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Hydrogen Sulfide Levels and Nuclear Factor-Erythroid 2-Related Factor 2 (NRF2) Activity Are Attenuated in the Setting of Critical Limb Ischemia (CLI)

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Background—Cystathionine γ-lyase, cystathionine β-synthase, and 3-mercaptopuruvate sulfurtransferase are endogenous enzymatic sources of hydrogen sulfide (H₂S). Functions of H₂S are mediated by several targets including ion channels and signaling proteins. Nuclear factor-erythroid 2-related factor 2 is responsible for the expression of antioxidant response element-regulated genes and is known to be upregulated by H₂S. We examined the levels of H₂S, H₂S-producing enzymes, and nuclear factor-erythroid 2-related factor 2 activation status in skeletal muscle obtained from critical limb ischemia (CLI) patients.

Methods and Results—Gastrocnemius tissues were attained postamputation from human CLI and healthy control patients. We found mRNA and protein levels of cystathionine γ-lyase, cystathionine β-synthase, and 3-mercaptopuruvate sulfurtransferase were significantly decreased in skeletal muscle of CLI patients as compared to control. H₂S and sulfane sulfur levels were significantly decreased in skeletal muscle of CLI patients. We also observed significant reductions in nuclear factor-erythroid 2-related factor 2 activation as well as antioxidant proteins, such as Cu, Zn-superoxide dismutase, catalase, and glutathione peroxidase in skeletal muscle of CLI patients. Biomarkers of oxidative stress, such as malondialdehyde and protein carbonyl formation, were significantly increased in skeletal muscle of CLI patients as compared to healthy controls.

Conclusions—The data demonstrate that H₂S bioavailability and nuclear factor-erythroid 2-related factor 2 activation are both attenuated in CLI tissues concomitant with significantly increased oxidative stress. Reductions in the activity of H₂S-producing enzymes may contribute to the pathogenesis of CLI. (J Am Heart Assoc. 2015;4:e001986 doi: 10.1161/JAHA.115.001986)

Key Words: antioxidant proteins • critical limb ischemia • hydrogen sulfide • NRF2 • oxidative stress

Critical limb ischemia (CLI) is a manifestation of peripheral arterial disease (PAD), affecting nearly 2 million people in United States.1–3 During PAD, arteries that supply blood to the legs are narrowed, typically due to atherosclerosis, and results in a number of symptoms called intermittent claudication. Patients with CLI have a 6-month risk of major amputation of 25% to 40% and an annual mortality of 20%.4 Patients with CLI share the same traditional risk factors as patients with atherosclerosis in other vascular territories. Patients with CLI usually have concomitant severe cardiac (20%) and cerebrovascular disease (50% to 75%).5–7 With an aging population and the rising incidence of diabetes and chronic kidney disease, the prevalence of CLI is likely to increase. Early recognition of PAD and aggressive risk factor modification is likely to ameliorate the severity of PAD presentation and will hopefully reduce the incidence of CLI.8

Hydrogen sulfide (H₂S), historically viewed as an environmental toxic gas,9–11 is now known to be an endogenous gaseous signaling molecule12–16 that is required for the maintenance of normal vascular function. The production of H₂S in mammalian systems has been attributed to at least 3 endogenous enzymes: cystathionine β-synthase (CBS), cystathionine γ-lyase (CSE), 3-mercaptopuruvate sulfurtransferase (3-MST).17–25 The rate of H₂S production in tissue homogenates is in the range of 1 to 10 pmol/s per mg of protein, resulting in low micromolar extracellular concentrations.26,27 At this physiological concentration, H₂S is cytoprotective in various models of cellular injury.28,29 As a gaseous signaling molecule, H₂S diffuses across cell membranes in a receptor-independent manner and activates various cellular...
targets. This distinct ability makes H2S an attractive pharmacological agent for the treatment of cardiovascular disease. Recent evidence suggests that H2S is a mediator of many physiological and pathological processes. While the exact mechanism of action of H2S is still under investigation, some chemical and biochemical catabolic reactions of H2S have been disclosed that may be responsible for its biological functions. This molecule provides significant protection in cardiac, neurologic, and hepatic models of ischemia reperfusion injury when delivered before an ischemic insult. H2S dilates most mammalian blood vessels; the possibility that a deficiency in the biosynthesis of this gas contributes to or predisposes to cardiovascular diseases is appealing. Over the past few years, numerous other cardiovascular effects of H2S have been noted. These include proangiogenesis in vitro and in vivo, positive vascular remodeling (ie, reduced vascular hyperplasia) in blood vessels of hypertensive rats, antioxidant activity of H2S may explain a number of the reported biological effects of this gas, including protection against (1) heart, liver, and intestinal damage following ischemia/reperfusion injury; (2) H2O2-induced damage in rat gastric epithelial cells; (3) myocardial and renal injury due to hyperhomocysteinemia in rats; (4) methionine-induced and homocysteine-induced oxidative stress; and (5) heme-mediated oxidation of low-density lipoprotein. H2S is known to increase intracellular reduced glutathione concentrations and to suppress oxidative stress in mitochondria. Coupled with the finding that mitochondria contain an H2S-producing enzyme, 3-MST is particularly interesting. The antioxidant activity of H2S may explain a number of the reported biological effects of this gas, including protection against (1) heart, liver, and intestinal damage following ischemia/reperfusion injury; (2) H2O2-induced damage in rat gastric epithelial cells; (3) myocardial and renal injury due to hyperhomocysteinemia in rats; (4) methionine-induced and homocysteine-induced oxidative stress; and (5) heme-mediated oxidation of low-density lipoprotein. H2S is known to increase heme oxygenase 1 levels. Based on these facts, it is clear that H2S is a potent reducing agent and is likely to be consumed by endogenous oxidant species, such as peroxynitrite, superoxide, and H2O2. Treatment with exogenous H2S or modulation of the endogenous production of H2S protects against acute myocardial ischemia/reperfusion injury and heart failure by attenuating oxidative stress, inhibiting apoptosis, and reducing inflammation. Recently, we have demonstrated that 1 mechanism by which H2S exerts cytoprotective actions is via upregulation of cellular antioxidants in a nuclear factor-erythroid-2-related factor 2 (NRF2)-dependent manner. NRF2 regulates the gene expression of a number of enzymes that serve to detoxify pro-oxidative stresses by binding to the antioxidant response element (ARE) found in the gene-promoter region. It is important to note here that most reactive oxygen species (ROS), such as superoxide, H2O2, lipid peroxides, and any other lipid radicals may not directly react with H2S but are presumably scavenged by either Cu, Zn-superoxide dismutase or catalase, and/or GPx1 or by their combined actions. On the other hand, H2S is known to activate NRF2 signaling, which is responsible for the upregulation of these antioxidant proteins.

The present study was undertaken to investigate the effects of CLI on endogenous levels of H2S, the expression of H2S-producing enzymes, and NRF2 and related downstream signaling pathways.

Materials and Methods
Collection of Skeletal Muscle From Human CLI and Healthy Controls
Gastrocnemius tissues were attained from human CLI patients and otherwise healthy individuals (non-CLI) who underwent leg amputations. All patients were consented to the use of their amputated tissue for research purposes and signed a HIPPA release for use of the medical record. Patients were segregated into normal perfusion or CLI according to pulse examination and objective imaging criteria.

Institutional Review Board approval with consent was obtained.

Immunoblot Analysis
Protein samples obtained from skeletal muscle of healthy controls and CLI patients were analyzed by immunoblotting using specific antibodies to CBS (Santa Cruz), CSE (Abcam), 3-MST, thioredoxin-1 and -2 and thioredoxin reductase 1 and 2 (Santa Cruz), catalase (Santa Cruz), glutathione peroxidase-1 (Santa Cruz), NRF2 (Santa Cruz), fibrillarin (Cell Signaling), and GAPDH (Santa Cruz).

RNA Isolation and Reverse Transcriptase Real Time q-Polymerase Chain Reaction
RNA was isolated from the skeletal muscle of CLI patients and otherwise healthy controls. One microgram of RNA was transcribed using an I-script cDNA synthesis kit from Bio-Rad. TaqMan primers for CBS, CSE, 3-MST, NRF2, TXN1 and 2, superoxide dismutase 1 and 2, catalase, and glutathione peroxidase-1 (GPx-1) from Life Technology were used to amplify q-polymerase chain reaction. For real-time q-polymerase chain reaction experiments, 18s was used as a housekeeping gene and values were corrected to 18s. 2A was used for the data analysis of all q-polymerase chain reaction.

Determination of Protein Carbonyl Content
Protein carbonyl (CO) contents in skeletal muscle from CLI patients and otherwise healthy controls were measured as described previously.

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Measurement of MDA Levels
Malondialdehyde (MDA) levels in skeletal muscle from CLI patients and healthy controls were assayed as described previously.65

Measurement of H2S and Sulfane Sulfur
H2S and sulfane sulfur levels were measured in protein extracts from skeletal muscle of healthy controls and CLI patients by gas chromatography chemiluminescence.66

Electrophoretic Mobility Shift Assay
Nuclear extracts (NE) were prepared from skeletal muscle of healthy controls and CLI patients. DNA-protein interaction was determined by utilizing electrophoretic mobility shift assay as described previously.64

Statistical Analysis
All data in this study are expressed as the mean±SEM. Differences in data between the groups were compared using Prism 6 (GraphPad Software) with nonparametric test (Wilcoxon rank sum test). A P value of <0.05 was considered statistically significant.

Results
Decreased Levels of H2S and Sulfane Sulfur in Skeletal Muscle of CLI Patients
H2S and sulfane sulfur levels were measured in samples obtained from skeletal muscle of CLI and healthy controls. As can be seen in Figure 1, both H2S (A) and sulfane sulfur (B) were significantly decreased in CLI patients as compared to control. Reduction in H2S (≈3-fold decrease) and sulfane sulfur may indicate reduced levels of H2S-producing enzymes such as CBS, CSE, and 3-MST in skeletal muscle of CLI patients.

Reduction in the Levels of CBS, CSE, and 3-MST in Skeletal Muscle of CLI Patients
Due to the decreased levels of H2S and sulfane sulfur in CLI patients, we next measured tissue levels of CBS, CSE, and 3-MST in skeletal muscle of CLI patients. Figures 2A through 2C and 3A through 3D show that both mRNA and protein levels of all 3 enzymes were significantly decreased in skeletal muscle of CLI patients, and it was clearly observed that the expression levels of CBS and CSE in CLI were reduced to about 2-fold or more as compared to control subjects. It is well appreciated that H2S increases cellular antioxidant levels.

Skeletal Muscle of CLI Patients Shows Decreased NRF2 Activity
NRF2 is a nuclear factor that binds to the ARE site in a specific promoter and thereby enhances the transcription of antioxidant protein genes. It was of next interest to determine the NRF2 activity in skeletal muscle of CLI patients. Figure 4A shows mRNA of NRF2 was markedly decreased in skeletal muscle of CLI patients as compared to control subjects. Nuclear level of NRF2 was also found to be decreased significantly (≈2-fold) in CLI patients compared to control subjects (Figure 4B and 4C). DNA binding activity of NRF2 to consensus ARE site was analyzed using electrophoretic mobility shift assay. Figure 4D shows that NRF2-DNA binding activity was markedly decreased in nuclear extracts of skeletal muscle of CLI patients as compared to control subjects. Figure 4E represents the binding specificity of NRF2 to ARE site.

Induction of Oxidative Stress in Skeletal Muscle of CLI Patients
One of the pathological consequences of chronic tissue ischemia is increased oxidative stress and cellular injury. Therefore, it was of interest to determine the levels of oxidative stress in the protein samples obtained from skeletal muscle of CLI patients. Oxidative stress was measured by determining the levels of protein CO contents and MDA formation. As can be seen in Figure 5A and 5B, both protein CO contents and MDA were significantly increased in skeletal muscle of CLI patients as compared to control subjects. Protein CO contents and MDA are considered biomarkers of
oxidative stress; therefore, based on the induction of protein CO \((\approx 2\text{-fold})\) and MDA \((\approx 2\text{-fold})\) formation in skeletal muscle of CLI patients, these data suggest that the cellular oxidative stress is increased in CLI patients.

**Skeletal Muscle of CLI Patients Exhibits Reduced Levels of Antioxidant Proteins**

We next determined the levels of antioxidant proteins in skeletal muscle of CLI patients. We determined both mRNA and protein levels of antioxidant protein in skeletal muscle of CLI patients as well as in control subjects. As can be seen in Figure 6, significant reductions \((\approx 2\text{-fold})\) in mRNA expression of catalase (A), glutathione peroxidase-1 (GPx-1) (B), and Cu, Zn-superoxide dismutase (SOD1) (C) were observed in skeletal muscle of CLI patients as compared to control subjects. Immunoblot analysis demonstrated significant reduction in protein levels of GPx-1 and superoxide dismutase in skeletal muscle of CLI patients as compared to control (Figure 7A, 7C, and 7D). These data clearly demonstrate reduced expression of antioxidant enzymes results in CLI patients in addition to increased levels of oxidative stress.

**Decreased Levels of Thioredoxins and Thioredoxin Reductases in Skeletal Muscle of CLI Patients**

Thioredoxins are proteins that act as antioxidants by facilitating the reduction of other proteins by cysteine thiol-disulfide exchange. Thioredoxin plays a central role in humans and is increasingly linked to medicine through their response to ROS. In order to define the roles of thioredoxins in CLI patients, both mRNA and protein levels of thioredoxin 1 and 2 were analyzed. As can be seen in Figure 8A through 8E, both mRNA and protein levels of thioredoxin 1 and 2 significantly decreased in CLI patients as compared to healthy control subjects. Furthermore, an experiment was also performed to analyze the expression of thioredoxin reductase 1 and 2. Thioredoxin reductases catalyze the NADPH-dependent reduction of thioredoxin 1 and 2 and other oxidized cellular components and thereby prevent cellular oxidative damage. Figure 9A and 9B shows that mRNA expression of thioredoxin reductases 1 and 2 were significantly decreased in skeletal muscle of CLI patients as compared to control subjects.

**Discussion**

In the present study, we provide novel insights into the biochemical and molecular derangements in isolated skeletal muscle in the setting of CLI. The major finding of this study is...
that the pathological conditions occurring in chronic CLI decrease H₂S bioavailability and expression of H₂S-producing enzymes in CLI patients. Furthermore, we have determined that NRF2 activity is also significantly attenuated in CLI patients as compared to control subjects. Uncontrolled oxidative stress resulting in decreased H₂S bioavailability and attenuated NRF2 activity may significantly contribute to the pathogenesis of CLI. Therapies aimed at restoring redox balance, thereby increasing H₂S, may prove beneficial for the treatment of CLI.

H₂S is synthesized in tissues throughout the body by enzymes located either in the cytosol (CBS, CSE) or in mitochondria (3-MST). Because H₂S is a highly diffusible gas, upon formation it is likely to be either sequestered or

Figure 4. Activity of NRF2 in skeletal muscle of CLI patients. A, Represents mRNA expression of NRF2 in the skeletal muscle of control and CLI patients. B, Represents immunobLOTS of nuclear levels of NRF2 and fibrillarin in the skeletal muscle of control and CLI patients. C, Quantitation of immunobLOTS in (B). D, NRF2-DNA binding activity as judged by EMSA; lanes 1 and 2, control; lanes 3 and 4, CLI. E, The binding specificity of NRF2; lane 1, free probe; lane 2, NE; lane 3, NE+W/T oligo (250× excess); lane 4, NE+NRF2 antibody; and lane 5, NE+non-specific antibody (non sp. ab). Circle with number inside bar denotes number of patients per group. CLI indicates critical limb ischemia; EMSA, electrophoretic mobility shift assay; NE, nuclear extracts; NRF2, nuclear factor-erythroid 2-related factor 2; W/T, wild type.

Figure 5. Induction of oxidative stress in skeletal muscle of CLI patients. Biomarkers of oxidative stress such as protein CO contents (A) and MDA (B) formation were significantly increased in skeletal muscle of CLI patients as compared to healthy controls. Circle with number inside bar denotes number of patients per group. CLI indicates critical limb ischemia; CO, carbonyl; MDA, malondialdehyde.

Figure 6. Decreased expression of antioxidant gene in skeletal muscle of CLI patients. A through D, Represent the mRNA expression of catalase, GPx-1, SOD1, and SOD2, respectively, in the skeletal muscle of control and CLI patients. Circle with number inside bar denotes number of patients per group. CLI indicates critical limb ischemia; GPx-1, glutathione peroxidase-1; NS, not significant; SOD1, Cu, Zn-superoxide dismutase.
catabolized rapidly. The molecular targets for H₂S are largely unknown, but recent experimental studies suggest that H₂S impacts on intracellular proteins, enzymes, transcription factors, as well as an array of membrane ion channels. H₂S produces anti-apoptotic, anti-inflammatory, antihypertrophic, cardioprotective, and anti-oxidant effects, which ultimately can lead to protection from cellular injury or oxidative damage. H₂S is also known to have protean effects, including the regulation of inflammation, vascular permeability and tone, and cellular metabolism. H₂S also activates prosurvival signaling cascades such as the reperfusion injury salvage kinases, which include PI3-kinase/Akt and extracellular signal-regulated kinase, among others.2,67 Previous studies clearly demonstrate that H₂S-releasing drugs significantly limit myocardial ischemia/reperfusion injury and myocardial injury in heart failure.2,4,68,69

![Figure 7. Antioxidant protein levels in skeletal muscle of healthy controls and CLI patients. A, Immunoblots for the levels of Cat, GPx-1, SOD1 and SOD2 of the skeletal muscle of CLI patients and healthy controls. B through E, Quantitation of immunoblots in (A). Circle with number inside bar denotes number of patients per group. Cat indicates catalase; CLI, critical limb ischemia; GPx-1, glutathione peroxidase-1; NS, not significant; SOD1, Cu, Zn-superoxide dismutase; SOD2, Mn-superoxide dismutase.](image1)

![Figure 8. Levels of TXN1 and 2 in skeletal muscle of healthy controls and CLI patients. A and B, Represent the mRNA expression of TXN1 and TXN2, respectively, in the skeletal muscle of control and CLI patients. C, Immunoblots of TXN1 and TXN2 for the protein samples obtained from skeletal muscle tissues from CLI patients and controls. D and E, Quantitation of immunoblots in (C). Circle with number inside bar denotes number of patients per group. CLI indicates critical limb ischemia; TXN, thioredoxin.](image2)
Hydrogen Sulfide Deficiency in CLI

A major mechanism by which H₂S exerts cytoprotective actions is via upregulation of cellular antioxidants in an NRF2-dependent manner.⁴ In addition, H₂S markedly reduces cellular ROS levels and suppresses oxidative stress in mitochondria via increasing intracellular reduced glutathione concentration.⁴⁴ Furthermore, mitochondrial are able to synthesize H₂S via 3-MST and thereby regulate redox balance and oxidant stress within the mitochondria.⁴⁴ Significant increases in oxidative stress that overwhelm endogenous antioxidant defenses result in potentially cytotoxic reactions with membrane phospholipids, proteins, nucleic acids, and other cellular components, and ultimately impair cell structure and function, leading to cell damage. In the present study, we determined that H₂S bioavailability was significantly reduced in the skeletal muscle of CLI patients. We also observed significant reductions in the protein expression of the H₂S-producing enzymes in CLI skeletal tissue. It is well appreciated that H₂S leads to NRF2 activation,⁷⁰,⁷¹ which attenuates oxidative damage by increasing the levels of antioxidant proteins. Previous reports have indicated that NRF2 deficiency is associated with enhanced oxidative stress and cell death.⁷²,⁷³ NRF2 is a family of nuclear basic leucine zipper transcription factors that regulate the gene expression of a number of antioxidant enzymes that serve to detoxify pro-oxidative stressors⁶³ including GPx-1 and heme oxygenase 1.⁴ NRF2 is widely expressed and is thought to translocate to the nucleus after treatment with xenobiotics and antioxidants, which stimulate its release from a repressor protein Kelch-like ECH (enoyl-CoA hydratase)-associated protein 1. Following translocation into the nucleus, NRF2 binds the ARE site in the promoter region of a gene and enhances the transcription of antioxidant proteins, which results in suppression of an oxidative stress response. Therefore, NRF2 controls the basal and inducible expression of antioxidant genes and other cytoprotective phase II detoxifying enzymes that are ubiquitously expressed throughout the cardiovascular system. Emerging evidence has revealed that NRF2 and its target genes are critical regulators of cardiovascular homeostasis via the suppression of oxidative stress/ROS production.⁷⁴ Similarly, oxidative stress/reactive oxygen species may also potentiate development of CLI.

In the present study, we found a significant induction of oxidative stress in skeletal muscle of CLI patients compared to controls, as judged by measuring the levels of protein CO contents and MDA formation (as protein CO contents and MDA formation are considered biomarkers of oxidative stress). Under basal conditions, the ability of NRF2 to induce endogenous antioxidants is tightly regulated when anchored in the cytoplasm, and is targeted for ubiquitination and proteasome degradation.⁷⁵ Only when there is a disruption in the binding of NRF2 to the cytosolic anchoring proteins (such as Kelch-like ECH-associated protein 1) is NRF2 able to translocate to the nucleus and protect the cells from oxidative damage through enhancing the transcription of antioxidant genes. Previous studies have indicated that H₂S exerts cytoprotective actions through upregulation of cellular antioxidants via the NRF2 signaling pathway.⁴

Medical management of CLI entails pain control, risk factors modification, proper wound care, and infection management. The management of this complex patient population often warrants a multidisciplinary approach with collaboration with an endovascular interventionist, vascular surgeons, podiatrist, infectious disease specialist, and wound-care specialist. Although data are limited, with regard to pharmacological interventions in CLI patients, most of the pharmacological measures are of proven benefit in reducing the risk of death, myocardial infarction, heart failure, and stroke in patients with atherosclerosis, and therefore the use of such therapy, despite the lack of evidence in the setting of CLI, is justified to limit adverse cardiovascular events.⁷⁶–⁸² Various reports have demonstrated that the use of “cardioprotective” medications such as statins, angiotensin-converting enzyme inhibitors and angiotensin receptor blockers, and antiplatelet agents is associated with a decreased cardiovascular event rate in patients with PAD.⁷⁷ However, little is known about the effectiveness of these drugs in patients with CLI. Effective new treatments for CLI are critically important, given the high incidence of major amputation and mortality seen in this population.⁸³ Increasing blood flow, tissue perfusion, and oxygen and nutrient delivery via new vessel growth (ie, angiogenesis) or collateralization could promote tissue regeneration, and/or delay or even prevent tissue necrosis and the development of conditions such as gangrene.

In the present study, we observed that levels of H₂S and H₂S-producing enzymes were significantly decreased in skeletal muscle of CLI patients as compared to healthy controls. We also found significant reductions in NRF2 activation and antioxidant proteins concomitant with increased skeletal muscle oxidative stress in CLI patients.

**Figure 9.** Expression of TXNRD1 and TXNRD2 in CLI patients. A and B, Represent the mRNA expression of TXNRD1 and TXNRD2 in CLI patients and healthy controls. Circle with number inside bar denotes number of patients per group. CLI indicates critical limb ischemia; TXNRD, thioredoxin reductase.

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In conclusion, the present study clearly demonstrates that induction of oxidative stress and attenuated NRF2 signaling appears to be involved in the pathogenesis of CLI and is associated with significant reductions in skeletal muscle H2S bioavailability. Given the recent elucidation of the potent cytoprotective, antioxidant, and pro-angiogenic properties of physiological or pharmacological levels of H2S, it is possible that H2S-based therapies or H2S-induced NRF2 activation may prove beneficial for the treatment of CLI. In the present study, we examined a small sample of control tissues, which represents a limitation of this research. However, it is very difficult to obtain these skeletal muscle samples from otherwise healthy individuals undergoing limb amputations. Clearly, additional studies of CLI in clinically relevant models are required to validate the therapeutic effects of H2S in this devastating disease.

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Disclosures
None.

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