# ORIGINAL ARTICLE

# Cystathionine gamma-lyase-deficient smooth muscle cells exhibit redox imbalance and apoptosis under hypoxic stress conditions

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**BACKGROUND:** Hydrogen sulphide ($H_2S$) has recently emerged as a novel and important gasotransmitter in the cardiovascular system, where it is generated mainly by cystathionine gamma-lyase (CSE). Abnormal metabolism and functions of the CSE/$H_2S$ pathway have been linked to various cardiovascular diseases including atherosclerosis and hypertension. An important role for $H_2S$ in regulating the balance between cellular growth and death has been demonstrated whereby inhibition of the endogenous CSE/$H_2S$ pathway results in greater apoptosis of vascular smooth muscle cells (SMCs). $H_2S$ is increasingly recognized as a critical regulator of vascular integrity, but its role in SMCs during hypoxia has not been explored in a model of CSE deficiency.
**METHODS:** Cell viability, apoptosis, redox status and mitochondrial activity in hypoxia-exposed (12 h at 1% $O_2$) SMCs derived from the mes-enteric artery of CSE-knockout (CSE-KO) mice were analyzed. These were compared with those from CSE-wild-type (CSE-WT) mice.
**RESULTS:** CSE-KO cells exhibited redox imbalance and aberrant mito-chondrial activity versus CSE-WT cells, indicating an essential regulatory role for the endogenous CSE/$H_2S$ pathway on SMC function. CSE-KO cells were also more susceptible to hypoxia-induced cell death, indicating a critical contribution of endogenous CSE/$H_2S$ pathway to the protective hypoxia stress response.
**CONCLUSION:** These findings support the concept that $H_2S$ is a crucial regulator of vascular homeostasis, the deficiency of which is associated with various pathologies, and provide further evidence that $H_2S$ is a potent vasculoprotectant.

**Key Words:** *Apoptosis; Cystathionine gamma-lyase; Hydrogen sulphide; Oxidative stress; Vascular smooth muscle cell*

Hydrogen sulphide ($H_2S$) has recently emerged as a novel and important endogenous gaseous signalling transmitter, or gaso-transmitter, in the cardiovascular system (1-4). Cystathionine γ-lyase (CSE), a pyridoxal-5′-phosphate-dependent enzyme that acts on L-cysteine in the trans-sulfuration pathway, is the principal $H_2S$-generating enzyme in vascular tissues. Abnormal metabolism and functions of the CSE/$H_2S$ pathway have been linked to various cardio-vascular diseases including atherosclerosis (5,6) and hypertension (2). Vascular CSE expression and $H_2S$ production were shown to be sig-nificantly decreased in hypertensive rats, wherein exogenous $H_2S$ was found to attenuate increased blood pressure and structural remodelling during the development of hypertension (7,8). CSE-deficient mice exhibited age-dependent hypertension and decreased endogenous $H_2S$ levels, and provided the first direct evidence that $H_2S$ is a physiologi-cal vasodilator and regulator of blood pressure (2).

Vascular remodelling contributes to increased peripheral resistance in hypertension, and involves smooth muscle cell (SMC) hypertrophy/ hyperplasia (9,10), wherein dysregulation of SMC proliferation is a causative factor (11). More recently, investigators have demonstrated that overexpression of CSE resulted in increased endogenous $H_2S$ pro-duction that stimulated SMC apoptosis (12); exogenous $H_2S$ has been shown to produce a similar effect (13,14). We recently reported that SMCs isolated from CSE-deficient mice featured increased prolifera-tion both in vitro and in vivo versus their CSE-wild-type (CSE-WT) counterparts (15), thereby elucidating a contributing mechanism to the observed hypertension (2). These studies indicate that decreased endogenous $H_2S$ due to suppression of CSE expression could be patho-genic in diverse cardiovascular diseases (16) – a concept that further investigation and characterization of the cellular effects of CSE defi-ciency promises to enlighten.

Elevated oxidative stress is a well-recognized factor in the develop-ment of many cardiovascular diseases including hypertension and atherosclerosis (17,18). $H_2S$ is, itself, known to have antioxidant effects

in the neuronal (19,20) and cardiovascular systems (21-26). Indeed, exogenous $H_2S$ has been shown to reduce homocysteine-induced levels of hydrogen peroxide, peroxynitrite and superoxide (23), and to decrease superoxide via inhibition of NADPH oxidase in vascular SMCs (25). Furthermore, $H_2S$ delayed the accumulation of lipid per-oxidation products in hemin-mediated oxidation in human umbilical vein endothelial cells (24). Such antioxidant effects of $H_2S$ may prove significant for vascular remodelling and the development of athero-sclerosis (5,26), particularly given the recent observations that a CSE/ $H_2S$ pathway deficiency was associated with atherosclerotic progres-sion, which was reversible via exogenous $H_2S$ (27).

Hypoxia is a hallmark of many cardiovascular pathologies includ-ing hypertension, atherosclerosis and myocardial infarction (28). Many studies have shown that $H_2S$ can mimic the cardioprotective effects of hypoxic pre- and postconditioning (29), and new interest in the relationship of $H_2S$ with $O_2$ homeostasis was recently spurred by the demonstration that the consumption of exogenous $H_2S$ by mito-chondria was $O_2$ dependent (30). In the present study, we analyzed cell viability and apoptosis, redox status, and mitochondrial activity in hypoxia-exposed SMCs derived from the mesenteric artery of CSE-knockout (CSE-KO) mice. We then compared them with those from CSE-WT mice. Our findings suggest a significant role for the endog-enous CSE/$H_2S$ pathway in the homeostatic regulation of SMC prolif-eration, redox balance and mitochondrial function, as well as in protecting SMCs against hypoxia-induced cell death.

## METHODS

### Cell culture and hypoxia
Single SMCs from the mesenteric artery of CSE-WT and CSE-KO mice were isolated and identifed as previously described (15). Cells were cul-tured in Dulbecco's modifed Eagle's medium containing 10% supple-mented fetal bovine serum, 100 U/mL penicillin and 100 mg/mL streptomycin. Cell counts and viability were assessed via automated

Trypan blue exclusion assay using a Vi-Cell XR Cell Viability Analyzer (Beckman Coulter, Canada). Doubling time analyses were completed as per convention, and cells were maintained at a passage density of 1:3 to 1:6. All experiments were completed when the cells reached 70% to 80% confluence with matched (ie, same passage number for both WT and KO) cultures between passages 6 and 12. A humidified hypoxia glove box chamber with automatic controller (Coy, USA) was used for the 12 h hypoxia exposure regimen, set at 37°C, 5% $CO_2$ and 1% $O_2$.

**Assessment of cell proliferation/viability**
Cell proliferation/viability was assessed via spectrophotometry using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay. Cells were seeded onto sterile flat-bottom 96-well plates (Corning, USA) and incubated overnight to achieve the desired confluence. With 4 h of treatment time remaining, MTT reagent (Thiazolyl Blue Tetrazolium Bromide; Sigma-Aldrich, USA) was added to plate wells to achieve a final concentration of 10% (v/v), and cells were incubated at 37°C for an additional 4 h, during which time the MTT reagent was converted to purple formazan crystals in living cells according to their metabolic activity. Following this, the incubation medium was aspirated and 50 μL of dimethylsulfoxide per well was added to solubilize the formazan crystals. Following 10 min of agitation on a Belly Dancer shaker (Stovall Life Science, USA) at its highest setting, absorbance was measured spectrophotometrically at a wavelength of 490 nm (650 nm correction wavelength) using a PowerWave XS Microplate Spectrophotometer (BioTek, USA).

**Apoptosis assay**
Active caspase-3/7 activity was assessed via flow cytometry using the CaspaTag Caspase-3/7 In Situ Assay kit (Chemicon International, USA). Cells were seeded onto sterile flat-bottom 25 cm$^2$ culture flasks (Corning) and grown overnight to achieve the desired confluence. Following treatment, cells were washed with buffer and suspended via trypsinization in phosphate buffered saline (PBS) to achieve $1×10^7$ cells/mL. Freshly prepared fluorochrome inhibitors of caspases reagent (10 μL) was added to 290 μL of cell suspension, mixed gently and incubated at 37°C for 1 h in the absence of light (gently mixing twice during this incubation). Following several wash and count steps, samples were immediately analyzed using the FL1-H channel of a BD FACSCalibur Flow Cytometer (BD Biosciences, USA) supported by BD CellQuest Pro software. A minimum of $1×10^4$ gated events were acquired per trial.

**Oxidative stress assay**
Intracellular reactive oxygen species (ROS) level was assessed via flow cytometry using the CM-H2DCFDA (5-[and-6]-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester) assay (Molecular Probes, USA). Cells were seeded onto sterile flat-bottom six-well plates (Corning) and grown overnight to achieve the desired confluence. Following treatment, cells were washed with PBS and stained for 30 min with CM-H2DCFDA under standard incubation conditions (including hypoxia samples). Stained cells were washed with PBS, detached from the plate surface using trypsin and suspended in PBS for analysis using the FL1-H channel of a BD FACSCalibur Flow Cytometer (BD Biosciences) supported by BD CellQuest Pro software. A minimum of $1×10^4$ gated events were acquired per trial.

**Antioxidant assay**
Superoxide dismutase (SOD) activity was assessed via spectrophotometry using the Superoxide Dismutase Assay (Trevigen, USA). Cells were seeded into sterile 150 cm$^2$ culture flasks (Corning) and grown overnight to achieve the desired confluence. Immediately following treatment, pelleted cell samples were lysed and their total protein quantitated via DC Protein Assay (Bio-Rad, USA). Samples were assayed as per the manufacturer's instructions and the absorbance was read at 550 nm using a PharmaSpec UV-1700 Visible Spectrophotometer (Shimadzu, USA). Briefly, SOD activity was
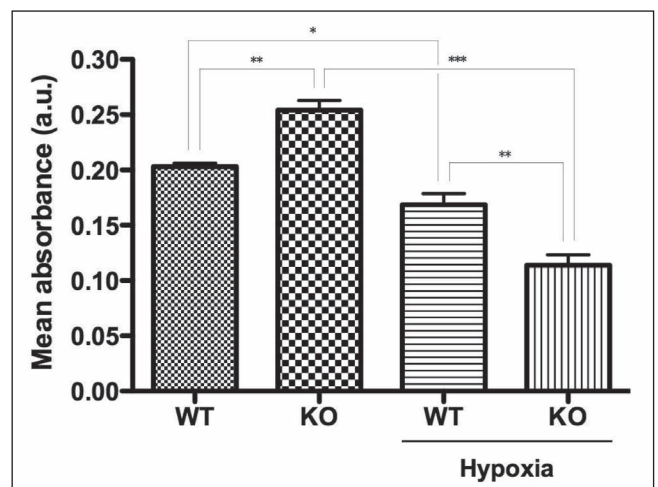


**Figure 1)** *Cellular proliferation/viability assay (MTT [3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide]). Colorimetric MTT assay indicating cystathionine γ-lyase (CSE)-wild-type (WT) and CSE-knockout (KO) smooth muscle cell proliferation at 12 h hypoxia. \*P<0.05; \*\*P<0.01; \*\*\*P<0.001. a.u. Arbitrary units*

calculated by measuring absorbance at 330 s subtracted by absorbance at 30 s; these values were converted to units of SOD per volume by reference to an SOD inhibition curve that was generated in parallel.

**Mitochondrial activity assay**
Mitochondrial membrane potential was assessed via flow cytometry using the MitoProbe JC-1 Assay Kit for Flow Cytometry (Molecular Probes). Cells were seeded onto sterile flat-bottom six-well plates (Corning) and grown overnight to achieve the desired confluence. Following hypoxia, cells were washed with PBS and stained for 30 min with JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-benzimidazolylcarbocyanineiodide). Stained cells were then detached from the plate surface via trypsinization, then were suspended in PBS for analysis via flow cytometry using the FL1-H (monomer) and FL2-H (aggregate) channels of a BD FACSCalibur Flow Cytometer (BD Biosciences) supported by BD CellQuest Pro software. A minimum of $1×10^4$ gated events were acquired per trial. The ratio of red fluorescence (potential-dependent generation of J-aggregates) to green fluorescence (nonconverted, monomeric form) was used as per the manufacturer's instructions to describe the relative status of mitochondrial electric potential.

**Statistical analysis**
Data were presented as mean ± SEM, and all data presented represent n≥3 independent experiments. Statistical analyses were performed using GraphPad Prism software (GraphPad Software Inc, USA). Student's *t* test was used throughout, with P<0.05 considered to be significant.
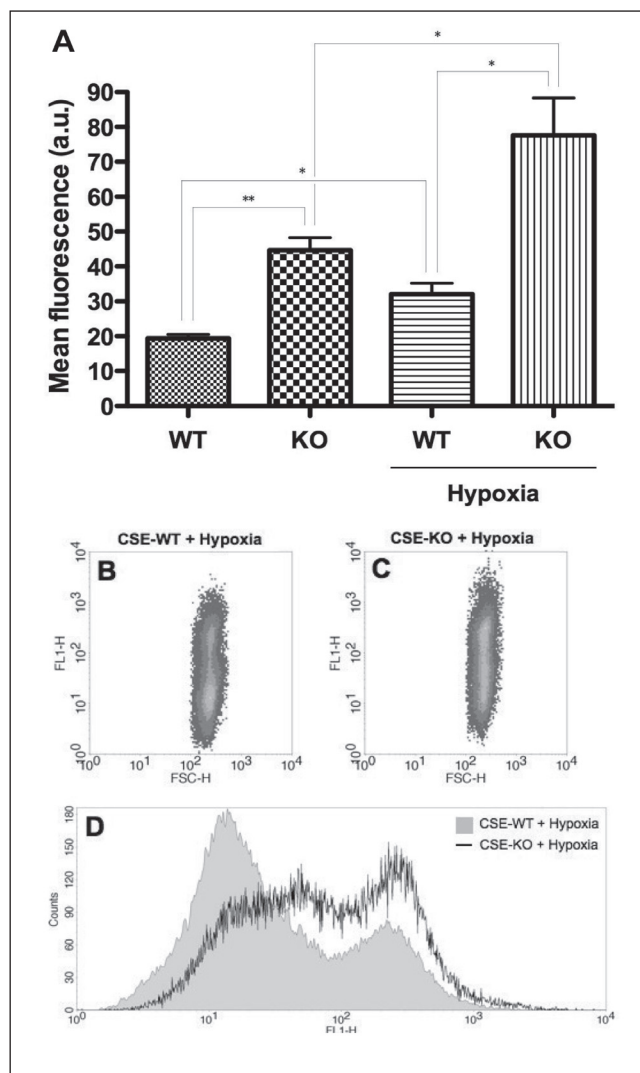
## RESULTS
The present study evaluated the responses of SMCs from CSE-WT and CSE-KO mice to hypoxic stress. Cell proliferation/viability was assessed via the MTT assay (Figure 1) – a quantitative index of the activity of dehydrogenases, which in living cells, reduce the yellow tetrazolium salt to produce a purple formazan dye that can be measured spectrophotometrically. CSE-KO SMCs exhibited significantly greater proliferation/viability versus their CSE-WT counterparts as evidenced by increased MTT reduction (125.4±5.9%, P<0.01) (Figure 1). By contrast, hypoxia (12 h, 1% $O_2$) caused significantly decreased MTT reduction in both cell lines versus their respective controls, but a much greater decrease was observed in the hypoxic CSE-KO (45.2±4.8%, P<0.001) than in the hypoxic CSE-WT cells (83.2±5.6%, P<0.05) (Figure 1).
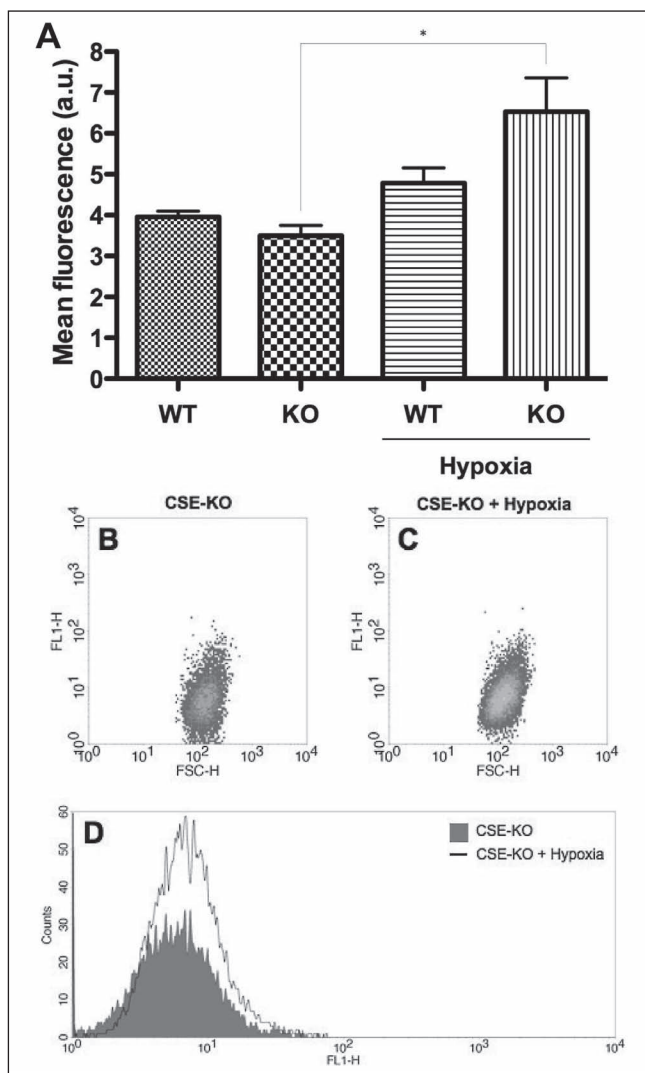
**Figure 2)** *Apoptosis assay (CaspaTag 3/7, Chemicon International, USA).* **A** *Fluorescence flow cytometry CaspaTag 3/7 assay indicating an abundance of active caspase-3 and caspase-7 in cystathionine γ-lyase (CSE)-wild-type (WT) and CSE-knockout (KO) smooth muscle cells at 12 h hypoxia. *P<0.05. a.u. Arbitrary units.* **B** *and* **C** *Representative dot plots indicating FL1 fluorescence versus forward scatter (FSC) in the cell populations of control and hypoxic KO samples.* **D** *Representative histogram indicating FL1 fluorescence versus cell counts of control and hypoxic KO samples. H Height*



**Figure 3)** *Intracellular reactive oxygen species (ROS) assay (CM-H2DCFDA).* **A** *Fluorescence flow cytometry CM-H2DCFDA assay indicating intracellular ROS levels in cystathionine γ-lyase (CSE)-wild-type (WT) and CSE-knockout (KO) smooth muscle cells at 12 h hypoxia. *P<0.05; **P<0.01. a.u. Arbitrary units.* **B** *and* **C** *Representative dot plots indicating FL1 fluorescence versus forward scatter (FSC) in the cell populations of hypoxic WT and KO samples.* **D** *Representative histogram indicating FL1 fluorescence versus cell counts of hypoxic WT and KO samples. H Height*

Apoptosis was measured via fluorescence flow cytometry using the CaspaTag Caspase-3/7 assay (Figure 2); this employs a cell-permeable, carboxyfluorescein-labelled fluoromethyl ketone peptide inhibitor that covalently binds to a reactive cysteine residue that resides on the large subunit of the active caspase heterodimer, thereby inhibiting further enzymatic activity and serving as a direct measure of the amount of active caspase-3/7 present in the cell. Consistent with the marked decrease in CSE-KO proliferation/viability on hypoxic insult, a significant increase in positive staining for active caspases-3/7 was evident in hypoxic CSE-KO cells versus control, but not in the CSE-WT cell line (Figure 2).

To explore whether oxidative stress was a mechanism underlying the observed susceptibility of CSE-WT SMCs to hypoxia, intracellular ROS level was assessed via fluorescence flow cytometry using the CM-H2DCFDA assay (Figure 3), wherein the namesake dye molecule remains nonfluorescent until the acetate groups are removed by intracellular esterases as oxidation occurs within the cell. It was found that even under basal conditions, CSE-KO SMCs featured greater ROS levels than CSE-WT cells (231.2±12.8%, P<0.01) (Figure 3). Furthermore, while hypoxia induced similar ROS increases in both the CSE-WT (165.4±8.5%, P<0.05) and CSE-KO cell lines (172.3±9.6%, P<0.05) versus their respective controls, hypoxic CSE-KO cells exhibited significantly greater absolute ROS levels versus hypoxic CSE-WT SMCs (243.6±28.1%, P<0.05) (Figure 3). In light of these findings, potential differences in antioxidant capacity were examined via spectrophotometric measurement of SOD activity (Figure 4). While hypoxia induced similar, significant increases in SOD activity in both CSE-WT (110.6±2.9%, P<0.05) and CSE-KO (109.4±2.9%, P<0.05) versus their respective controls, no inherent differences between the two cell lines were observed under basal or hypoxic conditions (Figure 4).

To investigate whether differences in mitochondrial activity contributed to the inherent redox imbalance and increased susceptibility of CSE-KO SMCs to hypoxia versus their CSE-WT counterparts,
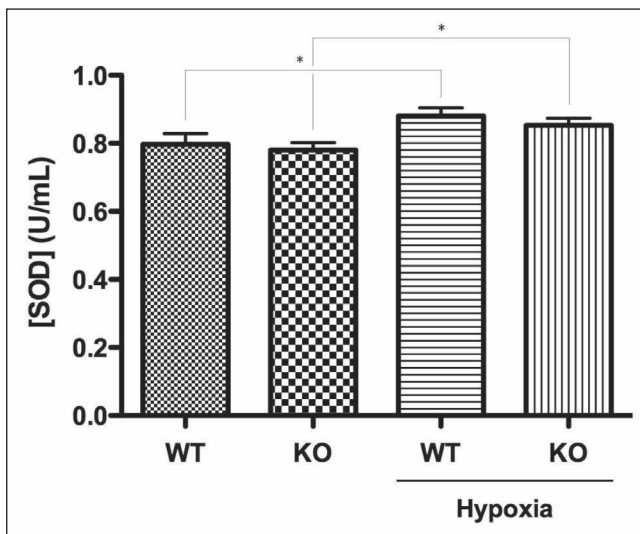
**Figure 4)** *Colorimetric superoxide dismutase (SOD) activity assay indicating SOD activity in cystathionine γ-lyase (CSE)-wild-type (WT) and CSE-knockout (KO) smooth muscle cells at 12 h hypoxia. *P<0.05*

mitochondrial membrane potential was assessed via fluorescence flow cytometry using the JC-1 assay (Figure 5). The cationic dye JC-1 exhibits potential-dependent accumulation in mitochondria. CSE-KO SMCs featured a significantly increased FL2:FL1 mean fluorescence ratio versus CSE-WT cells under both basal (169.6±11.7%, P<0.05) and hypoxic (164.2±19.9%, P<0.05) conditions (Figure 5). However, no significant hypoxia-induced increases in mitochondrial activity were observed in either cell line (Figure 5).

## DISCUSSION

The arrival of gasotransmitter biology has greatly influenced our understanding of vascular physiology and pathophysiology, from the Nobel prize-winning discovery that nitric oxide is the endothelium-derived relaxing factor to the recent discovery that the newest gasotransmitter, $H_2S$, may be an equally significant physiological vasorelaxant. Indeed, Yang et al (2) demonstrated that mice that were genetically deficient in CSE displayed marked hypertension comparable with that of endothelial nitric oxide synthase-deficient mice, and established CSE as the physiological source of $H_2S$ in multiple tissues including smooth muscle.

Using SMCs derived from the mesenteric artery of these CSE-WT and CSE-KO mice, we recently demonstrated that CSE-deficient SMCs lacked CSE messenger RNA and protein expression, and featured severely decreased $H_2S$ production (15). Given that abnormal CSE/$H_2S$ pathway had been demonstrated in vascular diseases including hypertension and atherosclerosis (2-8), we elected to compare the cellular responses of these CSE-WT and CSE-KO SMCs to hypoxia – a ubiquitous feature of cardiovascular disease and an important contributor to vascular remodelling in hypertension and atherosclerosis. Consistent with our previous finding of increased proliferation of CSE-deficient versus wild-type SMCs (15), CSE-KO cells exhibited significantly greater proliferation/viability under basal conditions versus their CSE-WT counterparts. Hypoxic insult caused significantly decreased proliferation/viability in both CSE-WT and CSE-KO cell lines, but a substantially larger decrease in the CSE-deficient SMCs. This hypoxia-induced change was also reflected as a significant increase in apoptosis of CSE-KO but not CSE-WT cells versus control. Taken together, these data indicate that the endogenous CSE/$H_2S$ pathway is essential for regulation of cell growth, and it plays a cytoprotective role against hypoxia-induced depression of metabolic activity and apoptosis.
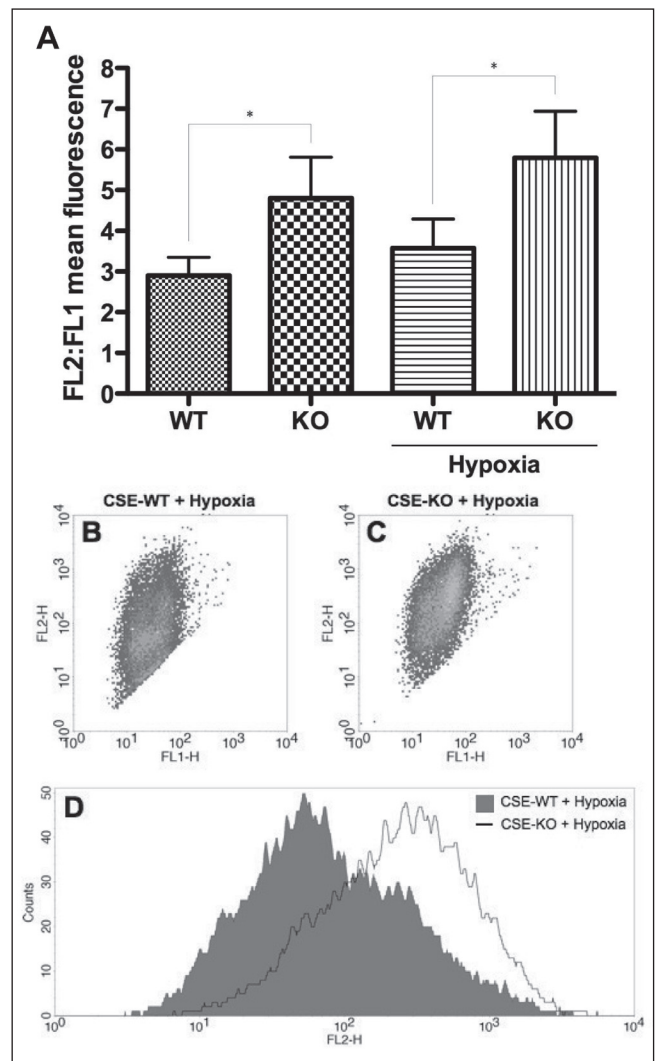


**Figure 5)** *Mitochondrial membrane potential assay (JC-1, Molecular Probes, USA). **A** Fluorescence flow cytometry JC-1 assay indicating mitochondrial membrane potential in cystathionine γ-lyase (CSE)-wild-type (WT) and CSE-knockout (KO) smooth muscle cells at 12 h hypoxia. *P<0.05. **B** and **C** Representative dot plots indicating FL2 versus FL1 fluorescence in the cell populations of hypoxic WT and KO samples. **D** Representative histogram indicating FL2 fluorescence versus cell counts of hypoxic WT and KO samples. H Height*

Both exogenously applied $H_2S$ and inhibition of endogenous $H_2S$ production have been shown to regulate cell growth and death in various systems including the cardiovascular (12,13,31), immune (32,33), pancreatic (34,35) and respiratory systems (36). Yang et al (13) demonstrated that $H_2S$ induced apoptosis of human aorta SMCs through phosphorylation of ERK1/2 and its subsequent activation of caspase-3 – an effect that was greatly enhanced on inhibition of the endogenous CSE/$H_2S$ pathway. Similarly, overexpression of CSE resulted in increased endogenous $H_2S$ that stimulated apoptosis of SMCs with inhibited basal $H_2S$ levels (12). Moreover, we recently showed that CSE-deficient SMCs featured reduced phosphorylation of ERK1/2, were more susceptible to $H_2S$-induced apoptosis, and exhibited greater proliferation than their CSE-WT counterparts (15). These CSE-deficient SMCs also showed decreased p21Cip/WAF-1 level and cyclin D1 expression (15), indicating $H_2S$-mediated regulation of cell cycle progression. Recent findings point to an essential role for the endogenous CSE/$H_2S$ pathway in maintaining SMC phenotype (16).

Thus, a phenomenon has been demonstrated whereby the endogenous $H_2S$ level is critical for the balance between apoptosis and cell proliferation in SMCs (26). Our present findings are consistent with this concept in that the lack of an endogenous CSE/$H_2S$ pathway rendered SMCs more susceptible to hypoxia-induced cell death. This susceptibility of CSE-deficient SMCs to hypoxia may be due to impairment of $H_2S$-mediated stimulation of stress-responsive, mitogen-activated protein kinases and phosphorylation of specific transcriptional factors including hypoxia-inducible factor (HIF)-1α and specificity protein-1 that regulate cellular processes of proliferation, differentiation and apoptosis. Indeed, given the established cytoprotective role for $H_2S$ in models of ischemic pre- and postconditioning (29), and the recent finding that $H_2S$ induces HIF-1 nuclear localization and transcription of HIF-1 targets (37), it is plausible that our findings indicate a key role for the endogenous CSE/$H_2S$ pathway in the protective hypoxic stress response in SMCs.

Oxidative stress is an important contributing factor in a variety of pathophysiological conditions including hypertension, atherosclerosis, ischemia-reperfusion injury, hypertrophy and heart failure (17,18). Considering the known antioxidant effects of $H_2S$ in the vasculature (23-25), and given the ROS-inducing nature of hypoxic stress (38), we investigated whether differences in redox status underlie the observed susceptibility of CSE-deficient SMCs to hypoxia. Even under basal conditions, CSE-KO cells featured much higher intracellular ROS levels versus CSE-WT cells. Hypoxia caused significantly increased ROS elaboration in both cell lines, but the stark difference between CSE-WT and CSE-KO cells persisted, with hypoxic CSE-KO SMCs exhibiting much higher intracellular ROS levels than hypoxic CSE-WT cells. To explore whether concomitant changes in antioxidant capacity were also present, the activity of SOD was also examined. We found that hypoxia induced similar increases in SOD activity in both CSE-WT and CSE-KO cells versus their respective controls, but no differences between the cell lines were evident under basal or hypoxic conditions. Taken together, these data suggest that the endogenous CSE/$H_2S$ pathway plays a role in regulating redox status, and that an inherent redox imbalance in the CSE-deficient SMCs may have contributed to the hypoxia-induced death of CSE-KO cells. Indeed, given the large body of evidence of ROS-induced apoptosis in cardiovascular disease (17,39), our observation of an inherent redox imbalance in the CSE-deficient SMCs could plausibly underlie the hypoxia-induced depression of metabolic activity and increased apoptosis therein. Importantly, this is consistent with our recent demonstration that hydrogen peroxide decreased cell proliferation in both the CSE-WT and CSE-KO cell lines, but significantly more so in the CSE-deficient SMCs, suggesting that these cells were more sensitive to oxidative stress (15).

At the cellular level, hypoxia limits $O_2$ availability for the electron transport chain, enhancing electron leak and causing mitochondria to accumulate oxidative damage, leading to dysfunction and disease (28,38). Increasing evidence points to an important regulatory role for $H_2S$ on mitochondrial function (19,21,40-42), impairment of which is associated with ROS-induced cell damage and death (21,38,40,41). To ascertain whether our findings could be traced to differences in mitochondrial activity, we measured mitochondrial membrane potential in the CSE-WT and CSE-KO SMCs. Remarkably, the CSE-deficient cells showed significantly greater mitochondrial activity than their CSE-WT counterparts under both basal and hypoxic conditions, but no significant hypoxia-induced changes were observed in either cell line. These data indicate aberrant mitochondrial activity in the CSE-deficient SMCs that paralleled the observed redox imbalance in those cells, suggesting a possible mitochondria-driven, ROS-enhancing mechanism at play. Commensurate with just such a scenario of increased cellular respiration and/or mitochondrial activity – and, thus, increased byproduction of ROS – both intracellular ROS levels and mitochondrial membrane potential were significantly higher in CSE-KO versus CSE-WT SMCs. Importantly, these data are consistent with the previously described protective roles for the CSE/$H_2S$ pathway in regulating redox balance (7,19,21,22,25) and mitochondrial function (19,21,40-42). Indeed, Elrod et al (41) demonstrated that $H_2S$ was cardioprotective against myocardial ischemia-reperfusion injury via stabilization of mitochondrial structure and function – a finding recently supported in a rat model of heart failure (40) and in an in vitro model of hypoxic rat cardiomyocytes (21). $H_2S$ was also shown to induce a suspended animation-like state in mice through its action on cytochrome c oxidase (43) that was protective against hypoxic damage (42). Moreover, Kimura et al (44) demonstrated that $H_2S$ protects neurons from oxidative stress by increasing both the production and redistribution of glutathione to mitochondria (19).

In summary, the present findings suggest a compromised ability of CSE-deficient vascular SMCs to regulate proliferation, redox levels and/or the byproduction of ROS that may result from deficient $H_2S$-mediated mitochondrial regulation versus their CSE-WT counterparts. CSE-KO cells were more susceptible to hypoxia-induced death than CSE-WT cells, indicating an essential contribution of the endogenous CSE/$H_2S$ pathway to the protective hypoxia stress response. These findings support the concept that $H_2S$ is a critical regulator of vascular homeostasis, a deficiency of which is associated with pathologies including atherosclerosis and hypertension.

## CONCLUSION

The results of our study indicate that vascular SMCs deficient in the $H_2S$-generating enzyme, CSE, exhibit redox imbalance and aberrant mitochondrial activity versus their CSE-WT counterparts. CSE-deficient SMCs were also more susceptible to hypoxia-induced cell death, indicating an essential contribution of the endogenous CSE/$H_2S$ pathway to the protective hypoxia stress response. These findings support the concept that $H_2S$ is a crucial regulator of vascular homeostasis, a deficiency of which is associated with various pathologies.

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