Hydrogen Sulfide and L-Cysteine Increase Phosphatidylinositol 3,4,5-Trisphosphate (PIP3) and Glucose Utilization by Inhibiting Phosphatase and Tensin Homolog (PTEN) Protein and Activating Phosphoinositide 3-Kinase (PI3K)/Serine/Threonine Protein Kinase (AKT)/Protein Kinase Cζ/λ (PKCζ/λ) in 3T3L1 Adipocytes

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Background: H2S and l-cysteine (LC) levels are low in diabetes. Results: H2S or LC inhibits PTEN and activates IRS1, PI3K, AKT, PKCζ/λ, GLUT4, and PIP3 levels and glucose utilization in cells exposed to high glucose. Conclusion: LC and H2S can activate insulin signaling cascades. Significance: LC has the potential to activate the insulin signaling pathway and to improve glucose metabolism.

This work examined the novel hypothesis that reduced levels of H2S or l-cysteine (LC) play a role in the impaired glucose metabolism seen in diabetes. 3T3L1 adipocytes were treated with high glucose (HG, 25 mM) in the presence or absence of LC or H2S. Both LC and H2S treatments caused an increase in phosphatidylinositol-3,4,5 trisphosphate (PIP3), AKT phosphorylation, and glucose utilization in HG-treated cells. The effect of LC on PIP3 and glucose utilization was prevented by propargylglycine, an inhibitor of cystathionine γ-lyase that catalyzes H2S formation from LC. This demonstrates that H2S mediates the effect of LC on increased PIP3 and glucose utilization. H2S and LC caused phosphatidylinositol 3-kinase activation and PTEN inhibition. Treatment with LC, H2S, or PIP3 increased the phosphorylation of IRS1, AKT, and PKCζ/λ as well as GLUT4 activation and glucose utilization in HG-treated cells. This provides evidence that PIP3 is involved in the increased glucose utilization observed in cells supplemented with LC or H2S. Comparative signal silencing studies with siAKT2 or siPKCζ revealed that PKCζ phosphorylation is more effective for the GLUT4 activation and glucose utilization in LC-, H2S-, or PIP3-treated cells exposed to HG. This is the first report to demonstrate that H2S or LC can increase cellular levels of PIP3, a positive regulator of glucose metabolism. The PIP3 increase is mediated by PI3K activation and inhibition of PTEN but not of SHIP2. This study provides evidence for a molecular mechanism by which H2S or LC can up-regulate the insulin-signaling pathways essential for maintenance of glucose metabolism.

Hydrogen sulfide (H2S) is gaining acceptance as a signaling molecule and has been shown to elicit a variety of biological effects that may mediate the protection of cardiovascular pathophysiology (1–3). However, elucidations of the mechanisms of action of H2S and its molecular targets are largely lacking. H2S is produced in vivo from l-cysteine (LC)2 by the action of two enzymes, cystathionine β-synthase and cystathionine γ-lyase (CSE) (4, 5). Both of these enzymes are dependent on pyridoxal 5′-phosphate. CSE is expressed mainly in the thoracic aorta, portal vein, ileum, heart, liver, kidney, and vascular smooth muscle, whereas cystathionine β-synthase is highly expressed in the central and peripheral nervous systems (4–6).

Recent studies have shown evidence for two other H2S-producing enzymes, 3-mercaptopuruvate sulfurtransferase and cysteine aminotransferase, which produce H2S in the brain as well as in vascular endothelium (7–9). H2S inhibits oxidative stress, promotes stimulation of KATP channels and relaxation and vasodilation in vascular smooth muscle cells, and relaxation of the human corpus cavernosum smooth muscle (4, 7–11). In vivo, H2S has been shown to inhibit leukocyte endothelial cell interactions and ischemia-reperfusion injury in liver and heart in animal studies (5). Genetic deletion of the CSE enzyme in mice markedly reduces H2S levels in the serum, heart, aorta, and other tissues, and mice lacking CSE display pronounced hypertension and diminished endothelium-dependent vasorelaxation (4). Furthermore, apolipoprotein E knock-out mice treated with as H2S donor showed reduced atherosclerotic plaque size compared with controls (12). These studies suggest that H2S is a physiological neuromodulator (13), vasodilator (4),...
and regulator of blood pressure (4). Human blood contains a significant amount of $H_2S$ (10–100 μM) (4, 14). Recent studies report lower blood levels of LC and altered cysteine homeostasis (15, 16) as well as lower $H_2S$ levels in diabetic patients (17, 18). Along with a host of proteins, LC is a precursor of glutathione, which is considered essential for the reduction of cellular oxidative stress (19). Dietary supplementation with N-acetylcysteine or whey protein and α-lactalbumin (cysteine-rich proteins) lowers the oxidative stress and insulin resistance induced by sucrose or fructose in rats and streptozotocin-treated diabetic mice (20–23). Oral supplementation with LC lowered glycemia, oxidative stress, and vascular inflammation markers in ZDF (Zucker diabetic fatty) rats, an animal model of type 2 diabetes (24). Recent studies report that LC supplementation lowered oxidative stress markers in type 2 diabetic patients and normal subjects (15, 25). These studies led to a novel hypothesis that reduced $H_2S$ or LC levels may play a direct role in modulating glucose metabolism in diabetes. Using an adipocyte cell model, this study demonstrates that $H_2S$, or its precursor LC, increases cellular phosphatidylinositol 3,4,5-trisphosphate (PIP3), a positive regulator of phospho-serine/threonine protein kinase (phospho-AKT) and glucose metabolism. The effect of LC supplementation on increased levels of PIP3 and glucose utilization was attenuated by the inhibitor of CSE that catalyzes $H_2S$ formation. In addition, exogenous treatment with PIP3 similarly increased AKT phosphorylation and glucose utilization. The increase in PIP3 by LC or $H_2S$ is mediated by the activation of PI3K and inhibition of phosphatase and tensin homolog (PTEN) but not SH2 domain containing inositol 5 phosphate phosphatase (SHIP2). This study provides evidence for a novel molecular mechanism by which $H_2S$ or LC supplementation can help improve glucose metabolism via activation of the PI3K/PIP3/phospho-AKT insulin signaling pathway using an adipocyte cell model.

**EXPERIMENTAL PROCEDURES**

**Materials**—PTEN, SHIP2, and glucose transporter 4 (GLUT4) antibodies were purchased from Abcam, Inc. (Cambridge, MA). AKT2, phospho-AKT (serine 473), phospho-NF-κB (p65) (serine 276), phospho-PKCζ (threonine 410/403), PKCζ, and PI3K (p85α) were purchased from Cell Signaling Technology (Beverly, MA). Insulin receptor substrate 1 (IRS1) and phospho-IRS1 (tyrosine 941) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). All other chemicals were purchased from Sigma unless otherwise mentioned.

**3T3L1 Cell Culture and Differentiation**—The murine 3T3L1 fibroblast cell line was obtained from American Type Culture Collection (ATCC, Manassas, VA). These cells were cultured in high glucose (HG) DMEM containing 10% (v/v) FCS, 100 units/ml penicillin, and 100 μg/ml streptomycin and maintained at 37 °C in a humidified atmosphere containing 5% (v/v) CO2. Three days after achieving confluent, to allow for differentiation into adipocytes, cells were incubated in HG DMEM containing 10% (v/v) FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin supplemented with 100 milliunits/ml insulin, 0.5 mM 3-isobutyl-1-methylxanthine, and 250 nM dexamethasone for 2 days. The cells were then placed in the same medium containing insulin but lacking any other supplements for an additional 2 days. The media were replaced every 2 days thereafter until >85% of the cells contained lipid droplets. Seven to 10 days after the induction of differentiation, 3T3L1 adipocytes were ready to be used in experiments (26). The cells were incubated with serum-free low glucose DMEM during the experimental incubation period.

**Treatment with HG, Mannitol, $H_2S$, and LC**—Cells were treated separately with normal glucose (7 mM) and HG (25 mM) without and with two different sources of $H_2S$, sodium sulfide ($Na_2S$) and LC. Mannitol was used as an osmolarity control. In the mannitol-treated group, cells were exposed to 18 mM mannitol and 7 mM glucose. In this study, cells were exposed to a HG concentration of 25 mM. Many previous studies have reported that glucose concentrations as high as 50 mM have been found in the blood of patients with uncontrolled diabetes (27). It is true that blood glucose levels in patients are not likely to stay as high as 25 mM for 24 h. However, tissue damage in diabetic patients occurs over many years of countless hyperglycemic episodes. Thus, the glucose concentration of 25 mM used in this cell culture study does not seem unreasonable. Cells were pre-treated with three different concentrations (100, 500, or 1000 μM) of LC and two different concentrations (10 or 100 μM) of $Na_2S$ for 2 h, followed by HG exposure for the next 20 h. Lipopolysaccharide (LPS) was used to stimulate the secretion of cytokines by HG in cell culture studies (28); cells were treated with LPS (2 ng/ml) at 37 °C either alone or along with the LC or $Na_2S$ supplementation against HG exposure. Thus, cells treated with LPS alone served as a control in this study. After treatment, cells were lysed in radioimmunoprecipitation assay buffer (50 mM Tris, pH 8, 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholic acid, 0.1% SDS) supplemented with protease and phosphatase inhibitors (1 mM PMSF, 5 μg/ml leupeptin, 2 μg/ml apronitin, 1 mM EDTA, 10 mM NaF, and 1 mM Na3VO4). Lysates were cleared by centrifugation, and total protein concentrations were determined using a BCA assay kit (Pierce/Thermo Scientific).

**Dose- and Time-dependent Studies with PIP3 against HG Exposure**—To investigate the role of PIP3, adipocytes were treated with normal glucose (7 mM) and HG (25 mM) without and with three different concentrations (1, 5, or 10 nM) of PIP3 for various time periods (2 and 4 h). After treatment, glucose utilization, the expression of AKT phosphorylation, and the intracellular PIP3 levels were investigated in experimental samples.

**siRNA Transient Transfection Studies**—siAKT2 and control siRNA were purchased from Cell Signaling Technology (Beverly, MA). siPKCζ was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). 3T3L1 adipocytes grown in 12-well plates were transiently transfected with 100 nM siRNA complex according to the manufacturer’s instructions. Briefly, 100 nM specified siRNA diluted in 100 μl of Opti-MEM (Invitrogen) was mixed with 4–6 μl of Lipofectamine 2000 transfection reagent (Invitrogen) and incubated for 30 min at room temperature to form siRNA complexes. The siRNA medium was formed by addition of 500 μl of growth medium to the siRNA complexes. Cells were incubated with siRNA medium for 5–6 h followed by incubation with fresh DMEM/10% FBS for 24 h.
After incubation the medium was aspirated, and cells were treated with PIP3, LC, or H₂S against HG exposure as mentioned above. After treatment, the supernatants were used for the glucose assay, and cell lysates were used for the immunoblotting studies with GLUT4 antibody. In parallel, lysates were immunoblotted with AKT2 and PKCζ antibodies to evaluate the efficiency of siRNA-mediated protein depletion.

Measurement of H₂S—In this study, the H₂S concentrations were measured in the cell culture medium following the method of Zhu et al. (29), which is based upon the old methylene blue methods. The limitation of this method is that this technique monitors not only free H₂S but also other species such as hydrosulfide anion (HS⁻) and sulfide (S²⁻). Briefly, 75 μl of medium was mixed with 250 μl of 1% (w/v) zinc acetate and 425 μl of distilled water, followed by the addition of 133 μl N-dimethyl-p-phenylenediamine sulfate (20 mm in 7.2 x HCl) and 133 μl of FeCl₃ in (30 mm in 1.2 M HCl) to the test tube. The mixture was incubated at room temperature for 10 min. The protein in the plasma was removed by adding 250 μl of 10% trichloroacetic acid to the reaction mixture which was pelleted by centrifugation. The absorbance of the resulting solution was measured at 670 nm in a 96-well plate with a microplate reader. The results are expressed as percentage change over control.

Detection of PIP3 Levels—The cellular PIP3 level was measured using the PIP3 Mass ELISA kit (Echelon Biosciences, Salt Lake City, UT). Appropriate controls and standards (specified by each manufacturer’s kit) were used each time. In addition to the ELISA method, PIP3 levels were also determined using flow cytometry. After treatment cells were washed in PBS-FCS buffer, fixed in 2% paraformaldehyde, permeabilized with 0.5% saponin in PBS, then washed and incubated overnight at 4 °C with anti-PIP3 antibody (Echelon) at a 1:40 dilution. The primary antibody was detected with a FITC-conjugated appropriate secondary antibody (Abcam). In each experiment, a minimum of 10,000 cells was analyzed (per treatment condition) by FACSCalibur flow cytometer (BD Biosciences) equipped with multicolor analysis capability. Gates were set to exclude nonviable cells, cell debris, and cells of abnormal size and shape.

Glucose Utilization, Cell Viability, GSH, and Cytokine Secretion—Glucose assays were done at 0 h and at other specified times. The glucose utilization level was determined by subtracting glucose values at specified times (leftover glucose) from the 0 h glucose level. All assays were done in duplicate at each time point (30). An advantage Accu-check glucometer (Roche Applied Science) was used for the glucose assay. Cell viability was determined using the Alamar Blue reduction bioassay (Alamar Biosciences, Salt Lake City, UT). Appropriate controls and standards (specified by each manufacturer) were used each time to check the variation from plate to plate on different days of analyses.

Immunoblotting—All samples contained approximately the same amount of protein (~20–40 μg) and were run as 8–10% SDS-PAGE and transferred to a nitrocellulose membrane. Membranes were blocked at room temperature for 2 h in blocking buffer containing 1% BSA to prevent nonspecific binding and then incubated with anti-PTEN (1:1000 dilution), anti-P38K (p85α) (1:1000), anti-AKT (AKT2) (1:1000 dilution), anti-NF-κB (p65) (1:1000 dilution), anti-PKCζ (1:1000), anti-GLUT4 (1:1000), anti-IRS1 (1:1000) and anti-phosphorylated IRS1 (tyrosine 941) (1:500), anti-phosphorylated AKT (serine 473) (1:500), anti-phosphorylated PKCζ/α (1:500) (threonine 410/403), and anti-phosphorylated NF-κB (p65) (serine 276) (1:500) primary antibodies at 4 °C overnight. The membranes were washed in TBS-T (50 mmol/liter Tris-HCl, pH 7.6, 150 mmol/liter NaCl, 0.1% Tween 20) for 30 min and incubated with the appropriate HRP-conjugated secondary antibody (1:5000 dilution) for 2 h at room temperature and developed using the ultrasensitive ECL substrate (Millipore, MA). The intensity of each immunoblotting band was measured using the histogram tool of Adobe Photoshop CS5.

Statistical Analysis—Data from cell culture studies were analyzed statistically using one-way analysis of variance (ANOVA) with Sigma Stat statistical software (Jandel Scientific, San Rafael, CA). When data passed a normality test, all groups were compared using the Student-Newman-Keuls method. A difference was considered significant at the p < 0.05 level.

RESULTS

Effect of LC and H₂S on PTEN, P38K, Phospho-AKT, PIP3, and Glucose Utilization in Adipocytes—Fig. 1 illustrates the effect of different concentrations of LC and of H₂S on PTEN, P38K, PIP3, phospho-AKT and glucose utilization levels in 3T3L1 adipocyte cells treated with HG. HG treatment caused a significant decrease in P38K activation (Fig. 1A) and increase in PTEN (Fig. 1B). Supplementation with LC prevented the decrease in P38K and increase in PTEN caused by HG. Similarly, treatment with H₂S caused a significant increase in P38K and a decrease in PTEN compared with treatment with HG alone. Fig. 1E illustrates that HG treatment decreased PIP3, which was replenished in cells supplemented with LC or H₂S. The increase in PIP3 mediated by LC in HG-treated cells was greater with increasing concentrations of LC. Notably, glucose utilization in cells was greater in the presence of LC or H₂S (Fig. 1F). HG treatment caused reduction in the AKT phosphorylation (Fig. 1D). Mannitol treatment did not have any significant effect on insulin signaling cascades or glucose utilization (data not shown). However, phosphorylation of AKT was significantly increased in the presence of different concentrations of LC or H₂S (Fig. 1D). Different treatments did not cause any change in cell viability (data not shown). High concentrations of substrate (glucose) are likely to increase glucose utilization.
When the intracellular glucose level surpasses its threshold value, cellular glucose auto-oxidation produces the reactive intermediate, which can cause oxidative stress, impair the PI3K/PTEN pathway, and suppress the cellular PIP3 level/AKT phosphorylation. These results demonstrate that supplementation with H2S and LC prevents the increase in PTEN and decrease in PI3K caused by HG and significantly increased AKT phosphorylation. The AKT phosphorylation parallels the increase in PIP3 levels (Fig. 1E) and glucose utilization (Fig. 1F) seen in adipocytes supplemented with LC or H2S against HG exposure.

Both PIP3 and glucose utilization levels were significantly higher in LC and H2S supplemented HG-treated adipocytes. The PIP3 concentration is up-regulated within cells by PI3K and is degraded by two specific phosphatases: PTEN and SHIP (31). PIP3 binds to the pleckstrin homology domain of AKT, promoting its translocation to the membrane. At the cell membrane AKT becomes phosphorylated, resulting in its activation. Our study did not observe any effect on SHIP2 levels in H2S- or LC-treated adipocytes (Fig. 1C). In addition to the ELISA method, the increase in PIP3 concentration was also confirmed using flow cytometric analysis (Fig. 1G). Our studies observed that LC or H2S supplementation prevented the HG-induced loss in intracellular PIP3 and thus shifted the corresponding histogram more closely to the control (right side of the diagram).
side corresponds to higher concentrations of cellular PIP3 (Fig. 1G).

Our findings are consistent with studies in the literature, which show that inhibition of PTEN expression using PTEN antisense oligonucleotides normalized blood glucose concentrations in ob/ob (obese) mice (32) and that overexpression of PTEN resulted in inhibition of insulin-induced PIP3 production and glucose uptake in 3T3L1 adipocytes (33). Many studies in the literature have implicated PTEN as a negative regulator of the insulin signaling pathway (34). Attenuation of PTEN expression by siRNA in 3T3L1 adipocytes enhanced insulin-stimulated AKT phosphorylation (35). Inhibition of PTEN has been a major target in the development of new drugs for the control of glycemia and its complications in diabetes. These experiments demonstrate that LC or H2S supplementation can potentially increase PIP3 and glucose utilization in adipocyte cell model.

Fig. 2 shows the effect of an inhibitor of CSE, which catalyzes H2S formation from LC, on PI3K, phospho-AKT (serine 473)/AKT (AKT2), and the levels of H2S and PIP3 as well as glucose utilization in adipocytes (3T3L1) exposed to HG. A, PI3K (p85α). B, H2S levels. C, PIP3 levels. D, phospho-AKT/AKT. E, glucose utilization. Cells were treated with PAG (10 mM) 5 min prior to LC (500 µM) supplementation followed by HG exposure. In PIP3 and glucose utilization assays, insulin was used as a positive control. Insulin (100 nM) was supplemented 2 h prior to HG (25 mM) exposure. Values are expressed as mean ± S.E. (error bars; n = 3). A difference was considered significant at the p < 0.05 level.
effects observed upon exogenous addition of standard H₂S per se in adipocytes. Insulin treatment used as a positive control increased both PIP3 and glucose utilization levels in adipocytes (Fig. 2, C and E). According to many reviews the concentrations of H₂S in human blood range from 10 to 100 nM (4, 12) even though some investigators suggest that tissue concentrations of H₂S may be lower. Human blood concentrations of LC range from 200 to 300 μM (36). LC at a concentration of 500 μM and 1000 μM is considered to be a pharmacological concentration. The medium used for cell culture studies does not contain any LC according to the analyses of medium provided by Sigma. These results demonstrate that supplementation with an exogenous source of H₂S can stimulate the PI3K/AKT/PIP3 insulin signaling pathway and glucose utilization in adipocytes.

**Role of PIP3 in Activation of AKT, IRS1, PKCζ/θ, GLUT4, and Glucose Utilization in Adipocytes**—PIP3 binds directly to the pleckstrin homology domain of AKT. This results in translocation of AKT to the plasma membrane. Likewise, phosphoinositide-dependent protein kinase 1 also contains a pleckstrin homology domain that binds directly to PIP3, causing it to translocate to the plasma membrane as well upon activation of PI3K. The co-localization of activated PI3K and AKT allows AKT to become phosphorylated on threonine 308 and serine 473, leading to the activation of AKT, which is required for the maintenance of glucose homeostasis. Phosphorylation of AKT and insulin signaling are critical components in the maintenance of whole body glucose homeostasis. The increased AKT activity is associated with increased PIP3 levels and diminished PTEN (37). PIP3 induces translocation, and subsequent activation of AKT is assessed by phosphorylation of its serine 473 residue (38). PIP3 plays a central role in the insulin signaling pathway and the maintenance of whole body glucose homeostasis.

Interestingly, no studies in the literature have examined whether PIP3 increases glucose utilization at the cellular level. Fig. 3 illustrates the effect of different concentrations of exogenous PIP3 on AKT phosphorylation and glucose utilization levels in adipocytes. Treatment with HG lowered AKT phosphorylation (Fig. 3A). However, supplementation with exogenous PIP3 increased both AKT phosphorylation (Fig. 3A) and glucose utilization (Fig. 3B). Fig. 3C shows that exogenous addition of PIP3 dose dependently increases the intracellular PIP3 concentration. The higher concentration of exogenous PIP3 is lower than its level in control cells. However, the decrease in intracellular PIP3 levels was maintained by the exogenous addition of PIP3 at 5 nM and 10 nM concentrations, so the 5 nM concentration of PIP3 was used for the subsequent experiments.

Fig. 4 illustrates the effect of PIP3, LC, or H₂S supplementation on the expression of IRS1, GLUT4, and PKCζ/θ phosphorylation in adipocytes. HG treatment alone significantly decreased the phosphorylation of IRS1 (Fig. 4A), PKCζ/θ (Fig. 4B), and GLUT4 (Fig. 4C) expression. However, supplementation with LC, H₂S, or PIP3 restored the phosphorylation of both
IRS1 and PKCζ/λ as well as GLUT4 activation. Comparative signal silencing studies with either siAKT2 or siPKCζ (siRNA of corresponding proteins AKT2 and PKCζ, respectively) are shown in Fig. 5. We observed that transient transfection with either siAKT2 or siPKCζ decreased glucose utilization (Fig. 5A). The effect of PKCζ silencing on glucose utilization was found to be more pronounced than the AKT2 silencing. The same effect was also observed in GLUT4 expression (Fig. 5B).

Insulin-stimulated glucose transport is regulated by the actions of two protein kinases, AKT/PKB (protein kinase B) (39) and atypical protein kinase C (aPKC) isoforms ζ and λ (40). Overexpression of a dominant negative mutant of PKCζ or PKCζ abrogates insulin-stimulated glucose uptake and GLUT4 translocation in adipose tissue (40, 41) and muscle cells (42, 43). Overexpression of dominant interfering PKB/AKT mutants or blocking antibodies is also reported to inhibit insulin-stimulated GLUT4 translocalization (39, 44). It has been reported that of these two aPKCs serve as terminal molecular switches that participate in turning on the glucose transport response, not only during insulin action but also during the actions of a number of other important agonists, including thiazolidones (45) and carbohydrates (46). It has been reported that binding of acidic lipids such as PIP3 with the regulatory domain of aPKC leads to molecular unfolding and increases enzymatic activity (47).

Thus, binding with PIP3 activates aPKC, which enhances more rapid GLUT4 translocalization. In our study, exogenous addition of PIP3, LC, or H₂S improved glucose utilization and GLUT4 activation in the adipocytes transfected with siAKT2 (see Fig. 5, left); however compared with those transfected with the siAKT2, no significant improvement was observed in the adipocytes transfected with siPKCζ (Fig. 5, right).

Effect of LC and H₂S on NF-κB, GSH, and Secretion of MCP-1 and Adiponectin in Adipocytes—The activation of transcription factor NF-κB has been well established in diabetic pathophysiology. Oh et al. (48) originally reported that NaHS inhibited the LPS-induced NF-κB activation in cultured RAW 264.7 macrophages. In agreement with the earlier findings (48), we also observed that pretreatment with either LC or H₂S could prevent transcriptional activity of NF-κB by increasing phosphorylation of the serine 276 residue of its p65 subunit activation in adipocytes treated with HG (Fig. 6A). GSH plays an important role in scavenging intracellular reactive intermediates. Recently, H₂S has been found to suppress oxidative stress in mitochondria by increasing the intracellular GSH concentration (11, 49). In this study we observed the same thing and found that either LC or H₂S supplementation prevented the

![FIGURE 4. Effect of PIP3, LC, and H₂S on the expression of phospho-IRS1/IRS1, phospho-PKCζ/λ/PKCζ, and GLUT4 in adipocytes (3T3L1) exposed to HG. A, phospho-IRS1 (tyrosine 941)/IRS1. B, phospho-PKCζ/λ (threonine 410/403)/PKCζ. C, GLUT4. Cells were pretreated with PIP3 (5 mM), LC (500 mM), or H₂S (100 mM) for 2 h prior to the HG (25 mM) exposure. Values are expressed as mean ± S.E. (error bars; n = 3). A difference was considered significant at the p < 0.05 level.](#)
This observation reveals the antioxidant role of LC or H$_2$S in intracellular GSH (Fig. 6B). This observation shows results with siAKT2 and the other panels expressed as mean ± S.E. (error bars; n = 3). A difference was considered significant at the p < 0.05 level.

**DISCUSSION**

Recent studies indicate that H$_2$S plays a potentially significant role in biological processes and that malfunctioning H$_2$S homeostasis may contribute to the pathogenesis of disease (1–3). Animal studies have demonstrated that supplementation with LC itself or protein rich in LC has improved glucose metabolism (20–24) and that H$_2$S or H$_2$S donor supplementation ameliorated atherogenic processes (23). However, elucidation of the mechanisms of action of H$_2$S or LC, a precursor of H$_2$S, is largely lacking. This study makes the novel observation that supplementation with LC or H$_2$S, a metabolite of LC, increases PIP3 and glucose utilization levels in an adipocyte cell model. The effect of LC or H$_2$S on PIP3 formation was mediated through activation of PTEN. Phosphatase PTEN dephosphorylates PIP3, and the PI3K enzyme generates the signaling lipid PIP3. It has been established that PIP3 plays a central role in the insulin signaling pathway and the maintenance of whole body glucose homeostasis (31, 53). However, to our knowledge, this study for the first time presents evidence that treatment with standard PIP3 per se increases the utilization of glucose at the cellular level in an adipocyte cell model. This study also demonstrated a positive effect on the activation of IRS1 and GLUT4 translocation in adipocytes pretreated with LC or H$_2$S. H$_2$S is synthesized from LC in mammals by four enzymes, cystathionine $\beta$-synthase, CSE, 3-mercaptoppyruvate sulfurtransferase, and cysteine aminotransferase (8, 9, 13), of which CSE is likely to be the most physiologically relevant (3). The inhibitor of CSE significantly lowered levels of H$_2$S, L-Cysteine, and PIP3 resistance (52). It has been reported that adiponectin inhibits hepatic glucose production by down-regulating the enzymes involved in gluconeogenesis, such as glucose 6-phosphatase and phosphoenolpyruvate carboxykinase, thereby decreasing blood sugar levels. Adiponectin treatment up-regulated the glucose uptake and fatty acid oxidation in skeletal muscle cells or a muscle cell line (52). Pretreatment with either LC or H$_2$S increased the adiponectin secretion in a dose-dependent manner in adipocytes exposed to HG (Fig. 6D). These results suggest an antioxidant and anti-inflammatory nature for LC and H$_2$S.

In our study we used insulin as a positive control. We observed that insulin treatment prevented the HG-induced alterations in the expression of PI3K (Fig. 7A), PTEN (Fig. 7B), GLUT4 (Fig. 7C), and the PIP3 level (Fig. 7D) as well as glucose utilization (Fig. 7E). Fig. 7 shows that both LC and H$_2$S have no effect on the cells not exposed with HG compared with control. We have also investigated the effect of PAG on the cells, either alone or with HG exposure (Fig. 7). It has been found that in both cases (with or without HG exposure) PAG treatment did not cause any alteration to the insulin signaling cascades or glucose utilization compared with the control or HG-treated groups, respectively.
GLUT4, and PI3K have documented that inhibition of PTEN and activation of PI3K and AKT signaling molecules are crucial in insulin signaling pathways and the maintenance of whole body glucose metabolism (54). Taken together, results from this study demonstrate that LC or H2S supplementation can increase cellular PIP3 levels and up-regulate insulin signaling pathways and glucose metabolism. These studies demonstrate the potential significant effect of circulating H2S and LC levels on glucose metabolism in the body.

The PI3K/PTEN pathway plays a central role in mediating insulin responses via insulin receptor tyrosine kinase, which phosphorylates insulin receptor substrate proteins (e.g. IRS1 and IRS2) that nucleate downstream signaling proteins. PI3K consists of a p110 catalytic subunit and a p85α regulatory subunit. The p85 subunit of PI3K modulates insulin sensitivity via effects on PTEN (55). Cells that lack PTEN show elevated PIP3 levels and activated AKT-dependent signaling (37). In addition to AKT signaling, recent studies in the literature suggest that aPKC (PKCζ/λ) are also the downstream regulators of PI3K, and their activation is required for insulin-stimulated glucose uptake, which involves translocation of the major insulin-responsive glucose transporter GLUT4 from intracellular sites to the cell membrane (40 – 42). This study also demonstrates that LC, H2S, or PI3P increased the phosphorylation of IRS1, AKT, and PKCζ/λ as well as GLUT4 activation in adipocytes. IRS1 is the major substrate of insulin receptor kinase and has many tyrosine phosphorylation sites, which provide binding sites for many kinases including the 85-kDa subunit of PI3K. Tyrosine phosphorylation of IRS1 also binds PI3K p85, thereby activating PI3K (56), leading to the activation of glucose transport into cells through glucose transporters. GLUT4 is the major glucose transporter protein involved in transport of glucose across adipocytes, from either inside or outside depending upon the insulin stimulus. Comparative signal silencing studies showed that either siAKT2 or siPKCζ could reduce glucose utilization but that transfection with siPKCζ caused a pronounced decrease in glucose utilization and GLUT4 activation compared with siAKT2 transfection. Treatment with LC, H2S, or PIP3 increased the glucose utilization and GLUT4 activation against HG exposure in the siAKT2-transfected cells, but the effect was significantly attenuated in the siPKCζ silencing study. This may be due to the high affinity of aPKC for the acidic lipid PIP3, a positive regulator of glucose metabolism, compared with the AKT/PKB binding (47). Thus, either LC or H2S can play a role in the metabolic actions of insulin by up-regulating the cellular PIP3, and thus any reduction in their blood levels may impair insulin action and glucose homeostasis.

Diabetes is associated with lower circulating levels of LC and H2S (15, 17, 18). Animal studies report that supplementation with dietary LC or compounds containing LC can improve glucose metabolism and lower hyperglycemia (20 – 24). Chemicals that inhibit PTEN or activate PI3K have been considered as potential therapeutic candidates for improvement of glucose metabolism in type 2 diabetes. Using an adipocytes cell model, this study provides evidence for a molecular mechanism by which impaired blood concentrations of H2S or LC can down-regulate insulin signaling pathways essential for the maintenance of glucose metabolism. Further studies are needed to determine the role of PTEN/PI3K/AKT/PKCζ/A and PIP3 levels in the liver or adipose tissue of diabetic animals after cys-
teinate supplementation. The in vivo studies are needed to demonstrate the clinical viability of our results. If this discovery leads to tangible clinical results, it would have an impact on the development of novel molecules, containing sulfide and cysteine moieties, for the improvement of glucose metabolism in diabetes.

LC is also a precursor of GSH. This study observed increases in cellular GSH in LC- or H₂S-supplemented adipocytes, which confirms previous reports that both LC and H₂S can decrease cellular oxidative stress. This can influence the expression of multiple genes in cells, including signaling molecules such as NF-κB and PIP3 (via inhibition of PTEN and PI3K activation). Some of these effects may be mediated through inhibition of oxidative stress, and others may be independent of oxidative stress. These genes regulate the production of proinflammatory cytokines and insulin sensitivity. LC and GSH are important components of redox signaling pathways and are implicated in the reduction of cellular oxidative stress and T cell functions (18, 57). Oxidative stress associated with diabetes activates NF-κB, which then can activate the insulin resistance cascade (58). Overexpression of MCP-1 causes inhibition of AKT phosphorylation and tyrosine phosphorylation in liver and skeletal muscle, macrophage recruitment, and insulin resistance in a P2-MCP-1 mouse model (59). MCP-1 can phosphorylate AKT independent of activation by the PI3K pathway. Adiponectin is a positive regulator of insulin sensitivity. Thus, inhibition of

FIGURE 7. Effect of LC, H₂S, PAG, and insulin on the expression of PI3K, PTEN, and GLUT4, PIP3 levels and glucose utilization in adipocytes (3T3L1). Effects of LC, H₂S, and PAG were investigated without HG exposures and the effects of insulin and PAG were investigated with HG exposure. The cell treatment procedure was the same as described previously. A. PI3K (p85α). B. PTEN. C. GLUT4. D. PIP3. E. glucose utilization. Values are expressed as mean ± S.E. (error bars; n = 4). A difference was considered significant at the p < 0.05 level.
MCP-1 and increased adiponectin secretion can also positively influence the metabolic actions of insulin in H₂S- or LC-supplemented adipocytes.

It is not yet completely understood how H₂S is metabolized in the body (2, 3). Some reports suggest that H₂S reacts readily with methemoglobin to form sulhemoglobin, which may act as a metabolic sink for H₂S. It can rapidly be oxidized to thiosulfate (SO₃²⁻) by mitochondria and is subsequently converted to sulfate (SO₄²⁻) and sulfite (SO₃⁻²). H₂S can also undergo methylation by thiol-S-methyltransferase to yield methanethiol (CH₃SH) and dimethylsulfide (CH₃SCH₃) and is also a substrate (especially in the colon) for rhodanese (thiosulfate:cysteine sulfurtransferase), which leads to the formation of thiocyanate (SCN⁻) and SO₄²⁻. In addition to the biochemical means by which H₂S is catabolized, H₂S is a powerful reducing agent by which H₂S is catabolized, H₂S is a powerful reducing agent. It can rapidly be oxidized to thiosulfate (SO₃²⁻) and sulfite (SO₃⁻²⁻) and is likely to be consumed by endogenous oxidant species in the mitochondria and is subsequently converted to sulfate (SO₄²⁻) and sulfite (SO₃⁻²⁻). H₂S can also undergo methylation by thiol-S-methyltransferase to yield methanethiol (CH₃SH) and dimethylsulfide (CH₃SCH₃) and is also a substrate (especially in the colon) for rhodanese (thiosulfate:cysteine sulfurtransferase), which leads to the formation of thiocyanate (SCN⁻) and SO₄²⁻. In addition to the biochemical means by which H₂S is catabolized, H₂S is a powerful reducing agent and is likely to be consumed by endogenous oxidant species in the vasculature, such as peroxynitrite, superoxide, and hydrogen peroxide (2, 3).

It appears that H₂S enters cells via active diffusion and without any facilitator (60). Studies using CSE knock-out mice, or pharmacological inhibition of CSE, which catalyzes formation of H₂S, demonstrate that H₂S plays an important physiological role in hypoxic sensing in the carotid body (61, 62). The beneficial role of H₂S in increasing thermotolerance and lifespan has been observed in Caenorhabditis elegans (63). The studies of Elrod et al. suggest that H₂S attenuates myocardial ischemia-reperfusion injury by preservation of mitochondrial function (64). Recent studies demonstrate that H₂S mediates the vaso-activity of garlic, which is also rich in sulfides and LC (65). Garlic supplementation has also been shown to lower hyperglycemia in streptozotocin-treated diabetic rats (66). An increasing number of studies in the literature indicate a potential benefit of H₂S with respect to some aspects of cardiovascular disease (1–3).

In conclusion, this is the first report to demonstrate that H₂S or its precursor LC can increase cellular levels of PI3K, a positive regulator of glucose metabolism. Fig. 8 outlines a schematic representation of a proposed mechanism by which H₂S or LC can positively modulate glucose metabolism. Diabetes is associated with hyperglycemia and increased oxidative stress, which can up-regulate PTEN and down-regulate PI3K. This results in a decrease in cellular PI3P, a positive regulator of insulin signaling pathways and glucose metabolism. LC or H₂S can lower oxidative stress and inhibit PTEN and activate PI3K, which leads to elevated levels of PI3P or AKT/PI3K/phosphorylation, IRS1 phosphorylation, and GLUT4 activation, resulting in up-regulation of the metabolic actions of insulin and an improvement in glucose metabolism. Using an adipocyte cell model, this work provides evidence for a molecular mechanism by which H₂S or LC may help improve glucose metabolism. Future studies are needed to validate the proposed biochemical mechanism by which LC or H₂S may up-regulate insulin signaling cascades and improve glucose metabolism in diabetic animals and diabetic patients.

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REFERENCES
1. Kabil, O., and Banerjee R. (2010) J. Biol. Chem. 285, 21903–21907
2. Wang, R. (2010) Antioxid. Redox Signal. 12, 1061–1064
3. Li, L., Rose, P., and Moore, P. K. (2011) Annu. Rev. Pharmacol. Toxicol. 51, 169–187
4. Yang, G., Wu, L., Jiang, B., Yang, W., Qi, J., Cao, K., Meng, Q., Mustafa, A. K., Mu, W., Zhang, S., Snyder, S. H., and Wang, R. (2008) Science 322, 587–590
5. Iha, S., Calvert, J. W., Duranski, M. R., Ramachandran, A., and Lefer, D. J. (2008) Am. J. Physiol. Heart Circ. Physiol. 295, H801–806
6. Singh, S., Padovano, D., Leslie, R. A., Chiku, T., and Banerjee R. (2009) J. Biol. Chem. 284, 22457–22466
7. Ishigami, M., Hiraki, K., Umemura, K., Ogasawara, Y., Ishii, K., and Kimura, H. (2009) Antioxid. Redox Signal. 11, 205–214
8. Shibuya, N., Tanaka, M., Yoshida, M., Ogahsawara, Y., Togawa, T., Ishii, K., and Kimura, H. (2009) Antioxid. Redox Signal. 11, 703–714
9. Shibuya, N., Mikami, Y., Kimura, Y., Nagahara, N., and Kimura, H. (2009) J. Biochem. 146, 623–626
10. d’Emmanuele di Villa Bianca, R., Sorrentino, R., Maffia, P., Miron, V., Imbimbo, C., Fusco, F., De Palma, R., Ignarro, L. J., and Cirino, G. (2009) Proc. Natl. Acad. Sci. U.S.A. 106, 4513–4518
11. Kimura, Y., goto, Y., and Kimura, H. (2010) Antioxid. Redox Signal. 12, 1–13
12. Wang, Y., Zhao, X., Jin, H., Wei, H., Li, W., Bu, D., Tang, X., Ren, Y., Tang, C., and Du, J. (2009) Arterioscler Thromb Vasc. Biol. 29, 173–179
13. Abe, K., and Kimura, H. (1996) J. Neurosci. 16, 1066–1071
14. Cai, W. J., Wang, M. J., Moore, P. K., Jin, H. M., Yao, T., and Zhu, Y. C. (2007) Cardiovasc. Res. 76, 29–40
15. Sekhar, R. V., McKay, S. V., Patel, S. G., Guthikonda, A. P., Reddy, V. T., Balasubramaniam, A. and Jahoof, F. (2011) Diabetes Care 34, 162–167
16. Darmoun, D., Smith, S. D., Sweeten, S., Hartman, B. K., Welch, S., and Mauras, N. (2008) Pediatr. Diabetes 9, 577–582
17. Jain, S. K., Bull, R., Rains, J. L., Bass, P. F., Levine, S. N., Reddy, S., McVie, R., and Bocchini, J. A. (2010) Antioxid. Redox Signal. 12, 1333–1337
18. Whitman, M., Gooding, K. M., Whatmore, J. L., Ball, C. I., Mawson, D., Skinner, M., Tooke, J. E., and Shore, A. C. (2010) Diabetologia 53, 1722–1726
19. Dröge, W. (2005) Philos. Trans. R. Soc. Lond. B Biol. Sci. 360, 2355–2372
20. Blouet, C., Mariotti, F., Mikogami, T., Tome, D., and Huneau, J. F. (2007) J. Nutr. Biochem. 18, 519–524
21. Blouet, C., Mariotti, F., Azzouz-Marniche, D., Mathé, V., Mikogami, T., Tomé, D., and Huneau, J. F. (2007) Free Radic. Biol. Med. 42, 1089–1097
