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Journal of Cellular Biochemistry 114:2334–2345 (2013)

Journal of Cellular Biochemistry

L-Cysteine and Hydrogen Sulfide Increase PIP3 and AMPK/PPARγ Expression and Decrease ROS and Vascular Inflammation Markers in High Glucose Treated Human U937 Monocytes

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ABSTRACT

Diabetic patients have lower blood levels of L-cysteine (LC) and hydrogen sulfide (H_2S) and a higher incidence of vascular inflammation. This study examined whether impaired LC or H_2S levels affect vascular inflammation markers in diabetes. Human U937 monocytic cells were treated with high-glucose (HG, 25 mM, 20 h) in the presence or absence of LC (100, 500, or 1,000 μM, an endogenous precursor of H_2S) or Na_2S (5 or 25 μM, an exogenous source of H_2S). Both LC and Na_2S supplementation decreased intracellular ROS production and increased cellular PIP3 (phosphatidylinositol-3,4,5-trisphosphate) in HG-exposed cells. The effect of LC on PIP3 was prevented by propargylglycine, an inhibitor of cystathionine-γ-lyase (CSE) that catalyzes H_2S formation from LC. Signal silencing studies with CSE siRNA also showed the inhibition of H_2S formation and PIP3 upregulation in LC-supplemented CSE knockdown cells exposed to HG. This demonstrates that H_2S plays a role in mediating the effect of LC on increased PIP3. Using the PI3K specific inhibitor LY294002, this study demonstrated that PI3K activation mediates the effect of LC and Na_2S on PIP3 upregulation. Results showed that supplementation with LC and Na_2S reduced NF-κB phosphorylation and the secretion of TNF-α, MCP-1, IL-8, IL-1β, and IP-10. Treatment with LC (500 μM), Na_2S (25 μM), and PIP3 (5 nM) increased the AMPK phosphorylation and PPARγ expression in cells exposed to HG. This study reports for the first time a novel molecular mechanism by which Na_2S or LC supplementation can lower oxidative stress and various markers of vascular inflammation in diabetes. J. Cell. Biochem. 114: 2334–2345, 2013. © 2013 Wiley Periodicals, Inc.

KEY WORDS: L-CYSTEINE; H_2S; PIP3; DIABETES; VASCULAR INFLAMMATION

The increasing prevalence of diabetes worldwide and its associated complications is a significant burden to human health. Inflammatory processes have been implicated in the onset of diabetes and the progression of its complications [Shoelson et al., 2006]. The existence of chronic inflammation in diabetes is demonstrated by increased plasma concentrations of pro-inflammatory cytokines, such as TNF-α, MCP-1, IL-8, IL-1β, etc. [Hotamisligil, 2006; Shoelson et al., 2006; Jain et al., 2007; Li et al., 2010]. Vascular inflammation and activation of monocytes may cause loss of insulin secretory function by islet cells in type 1 diabetes and may also play an important role in enhancing insulin resistance in type 2 diabetes [Shurtz-Swirski et al., 2001; Devaraj et al., 2007; King, 2008]. Several earlier lines of investigation suggest that oxidative stress plays a role in the pathogenesis of vascular inflammation in diabetes and its complications [Huerta and Nadler, 2004; Rains and Jain, 2011].

Cysteine, a well known conditionally essential sulfur-containing amino acid, plays an important role in controlling protein structure and stability [Stipanuk et al., 2006]. In addition to its role in protein synthesis, cysteine is also used in the synthesis of coenzyme A, taurine, and glutathione, an essential molecule for the reduction of cellular oxidative stress [Stipanuk et al., 2006]. Recent studies report that diabetic patients experience altered cysteine homeostasis, which causes lower blood levels of LC [Darmaun et al., 2008; Sekhar

The authors have declared that no conflict of interest exists.

Grant sponsor: NIDDK; Grant sponsor: Office of Dietary Supplements of the National Institutes of Health; Grant number: RO1 DK072433; Grant sponsor: Malcolm Feist Endowed Chair in Diabetes; Grant sponsor: Malcolm Feist Cardiovascular Research Endowment; Grant sponsor: LSU Health Sciences Center, Shreveport.

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Manuscript Received: 15 March 2012; Manuscript Accepted: 18 April 2013

Accepted manuscript online in Wiley Online Library (wileyonlinelibrary.com): 4 June 2013

DOI 10.1002/jcb.24578 • © 2013 Wiley Periodicals, Inc.

et al., 2011]. A number of previous studies have shown that dietary supplementation with *N*-acetyl cysteine (NAC) or whey protein and α-lactoalbumin (cysteine rich proteins) lowers the oxidative stress and insulin resistance induced by sucrose or fructose in rats and streptozotocin-treated diabetic mice [Haber et al., 2003; Song et al., 2005; Diniz et al., 2006; Blouet et al., 2007a,b]. Oral supplementation with LC lowered oxidative stress and vascular inflammation markers in ZDF rats, an animal model of type 2 diabetes [Jain et al., 2009]. Studies with type 2 diabetic patients and normal subjects revealed that LC supplementation lowered oxidative stress markers in the diabetic population compared to those found in the normal population [Darmaun et al., 2008; Sekhar et al., 2011]. Recent studies report that diabetic patients and diabetic animals also have reduced blood levels of H_2S [Brancaleone et al., 2008; Jain et al., 2010; Whiteman et al., 2010]. H_2S is increasingly being recognized as an important signaling molecule and has been shown to modulate a variety of biological effects that may mediate the protection of various organ dysfunctions [Szabó, 2007; Whiteman et al., 2011; Jain et al., 2012a,b; Manna and Jain, 2012a]. Studies in the literature report the protection of pancreatic β-cells from oxidative stress by H_2S via an anti-oxidative mechanism and the activation of AKT signaling [Kaneko et al., 2009; Taniguchi et al., 2011]. H_2S is produced *in vivo* from LC via the action of enzymes, including cystathionine β-synthase (CBS), cystathionine-γ-lyase (CSE) and 3-mercaptopyruvate sulfur transferase (3-MST) [Hosoki et al., 1997; Yang et al., 2008; Shibuya et al., 2009]. There is no previous study in the literature describing whether impaired blood levels of LC or H_2S affect vascular inflammation in diabetes. Using a U937 human monocyte cell model, this study reports that both LC and Na_2S supplementation can inhibit ROS, increase PIP3, upregulate AMPK phosphorylation and PPARγ expression, and lower the levels of pro-inflammatory cytokines in high-glucose treated cells.

MATERIALS AND METHODS

MATERIALS

CSE, PTEN and phospho NF-κB (p65) (serine 276) antibodies were purchased from Abcam, Inc. (Cambridge, MA). Phospho AMPKα1/2 (Thr 172), PI3K (p85α), and PPARγ antibodies were purchased from Cell Signaling Technology (Beverly, MA). AMPKα1/2 and NF-κB (p65) antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise mentioned.

HUMAN PRO-MONOCYTIC CELL LINE

The U937 monocytic cell line was obtained from American Type Culture Collection (ATCC, Manassas, VA). These cells were maintained at 37°C in RPMI 1640 medium containing 7 mM glucose, 10% (v/v) heat-inactivated FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 12 mM sodium carbonate, 12 mM HEPES, and 2 mM glutamine in a humidified atmosphere containing 5% (v/v) CO_2. For treatments, cells were washed once in plain RPMI 1640 before being suspended in fresh medium (complete) containing serum and other supplements [Jain et al., 2002]. For treatments, the cells were counted on a hemocytometer using Trypan Blue exclusion; approximately one million cells/mL were used in each treatment group.

TREATMENT WITH HIGH GLUCOSE (HG), MANNITOL, L-CYSTEINE (LC), AND SODIUM SULFIDE (NA_2S)

Cells were treated with normal glucose (7 mM) and HG (25 mM) with and without LC (endogenous precursor of H_2S) and sodium sulfide (Na_2S, an exogenous source of H_2S). In this study control cells were exposed to media with a concentration of 7 mM glucose. In our bodies glucose is continuously degraded and formed to maintain a 5 mM blood glucose level. However, in cell culture studies, we observed that incubating cells in media with a concentration of 5 mM glucose for 24 h caused a decrease in glucose concentration to levels lower than 2 mM. In cell culture studies, glucose gets metabolized but is not replaced. For this reason we chose a concentration of 7 mM, since in our experience glucose concentration of 7 mM does not lead to glucose deficiency at 24 h incubation. In high glucose studies, cells were exposed to a high glucose 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 [Candiloros et al., 1995]. 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 pretreated with three different concentrations of LC (100, 500, or 1,000 μM) and two concentrations of Na_2S (5 or 25 μM) for 2 h followed by HG exposure for the next 20 h. Mannitol was used as an osmolarity control. In the mannitol treated group, cells were exposed to 18 mM mannitol since the media contains 7 mM glucose. LPS (lipopolysaccharide) was used to stimulate the secretion of cytokines by HG in cell culture studies [Guha et al., 2000]. Thus, in this study, cells were treated with LPS (2 ng/mL) at 37°C either alone or in conjugation with the LC or Na_2S supplementation against HG exposure. Cells treated with LPS alone served as the control for this study. After treatment, cells were lysed in radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris pH 8, 150 mM NaCl, 1% NP-40, 0.5% deoxycholic acid, 0.1% SDS) supplemented with protease and phosphatase inhibitors (1 mM PMSF, 5 μg/mL leupeptin, 2 μg/mL aprotinin, 1 mM EDTA, 10 mM NaF, and 1 mM $NaVO_4$). Lysates were cleared by centrifugation and total protein concentrations were determined using the BCA assay (Pierce/Thermo Scientific, Rockford, IL). The medium used for cell culture studies does not contain any LC according to the analyses of medium provided by the Sigma Chemical Co. (St. Louis, MO). Although this pretreatment protocol is artificial in the context of diabetes, the study protocol is in agreement with the other studies in the literature [Guan et al., 2012; Lee et al., 2012].

TREATMENT WITH EXOGENOUS PIP3 AGAINST HG EXPOSURE

To investigate the effect of PIP3 against HG exposure, cells were treated with normal glucose (7 mM) and HG (25 mM, 20 h) with and without exogenous PIP3 supplementation (5 nM, 4 h) [Manna and Jain, 2011]. Anionic phosphatidylinositol phosphate derivatives were delivered across the cell membrane by complexing with a positive lysine-rich histone carrier following the procedure described by the manufacturer (Echelon Biosciences, Inc., Salt Lake City, UT).

Unlabeled Histone H1 (Echelon Biosciences) and PIP3 (Echelon Biosciences) were mixed at a molar ratio of 1:1, vortexed, subjected to sonication, and incubated for 10 min at room temperature. The mixture was then added to the medium of monocytes to a final concentration of 5 nM. After treatment, cells were lysed in RIPA supplemented with protease and phosphatase inhibitors. Lysates were cleared by centrifugation and total protein concentrations were determined using a BCA assay kit (Pierce/Thermo Scientific, Rockford, IL).

SIGNAL SILENCING STUDIES

CSE siRNA and control siRNA were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Cells were transfected with 100 nM siRNA complex following the method described earlier [Manna and Jain, 2011]. Briefly, 6 μL 10 μM specified siRNA diluted in 100 μL transfection media (Santa Cruz) was mixed with 2 μL Lipofectamine™ 2000 transfection reagent (Invitrogen, Carlsbad, CA) and incubated for 30 min at room temperature to form siRNA complexes. The siRNA medium was formed by addition of 500 μL transfection media to the siRNA complexes to yield a concentration of 100 nM specified siRNA. Cells were incubated with siRNA medium for 5–6 h followed by incubation with fresh RPMI/10% FBS media for the next 24 h. After incubation the medium was aspirated and the cells were treated with LC followed by HG described a bone. After treatment, the supernatants were used for the H_2S assay and the cell lysates were used for the immunoblotting studies with CSE antibody (1:1,000) and the PIP3 assays.

MEASUREMENT OF CELLULAR PIP3 LEVELS

Cellular PIP3 concentrations were measured using a PIP3 Mass ELISA Kit (Echelon Biosciences, Inc., Salt Lake City, UT). Appropriate controls and standards (specified by each manufacturer's kit) were used every time. The reactivity of the ELISA plate with rsprvt to PIP3 has been verified by using different polyphosphoinositides [e.g., PI (3,4)P2 and PI(4,5)P2] as well a inhibitor of PI3K.

DETECTION OF INTRACELLULAR ROS LEVELS

Intracellular reactive oxygen species (ROS) levels were measured using the fluorescent dye H_2DCFDA (2′,7′-dichlorofluorescein diacetate) [Rubinstein et al., 2008]. After treatment, cells were washed once with PBS and then loaded with 5 μM H_2DCFDA in PBS with 4% FBS. The cells were incubated at 37°C for 30 min in the dark and subsequently washed with PBS, harvested in PBS with 0.5% Triton X-100, centrifuged at 12,000*g* for 10 min at 37°C, and the supernatant collected. The intensity of DCF fluorescence in the supernatant was read at excitation and emission wavelengths of 488 and 530 nm, respectively, using a multidetection microplate reader (Synergy HT, BIOTEK). The change in intracellular ROS levels was plotted as mean fluorescence intensity (MFI). The oxidative stress sensitive dye DCFH-DA diffuses passively through the cellular membrane. Intracellular esterase activity causes the formation of DCFH, a nonfluorescent compound, which emits fluorescence when it is oxidized to DCF. Several studies [Wang and Joseph, 1999; Myhre et al., 2003] in the literature reported that DCFH is sensitive towards the oxidation by peroxynitrite ($ONOO^-$), hydrogen peroxide (H_2O_2) (in combination with cellular peroxidases), peroxidases alone, hydroxyl radical ($OH^{\cdot}$), nitric oxide (NO), hypochlorous acid (HOCl), and superoxide ($O_2^{\cdot -}$). Among them, $OH^{\cdot}$ and $ONOO^-$ oxidize DCFH within seconds or a few minutes whereas the other oxidants need higher concentrations and more time. Also, the simultaneous presence of cellular peroxidases is necessary for DCFH oxidation by H_2O_2. Furthermore, there are conflicting findings regarding the inhibition of DCF formation by superoxide dismutase; some studies report an inhibitory effect [Atlante et al., 1997; Scott et al., 1988], while others show no effects [Takeuchi et al., 1996; Yang et al., 1997]. Although ROS measurement by DCFH-DA is nonspecific, this dye has been widely used to measure the formation of overall intracellular reactive intermediates.

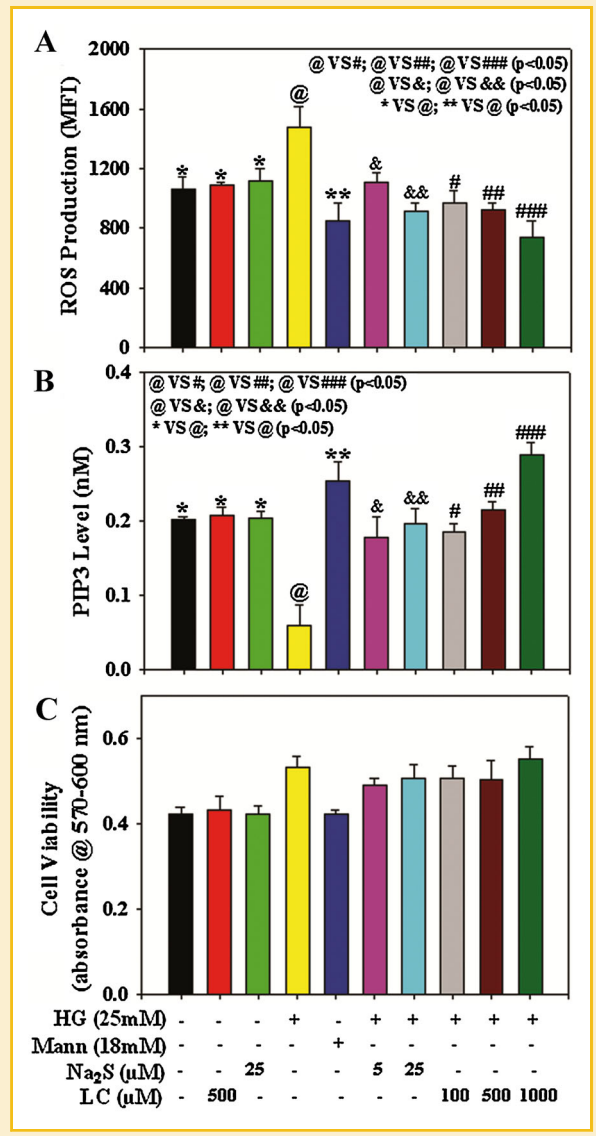

Fig. 1. Effect of L-cysteine (LC) and sodium sulfide (Na_2S) on the intracellular ROS production and PIP3 levels in human U937 monocytes either alone or with high glucose (HG). A: ROS production, (B) PIP3 levels, and (C) cell viability. Cells were pretreated with LC (100, 500, or 1,000 μM) or Na_2S (5 or 25 μM) for 2 h followed by HG (25 mM) exposure for the next 20 h. Values are expressed as mean ± SE (n = 3). A difference was considered significant at the $P < 0.05$ level.

CELL VIABILITY AND CYTOKINE STUDIES

Cell viability was determined using the Alamar Blue reduction bioassay (Alamar Biosciences, Sacramento, CA). This method is based upon Alamar Blue dye reduction by live cells. IL-8 and IL-1β levels in the supernatants of treated cells were determined by the sandwich ELISA method using a commercially available kit from Fisher Thermo Scientific Co. (Rockford, IL). MCP-1 and IP-10 levels were determined using commercially available kits from R&D Systems, Inc. (Minneapolis, MN). TNF-α levels were determined using a commercially available kit from eBiosciences (Vienna, Austria). All appropriate controls and standards as specified by each manufacturer's kit were used. In the cytokine assay, control samples were analyzed each time to check the variation from plate to plate on different days of analysis.

MEASUREMENT OF H_2S CONCENTRATION

H_2S concentrations in the cell culture supernatant were measured following the method of Zhu et al. [2007], which is based upon the formation of Methylene Blue. In this study, approximately 1 million cells/ml were used in each treatment group. After treatment the cell suspensions were centrifuged, the cells pelleted, and the supernatants were used for the H_2S assay. Briefly, 200 μl supernatant was mixed with 600 μl 1% (w/v) zinc acetate to trap H_2S and form zinc sulfide, followed by the addition of 400 μl *N*-dimethyl-*p*-phenylenediamine sulfate (20 mM in 7.2 M HCl) and 400 μl $FeCl_3$ (30 mM in 1.2 M HCl) to the test tube. After the reaction mixture was incubated in the dark at room temperature for 20 min, 250 μl of 10% (w/v) tricholoacetic acid was added to precipitate any protein that might be present in the culture media. Subsequently the reaction mixture was centrifuged at 10,000*g* for 10 min. The absorbance of the resulting solution was determined at 670 nm with a microplate reader. H_2S concentration was calculated using a calibration curve for standard Na_2S. The results were expressed as nmol/mg protein/h.

IMMUNOBLOTTING

All samples, which contained approximately the same amount of protein (~20 to 40 μg), were run on 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

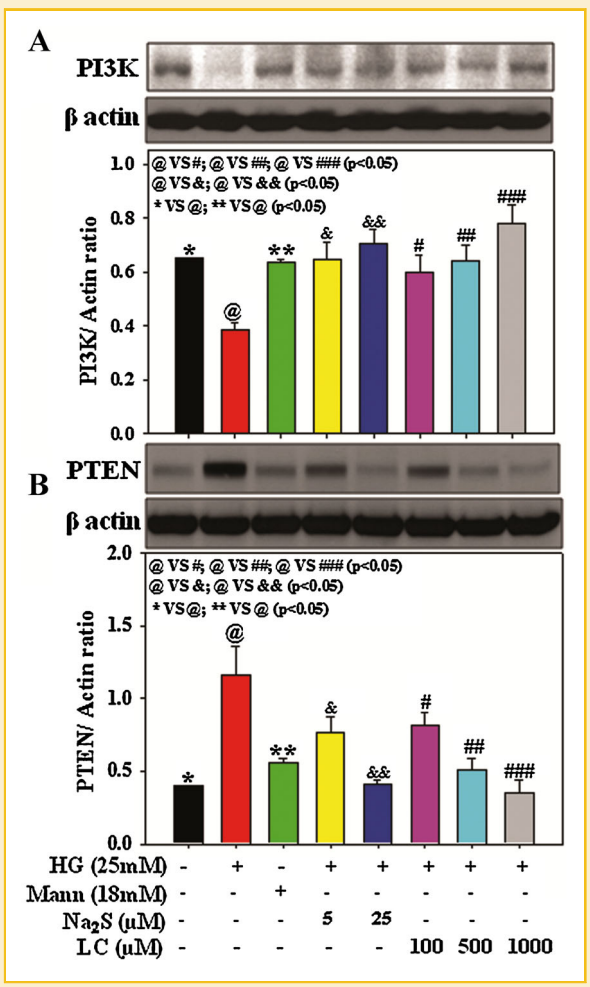

Fig. 2. Effect of L-cysteine (LC) and sodium sulfide (Na_2S) on the expression of PI3K and PTEN in U937 monocytes exposed to high glucose (HG). A: PI3K and (B) PTEN. Cells were pretreated with LC (100, 500, or 1,000 μM) or Na_2S (5 or 25 μM) for 2 h followed by HG (25 mM) exposure for the next 20 h. Values are expressed as mean ± SE (n = 3). A difference was considered significant at the $P < 0.05$ level.

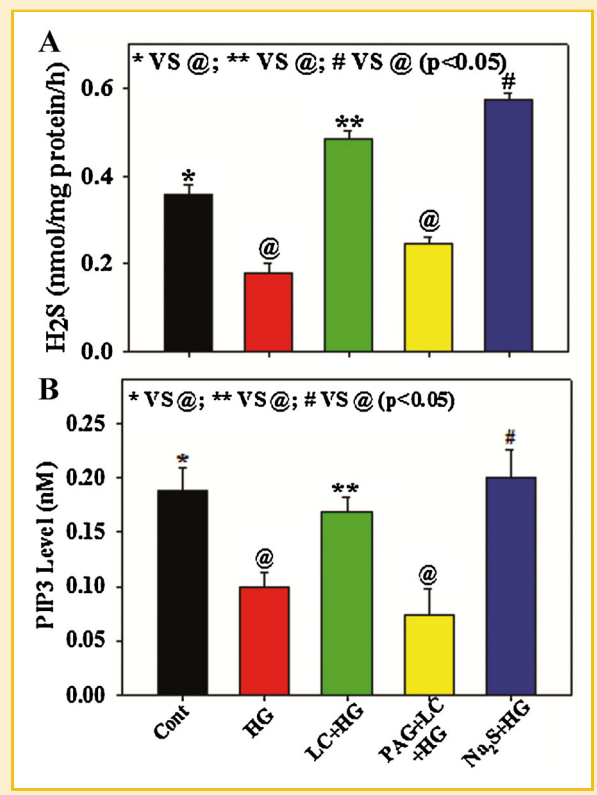

Fig. 3. Effect of propargylglycine (PAG), an inhibitor of CSE (enzyme that catalyzes H_2S formation from LC), on H_2S formation and PIP3 levels in U937 monocytes. A: H_2S formation and (B) PIP3 levels. Cells were treated with PAG (10 mM) 5 min prior to LC (500 μM) supplementation followed by HG exposure. C: represents the effect of one PI3K inhibitor, LY294002 (30 μM), on PIP3 levels in cells exposed to LC (500 μM) or Na_2S (25 μM) followed by HG exposure. Values are expressed as mean ± SE (n = 3). A difference was considered significant at the $P < 0.05$ level.

prevent nonspecific binding and then incubated with anti-PTEN (1:1,000 dilution), anti-PI3K (p85 alpha; 1:1,000), anti-NF-κB (p65; 1:500), anti-AMPKα1/2 (1:500 dilution), anti-PPARγ (1:1,000 dilution), anti-phosphorylated NF-κB (p65; serine 276; 1:500), or anti-phosphorylated AMPKα1/2 (Thr 172; 1:500) primary antibodies at 4°C overnight. The membranes were washed in TBS-T (50 mmol/L Tris–HCl, pH 7.6, 150 mmol/L NaCl, 0.1% Tween 20) for 30 min and incubated with the appropriate HRP conjugated secondary antibody (1:5,000 dilution) for 2 h at room temperature, then 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.

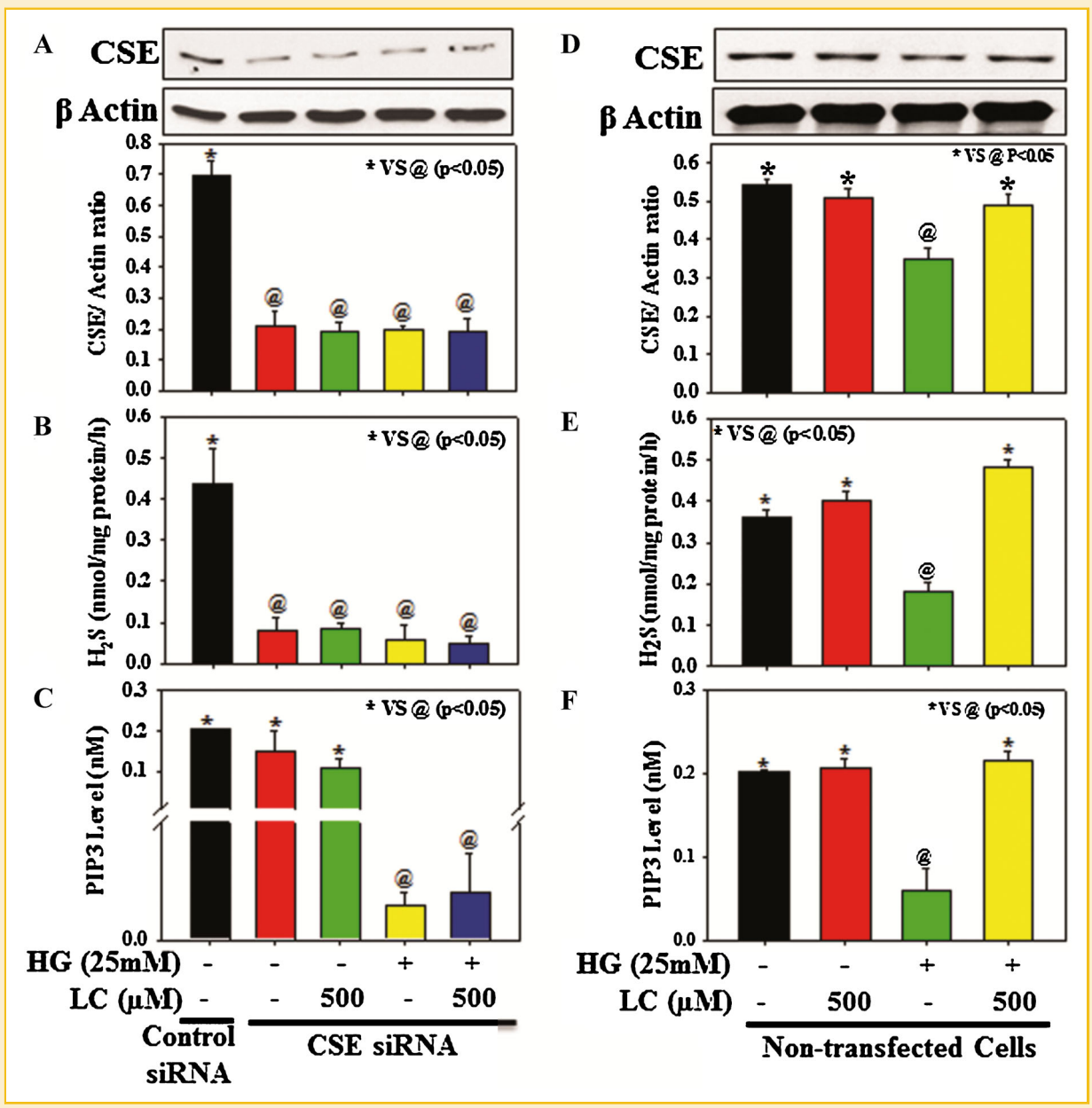

Fig. 4. Effect of L-cysteine (LC) supplementation on H_2S formation and PIP3 levels in either CSE siRNA transfected (left panel) or non-transfected (right panel) U937 monocytes exposed to high glucose (HG). A,D: CSE protein expression; B,E: H_2S formation; and C,F: PIP3 levels. Cells transfected with CSE siRNA (100 nM) or non-transfected cells were treated with LC (500 μM) for 2 h followed by HG (25 mM) exposure for the next 20 h. Control siRNA is a scrambled nonspecific RNA duplex with no sequence homology with any of the genes. Values are mean ± SE (n = 3). A difference was considered significant at the $P < 0.05$ level.

RESULTS

EFFECT OF LC AND Na_2S ON ROS PRODUCTION AND PIP3 LEVELS IN HG-TREATED U937 MONOCYTES

Figure 1 illustrates the effect of LC and Na_2S supplementation on ROS production and PIP3 levels in HG-treated human U937 monocyte cells. It shows that HG exposure caused a significant ($P < 0.05$) increase in ROS production and decrease in cellular PIP3 levels compared to those of control. However, treatment with both LC (100, 500, or 1,000 μM) and Na_2S (5 or 25 μM) prevented intracellular ROS production (Fig. 1A) and augmented the PIP3 levels (Fig. 1B) in cells exposed to HG. It has been observed that treatment with both LC (500 μM) and Na_2S (25 μM) have no effect on ROS production and PIP3 levels in cells exposed to normal glucose (7 mM) compared to those of control cells. Different treatments did not cause any change in cell viability (Fig. 1C).

Cellular PIP3 concentration is regulated by the PI3K/PTEN equilibrium. Figure 2 shows that HG treatment caused a decrease in PI3K expression and increase in PTEN expression, which explains the reduction in cellular PIP3 levels in cells exposed to HG. Supplementation with LC prevented the decrease in PI3K (Fig. 2A) and increase in PTEN (Fig. 2B) caused by HG. Similarly, treatment with Na_2S caused a dose dependent increase in PI3K (Fig. 2A) and a decrease in PTEN (Fig. 2B) expression compared to treatment with HG alone.

Figure 3 shows the effect of an inhibitor of CSE, an enzyme that catalyzes H_2S formation from LC, on H_2S formation and PIP3 levels in cells cultured with high glucose medium. The role of H_2S was demonstrated when the presence of PAG (an inhibitor of CSE) lowered both H_2S formation (Fig. 3A) and PIP3 levels (Fig. 3B) in LC-supplemented cells exposed to HG. This is consistent with effects observed upon exogenous addition of standard Na_2S (a source of H_2S) per se in monocytes.

Recent studies in the literature suggest that use of the inhibitors of CSE such as PAG or BCA (β-cyanoalanine) may be problematic since they also target other PLP dependent enzymes [Whiteman et al., 2011]. In vivo studies in the literature report that PAG supplementation caused significant cardiac complications and hepatosplenomegaly, as well as perturbation of taurine, glycine, asparagines, and citrulline metabolism [Cho et al., 1991], inhibited other PLP dependent enzyme activity, such as that of L-alanine transaminase, a clinical marker of liver function [Burnett et al., 1997], and also inhibited in vivo cyanide metabolism [Porter et al., 1996]. Konno et al. [2000] also reported that the nephrotoxic effect of PAG is mediated via its metabolite, which is produced by the D-amino acid oxidase reaction. In this study, the concentration of PAG used to inhibit CSE and investigate the effect of LC supplementation on H_2S production and PIP3 levels may also affect other PLP dependent enzymes, especially those in the mitochondria. Thus reduction in H_2S concentrations and PIP3 levels in cells treated with PAG may account for the inhibition of both CSE and other PLP dependent enzymes.

Due to the nonspecific nature of the CSE inbibitors, signal silencing studies with antisense CSE were carried out to investigate the effect of CSE inhibition on H_2S and PIP3 formation in cells treated with LC. Figure 4 demonstrates that transfection with CSE siRNA decreased CSE protein expression (Fig. 4A) and H_2S formation (Fig. 4B) significantly compared to those in cells transfected with control siRNA. Treatment with HG caused a reduction in PIP3 levels (Fig. 4C) in CSE siRNA transfected cells compared to those of CSE siRNA transfected control cells. However, the effect of HG on PIP3 could not be reversed by LC supplementation in CSE siRNA transfected cells. Interestingly, PIP3 levels in CSE siRNA transfected control cells and LC-treated cells were not significantly different from those seen in control siRNA transfected cells. The effects of LC and HG in non-transfected cells have also been included in Figure 4. It shows that HG treatment reduced the CSE protein expression (Fig. 4D), H_2S formation (Fig. 4E), and PIP3 concentration (Fig. 4F). In contrast to CSE-siRNA transfected cells, LC supplementation caused an increase in CSE protein expression, H_2S formation, and PIP3 levels in HG-treated non-transfected cells. LC supplementation alone did not cause any significant alteration in CSE protein expression, H_2S formation, or PIP3 concentration compared to those seen in non-transfected control cells.

In order to investigate whether the observed effect on PIP3 levels is specific to PI3K activity, additional experiments were performed using LY294002, a specific inhibitor of PI3K. Results shown in Figure 5 demonstrate that treatment with LY294002 alone can decrease cellular PIP3 levels, which suggests a role for PI3K activity in the formation of cellular PIP3. It has also been observed that in the presence of LY294002, supplementation with neither LC nor Na_2S could augment the HG induced lower PIP3 levels. Taken together, the inhibitor studies as well as the signal silencing studies demonstrate that both LC and Na_2S cause an increase in cellular PIP3 mediated via PI3K activation and that the effect of LC on PIP3 is mediated by H_2S in cells exposed to HG.

The medium used for cell culture studies does not contain any LC according to the analyses of medium provided by the Sigma Chemical Co. (St. Louis, MO). Mannitol was used as an osmolarity control and mannitol treatment did not have any significant effect on ROS production or PIP3 levels compared to control.

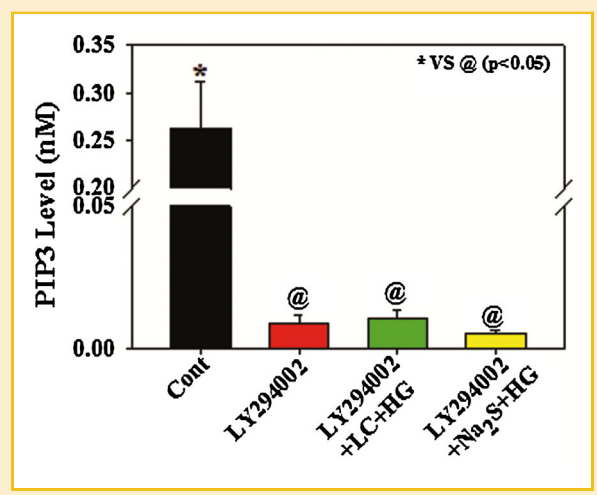

Fig. 5. Effect of the PI3K inhibitor LY294002 on PIP3 levels in cells exposed to L-cysteine (LC) and sodium sulfide (Na_2S) followed by HG exposure. Cells were treated with LY294002 (50 μM) 30 min after supplementation with LC (500 μM) or Na_2S (25 μM) followed by HG exposure. Values are expressed as mean ± SE (n = 3). A difference was considered significant at the $P < 0.05$ level.

EFFECT OF LC AND Na_2S ON NF-κB ACTIVATION AND THE LEVELS OF PRO-INFLAMMATORY MOLECULES IN HG-TREATED CELLS

Figure 6 demonstrates the effect of LC and Na_2S supplementation on NF-κB activation and the levels of pro-inflammatory molecules in cells exposed to HG. It was observed that HG exposure increased the NF-κB phosphorylation and the vascular inflammation as demonstrated by increased levels of pro-inflammatory cytokines, namely TNF-α, MCP-1, IL-8, IL-1β, and IP-10, compared to levels in control cells. Treatment with both LC and Na_2S decreased NF-κB phosphorylation (Fig. 6A) and reduced the levels of pro-inflammatory molecules (Fig. 6B–F), which demonstrates the anti-inflammatory properties of LC and H_2S in the pathogenesis of diabetic complications.

EFFECT OF LC, Na_2S, AND PIP3 ON THE ACTIVATION OF AMPK AND PPARγ IN CELLS EXPOSED TO HG

Both AMPK and PPARγ play an important role in the regulation of vascular inflammation in diabetes. Figure 7 shows the effect of LC, Na_2S, and PIP3 on the expression of both AMPK and PPARγ in cells exposed to HG. Results show that HG treatment decreased AMPK phosphorylation and PPARγ expression compared to those in control

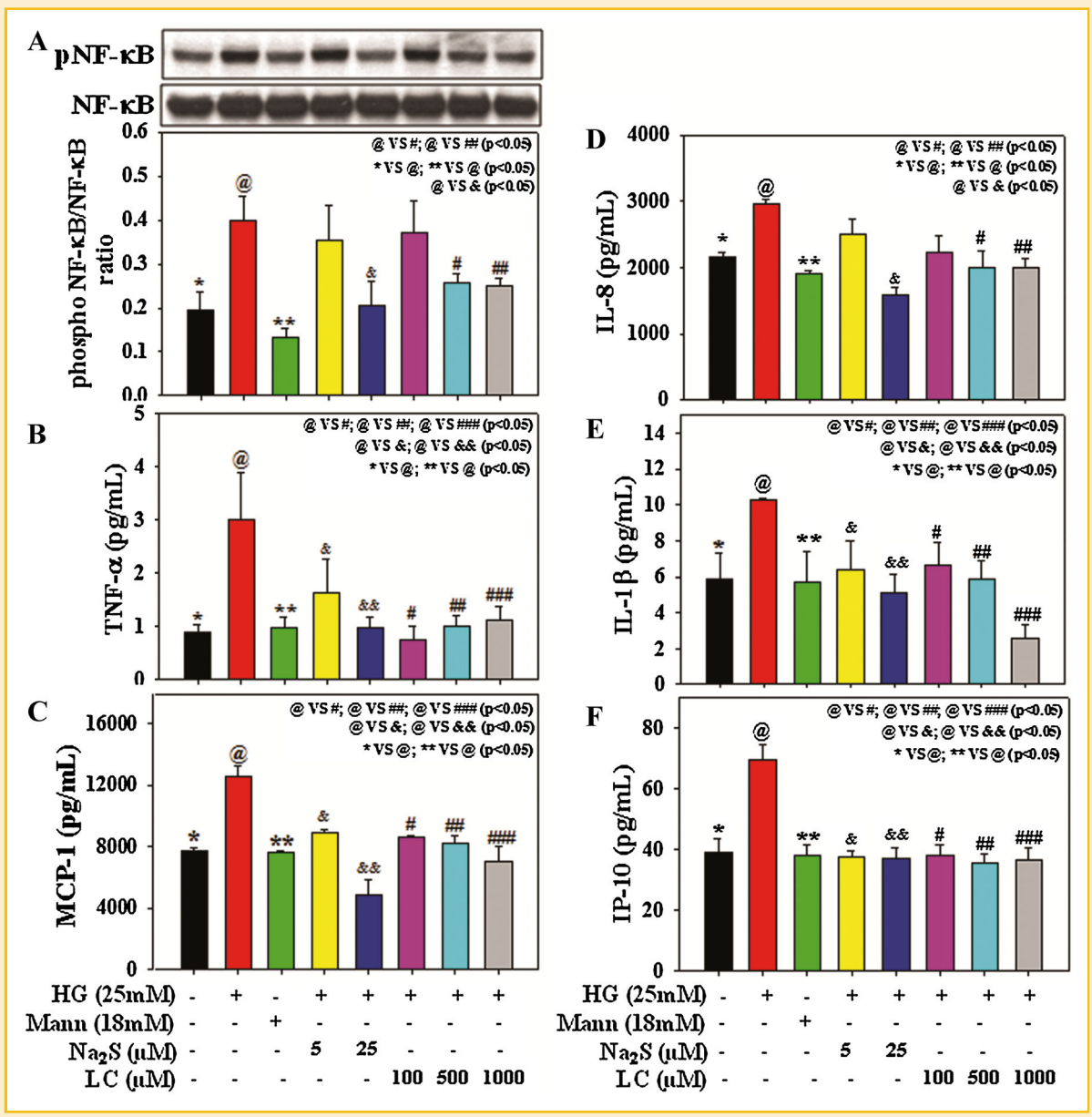

Fig. 6. Effect of L-cysteine (LC) and sodium sulfide (Na_2S) supplementation on the activation of NF-κB and levels of pro-inflammatory molecules in U937 monocytes exposed to high glucose (HG). A: phospho NF-κB (serine 276)/NF-κB; (B) TNF-α; (C) MCP-1; (D) IL-8; (E) IL-1β; and (F) IP-10. Cells were pretreated with either LC (100, 500, or 1,000 μM) or Na_2S (5 or 25 μM) for 2 h followed by HG (25 mM) exposure for the next 20 h. Values are expressed as mean ± SE ($n = 3$). A difference was considered significant at the $P < 0.05$ level.

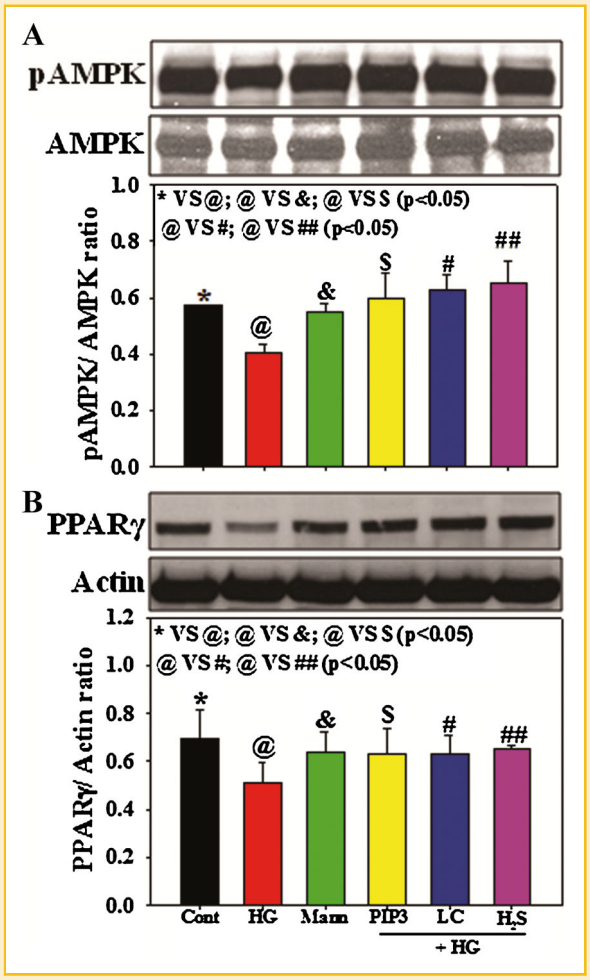

Fig. 7. Effect of L-cysteine (LC), sodium sulfide (Na_2S), and PIP3 on the expression of phospho AMPKα1/2 (Thr 172)/AMPKα1/2 and PPARγ in U937 monocytes exposed to high glucose (HG). A: phospho AMPKα1/2 (Thr 172)/ AMPKα1/2 and (B) PPARγ. Cells were pretreated with LC (500 μM), Na_2S (25 μM), or PIP3 (5 nM) followed by HG (25 mM) exposure. Values are expressed as mean ± SE (n = 3). A difference was considered significant at the $P < 0.05$ level.

cells. Conversely, both LC and Na_2S supplementation increased the expression of phospho AMPK (Fig. 7A) and PPARγ (Fig. 7B) in cells exposed to HG. Interestingly, supplementation with exogenous PIP3 (5 nM) also increased the AMPK phosphorylation and PPARγ expression in HG-treated cells. These results reveal that HG-induced reduction in PIP3 may be linked to impaired activation of AMPK and PPARγ and increased vascular inflammation. However, treatment with both LC and Na_2S increased cellular PIP3 levels, upregulated AMPK phosphorylation and PPARγ expression, and reduced the levels of pro-inflammatory molecules, thus preventing vascular inflammation in diabetes.

DISCUSSION

Recent studies indicate that H_2S plays a significant role in biological processes and that its malfunctioning may contribute to the pathogenesis of disease [Olson, 2009; Kabil and Banerjee, 2010; Wang, 2010; Manna and Jain, 2011]. There is no study in the literature examining the effect of impaired LC or H_2S levels on the excess vascular inflammation associated with diabetes. This study reports that supplementation with LC and Na_2S can decrease oxidative stress, increase cellular PIP3 levels, upregulate AMPK phosphorylation and PPARγ expression, and lower the levels of pro-inflammatory cytokines in monocytes exposed to high glucose.

Oxidative stress is a widely accepted as a risk factor in the development and progression of vascular inflammation in diabetes [Huerta and Nadler, 2004]. Numerous studies in the literature report that H_2S can inhibit the production of various reactive intermediates, such as nitric oxide, peroxynitrite, hypochlorous acid, etc. [Whiteman et al., 2004; Whiteman et al., 2005; Oh et al., 2006; Carballal et al., 2011]. Recent studies report that H_2S plays a protective role against high-glucose induced oxidative stress [Kaneko et al., 2009; Manna and Jain, 2011; Taniguchi et al., 2011; Guan et al., 2012]. Polyphosphoinositides (PIs) play an important role in diverse cellular functions depending upon the phosphorylation status of their inositol group [Pendaries et al., 2003]. Among them, PIP3 controls a complex intracellular signaling network that regulates many cellular processes, including cell growth, proliferation, and survival [Hinchliffe, 2001]. Dysregulation of PIP3 metabolism has been implicated in the pathophysiology of various diseases including diabetes [Beeson et al., 2003; Kanoh et al., 2003; Beeson et al., 2004; Chagpar et al., 2010; Manna and Jain, 2011; Manna and Jain, 2012b]. This study observed that HG treatment caused an increase in intracellular ROS production and a decrease in cellular PIP3 levels, and that supplementation with LC and Na_2S per se decreased the ROS production and increased the PIP3 levels.

The activation of Class I PI3K causes phosphorylation of PIP2 (phosphatidylinositol-4,5-biphosphate) at position 3 of its inositol head group, leading to the formation of PIP3, but PTEN activation causes the degradation of PIP3 [Pendaries et al., 2003]. This means that the cellular PIP3 concentration is regulated by the PI3K/PTEN equilibrium. This study demonstrates that the LC or Na_2S induced increase in cellular PIP3 levels is mediated by an increase in PI3K expression and a decrease in PTEN expression. This is in agreement with our earlier study in an adipocyte cell model [Manna and Jain, 2011]. H_2S is endogenously synthesized from LC via the action of enzymes, cystathionine γ-lyase (CSE), cystathionine β-synthase (CBS), 3-mercaptopyruvate sulfurtransferase (3MST), and cysteine aminotransferase (CAT) [Szabó, 2007; Whiteman et al., 2011], of which CSE is likely to be the most physiologically relevant [Pan et al., 2012]. In the present study additional experiments done with the inhibitor of CSE shows significantly lower levels of H_2S formation as well as PIP3 concentrations in cells treated with LC followed by HG exposure. Additional signal silencing studies were carried out with CSE siRNA to investigate the effect of LC on H_2S and PIP3 formation in HG-treated CSE knockdown monocytes. Signal silencing studies with CSE siRNA showed a significant decrease in CSE protein expression and H_2S formation compared to cells transfected with control siRNA. Results demonstrate that HG treatment caused a decrease in PIP3 in CSE siRNA transfected cells compared to those seen in CSE siRNA transfected control cells. LC supplementation however, could not augment PIP3 in HG-treated CSE siRNA

transfected cells compared to CSE siRNA transfected control cells. Interestingly, LC supplementation had no effect on PIP3 in CSE siRNA transfected control cells compared to control siRNA transfected cells. This suggests that the effect of LC on PIP3 upregulation in HG-treated cells is mediated via the endogenous production of H_2S from LC. In addition, studies with a specific inhibitor of PI3K (LY294002) suggest that PI3K activation mediates the effect of LC and Na_2S on cellular PIP3.

The activation of transcription factor NF-κB has been well established in the development of diabetic pathophysiology. Upon stimulation, NF-κB induces the secretion of various pro-inflammatory molecules, such as TNF-α, MCP-1, IL-1β, IL-8, and IP-10 [Singh et al., 2005; Wellen and Hotamisligil, 2005]. Tamizhselvi et al. [2009] reported that H_2S-induced activation of the PI3K/AKT pathway negatively regulates the phosphorylation of NF-κB and the cytokine secretion in caerulein-treated mouse pancreatic acinar cells. In addition, different studies in the literature strongly suggest that H_2S is a potent anti-inflammatory molecule in various models [Oh et al., 2006; Hu et al., 2007; Wang et al., 2009; Pan et al., 2011; Taniguchi et al., 2011]. In line with these earlier investigations, this study demonstrates that both LC and Na_2S supplementation reduced NF-κB phosphorylation and the levels of pro-inflammatory cytokines, thus reducing the risk factors that lead to the development of vascular inflammation in diabetes.

AMPK and PPARγ each play an important role in the regulation of vascular inflammation in various metabolic disorders including diabetes [Jiang et al., 1998; Ricote et al., 1998; Fullerton et al., 2013]. Sag et al. [2008] reported that downregulation of AMPK increased the transcriptional expression of TNF-α and IL-6 in response to LPS treatment. Conversely, treatment with AICAR or constitutively active AMPK attenuated these pro-inflammatory responses [Yang et al., 2010]. It has been reported that activation of PPARγ attenuated TNF-α induced VCAM-1 and ICAM-1 expression in endothelial cells and reduced monocyte/macrophage homing to atherosclerotic plaques in apolipoprotein E-deficient mice [Pasceri et al., 2000]. It has been reported that activation of both AMPK and PPARγ leads to a significant suppression of NF-κB phosphorylation, which regulates the gene expression of various pro-inflammatory molecules responsible for the pathogenesis of diabetic complications [Ricote et al., 1998; Cacicedo et al., 2004]. In our study we observed a reduction in AMPK phosphorylation and PPARγ expression in cells exposed to HG, and that supplementation with LC and Na_2S per se upregulated the expression of both phospho AMPK and PPARγ. Interestingly, treatment of cells with exogenous PIP3 similarly increased AMPK phosphorylation and PPARγ expression. This demonstrates that upregulation of AMPK phosphorylation and PPARγ expression by LC and Na_2S treatment is associated with an increase in cellular PIP3. Like other studies in the literature [Guan et al., 2012; Lee et al., 2012], in our study cells were supplemented with a single addition of Na_2S (a source of H_2S). H_2S is an out-going gas and easily auto-oxidized as well as decomposed by intracellular ROS. Thus the observed effect of H_2S may be mediated via its effect on very early steps in cellular signaling.

The monocyte cell culture model is widely used to investigate the various mechanisms that contribute to the development of the vascular inflammation associated with diabetes. Using a U937

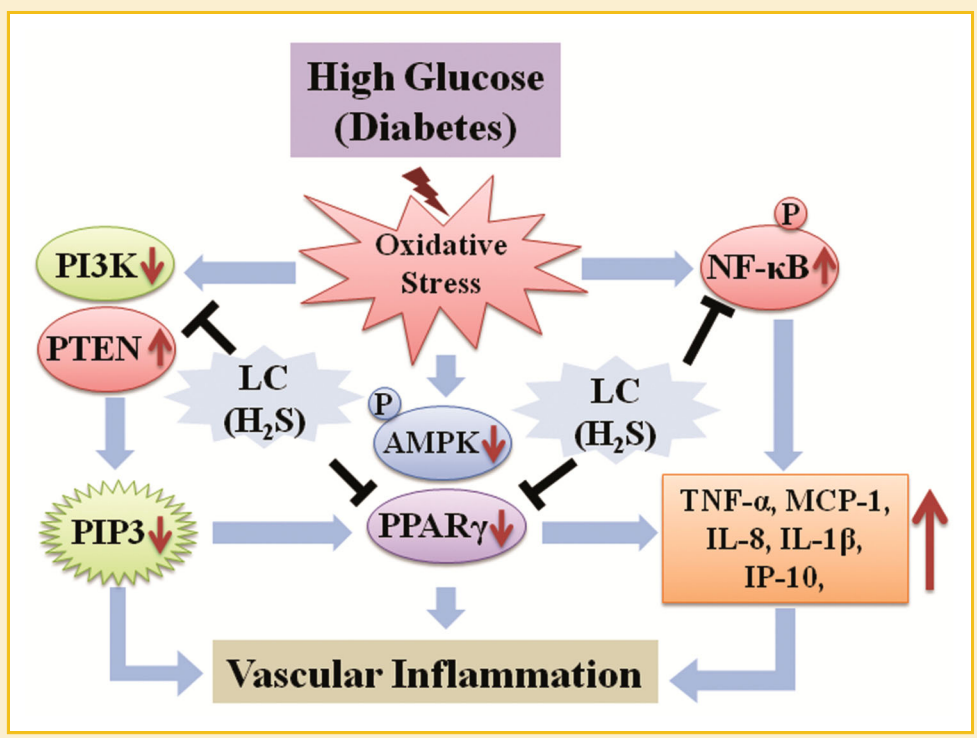

Fig. 8. Schematic diagram of proposed mechanism for LC or H_2S effects on vascular inflammation in diabetes.

monocytic cell model, this study demonstrates for the first time that supplementation with both LC (endogenous precursor of H_2S) and Na_2S (a sourcer of H_2S) can lower oxidative stress, increase PIP3, upregulate AMPK and PPARγ, and lower the levels of pro-inflammatory cytokines (Fig. 8). This study provides evidence for a novel molecular mechanism by which impaired blood concentrations of H_2S or LC can induce vascular inflammation in diabetes. Further studies are needed to examine whether supplementation with LC indeed helps to lower markers of vascular inflammation in diabetic patients.

ACKNOWLEDGMENTS

The authors thank Ms. Georgia Morgan for excellent editing of this manuscript. The authors are supported by grants from NIDDK and the Office of Dietary Supplements of the National Institutes of Health RO1 DK072433 and the Malcolm Feist Endowed Chair in Diabetes. This study is also funded by a fellowship from the Malcolm Feist Cardiovascular Research Endowment, LSU Health Sciences Center, Shreveport.

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