Hydrogen sulfide
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CHAPTER

CYSTATHIONINE γ-LYASE PROTECTS AGAINST RENAL ISCHEMIA-REPERFUSION BY MODULATING OXIDATIVE STRESS

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CHAPTER 4

ABSTRACT

Hydrogen sulfide (H₂S) is now recognized as the third endogenous gasotransmitter, with similar physiological functions to nitric oxide and carbon monoxide. In this study, we investigated whether endogenous production of H₂S by cystathionine γ-lyase (CSE) has protective effects – similar to treatment with exogenous H₂S – in a renal ischemia setting. We found that CSE is abundantly expressed in the kidney, while CSE knockout animals had markedly reduced renal production of H₂S. CSE deficiency was associated with increased damage and mortality after renal ischemia/reperfusion injury (IRI). Treatment with exogenous H₂S rescued CSE knockout mice from mortality and injury associated with renal ischemia. In addition, overexpression of CSE in vitro reduced the amount of reactive oxygen species produced during stress. Finally, CSE expression in renal transplant donors was positively associated with superior outcome after transplantation. These results indicate a role for CSE as a modulator of oxidative stress through the production of H₂S.
INTRODUCTION
In recent years, the fundamental physiological role of hydrogen sulfide (H₂S) has gradually been uncovered. H₂S is now acknowledged as the third endogenously produced gaseous signaling molecule, in addition to nitric oxide (NO) and carbon monoxide (CO)¹. H₂S is generated from the amino acid L-cysteine by three distinct enzymes: cystathionine γ-lyase (CSE), cystathionine β-synthase (CBS) and 3-mercaptopyruvate sulfurtransferase (MPST)². In the cardiovascular system, CSE is the most abundantly expressed protein, and is responsible for the majority of endogenous H₂S production³. The physiologic properties of endogenously produced H₂S are remarkably similar among the different gasotransmitters. H₂S functions as an endothelial-cell derived relaxing factor (EDRF) similar to NO⁴. Accordingly CSE deficient mice (CSE⁻/⁻) develop hypertension⁵, analogous to mice lacking endothelial nitric oxide synthase (eNOS)⁶ and CBS⁺/⁻ mice⁷. In addition, endogenously produced H₂S is involved in cellular proliferation⁷, angiogenesis⁸, inflammation⁹,¹⁰ and regulation of protein activity through S-sulfhydration¹¹-¹³.

Exogenous treatment with H₂S can induce a reversible hypometabolic, hibernation-like state¹⁴. The proposed mechanism behind H₂S-induced hypometabolism is through the reduction of mitochondrial activity by reversible binding to cytochrome c oxidase, the terminal enzyme in the electron transport chain¹⁵. During hypoxia, H₂S treatment ameliorates the reduction in the function and integrity of mitochondria¹⁶-¹⁸. The lower demand for oxygen during hypometabolism can protect animals from hypoxia or shock, and organs from ischemia/reperfusion injury¹⁶,¹⁷,¹⁹,²⁰. Exogenous H₂S can play a detoxifying role during oxidative stress by directly scavenging reactive oxygen/nitrogen species (ROS/RNS) as well as increasing the formation of the antioxidant glutathione (GSH)²¹,²².

In this study we found that CSE deficiency in mice led to reduced renal H₂S production and was associated with increased mortality and severity of damage after renal ischemia/reperfusion injury. Administering exogenous H₂S rescued these mice from mortality and injury associated with renal ischemia. Our in vivo experiments together with in vitro cell studies in which CSE is overexpressed highlight the fundamental role CSE has in regulating the amount of ROS associated with hypoxic stress. In addition, we notably found that CSE mRNA and protein levels were shown to be associated with kidney outcome after transplantation. Together, our data reveal CSE as a modulator of oxidative stress induced following renal ischemia.

RESULTS
Localization of CSE in control human renal tissue
In human renal tissue, CSE protein was localized to glomeruli and tubulo-interstitium. Glomeruli were homogenously positive for CSE. Non-endothelial (CD31-negative) glomerular cells showed positive staining in addition to (CD31-positive) endothelial cells, indicating expression in mesangial cells and/or podocytes. (Figure 1A). CSE was seen in tubular epithelium – proximal as well as distal (Figure 1B), peritubular capillaries
CHAPTER 4

(Figure 1C), and vascular endothelium (Figure 1D). The protein was observed in an unidentified intracellular staining pattern, showing a distinct line-like staining in the cytosolic compartments of tubules and non-glomerular endothelial cells (Figure 1B). TissueFAXS analysis showed that 75% of renal cells were positive for CSE (Figure 1E). Moreover, when we investigated endothelial cells, we found that 87% of endothelium was positive for CSE (Figure 1F). Transplant biopsies showed similar localization and staining pattern as the control renal tissue (data not shown).

CSE mRNA levels are modulated after renal ischemia in rats

The expression of CSE mRNA was modulated after ischemia in control rat kidneys. Early time points show an increase in renal CSE mRNA (90 minutes), after which expression decreased to significantly lower levels compared to basal levels (Figure 2A). At 1, 2 and 4 days after ischemia mRNA levels were decreased after which mRNA levels normalized to basal values after 9 days and afterwards (Figure 2A). The expression of CBS was modulated in a temporally similar pattern (Figure 2B).

CSE but not CBS mRNA expression levels were altered during the transplant process

CSE mRNA levels were significantly increased after reperfusion (p<0.01) (Figure 2C). Expression levels were (Median [range]): procurement 0.67 [0.03-1.66], pre-implantation 0.41 [0.08-1.75], reperfusion 0.93 [0.15-3.65]. CBS mRNA levels were not modulated during the transplant process (Figure 2D): Procurement 0.20 [0.001-1.99], pre-implantation 0.23 [0.007-1.09], reperfusion 0.20 [0.02-0.86].

Modulation of CSE protein levels during the transplant process

CSE immunofluorescence was performed on a limited number of renal transplant biopsies (n=27). No differences in protein levels were detected between pre-implantation and reperfusion time points (Figure 2E).

CSE deficient mice have reduced renal H\textsubscript{2}S production

Kidneys from untreated wildtype (WT) and CSE knockout (CSE\textsuperscript{-/-}) animals were examined to confirm that CSE deficiency caused reduced production of H\textsubscript{2}S in renal tissue. Renal weight did not significantly differ between WT and CSE\textsuperscript{-/-} mice (data not shown). CSE\textsuperscript{-/-} mice had a 91% reduction in renal H\textsubscript{2}S production compared to WT mice (p<0.05, Figure 3A).

Increased mortality after bilateral renal ischemia in CSE deficient mice

Mortality after IRI was significantly higher in the CSE\textsuperscript{-/-} animals, where 35% (5/14) of animals died within the first 24 hours, while WT animals showed 0% (0/14) mortality during this
Figure 1 - Localization of CSE in control human renal tissue. Representative examples of the localization of CSE (green) and the colocalization with CD31 positive cells (red). CSE is localized in (A) the glomerulus (endothelial and mesangial cells), (B) tubules, (C) peritubular capillaries (colocalization of CSE with CD31 is seen as yellow in the merged images, and is marked by white arrowheads. Image is a magnification from (B)) and (D) vascular endothelial cells (white arrowheads). In (D), aspecific autofluorescence of the elastic laminae is demarcated in dashed lines. CSE is expressed in proximal and distal renal tubules (A,B,C) in an unidentified subcellular staining pattern. Original magnification: 630X. (E) TissueFAXS analysis of the total amount of CSE-expressing cells in the kidney of six control kidneys, indicating that 75% of renal cells express CSE. (F) Of all renal endothelial cells, the large majority express CSE. (G) The variation between control patients in the abundance of CSE and CD31 expressing cells.
Figure 2 – Time-course expression of CSE/CBS after ischemia/reperfusion. Renal expression of (A) CSE and (C) CBS at various time points in the first 21 days after renal ischemia in rats (n=5-6 animals per group). CSE mRNA is increased in the acute stage, and both are reduced in the medium-long term, and normalized after 9 days (* - p<0.05; ** - p<0.01; *** - p<0.001). (B) CSE protein abundance as measured by Western blot, showing significantly reduced CSE protein at 4, 14 and 21 days (* - p<0.05; ** - p<0.01, n=3-5 animals per group). Under each bar representative protein bands, all from the same blot. Expression of CSE mRNA (D) and CBS mRNA (F) in human donor biopsies at different time points during the transplant process. CSE expression at reperfusion is significantly increased compared to pre-implantation (** - p<0.01). (E) No significant difference in CSE protein levels between procurement and reperfusion time points in human renal transplantation was found.
CSE MODULATES OXIDATIVE STRESS

period (p<0.05, Figure 3B). CSE\(^{-}\) animals could be rescued by pretreatment with NaHS, with 11% (1/9) mortality compared to 0% (0/9) in WT animals (p=ns, Figure 3C).

CSE deficient mice display more severe kidney damage and decreased renal function after IRI compared to WT mice

No necrosis was detected in kidneys of sham-operated mice, while all kidneys subjected to IR showed tubular necrosis in the cortico-medullary transition area. CSE\(^{-}\) animals showed 60% higher levels of necrosis compared to WT animals (p<0.01, Figure 3D, representative examples in Figure 3F, representative photomicrographs in Supplementary figure 1). The amount of necrosis was reduced by treatment with NaHS regardless of genotype. Renal function as measured by plasma creatinine levels showed a similar pattern, with a 51% higher level of creatinine in CSE\(^{-}\) animals compared to WT animals after IRI. Pretreatment with NaHS significantly improved renal function in CSE\(^{-}\) animals (p<0.001, Figure 3E). In sham-operated animals creatinine levels were similar, indicating that renal function in untreated CSE\(^{-}\) is not impaired (Figure 3E).

Renal inflammation after renal ischemia/reperfusion

Granulocyte influx as measured by Ly-6G immunohistochemistry showed an increase in granulocytes after ischemia in both WT and CSE\(^{-}\) animals, which was abrogated by NaHS treatment regardless of genotype. There was no difference in the amount of granulocytes between WT and CSE\(^{-}\) animals (Figure 3G).

CSE mice display increased levels of DNA damage after ischemia/reperfusion

In order to determine the extent of oxidative damage after IRI we carried out immunofluorescence staining for yH2AX, a marker for DNA double strand breaks (DSBs) on kidney sections from CSE deficient and WT mice. yH2AX plays an important role in the DNA damage response and is necessary for the initial rapid phase of DSB repair. pyH2AX is a proven marker of oxidative stress\(^2\). We found phosphorylated yH2AX positive cells in the cortical tubular cells of mice after IRI the amount of positive cells was significantly higher in CSE\(^{-}\) mice compared to WT mice (Figure 4A). Pretreatment with NaHS reduced the amount of pyH2AX positive cells in both WT and CSE deficient mice but did not reduce the amount of positive cells to sham injury levels (Figure 4A).

Proliferation after renal ischemia/reperfusion

Ki67 staining indicated that CSE\(^{-}\) animals did not have increased proliferation after IRI (Figure 4B). The amount of Ki67 positive nuclei was significantly increased after NaHS treatment in combination with IRI in CSE\(^{-}\) animals, but not in the WT animals (Figure 4B).
Figure 3 – Renal H$_2$S production, survival, renal function, necrosis and inflammation in CSE$^{-/-}$ mice after ischemia/reperfusion. (A) Renal H$_2$S production rate is significantly lower in CSE$^{-/-}$ mice compared to WT mice (* - p<0.05, n=4 per group). (B) Animal survival after renal IRI is impaired in the CSE$^{-/-}$ animals (p<0.05, n=14 per group). (C) Treatment with NaHS rescues CSE$^{-/-}$ mice from IRI-induced mortality (p=ns, WT: n=7; CSE$^{-/-}$: n=9). (D) The amount of renal cortical necrosis is significantly higher in CSE$^{-/-}$ mice (n=9) compared to WT (n=14), while pretreatment with NaHS reduces necrosis in both WT (n=7) as well as CSE$^{-/-}$ mice (n=8) (** - p<0.01; *** - p<0.001, WT Sham: n=9; CSE$^{-/-}$ Sham: n=8). (E) Plasma creatinine levels indicate reduced renal function after ischemia in CSE$^{-/-}$ (n=9) compared to WT mice (n=14). NaHS treatment protected CSE$^{-/-}$ mice (n=8) from the IRI-induced decline in renal function (* - p<0.05; *** - p<0.001, WT Sham: n=9; CSE$^{-/-}$ Sham: n=8). (F) Representative examples of PAS-stained coronal renal sections from WT and CSE$^{-/-}$ animals with the necrotic area artificially colored red. (G) Influx of Ly-6G-positive granulocytes was not affected by CSE deficiency (CSE$^{-/-}$: n=9, WT: n=14). Granulocyte influx was reduced in both WT (n=7) and CSE$^{-/-}$ animals (n=8) after NaHS pretreatment (** - p<0.01; *** - p<0.001, WT Sham: n=9; CSE$^{-/-}$ Sham: n=8).
Figure 4 – Expression of pyH2AX and Ki67 in WT and CSE−/− animals after ischemia/reperfusion. (A) Abundance of the phosphorylated DNA-repair protein γH2AX is significantly higher in CSE−/− animals after IRI (n=9) compared to WT animals (n=14), indicating an increased amount of DNA double strand breaks, possibly related to oxidative DNA damage. (B) After IRI, the expression of the nuclear proliferation marker Ki67 is not increased in CSE−/− animals (n=9) compared to WT (n=14), but significantly increased after NaHS pretreatment and IRI combined (n=8, WT Sham: n=9; CSE−/− Sham: n=8).

Renin is not differentially expressed in wildtype and CSE−/− mice

Immunohistochemistry for renin showed its expression in the juxtaglomerular cells. When the amount of glomeruli with renin-positive juxtaglomerular cells were counted, no significant differences between the groups were found (Figure 5A). The number of renin-positive juxtaglomerular cells per glomerulus did not differ between groups (Figure 5B). The lack of renal H2S production in CSE−/− mice does not affect renin levels, nor does the treatment of wildtype or CSE−/− animals with NaHS. IRI also does not affect renin levels 24 hours after ischemia.

Cell viability, EGFP fluorescence and CSE protein abundance after transfection

Transfection with the pIRES2-EGFP or pIRES2-EGFP-CSE plasmids did not affect viability of HEK293 cells between 1 and 4 days after transfection (Figure 6A). Both plasmids increased the amount of EGFP fluorescence between 1 and 5 days after transfection, reaching a peak between 3-4 days (Figure 6B). The amount of EGFP fluorescence was not significantly different between the two plasmids. Western blot at 72 hours after transfection showed no effect of mock-transfection with pIRES2-EGFP on CSE protein, while the pIRES2-EGFP-CSE plasmid induced a 6-fold increase in the band density for CSE (Figure 6C). The amount of H2S in the supernatant medium of the pIRES2-EGFP-CSE transfected cells did not significantly differ from the H2S levels in the control and pIRES2-EGFP transfected groups (Figure 6D).
CHAPTER 4

Figure 5 – No difference in renin expression between genotypes or between treatment groups. (A) Percentage of glomeruli which had renin-positive juxtaglomerular cells and (B) amount of renin-positive juxtaglomerular cells per glomerulus in the different groups did not differ. (C) Representative examples of glomeruli with renin-positive juxtaglomerular cells. Original magnification 200x.

Effects of exogenous NaHS and CSE overexpression on mitochondrial and overall superoxide

Antimycin A-induced oxidative stress as measured by DHE fluorescence in HEK293 cells was concentration dependently reduced by treatment with NaHS. Antimycin induced a 48 fold increase in DHE fluorescence which was significantly attenuated by NaHS at 10 μM, 100 μM and 1 mM (Figure 6E). Mitochondrial superoxide production as assessed by MitoSOX fluorescence showed a similar pattern, with a 13.5 fold increase in fluorescence, which was significantly lower when treated with 10 μM, 100 μM or 1 mM NaHS (Figure 6G).

Treatment with Antimycin A showed an increase in DHE (Figure 6F) and MitoSOX (Figure 6H) fluorescence in all groups. Fluorescence intensity was similar in control and pIRES2-EGFP transfected groups, while cells overexpressing CSE showed a significantly reduced amount of fluorescence. The reduction was 60% when measured using DHE (Figure 6F, red bar), and 74% using MitoSOX (Figure 6H, red bar). Treatment
with 1 mM of NaHS reduced the amount of fluorescence of both probes in all groups except the CSE overexpressing cells loaded with MitoSOX (Figure 6H, red bars).

Association of CSE and CBS mRNA expression pre-transplantation and renal function after transplantation
CSE mRNA levels at organ procurement were positively associated with renal function 14 days after transplantation, as measured by glomerular filtration rate (GFR). Linear regression analysis showed that the association significantly deviated from zero \( p=0.007 \), and the goodness of fit \( R^2 \) was 0.3146 (Figure 7A). Relative CBS mRNA levels did not associate with renal function after transplantation, showing no significant deviation from zero \( p=0.16 \) and low goodness of fit with an \( R^2 \) of 0.0262 (Figure 7B).

DISCUSSION
Over the past decades, the perceived image of \( \text{H}_2\text{S} \) has transformed from that of a dangerously toxic molecule to that of an endogenously produced gas affecting many physiological processes. The similarities between \( \text{H}_2\text{S} \) and the other gasotransmitters are numerous\(^{1,2} \). In this study, we show that the endogenous production of \( \text{H}_2\text{S} \) reduces the damage associated with renal ischemia/reperfusion injury. In previous papers, others and we have previously demonstrated the highly protective effects of exogenously applied \( \text{H}_2\text{S} \) treatment in similar models of ischemia\(^{16,17,19,24-27} \). From the present study it now becomes clear that after renal hypoxic stress, CSE functions as an endogenous mediator of the antioxidant response, most likely through the production of \( \text{H}_2\text{S} \).

This study shows that CSE is abundantly present in the normal human kidney, with widespread expression in the tubules. We found that the majority of renal tubules stain positive for CSE, which aligns with a previous study indicating that CSE activity was found in all segments of the renal tubule, with the highest activity in the proximal straight and distal tubules\(^{28} \). The large majority of renal endothelial cells express CSE. In CSE deficient mice, renal \( \text{H}_2\text{S} \) production was reduced by more than 90\%, indicating that CSE is the most essential \( \text{H}_2\text{S} \) producing enzyme in the kidney.

There was a clear association between the absence of CSE expression in the CSE\(^{-/-} \) mice and an increase in renal damage after IRI. Mortality, renal failure and tubular necrosis were significantly increased when renal \( \text{H}_2\text{S} \) production was low. Treatment with exogenous NaHS rescued these animals from death and renal failure. The absence of CSE expression did not influence the inflammatory response. This indicates that endogenous production of \( \text{H}_2\text{S} \) does have the anti-inflammatory effects that the concentrations afforded by exogenous \( \text{H}_2\text{S} \) treatment has in this model\(^{16} \). Others have shown the inhibitory effects of endogenously produced \( \text{H}_2\text{S} \) by CSE on leukocyte adhesion\(^{9} \). We have no indication of the actual plasma concentrations and, perhaps more relevant, the intracellular concentrations caused by CSE/CBS/MPST activity relative to those brought by exogenous \( \text{H}_2\text{S} \). This means that, in this model,
CHAPTER 4

A. Cell viability

B. EGFP Expression

C. CSE protein

D. H₂S level in medium

E. ROS production

F. ROS production

G. Mitochondrial superoxide production

H. Mitochondrial superoxide production
Figure 6 – CSE overexpression in vitro and production of ROS in HEK293 cells. (A) Cell viability was not affected by transfection with pIRES2-EGFP or pIRES2-EGFP-CSE vectors. (B) EGFP fluorescence intensity after transfection, showing peak values between 72-96 hours after transfection with both control and CSE vector. (C) CSE protein as measured by western blot showed an increase of 6x after 72 hours after transfection with pIRES2-EGFP-CSE vector (** - *p<0.001), while there was no increase in CSE expression in the pIRES2-EGFP transfected cells compared to controls (representative bands from the same gel are shown). (D) No differences in supernatant H2S were measured. (E) Antimycin induced cytoplasmatic ROS production as measured by DHE fluorescence was significantly and concentration dependently attenuated by treatment with NaHS (### - *p<0.001 vs. -NaHS, -Antimycin; * - *p<0.05; *** - *p<0.001 vs. -NaHS, +Antimycin). (F) DHE fluorescence is not affected by transfection with the pIRES2-EGFP vector, but significantly reduced by transfection with pIRES2-EGFP-CSE. Treatment with NaHS further reduces DHE fluorescence intensity in all groups (* - p<0.05; *** - *p<0.001). (G) Mitochondrial superoxide production, as measured with the fluorescent MitoSOX probe, is significantly and concentration dependently reduced by NaHS treatment (# - p<0.05; ### - *p<0.001 vs. -NaHS, -Antimycin; ** - p<0.01 vs. -NaHS, +Antimycin). (H) pIRES2-EGFP transfection does not affect MitoSOX fluorescence after antimycin treatment, while pIRES2-EGFP-CSE transfection significantly reduces the amount of mitochondrial superoxide produced (* - p<0.05; *** - *p<0.001). Addition of NaHS did not produce significant additional effects in the pIRES2-EGFP-CSE transfected cells. Data are representative of at least three independent experiments.

Figure 7 – Association between renal CSE/CBS mRNA and outcome after human renal transplantation. (A) Renal CSE mRNA level at organ procurement is associated with the glomerular filtration rate at 14 days after transplantation, with higher expression associating with better renal function (R²=0.3146, p=0.0007). Renal CBS levels at procurement are not associated with outcome after renal transplantation (R²=0.0262, p=ns). Lines denote best fit, with the dashed lines representing the 95% confidence interval for best fit.

The concentrations needed to produce anti-inflammatory effects might be too high to be reached by endogenous H2S production. The known inhibitory effects of H2S on inflammatory processes are likely absent in the CSE−/− mice, so increased influx of leukocytes was expected in this model, especially with the increased renal damage in the CSE−/− mice. The results indicate that CSE deficiency can also influence the inflammatory response to injury through an unknown mechanism.
Excessive generation of ROS following injury damages proteins, DNA, mitochondria and lipids and can stimulate the immune system leading to organ damage. Hydroxyl radicals can also react with nearby tissues, resulting in cellular DNA damage. Phosphorylated γH2AX is a well-known marker of DNA damage and, in particular, of DSBs. Phosphorylated γH2AX is the first step in recruiting and localizing DNA repair proteins. We found more DSBs as detected by γH2AX staining in the kidneys of CSE mice after IRI compared to WT. Pretreatment of NaHS was associated with reduced amounts of DNA damage together with all the other parameters of renal injury. Our present results show severe DNA damage in CSE<sup>-/-</sup> mice was associated with a reduced but not significant number of Ki67 positive cells (hence; cell proliferation) when compared to WT mice.

Proliferation is affected by CSE, as Ki67 immunofluorescence showed. CSE activity or the availability of H<sub>2</sub>S seems to be a necessity for the induction of proliferation in this model, as IRI by itself does not induce Ki67 expression in CSE<sup>-/-</sup> animals, but the combination of IRI and NaHS treatment induces high expression of this protein. Interestingly, treatment of wildtype animals with NaHS during IRI seems to inhibit proliferation, which correlates with the amount of renal cell damage in this experiment, indicating that the protective effects seen in wildtype animals are not due to an increase in regeneration. These results contrast earlier work, where CSE deficiency caused overproliferation in vascular smooth muscle cells.

Since CSE<sup>-/-</sup> animals develop higher blood pressure compared to wildtype after 6 weeks (a rise of about 10 mmHg at 7 weeks), the differences between groups could be related to differences in renal perfusion before or after the ischemic event. To investigate this, we measured renin expression in the juxtaglomerular apparatus (Figure 5 A-C). We found no differences between wildtype and CSE<sup>-/-</sup> mice, nor between IRI and NaHS treated groups. This indicates that there were no large differences in renal perfusion between the groups, and that the increase in renal damage after IRI in the CSE<sup>-/-</sup> animals, nor the protective effects of NaHS can be explained by altered perfusion. Ming et al. recently showed that H<sub>2</sub>S can inhibit renin expression in a 2K1C model, but it did not affect expression in sham or unclipped kidneys. We noted no effect of NaHS on renin expression, suggesting that H<sub>2</sub>S can inhibit an increase in renin levels, but does not affect normal renin levels. However, renin protein levels have to be regarded as a crude marker for renal perfusion, for which changes can only be detected with large perfusion differences.

In vitro experiments showed that exogenous H<sub>2</sub>S treatment concentration-dependently reduced the amount of intracellular ROS production when stimulated with Antimycin. A significant effect was reached at low concentrations. Overexpression of CSE showed a reduction in oxidative stress that was similar to ~10 μM of NaHS. This concentration is close to the most quoted physiological range of 30-300 μM in human serum, but the actual physiological concentration in mammals is still up for discussion. The addition of 1 mM NaHS showed a further reduction in DHE fluorescence, which might indicate that overexpression of CSE did not induce supraphysiological concentrations in this particular model. The fact that we did not find a significant
increase in supernatant H$_2$S concentration after CSE overexpression is not unexpected, since H$_2$S rapidly evaporates from solution at physiological pH$^{34}$ or from serum$^{33}$. Whether H$_2$S directly scavenges ROS, increases the intracellular GSH levels or reduces the amount of ROS produced through modulation of mitochondrial ROS production was not assessed by this study. H$_2$S can exert all these effects$^2$, so the protective and antioxidant effects demonstrated here could be the result of multiple mechanisms acting simultaneously. One previous study assessed the effects of CSE overexpression in a murine model of myocardial infarction. Myocardium-specific overexpression of CSE nearly doubled H$_2$S production and reduces myocardial infarct size by 47%$^{17}$. Other studies have shown the effects of inhibitors of CSE and CBS in ischemic models, but the specificity of these inhibitors is subject to discussion$^{35}$. Most studies using these inhibitors imply protective effects afforded by endogenous H$_2$S$^{25,36-38}$. Interestingly, recent data suggests that under stress CSE can translocate to mitochondria, and H$_2$S produced by mitochondrially located CSE can be used as a substrate for the production of ATP$^{39}$. This might be an additional protective mechanism in the setting of ischemia/hypoxia.

The association between the mRNA expression of CSE and the outcome after human renal transplantation is an indication that CSE could have protective effects in human ischemia/reperfusion injury similar to those we have found in murine models. The observation that CBS is not differentially expressed during the transplant process, nor is associated with outcome after transplantation, indicates a central role for CSE in human renal tissue. We found an increase in mRNA expression of CSE shortly after reperfusion in humans, which was similar to the results from rats. The decrease in CSE expression found at the later time points after reperfusion in the murine model could not be confirmed in the human study due to the absence of biopsies late after reperfusion. The question remains whether this early decrease is associated with a functional mechanism where CSE is downregulated or inhibited during renal repair and regeneration, or the inflammatory response to injury.

Taken together, our data reveal a key role for CSE in the regulation of oxidative stress induced following renal ischemia, most likely through the production of H$_2$S. The modulation of CSE expression and activity may be a therapeutic target in settings of hypoxia.

Table 1 – Characteristics of the human kidney donors analyzed in the different analyses performed in this paper

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
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<td>Sex (Male:Female)</td>
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<tr>
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<tr>
<td>Donor type (Living:Brain dead)</td>
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<td>Delayed graft function</td>
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<td>Rejection (1 year)</td>
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CONCISE MATERIALS AND METHODS

Collection of human material

Human renal transplant biopsies were obtained from brain-dead or living donors (n = 50) at three different time points: just before donation (before start of preservation), at the end of cold ischemia and approximately 45 min after reperfusion in the recipient. Control kidneys were taken from the unaffected part of kidneys from patients undergoing nephrectomy for renal cell carcinoma (n=6).

Animals

CSE deficient mice (CSE-/-) were generated using conventional techniques as described before3. Wildtype C57BL/6J littermates (WT) were used as control animals. Male Wistar rats (250–300 g) were used for time-course experiments.

IRI protocol

Renal ischemia/reperfusion in mice was performed as described previously16. In short, both renal pedicles were clamped for 30 minutes using non-traumatic vascular clamps through a midline abdominal incision under general anesthesia (ketamine/xylazine). Core body temperature was maintained at 37°C in all groups using heat pads and lamps. NaHS injection (1 mg/kg) was given intraperitoneally 15 minutes before clamping. Mice were terminated after 1 day of reperfusion and samples were collected.
Cell culture experiments
To generate CSE overexpressing cells, human embryonic kidney 293 (HEK293) cells were transfected with the pIRES2-EGFP (mock) or pIRES2-EGFP-CSE vector in 24- or 96-well plates. For induction of ROS, Antimycin A (50 μg/mL) with or without NaHS (1-1000 μM) was given for 30 min. Subsequently, cells were incubated with 15 μM dyhidroethidine or 5 μM MitoSOX for 15 minutes.

Renin measurement
Renin immunohistochemistry was performed as described before\textsuperscript{41}. Subsequently, the amount of glomeruli with renin-positive juxtaglomerular cells were counted. In a second analysis, the amount of renin positive cells per glomerulus were counted. For both measurements, the complete cortex was analyzed in a blinded fashion.

TissueFAXS analysis
CSE-Alexa 488 / CD31-TRITC / DAPI triple stained sections were scanned using a fluorescent microscope fitted with an automated acquisition system (TissueFAXS, TissueGnostics GmbH, Vienna, Austria). Percentages of CSE+, CD31+ and CSE+CD31+ cells were measured using TissueQuest software (TissueGnostics), using an algorithm based on the recognition of nuclei and their associated cytoplasm. Ki67 and γH2AX stained sections were quantified in this manner as well.

Statistical analysis
Data were analyzed using Mann-Whitney U tests, One-Way ANOVA or Kruskall Wallis tests where appropriate. Bonferroni or Dunns postcorrection was applied where multiple comparisons were made. Normality was tested using the Kolmogorov–Smirno test. A value of \( p<0.05 \) was considered statistically significant. Data are expressed as the mean ± SEM (Standard Error of the Mean) unless otherwise indicated.

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CSE MODULATES OXIDATIVE STRESS


CHAPTER 4

FULL MATERIALS AND METHODS

Collection of human renal transplant biopsies

Cortical kidney needle biopsy specimens were taken from donors (n=50) during procurement, pre-implantation, and ~45 min after reperfusion. Biopsy specimens were taken using a 16-gauge needle (Acecut, TSK Laboratory, Japan). Biopsies were fixed in 4% formalin for paraffin embedding or snap frozen in liquid nitrogen and stored at ~80°C for mRNA isolation. Some donor kidneys were obtained outside our medical center, which impeded the collection of some biopsies at the procurement time point. Some kidneys were retrieved at our hospital, but allocated outside our medical center, obstructing the collection of some pre-implantation and reperfusion biopsies. Subjects were included when the following criteria were fulfilled: donor and recipient age between 18 and 70 years; cold ischemic time less than 25 hours; anastomosis time less than 60 min; no reclamping after completing the anastomosis; and first kidney recipients.

Collection of control renal tissue

Control human renal tissue used for analysis of normal CSE expression patterns was taken from the unaffected part of kidneys removed for the treatment of renal cell carcinoma (n=6).

RNA isolation and real-time PCR analysis

A nucleosin II RNA isolation kit (Macherey-Nagel GmbH, Germany) was used. RNA was eluted in 20 µl of RNase-free water. RNA concentration was measured using a Nanodrop spectrophotometer. RNA quality was confirmed by agarose gel electrophoresis. For cDNA synthesis, 1 µg of RNA was used and reverse transcription was performed with a Quantitect Reverse Transcription kit (Qiagen GmbH, Germany). Gene expression assays were bought from Applied Biosystems. (Foster City, Ca, USA). For human PCR; CBS: Hs00163925_m1, CSE: Hs00542284_m1. HPRT was custom made, with sequence: F: 5’-GGCAGTATAATCCAAAGATGTCAA-3’; R: 5’-GTCTGGCTTATATCCAACACTCGT-3’. For rat material; CBS: Rn00560948_m1, CSE: Rn00567128_m1. HPRT was custom made, with sequence: F: 5’-GCCCTTGACTATAATGAGCACTTCA-3’; R: 5’-TCTTTTAGGCTTTGTACTTGGCTTTT-3’. Changes in CSE, CBS and HPRT mRNA were confirmed by two-step quantitative RT-PCR on an AB Prism 7900HT Sequence detector (Applied Biosystems). The PCR profile consisted of 15 min at 95°C, followed by 40 cycles with heating of 95°C for 15 s and cooling to 60°C for 1 min. Data were analysed with SDS2.3 (Applied Biosystems). All results were normalized to the expression of HPRT in the sample.

Immunofluorescence

Immunofluorescent labeling was performed as described previously. Briefly, Cryosections (4 µm) were dried and acetone fixed. Primary antibodies were applied for 1 hour, and thereafter incubated with appropriate secondary antibodies for 30 minutes. Slides were
mounted in Aqua/polymount (Polysciences Inc., Warrington, PA, USA) containing DAPI (1.5 μg/ml). The following primary antibodies were used: mouse monoclonal CSE (kindly donated by dr. N. Nishi, Kagawa Medical School, Japan). Mouse monoclonal CD31 (Dako), Rabbit polyclonal Ki67 (Abcam), and mouse monoclonal γH2AX (Millipore). Secondary antibodies include Alexa Fluor 488 rabbit anti-mouse IgG2a (Invitrogen), TRITC-conjugated rabbit anti mouse IgG1 (Southern Biotech), Alexa Fluor 488 goat anti-mouse IgG1 (Invitrogen), Cy3-conjugated goat anti-rabbit IgG (Invitrogen).

**TissueFAXS analysis**
CSE-Alexa 488 / CD31-TRITC / DAPI triple stained sections were scanned using a fluorescent microscope (Zeiss AxioObserver.Z1, Carl Zeiss AG, Oberkochen, Germany) fitted with an automated acquisition system (TissueFAXS, TissueGnostics GmbH, Vienna, Austria). Non-representative images of the section were removed (e.g. folds, edges). Percentages of CSE+, CD31+ and CSE+CD31+ cells were measured using TissueQuest software (TissueGnostics), using an algorithm based on the recognition of nuclei and their associated cytoplasm. In human renal transplant biopsies, intensity of the fluorescence signal was measured in the cytoplasm using the TissueQuest software. In addition, Ki67 and γH2AX stained sections were quantified in this manner. Random backgating analysis was performed in each sample, where negativity, positivity, and double positivity of cells in the scatterplots was confirmed by manual investigation of the associated photomicrograph.

**Ly-6G and renin immunohistochemistry**
Immunohistochemical staining for Ly-6G/C positive granulocytes was performed as described previously\(^\text{15}\). In short, paraffin embedded sections were stained for Ly-6G using rat-anti-mouse Ly6G/C-FITC IgG2b antibody, followed by rabbit-anti-FITC and HRP-conjugated goat-anti-rabbit antibodies. Full slide images were captured at 40x magnification using a Hamamatsu NanoZoomer 2.0HT, and images were analysed using HistoQuest software (TissueGnostics). For renin, we used a polyclonal antibody as described before\(^\text{16,17}\).

**Measurement of necrosis**
Formalin fixed, paraffin embedded coronal renal sections were stained using the Periodic Acid Schiff (PAS) method using standard procedures. Whole slides were digitized using an Aperio ScanScope digital slide scanner (Aperio Technologies inc, Vista, Ca, USA) at 20x magnification and the accompanying Aperio Imagescope software was used to measure the necrotic renal cortical area relative to the total renal cortical area.

**Plasma biochemical analysis**
Creatinine measurements were performed by our hospital clinical chemistry research services using conventional methods.
CHAPTER 4

Renin measurement
Subsequently, the amount of glomeruli with renin-positive juxtaglomerular cells were counted. In a second analysis, the amount of renin positive cells per glomerulus were counted. For both measurements, the complete cortex was scanned in a blinded fashion.

Animals
For renal IRI and H₂S production rate measurement studies, CSE deficient mice (CSE⁺⁻) were generated using conventional techniques as described before⁵. Wildtype C57BL/6J littermates (WT) were used as control animals. Time-course expression of CSE mRNA after IRI was examined in male Wistar rats (250–300 g; Harlan, Zeist, the Netherlands, n=6 per group).

IRI protocol
In mice, both renal pedicles of WT or CSE⁺⁻ animals (n=9-14 per group) were clamped for 30 minutes using non-traumatic vascular clamps through a midline abdominal incision under general anesthesia (100 mg/kg ketamine, 8 mg/kg xylazine). After removing the clamps, kidneys were inspected for restoration of blood flow and the muscle and skin layers were sutured with 5-0 vicryl stitches (Ethicon, New Jersey, USA). Body temperature was maintained at 37°C using heat pads and lamps. Sham-operated animals received the exact same procedure, without placement of the clamps on the renal pedicles. Treatment with NaHS (1 mg/kg, freshly dissolved in sterile phosphate buffered saline (PBS)) was given 15 minutes before application of the clamps through an intraperitoneal injection. Control animals received an injection of PBS at that time. Mice were sacrificed 24 hours after reperfusion.

In the Wistar rat model, a unilateral approach was used. Animals were anesthetized with 2% isoflurane with a flow of 0.6 l/min of 100% oxygen and a left-flank incision was made. Renal vessels of the left kidney were dissected and clamped with non-traumatic vascular clamps for 45 min. After removal of the clips, renal reperfusion was confirmed visually and muscle and skin layers were sutured using 4-0 vicryl stitches (Ethicon, New Jersey, USA). Reperfusion times were 30 min, 90 min, 6 h, 1 day, 4 days, 9 days, 14 days or 21 days (n=5-6 per group).

Cell culture
Human embryonic kidney 293 (HEK293) cells were used for in vitro experiments, cultured under standard conditions using Dulbecco’s Modified Eagle Medium (DMEM, Gibco) with 10% fetal bovine serum. All experiments were independently reproduced at least three times.
Transfection
For experiments utilizing overexpression of CSE, HEK293 cells were transfected with the pIRES2-EGFP (mock) or pIRES2-EGFP-CSE vector. For this purpose, cells were seeded in 24- or 96-well plates and grown until subconfluent. Subsequently, transfection was performed according to manufacturers’ instructions using Lipofectamine 2000 reagent (Invitrogen). Transfection efficiency and time course of expression was determined using fluorescence microscopy for EGFP. For experiments investigating oxidative stress and the protein expression of CSE, cells were used or harvested 72 hours after transfection.

Cell viability measurement
Alamar Blue reagent (Invitrogen) was used according to manufacturers’ protocol to measure cellular viability in the first 96 hours after transfection.

H$_2$S production rate measurement
Whole kidneys were taken from untreated CSE$^{-/-}$ or WT mice and snap frozen in liquid nitrogen. For measurement of H$_2$S production, renal tissue was homogenized and measurement was performed as described previously$^{18}$.

H$_2$S measurement in medium
Supernatants of control, pIRES2-EGFP and pIRES2-EGFP-CSE transfected cells was collected 72 hours after transfection and H$_2$S measurement was performed as described previously using the methylene blue assay$^{19}$.

Western Blot – rat tissue
Total protein lysates in RIPA buffer (Thermo Scientific, MA, USA) were supplemented with protease inhibitor cocktail (Sigma–Aldrich, MA, USA). Protein concentrations were determined using the BioRad DC protein assay (Bio-Rad, VA, USA), according to manufacturer’s protocol. Equal amounts of protein were loaded onto 12% SDS-polyacrylamide gels. Aspecific binding of the antibodies was prevented using 5% milk in Tris-buffered saline-0.1% Tween-20 (TBST). Then membranes were incubated with the primary antibody solution in 5% milk in TBST, overnight at 4°C (for CSE: Proteintech rabbit polyclonal CSE antibody 12217-1-AP (1:1000), for Ywhaz (which was used as a housekeeping protein): Abcam anti 14-3-3 zeta rabbit polyclonal antibody ab51129 (1:500)). Afterwards membranes were incubated with secondary antibody (goat anti-rabbit IgG horseradish peroxidase) 1:500 in 5% milk in TBST. All antibody incubations were followed by washing with TBST. Membranes were immediately developed by luminol-chemiluminescence according to manufacturer's instructions, and digital images taken with the Bio-Rad ChemiDoc MP system quantified using Bio-Rad Image Lab 4.01.
CHAPTER 4

Western Blot – cultured cells

Cultured HEK293 cells were obtained 72 hours after transfection and lysed in lysis buffer (0.5 M EDTA, 1 M Tris-Cl, pH 7.4, 0.3 M sucrose, 1 μg/ml antipain hydrochloride, 1 mM benzamidine hydrochloride hydrate, 1 μg/ml leupeptin hemisulfate, 1 mM 1,10-phenanthroline monohydrate, μ1 M pepstatin A, 0.1 mM phenylmethylsulfonyl fluoride, and 1 mM iodoacetamide). Equal amounts of protein were boiled and electrophoretically separated by SDS-PAGE and transferred to nitrocellulose membrane as described previously. The dilutions of primary antibody were 1:5000 for α-Tubulin and 1:1000 for CSE. Horseradish peroxidase-conjugated antibody was used as secondary antibody. The immunoreactions were visualized by ECL and exposed to X-ray film (Kodak Scientific Imaging film).

Model of Antimycin A induced oxidative stress and measurement of reactive oxygen species

HEK293 cells were cultured in 96-well plates until subconfluent and starved in serum-free medium for 24 hours. Antimycin A (50 μg/mL) with or without NaHS (1-1000 μM) was given for 30 min to induce intracellular production of ROS. Subsequently, cells were washed two times with PBS to remove Antimycin and NaHS and incubated with 15 μM dyhydroethidine (DHE, Invitrogen) or 5 μM MitoSOX (Invitrogen) for 15 minutes and washed three times with PBS. Images were directly made using an inverted fluorescent microscope and analyzed for intensity of fluorescence.

Statistical analysis

Data were analyzed using GraphPad PRISM 5.0 (GraphPad, San Diego, USA) or SPSS 16.0 (SPSS Inc., Chicago, IL, USA) using Mann-Whitney U tests, One-Way ANOVA or Kruskall Wallis tests where appropriate. Bonferroni or Dunns postcorrection was applied where multiple comparisons were made. Normality was tested using the Kolmogorov–Smirno test. Association between mRNA and protein levels and GFR were analysed using linear regression. For histopathological scoring, both kidneys of each animal were analysed and a mixed-effects model was used taking the animal of origin into account. A value of p<0.05 was considered statistically significant. Data are expressed as the mean ± SEM (Standard Error of the Mean) unless otherwise indicated.

Approval

For clinical studies, all procedures and use of anonymized human tissue were performed according to recent national guidelines, and were approved by our institutional review board. For the animal studies, experimental procedures were in agreement with institutional and legislator regulations and approved by the local committee for animal experiments.
REFERENCES FOR THE FULL MATERIALS AND METHODS


