Cystathionine γ-Lyase Overexpression Inhibits Cell Proliferation via a H₂S-dependent Modulation of ERK1/2 Phosphorylation and p21⁠<sub>Cip/WAK</sub>-1*

Guangdong Yang‡§, Kun Cao‡§, Lingyun Wu***, and Rui Wang‡ ‡‡

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Cystathionine γ-lyase (CSE) is a key enzyme in the trans-sulfuration pathway. CSE uses L-cysteine as a substrate to produce hydrogen sulfide (H₂S). The CSE/H₂S system has been shown to play an important role in regulating cellular functions in different systems. In the present study, we used CSE stably overexpressed HEK-293 cells to explore the effect of the CSE/H₂S system on cell growth and proliferation. The overexpression of CSE resulted in increases in CSE mRNA levels, CSE proteins, and intracellular H₂S production rates, as well as the inhibition of cell proliferation and DNA synthesis. These effects were accompanied by a sustained ERK activation and up-regulation of the cyclin-dependent kinase inhibitor p21⁠<sub>Cip/WAK</sub>-1. Blocking the action of ERK with U0126 inhibited the induction of p21⁠<sub>Cip/WAK</sub>-1, suggesting that ERK activation functions upstream of p21⁠<sub>Cip/WAK</sub>-1 activation to initiate the CSE overexpression-induced cell growth inhibition. The antiproliferative effect of CSE is likely mediated by endogenously produced H₂S because the H₂S scavenger methemoglobin (10 µM) significantly decreased the H₂S production rate and reversed the antiproliferative effect afforded by CSE. Exogenous H₂S (100 µM) also inhibited cell proliferation. However, the other CSE-catalyzed products, ammonium and pyruvate, failed to inhibit cell proliferation. Methemoglobin also abolished the inhibitory effect of exogenous H₂S on cell proliferation. Moreover, exogenous H₂S induced a sustained ERK and p21⁠<sub>Cip/WAK</sub>-1 activation. These findings support the hypothesis that endogenously produced H₂S may play a fundamental role in cell proliferation and survival.

The endogenous production of hydrogen sulfide (H₂S) and its physiological functions, including membrane hyperpolarization and smooth muscle cell relaxation, place this gas in the family of gas transmitters, together with nitric oxide and carbon monoxide (1, 2). Two pyridoxal-5′-phosphate-dependent enzymes, cystathionine β-synthase (EC 4.2.1.22) and cystathionine γ-lyase (CSE) (EC 4.4.1.1), are responsible for the endogenous production of H₂S in mammalian tissues, which use L-cysteine as the main substrate (3–5). Cystathionine β-synthase is a predominant H₂S-generating enzyme in the brain and nervous system (6), and CSE is mainly expressed in liver, kidney, and vascular smooth muscles (7, 8). Cystathionine β-synthase and CSE are important for the metabolism of sulfur-containing amino acids (e.g. cystathionine), as well as the production of H₂S, ammonium, and pyruvate from L-cysteine.

H₂S inhibits cell proliferation (9) and induces cell death predominantly by an apoptotic mechanism in polymorphonuclear cells (10). H₂S treatment has also been shown to lead to nasal lesions and olfactory epithelial necrosis (11). On the other hand, H₂S induces serum-independent cell cycle entry in rat intestinal epithelial cells and increases the fraction of colonic mucosa cells in the S phase (12, 13). However, little is known about the cellular consequences of an elevated CSE expression or about the associated increase in endogenously produced H₂S. In the present study, we overexpressed the CSE gene using a highly effective expression system. The successful overexpression of CSE was confirmed by measuring the CSE protein contents, CSE mRNA expression level, and endogenous H₂S production rate. Proliferation of the transfected HEK-293 cells was monitored. We determined whether the CSE overexpression-induced cellular changes were because of overproduced H₂S. Finally, the effect of both the cyclin-dependent kinase (cdk) inhibitor p21⁠<sub>Cip/WAK</sub>-1 and the ERK/mitogen-activated protein kinase (MAPK) pathway on the CSE/H₂S system was examined. Our findings support the hypothesis that endogenously produced H₂S may play a fundamental role in cell proliferation and survival.

** Experimental Procedures**

**Cell Culture and Measurement of H₂S Production**—HEK-293 cells (American Type Culture Collection, Manassas, VA) were cultured at 37 °C in a humidified incubator with 95% air and 5% CO₂ in minimal essential medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Invitrogen), 100 units of penicillin, and 100 µg of streptomycin/ml. The cultured cells were subjected to gene transfection when they had grown to 70–80% confluence. The H₂S production rate in CSE stably transfected HEK-293 cells was measured as described previously (8).

**Cloning of CSE cDNA and Stable Transfection**—PCR was used to amplify the open reading frame of CSE (GenBank™ accession number AF032875) from rat vascular tissues using the primers 5′-CGTCCAAGAGAAGTTAA-3′ and 5′-CAGTTATTCGAAAGTCTGCTGAG-3′.

* The abbreviations used are: CSE, cystathionine γ-lyase; cdk, cyclin-dependent kinase; ERK, extracellular signal-regulated kinase; FBS, fetal bovine serum; EGFP, enhanced green fluorescent protein; HEK, human embryonic kidney; MAPK, mitogen-activated protein kinase; PCR, polymerase chain reaction.
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The amplified open reading frame of CSE was subcloned into TA cloning vector (PCR4-TOPO). Positive clone containing CSE open reading frame insert was sequenced to confirm the accuracy of the inserted CSE sequence.

The constructs containing CSE cDNA were cleaved and subcloned into the mammalian expression vector pIRE2-EGFP (Clontech), which contained the human cytomegalovirus immediate early promoter/enhancers and the SV40 poly(A) signal. For stable transfection, the constructs were linearized with KpnI (MBI Fermentas) and then subjected to phenol-chloroform extraction and ethanol precipitation. Linearized constructs were mixed with a FuGENE 6 transfection reagent (Roche Applied Science) in a ratio of 1 μg to 3 μl in 100 μl of PBS-free minimal essential medium (14). After incubating for 45 min at room temperature (20–22 °C), the mixture was added to HEK-293 cells in 2 ml of PBS-free minimal essential medium. After 48 h of transfection, the cells were trypsinized, counted, and replated at 1 × 10^5 cells per plate in 35-mm plates with 6 μl of 150 μM G418 for 3 days. Mock (empty vector) transfection was also performed. In the pIRE2-EGFP vector, the EGFP gene (which encodes the enhanced green fluorescent protein) was expressed separately from the gene of interest and was used as a transfection marker. Non-transfected HEK-293 cells were included as the negative control for antibiotic selection. After 5 weeks of the antibiotic selective culturing, survival-transfected cells were harvested and grown to establish the sublines, which were subsequently examined for the presence of the CSE gene. Stable transfectants showing passage numbers <15. Wild-type HEK-293 cells were maintained in identical conditions but without selection antibiotic G418 treatment.

Western Immunoblotting—Cultured cells (3 × 10^5) were harvested and lysed in a lysis buffer (0.5 M EDTA, 1 M Tris-Cl, pH 7.4, 0.3 M sucrose, 1 μg/ml antipain hydrochloride, 1 μg/ml leupeptin hemisulfate, 1 μg/ml pepstatin A, 0.1 mM phenylmethylsulfonyl fluoride, and 1 mM iodoaceticamide). The extracts were clarified by centrifugation at 14,000 × g for 15 min at 4 °C. SDS-PAGE and Western blot analysis were performed as described previously (15). The primary antibody 1:1000 for phosphorylated or total ERK, p85 MAPK, and c-Jun NH2-terminal kinase, 1:500 for pi21Cip/WAF-1 and cyclin D1, and 1:500 for actin or an identical empty vector lacking a cDNA insert as a control or an endogenous reference cDNA. Mock (empty vector) transfection was also performed. In the pIRE2-EGFP vector, the EGFP gene (which encodes the enhanced green fluorescent protein) was expressed separately from the gene of interest and was used as a transfection marker. The CSE antibody was kindly provided by Dr. N. Nishi (Kagawa Medical School). The MAPK antibody, ERK/MEK (molecular-activated protein kinase/extracellular signal-regulated kinase inhibitor U0126, and pi21Cip/WAF-1) antibodies were obtained from New England Biolabs (Camarillo, CA). Cyclin D1 antibody was from Lab Vision Corporation (Fremont, CA). [3H]Thymidine was purchased from Amer sham Biosciences. Methemoglobin, ammonium hydroxide, and sodium pyruvate were from Sigma. Horseradish peroxidase-conjugated goat anti-rabbit IgG antibody was from Bio-Rad.

Statistical Analysis—All data are expressed as means ± S.E. and represent at least three independent experiments. Statistical comparisons were made using the Student’s t test or one-way analysis of variance followed by a post hoc analysis (Tukey test) where applicable. The level of significance was set at p < 0.05.

RESULTS

Overexpression of CSE cDNA in HEK-293 Cells—HEK-293 cells were transfected with a CSE cDNA/pIRE2-EGFP construct or an identical empty vector lacking a cDNA insert as a control (mock). The transfection of HEK-293 cells with CSE cDNA or the control empty vector did not change the morphological characteristics of the cell. In HEK-293 cells transfected with CSE cDNA or the control empty vector, green fluorescence emitted by green fluorescent protein was observed. In contrast, green fluorescence was not detectable in wild-type HEK-293 cells (data not shown). The expression of CSE was verified by Western blot analysis, real time PCR analysis, and the H2S production rate. Western blot and real time PCR analysis revealed that transfection of CSE cDNA, but not of the mock cDNA, resulted in significant increases in both CSE protein expression and CSE mRNA levels compared with wild-type HEK-293 cells (Fig. 1, A and B). Transfection of HEK-293 cells with CSE cDNA also resulted in a marked increase in the H2S production rate (1.51 ± 0.12 nmol/g/min) compared with mock-transfected cells (0.41 ± 0.06 nmol/g/min, p < 0.05) (Fig. 1C).

Suppressed Proliferation and DNA Synthesis in CSE-transfected HEK-293 Cells—To determine whether overexpression of the CSE gene affects proliferation and DNA synthesis of HEK-293 cells, cell growth curves were constructed. Compared with mock-transfected or wild-type cells, HEK-293 cells transfected with CSE cDNA exhibited a significantly reduced cell growth rate on the second day. At the fourth day, the number of CSE-overexpressed cells was only 72.2 ± 2.8% of the mock-transfected cells (Fig. 2A).

An antiproliferative effect of CSE overexpression was also evidenced by the extent of [3H]thymidine incorporation. As shown in Fig. 2B, HEK-293 cells transfected with CSE cDNA...
had less [3H]thymidine incorporation (19,181 ± 405 cpm) than cells transfected with mock (28,075 ± 1748 cpm) or wild-type cells (30,121 ± 1115 cpm) (p < 0.05).

Sustained Activation of MAPK and Up-regulation of p21Cip/WAF-1 by CSE Overexpression—The MAPK family, including ERK, p38 MAPK, and c-Jun NH2-terminal kinase, plays an important regulatory role in the proliferation and differentiation of cells. Transfection of HEK-293 cells with CSE cDNA, but not the mock cDNA, resulted in a marked accumulation of phosphorylated ERK and p38 MAPK (Fig. 3A). Neither the phosphorylation of c-Jun NH2-terminal kinase nor total amounts of MAPK were affected. Incubating cells with 10 μM U0126 or 20 μM SB203580 (the p38 MAPK inhibitor) for 24 h significantly decreased the expression of phosphorylated ERK and p38 MAPK (Fig. 3B).

p21Cip/WAF-1 is a cdk inhibitory protein, and it can prevent cell cycle progression by directly binding with cyclin and cdk complexes or binding to a proliferating cell nuclear antigen (18, 19). Increased p21Cip/WAF-1 expression is an important indicator for MAPK-dependent cell growth arrest (20–22). As shown in Fig. 4, the expression of p21Cip/WAF-1 increased significantly in CSE-transfected HEK-293 cells (~3-fold that in mock-transfected or wild-type cells). To verify the correlation of MAPK activation with CSE overexpression-mediated p21Cip/WAF-1 up-regulation, we tested the effects of U0126 and SB203580 on the expression of p21Cip/WAF-1. After incubating cells with 10 μM U0126, the expression of p21Cip/WAF-1 in CSE-transfected HEK-293 cells decreased almost to the basal level, whereas SB203580 (20 μM) had little effect. This suggests that p21Cip/WAF-1 induction by CSE overexpression may result from activation of the ERK pathway.

Cyclin D1 is an activator of cdk4 and cdk6, which limit the rate of cell cycle reentry (23). p21Cip/WAF-1 can inhibit cyclin D1 nuclear export by binding to Thr-286-phosphorylated cyclin D1, therefore preventing cyclin D1/CRM1 association (24). Our results showed that cyclin D1 expression was unchanged in all
kinds of cells, and U0126 and SB203580 had little effect on cyclin D1 expression (Fig. 4).

Inhibitory Effects of CSE Overexpression on Cell Proliferation Mainly Due to Endogenously Produced H2S—To determine the mechanism by which CSE overexpression inhibited cell growth, we assessed the effects of H2S, ammonium, and pyruvate, three products of CSE-catalyzed cysteine degradation, on cell growth. When confluent serum-starved wild-type HEK-293 cells were stimulated with 10% FBS in the presence of H2S (100 μM), DNA synthesis decreased significantly. However, 100 μM ammonium hydroxide and 100 μM sodium pyruvate failed to inhibit DNA synthesis (Fig. 5A). Furthermore, we tested the effect of the H2S scavenger methemoglobin (1, 25) on CSE overexpression-mediated cell growth inhibition. As shown in Fig. 5B, methemoglobin at 10 μM partly but significantly reversed the antiproliferative effect of CSE (p < 0.05). Pretreating wild-type HEK-293 cells with 10 μM methemoglobin for 1 h prior to adding 100 μM H2S significantly abolished the antiproliferative effect of H2S (Fig. 5A). Furthermore, we tested the effect of the H2S scavenger methemoglobin (1, 25) on CSE overexpression-mediated cell growth inhibition. As shown in Fig. 5B, methemoglobin at 10 μM partly but significantly reversed the antiproliferative effect of CSE (p < 0.05). Pretreating wild-type HEK-293 cells with 10 μM methemoglobin for 1 h prior to adding 100 μM H2S significantly abolished the antiproliferative effect of H2S (Fig. 5A). Decreased H2S production in CSE-overexpressed cells by methemoglobin also provided evidence that methemoglobin scavenged the endogenous H2S (Fig. 5C). To assess the role of exogenous H2S on MAPK and cell cycle protein expression, we tested the status of ERK and p21Cip/WAF-1 in wild-type HEK-293 cells after exposure to 100 μM H2S. The expression of ERK and p21Cip/WAF-1 increased after incubating wild-type HEK-293 cells with H2S for 2 h (p < 0.05) (Fig. 6), indicating that H2S likely mediates the antiproliferative effect of CSE.

DISCUSSION

Our present study demonstrated that the CSE expression level is an important regulatory element for cell growth. By increasing the endogenous production of H2S, the overexpression of CSE significantly inhibited cell proliferation. In this cascade of signal transduction, ERK and p21Cip/WAF-1 were consequentially activated by H2S, leading to cell growth inhibition.

CSE is a key enzyme of the trans-sulfuration pathway, which interconverts L-methionine and L-cysteine. It also uses L-cysteine as an alternative substrate to form H2S (4). As a pyridoxal phosphate-dependent enzyme, CSE is expressed in a range of mammalian cells and tissues, and it seems to be the main H2S-forming enzyme in the liver, kidney, and cardiovascular system (7, 8). Deficiency of H2S-producing enzymes results in some disorders such as homocystinuria, which are characterized by mental retardation, skeletal abnormalities, increased urine homocysteine, increased risks of thromboembolism, and early onset of atherosclerosis (9, 25, 26).

Many previous studies on H2S focused on the toxicological profile rather than the physiological function of the gas. Recent studies, however, have demonstrated the biological functions of H2S, including hyperpolarization of cell membranes, relaxation of smooth muscle cells, and increased neuronal excitability (1,
Little is known about the modulatory effect of endogenous CSE/H₂S on cell growth and proliferation. In the present study, we used CSE stably transfected HEK-293 cells to explore the effects of CSE overexpression on cell growth and proliferation. Our results indicate that the CSE overexpression increased intracellular H₂S production, inhibition of cell proliferation, and DNA synthesis (Figs. 1 and 2). CSE induced the expression of cdk inhibitor p21^Cip/WAF-1 following sustained ERK activity, suggesting a cell cycle arrest. The antiproliferative effect of CSE is likely mediated via the release of H₂S because the H₂S scavenger methemoglobin (1, 25) partly and significantly reversed the antiproliferative effect of CSE (Fig. 5B). Exogenous H₂S alone also inhibited cell proliferation, evidenced by decreased [³H]thymidine incorporation. The other CSE products, ammonium and pyruvate, failed to inhibit cell proliferation. Pretreatment of wild-type HEK-293 cells with...
Exogenous H2S increases the ERK and p21cip/WAF-1 activity. The quiescent wild-type HEK-293 cells were cultured in the presence or absence of H2S (100 μM) for the indicated times, and Western blot analysis was used to determine the activation of ERK (A) and p21cip/WAF-1 (B). The histograms represent the ratio of phosphorylated ERK optical density to total ERK (A) or p21cip/WAF-1 optical density to β-actin (B) from three independent experiments. *, p < 0.05.

Fig. 6. Exogenous H2S increases the ERK and p21cip/WAF-1 activity. The quiescent wild-type HEK-293 cells were cultured in the presence or absence of H2S (100 μM) for the indicated times, and Western blot analysis was used to determine the activation of ERK (A) and p21cip/WAF-1 (B). The histograms represent the ratio of phosphorylated ERK optical density to total ERK (A) or p21cip/WAF-1 optical density to β-actin (B) from three independent experiments. *, p < 0.05.

methemoglobin for 1 h prior to the addition of H2S abolished the antiproliferative effect of H2S (Fig. 5A). Moreover, exogenous H2S induced sustained ERK and p21cip/WAF-1 activation (Fig. 6). These findings support the hypothesis that endogenously produced H2S plays an important role in cell proliferation and survival.

The mitogen-activated protein kinase cascade plays a crucial role in transducing extracellular signals into responses governing growth and differentiation. The ERK pathway is involved in the stimulation of cellular proliferation (29–31), although MAPK-induced growth inhibition has also been reported (32, 33). Here, we provide evidence that a sustained increase in ERK activity induced by CSE overexpression leads to inhibited cell growth (Fig. 3A). The seemingly conflicting results can partially be explained by the fact that the MAPK pathway plays roles in both progression and inhibition of cell proliferation (21, 34). The final cellular response (i.e., cell cycle arrest or cellular proliferation) to activation of the ERK/MAPK pathway depends on the strength and duration of the MAPK signal. Transient or cyclic activation may contribute to cell cycle progression, whereas sustained high levels of ERK may lead to cell growth inhibition. Because active MAPK accumulates in the nucleus, it has been suggested that the duration and magnitude of MAPK activation will direct qualitative changes in gene expression, which in turn will determine whether a cell re-enters the cell cycle, undergoes cell cycle arrest, or remains quiescent. Although p38 MAPK was also increased in CSE-transfected cells, it may not be involved in CSE overexpression-induced p21cip/WAF-1 up-regulation (Figs. 3B and 4) because the inhibition of p38 activity by SB203580 did not change the expression of p21cip/WAF-1.

p21cip/WAF-1 is one of the cdk inhibitory proteins, and it plays an important role in growth arrest, cellular differentiation, DNA repair, cell senescence, and apoptosis (35, 36). Increased p21cip/WAF-1 expression is also an important indicator for MAPK-dependent cell growth arrest (20, 21, 37, 38). Our data provide evidence that the inhibition of growth by CSE overexpression in HEK-293 cells was accompanied by an induction of the cdk inhibitor p21cip/WAF-1, which was dependent on the ERK activation (Fig. 4). Correlation between p21cip/WAF-1 induction, prolonged ERK activation, and the inhibition of p21cip/WAF-1 by U0126 confirmed that p21cip/WAF-1 induction occurs after prolonged ERK activation. In the progression of the cell cycle, activation of cdk's has been demonstrated (23). The kinase activity of cdk's is negatively regulated by cdk inhibitors such as p21cip/WAF-1. p21cip/WAF-1 has been shown to directly bind with cyclin, cdk complexes, and DNA polymerase δ cofactor, a proliferating cell nuclear antigen (18). Blocking the activation of ERK blocked the induction of p21cip/WAF-1 expression, suggesting that ERK activation is correlated with CSE overexpression-induced up-regulation of p21cip/WAF-1 and cell growth inhibition. Several studies indicate that sustained ERK activation allows for the induction of cyclin D1 (20, 21). p21cip/WAF-1 can inhibit cyclin D1 activation and thereby prevent cyclin D1/CRM1 association (24). However, our study demonstrated that cyclin D1 was not involved in CSE overexpression-induced cell growth inhibition. Although ERK and p21cip/WAF-1 likely play roles in CSE overexpression-mediated cell growth inhibition, it is also possible that some other molecules that were not tested here are involved. Furthermore, the linkage between sustained ERK activation and expression of p21cip/WAF-1 needs to be identified.

In conclusion, this study provides evidence for the first time that CSE regulates cell proliferation through a H2S-mediated phosphorylation of ERK and p21cip/WAF-1. Collectively, the ability of the CSE/H2S system to inhibit cell growth suggests that genetic approaches to manipulate CSE expression and H2S production may provide a novel therapeutic avenue in the treatment of CSE/H2S disorder-related diseases.

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Guangdong Yang, Kun Cao, Lingyun Wu and Rui Wang

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