling carbonylation of protein in the *MsrA*^{-/-} mice.

FIG. 5. Levels of H₂S in plasma (A) and kidneys (B). *MsrA*^{+/+} and *MsrA*^{-/-} male mice were subjected to either 30 min of bilateral renal ischemia or sham operation. Samples of kidney and plasma were collected at 24 h after the surgeries, and H₂S levels in those samples were assessed. Results are expressed as means $\pm$ SE ($n=10$).

FIG. 6. Expression and activities of CBS and CSE. *MsrA*^{+/+} and *MsrA*^{-/-} male mice were subjected to either 30 min of bilateral renal ischemia (Ischemia) or sham-operation (Sham). Kidneys were harvested 24 h after the surgeries. **(A)** Levels of CBS and CSE expression were determined via Western blot analysis using anti-CBS or -CSE antibodies in the kidney; β -actin expression was determined as an equal loading marker. Protein size is indicated on the left of the figure. The densities of blots were quantified using the Lab Works program. Results are expressed as means $\pm$ SE ($n=4$). **(B, C)** Activities of CBS (**B**) and CSE (**C**) for H₂S generation were determined as described in the Materials and Methods section. Results are expressed as means $\pm$ SE ($n=4$).

In wild-type mice, I/R attenuated the expression and activity of MsrA, but did not reduce the expression of MsrB1, which is a main enzyme of the MsrB family. Although the expression level of MsrB1 was not altered by I/R in the kidneys of *MsrA*^{+/+} mice, a significant reduction in MsrB activity was noted. The reduced MsrB activity may be due to the inactivation of the MsrB1 protein resulting from the increased oxidation status induced by I/R injury. In contrast, in the *MsrA*^{-/-} mice, I/R markedly reduced MsrB1 expression and MsrB activity. It remains unclear as to why the effect of I/R on the expression of MsrB1 is so dependent on the MsrA protein. Since MsrB1 functions as an antioxidant, the substantial I/R-induced reductions in its expression and activity in the *MsrA*^{-/-} mice can exacerbate the oxidative damage.

H₂S has been recognized as a potent antioxidant, anti-inflammatory, and physiological gaseous molecule (1, 4, 42, 48). Recent evidence from human and animal studies has demonstrated that H₂S is directly involved in various dis-

eases, including hypertension, atherosclerosis, and end-stage renal disease (42). The cytoprotective effects of H₂S have been reported in a variety of organs, including the heart, brain, liver, and kidney, against I/R injury (5, 37, 47). In this study, we determined that the deletion of MsrA induced a reduction in the levels of plasma H₂S and accelerated the post-I/R decrease of H₂S in plasma and kidney tissue. Consistent with the reduced H₂S levels, homocysteine levels were lower in *MsrA*^{-/-} than in *MsrA*^{+/+} mice. Recently, Bearden *et al.* reported that extracellular trans-sulfuration from homocysteine to H₂S protects the endothelium against redox stress (2). Our data suggest that MsrA deficiency alters methionine metabolism and results in reduced homocysteine levels, leading to a sequential reduction in H₂S production. Therefore, the reduced production of H₂S in *MsrA*^{-/-} mice may increase the sensitivity of the kidney to I/R.

Homocysteine is an intermediate amino acid formed during the metabolism of methionine to cysteine. It can be

FIG. 7. Levels of homocysteine in plasma and kidney. *MsrA*^{+/+} and *MsrA*^{-/-} male mice were subjected to either 30 min of bilateral renal ischemia (Ischemia) or sham-operation (Sham). Plasma (A) and kidney (B) were collected 24 h after the surgeries. Levels of homocysteine in the plasma and kidney were determined as described in the Materials and Methods section. Results are expressed as means $\pm$ SE ($n=6$).

converted to methionine, but is largely metabolized to cysteine (about 78% of homocysteine) in the proximal tubule of the kidney by the sequential reactions of CBS and CSE in the trans-sulfuration pathway (10). CBS and CSE generate H₂S via a multitude of reactions and substrate combinations. Recent studies have demonstrated that deficiencies in the CBS and CSE enzymes increase the susceptibility of organs to I/R injury, whereas the overexpression of those enzymes reduces it (4, 18). Wu *et al.* reported that renal I/R inhibits CBS gene expression mediated by a transcription factor Sp1, leading to the accumulation of homocysteine and the reduction of H₂S in the rat kidney (46). Previous studies reported that I/R reduces CBS and CSE activities and H₂S production, leading to increased kidney damage and oxidative stress (37, 47). In the heart, Calvert *et al.* reported that CSE gene-transfer and exogenous H₂S treatment protected the heart against I/R injury by attenuating oxidative stress (5).

In this study, I/R reduced homocysteine levels in the plasma and, furthermore, *MsrA* gene deletion exacerbated

these reductions. The homocysteine level in the kidney did not change in the *MsrA*^{+/+} mouse after I/R injury, but was significantly reduced in the *MsrA*^{-/-} mice. Additionally, both CBS and CSE activities were significantly reduced after I/R, and the degree of reduction was greater in the *MsrA*^{-/-} mice than in the *MsrA*^{+/+} mice. Consistent with the reduced CBS and CSE activities, the expression levels of those enzymes were significantly reduced after I/R. Also, the degree of reduction was greater in the I/R-subjected *MsrA*^{-/-} mice than in the I/R-subjected *MsrA*^{+/+} mice. From our results, we speculate that *MsrA* gene deletion inhibits methionine metabolism to homocysteine by restricting methionine availability, as judged from the reduced homocysteine levels, and this reduction in homocysteine, a substrate of CBS, results in an additional reduction in H₂S production. Furthermore, more profound reductions in the activity and expression of both CBS and CSE in the I/R-subjected *MsrA*^{-/-} mouse kidney may induce a further reduction of H₂S, leading to greater oxidative stress, inflammatory responses, and kidney injury.

Kidney oxidative stress induces tissue damage and also accelerates inflammatory responses, including neutrophil accumulation into the kidney tissue, which triggers ROS production, thereby leading to further tissue and cell damage (15, 17, 36). Inflammatory responses perform an important role in kidney I/R injury. During I/R injury, leukocytes adhere to endothelial cells and infiltrate into the tissues. Accumulating evidence has demonstrated that the inhibition of leukocytic adhesion to endothelial cells protects the kidneys against I/R injury (12, 35, 36, 40). Many studies have demonstrated that H₂S inhibits the production of inflammatory cytokines as well as leukocyte-endothelial interactions (20, 31, 49, 50). Yang *et al.* reported that the reduction of H₂S induced by CSE deletion diminishes endothelium-dependent vasodilation (48). In this study, we determined that I/R increases kidney congestion and leukocyte infiltration, and those changes were enhanced by *MsrA* deletion. Therefore, it can be speculated that the higher kidney congestion and leukocyte infiltration noted in *MsrA*^{-/-} I/R mice may be associated with the attenuation of vasodilation in the renal vessels and with increased inflammatory responses, both of which result from the reduction of H₂S.

Materials and Methods

Animal preparation

All experiments were conducted using 8- to 10-week-old *MsrA* gene-deleted (*MsrA*^{-/-}) or wild-type (*MsrA*^{+/+}) male mice weighing 20 to 25 g. *MsrA*^{-/-} and *MsrA*^{+/+} mice used in this study were generated by breeding heterozygous *MsrA* knockout mice (23) that were of mixed background of 129/SvJ and C57BL/6. This study was conducted wholly in accordance with the guidelines of the Institutional Animal Care and Use Committee of Kyungpook National University and of Yeungnam University. Kidney ischemia was induced as described previously (35).

Renal functional parameter

PCr concentrations were determined using a Beckman Analyzer II, as described previously (34).

Histology

Paraffin sections of kidneys were stained with PAS to evaluate histological changes. Kidney tubular damage level was scored described previously (35).

MsrA and MsrB activity

The reaction mixture (100 μ l) contained 50 mM sodium phosphate (pH 7.5), 50 mM NaCl, 20 mM dithiothreitol, 200 μ M dabsylated methionine-S-sulfoxide (for MsrA) or methionine-R-sulfoxide (for MsrB), and 200 μ g crude protein. The reaction was conducted at 37°C for 30 min, and the reaction product, dabsyl-Met, was analyzed via HPLC.

H₂S concentration in the plasma and kidney

Fifty μ l of plasma was mixed with 187.5 μ l of 1% zinc acetate and 12.5 μ l of 12% NaOH solution and then incubated for 10 min at 37°C. 1.75 ml of distilled water, 0.2 ml of 20 mM *N,N*-dimethyl-*p*-phenylenediamine dihydrochloride in 7.2 M HCl, and 0.2 ml of 30 mM FeCl₃ in 1.2 M HCl were added and incubated for 10 min in a dark box at room temperature. Optical density at 670 nm was measured using a spectrophotometer (Ultraspec2100, Tecan). The H₂S concentration was calculated against the standard curve of NaHS solution.

For the kidney, kidney tissues were homogenized in ice-cold 100 mM potassium phosphate buffer (pH 7.4) with protease inhibitors. 100 μ l of homogenate was mixed with 100 μ l of 1% zinc acetate, 100 μ l of borate buffer (pH 10.0), 200 μ l of 20 mM *N,N*-dimethyl-*p*-phenylenediamine dihydrochloride in 7.2 M HCl, and 200 μ l of 30 mM FeCl₃ in 1.2 M HCl and incubated for 15 min at 37°C. The sample was centrifuged for 5 min at 10,000 rpm at 4°C. The supernatant was taken and optical density was measured at 670 nm.

CBS and CSE enzyme activity

The kidney homogenate was prepared as described above. CBS and CSE activities were determined according to the methods described previously (6, 47). 120 μ l of homogenate was mixed with 20 μ l of 10 mM L-cysteine, 20 μ l of 10 mM DL-homocysteine, 20 μ l of 0.05 mM S-adenosylmethionine, and 20 μ l of 2 mM pyridoxal 5'-phosphate and incubated for 90 min at 37°C. Then, 100 μ l of 1% zinc acetate, 100 μ l of 10% trichloroacetic acid, 350 μ l of 40 mM *N,N*-dimethyl-*p*-phenylenediamine dihydrochloride and 350 μ l of 60 mM FeCl₃ were added and incubated for an additional 15 min at 37°C. The reaction mixture was centrifuged for 5 min at 10,000 rpm at 4°C and the optical density at 670 nm was measured. For the CSE activity assay, 20 μ l of 10 mM L-cysteine and 20 μ l of 2 mM pyridoxal 5'-phosphate were used for H₂S production.

Homocysteine measurements

Homocysteine levels in the plasma were measured using a homocysteine ELISA kit (MyBiosource) according to the manufacturer's protocols. For the kidney, kidney was smashed in 1 ml PBS, spun down at 5000 rpm for 1 min to wash, and then the pellets were resuspended in 1 ml of distilled water. The resuspended kidney was sonicated with an ultrasonic processor (Sonic and Materials Inc., Newton, CT). After centrifugation, 100 μ l of the supernatant was used for homocysteine measurement using the homocysteine ELISA kit.

Lipid peroxidation, hydrogen peroxide, and protein-carbonyl content

Thiobarbituric acid-reactive substances (TBARS; Sigma-Aldrich) were used to measure lipid peroxidation (17). H₂O₂ levels were measured using the ferric sensitive dye, xylenol orange, as previously described (17). Protein-carbonyl content was measured using an OxyBlot protein oxidation detection kit (Chemicon International) according to the manufacturer's recommendations.

Western blot analysis

Western blot analysis was conducted as described previously (35). Polyclonal rabbit anti-mouse MsrA antibodies were generated. Polyclonal anti-mouse MsrB1 were described elsewhere (8). Other antibodies used were: Ly6G (Sigma-Aldrich), β -actin (Sigma-Aldrich), CBS (Santa Cruz), and CBE (Santa Cruz).

Analysis of MsrA and MsrB1 mRNA levels by reverse transcription-polymerase chain reaction

Total RNA from chopped kidneys was extracted with TRI-Solution (Bioscience). The isolated RNA was reverse-transcribed, followed by a PCR reaction to amplify MsrA or MsrB1 genes. β -actin was used to normalize the amount of RNA in each sample. The primers used were: 5'-GAACCTTCCCCAGAGGGAACAC-3' and 5'-CTCAGGTACTGCTGGTGGTAGTC-3' for MsrA, 5'-GCCAGGTGTCTACGTGTGTG-3' and 5'-TTCTTTGCCTTTAGGGACGA-3' for MsrB1, and 5'-GAGACCTTCAACACCCACAGC-3' and 5'-CACATCTGCTGGAAGGTGGAC-3' for β -actin.

Statistical analysis

The results were expressed as means $\pm$ S.E. Statistical differences among groups were calculated using analysis of variance (ANOVA) followed by the least significant difference (LSD) comparisons using SPSS 12.0. A *p* value of <0.05 was considered significant.

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Author Disclosure Statement

No competing financial interests exist

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Address correspondence to:

Prof. Kwon Moo Park

Department of Anatomy

Kyungpook National University School of Medicine

101 Dongdong-2ga

Junggu

Daegu 700-422

Republic of Korea

E-mail: kmpark@knu.ac.kr

Prof. Hwa-Young Kim

Department of Biochemistry and Molecular Biology

Yeungnam University College of Medicine

170 Hyeonchungno

Namgu

Daegu 705-717

Republic of Korea

E-mail: hykim@ynu.ac.kr

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Abbreviations Used

CBS = cystathionine-β-synthase

CSE = cystathionine-γ-lyase

H₂O₂ = hydrogen peroxide

H₂S = hydrogen sulfide

I/R = ischemia/reperfusion

Ly6G = lymphocyte antigen 6 complex

MDA = malondialdehyde

Msr = methionine sulfoxide reductase

Msr +/- = methionine sulfoxide reductase gene wild-type

Msr -/- = methionine sulfoxide reductase gene knock-out

MsrA = methionine sulfoxide reductase A

MsrB = methionine sulfoxide reductase B

PAS = periodic acid Schiff

PBS = phosphate buffered saline

PCr = plasma creatinine

ROS = reactive oxygen species

RT-PCR = reverse transcription-polymerase chain reaction

TBARS = thiobarbituric acid-reactive substances