Laminar shear stress inhibits lipid peroxidation induced by high glucose plus arachidonic acid in endothelial cells

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1Department of Molecular Medicine and Cell and Matrix Research Institute, BK21 Medical Education Program for Human Resources, Kyungpook National University School of Medicine, Daegu, Republic of Korea; 2Department of Molecular Biology and Institute of Nanosensor and Biotechnology, BK21 Graduate Program for RNA Biology, Dankook University, Seoul, Republic of Korea; and 3Wallace H. Coulter Department of Biomedical Engineering at Georgia Institute of Technology and Emory University, Atlanta, Georgia

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Mun GI, An SM, Park H, Jo H, Boo YC. Laminar shear stress inhibits lipid peroxidation induced by high glucose plus arachidonic acid in endothelial cells. Am J Physiol Heart Circ Physiol 295: H1966–H1973, 2008. First published September 12, 2008; doi:10.1152/ajpheart.00727.2008.—Endothelial dysfunction has been associated with the incidence of cardiovascular disease. In contrast, laminar shear stress (LSS) plays a critical role in maintaining vascular health. The present study examined the mechanism for the antioxidant effect of LSS attenuating the oxidative stress induced by high glucose (HG) and arachidonic acid (AA) in human umbilical vein endothelial cells. HG and AA synergistically decreased cell viability and increased glutathione (GSH) oxidation and lipid peroxidation. The lipid peroxidation was markedly prevented by LSS as well as tetrahydrobiopterin (BH4) and GSH. LSS increased BH4 and GSH contents, and expression of GTP cyclohydrolase-I and glutamylcysteine ligase (GCL) involved in their biosynthesis. Inhibition of GCL activity by DL-buthionine-(S,R)-sulfoximine and small-interfering RNA-mediated knockdown of GCL lessened the antioxidant effect of LSS. Therefore, it is suggested that LSS enhances antioxidant capacity of endothelial cells and thereby attenuates the oxidative stress caused by cardiovascular risk factors.

Laminar shear stress; high glucose; arachidonic acid; lipid peroxidation; glutamylcysteine ligase

SHEAR STRESS, A HEMODYNAMIC force generated by blood flow, is known to regulate various vascular functions as well as gene expression in a magnitude- and flow pattern-dependent manner (22). The regions of arteries experiencing laminar shear stress (LSS) due to orderly blood flow are usually protected from atherosclerotic lesion formation, and thus LSS of relatively high level has been proposed to play anti-atherogenic roles. Differently from oscillatory shear stress, LSS usually does not stimulate inflammatory reactions but suppresses them probably by increasing nitric oxide (NO) production rather than reactive oxygen species (ROS) (8) or by induction of enzymes associated with antioxidant defense (25).

Chronic LSS enhances endothelial NO production by inducing endothelial nitric oxide synthase (eNOS) expression (5). A recent study demonstrated that LSS increases the content of tetrahydrobiopterin (BH4), an essential cofactor of eNOS, by activating GTP cyclohydrolase-I (GTPCH-I), a rate-limiting enzyme for de novo synthesis of this cofactor, providing an additional mechanism for the increased NO production by LSS (34). However, BH4 is very prone to oxidation by ROS and can be depleted under oxidative stress conditions. Then, eNOS reaction can be “uncoupled” and produce ROS instead of NO, causing further increase of oxidative stress, rather than attenuation of oxidative stress.

Plasma free fatty acids are elevated in diabetic patients and play a role in the pathogenesis of diabetic vascular complications (35). High blood glucose and free fatty acids can cause the activation of protein kinase C and NAD(P)H oxidase, leading to ROS formation and inactivation of eNOS (15, 30). Indeed, high glucose (HG) and free fatty acids have been shown to produce an endothelial dysfunction by increasing ROS formation and decreasing antioxidant defenses (10). However, LSS effects on the oxidative stress induced by HG and free fatty acids are unknown. Therefore, assuming the potential prooxidant effect of HG plus free fatty acids and antioxidant effect of LSS, the present study investigated how LSS attenuates the oxidative stress induced by HG and arachidonic acid (AA) in human umbilical vein endothelial cells (HUVECs).

MATERIALS AND METHODS

Reagents. Glucose, nitro-l-arginine methyl ester (l-NAME), glutathione (GSH), glutathione disulfide (GSSG), N-acetyl-l-cysteine (NAC), DL-buthionine-(S,R)-sulfoximine (BSO), 2-thiobarbituric acid (TBA), 1,1,3,3-tetramethoxypropane,3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), 5,5′-dithiobis[2-nitrobenzoic acid], 1-methyl-2-vinyl-pyridium trifluoromethane sulfonate (M2VP), NADPH, GSH reductase, and dihydroorhodamine 123 (DHR 123) were purchased from Sigma-Aldrich-Fluka (St. Louis, MO). Apocynin was purchased from Calbiochem (San Diego, CA). AA and BH4 were purchased from Cayman Chemical (Ann Arbor, MI).

Cell culture and treatments. HUVECs purchased from Clonetics Cambrex (Rockland, ME) were cultured on 0.2% gelatin-coated 100-mm tissue culture dishes (BD Biosciences, San Jose, CA) at 37°C and 5% CO2 in endothelial cell growth medium EBM-2 (Clonetics Cambrex) with a normal level of glucose (5.0 mM glucose, NG) supplemented with endothelial growth supplements and 10% FBS (GIBCO-BRL, Grand Island, NY).

In the first set of experiments, the effect of HG and AA inducing oxidative stress was examined. Cells were plated on a six-well plate at a density of 2 × 104 cells/cm and grown in a growth medium with a normal level of glucose (5.0 mM glucose, NG) for 48 h to reach ~80% confluence. Cells were pretreated with test materials in a fresh...
medium for 1 h followed by treatments with additional 25 mM glucose (total 30 mM glucose, HG) and/or 20-40 μM AA for 48 h. In the second set of experiments, the antioxidant effect of LSS was examined. Cells on 100-mm culture dishes were exposed to LSS at 12 dyn/cm² for 24 h. LSS was provided by rotating a Teflon cone (0.5°Cone angle) mounted on a culture dish, as described previously (2, 28). When specified, small-interfering RNA (siRNA) were transfected into HUVEC at 25 nM, according to the manufacturer’s instruction. Cells at ~50% confluency were treated with a mixture of siRNA and Lipofectamin RNAiMAX (Invitrogen, Carlsbad, CA) in Opti-MEM (Invitrogen) for 5 h, followed by incubation in a growth medium for 36 h. Human GCLm siRNA (no. 1299001) and control duplex oligoribonucleotide (no. 12935200) were purchased from Invitrogen. GCLm siRNA sequences were as follows: 5’-CCA GAU GUC UUG GAA UGC ACU GUA U-3’ (sense) and 5’-AUA CAG UGC AGU CCA AGA CAU CUG G-3’ (antisense). Cell morphology was examined under an Eclipse TS100 inverted-phase microscope from Nikon (Melville, NY). Cell viability was assayed using MTT (6).

Analysis of lipid peroxidation. The analysis of 2-thiobarbituric acid-reactive substances (TBARS) as a marker of lipid peroxidation was carried out as described previously (23). Briefly, cells were treated in a lysis buffer (20 mM Tris-Cl, 2.5 mM EDTA, and 1.0% SDS, pH 7.5). Cell lysates (100 μl) were mixed with 900 μl of 1.0% phosphoric acid and 1.0 ml of 0.9% TBA and then heated on a boiling water bath for 45 min. Standard solutions of 1,1,3,3-tetramethoxypropane, a precursor of malondialdehyde (MDA), were treated in the same way as cell lysates. After cooling, 1.5 ml of 1-butanol was added, and the mixture was centrifuged at 13,000 rpm for 15 min to separate into two layers. TBARS contents of the 1-butanol layer were spectrophotometrically determined at 532 nm. Additionally, TBA-MDA adducts were quantified by an HPLC method (13). The HPLC system (Waters, Milford, MA) consisted of a SAT/IN module, a 515 isocratic pump, a Rheodyne 7725i injector with a 20-μl sample loop, a 2487 ultraviolet (UV) detector, and a 2475 electrochemical detector. Separation was carried out on a 5 μm Agilent RP-18 (4.6 mm × 250 mm). The mobile phase consisted of 50% (vol/vol) 50 mM potassium phosphate (pH 6.3) and 50% (vol/vol) methanol. The flow rate was 1.0 ml/min. TBA-MDA adducts were detected with a UV detector at 532 nm. Protein content of cell lysates was determined by a DC protein assay kit (Bio-Rad, Hercules, CA).

Detection of ROS. The production of ROS was determined by using oxidant-sensitive probe DHR 123 as described before (31). HUVECs were pretreated with or without 1.0 mM apocynin for 30 min and then loaded with 1.0 μM DHR 123 for 30 min before the treatment with HG and AA. After incubating the treated cells for 3 h, images of cells fluorescing due to the oxidation of DHR 123 to rhodamine 123 were obtained with the use of a Nikon Eclipse TE2000-U microscope. To extract the formed rhodamine 123, cells were ruptured in ice-cold...
70% ethanol that contained 0.1 M HCl (31). The precipitated proteins were removed by centrifugation at 13,000 rpm for 15 min. The supernatants were neutralized with NaHCO₃ and centrifuged again to remove precipitates. The fluorescence intensity of clear supernatant was measured at an emission wavelength of 580 nm (excitation at 485 nm) in a FLUO-star OPTIMA multidetection microplate reader (BMG Labtechnologies, Offenburg, Germany).

Analysis of GSH and GSSG. Total GSH content (GSH + GSSG) was quantified by an enzymatic cycling assay method (1). GSSG content was determined by the same method after trapping GSH with iVdyte 100-bp DNA ladder (GenDEPOT, Barker, TX) as a size marker. The gel was ethidium bromide-stained, and band intensities were quantified using a Gel Doc system (Bio-Rad). The sequences of the primers were as follows: GTPCH-1 (GeneBank accession no., NM000161.2) 5'-TTG GTT ATC TTC CTA ACA AG-3' (sense) and 5'-GTG CTG ATC ACA GTT TTG CT-3' (antisense); eNOS (GeneBank accession no., NM000603.3) 5'-TGC CAT ACA GGA CTC AG-3' (sense) and 5'-TAG TTG GGG TTG TCA GGA-3' (antisense); GCLc (GenBank accession no., NM001498.2) 5'-CTG GGG AGT GAT TTC TGC AT-3' (sense) and 5'-AGG AGG GGG CT-3' (antisense); glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (GeneBank accession no., NM002061.2) 5'-GCC AAA AGG GTC ATC ATC TC-3' (antisense) and 5'-GTA GAG GCA GGG ATG TTG CT-3' (antisense). Statistical analysis. Data are presented as means ± SE of three or more independent experiments. The statistical analyses were performed using the Sigma Stat 3.1 software program. Significant differences among the groups were determined by a one-way ANOVA. Duncan’s multiple-range test was performed if differences were identified between the groups at P < 0.05.

RESULTS

The study first examined if HG or AA can induce oxidative stress in cultured HUVECs. Cells were treated in a growth medium with a normal level of glucose (5.0 mM glucose, NG) or high glucose medium (30 mM glucose) containing different concentrations (0, 20 or 40 μM) of AA for 48 h. HG and AA decreased cell viability in a synergistic manner (Fig. 1A). GSSG content was also synergistically increased by HG and AA (Fig. 1B). Of interest, HG and AA increased the glutathione pool significantly (Fig. 1C). Therefore, it was indicated...
that HG plus AA increased both prooxidant generation and antioxidant synthesis.

TBARS content, a marker of lipid peroxidation, was dose-dependently increased by AA in both the NG and HG media (Fig. 1D). HG tended to enhance the TBARS formation. The lipid peroxidation was significantly attenuated by both an inhibitor (l-NAME) and cofactor (BH4) of nitric oxide synthase (Fig. 1E), implicating uncoupled eNOS activity might be involved. As expected, GSH and its precursor, NAC, prevented the lipid peroxidation induced by HG plus AA significantly (Fig. 1F).

HG plus AA also markedly increased ROS formation detected by using DHR 123 (Fig. 2). As expected, ROS formation due to HG + AA was significantly attenuated by apocynin, an inhibitor of NAD(P)H oxidase (Fig. 2), confirming an involvement of NAD(P)H oxidase in oxidative stress under HG/AA conditions (15, 30).

The next experiment tested if LSS could provide an antioxidant effect in this model system. HUVECs were exposed to an arterial level of LSS (12 dyn/cm²) in an NG medium or HG/AA medium. LSS treatment for 24 h resulted in an alignment of the cells along the direction of fluid movement in both cases (Fig. 3A). LSS also reduced the cytotoxic effect of HG + AA observed at static conditions, in agreement with the anti-apoptotic effect of LSS against oxidative stress (7). Both TBARS and TBA-MDA were increased by HG + AA treatments, and these changes were suppressed markedly by LSS (Fig. 3, B and C), demonstrating a unique effect of LSS attenuating lipid peroxidation under oxidative stress.

Because BH₄ and GSH prevented the lipid peroxidation (Fig. 1, E and F), the effects of LSS on the cellular contents of these molecules were then examined. LSS significantly increased BH₄ content by three- to approximately fourfold (Fig. 3D) and GSH + GSSG content by approximately twofold (Fig. 3E) in both NG and HG + AA media. GSH contents determined by an HPLC method (Fig. 3F) were very close to GSH + GSSG contents determined by an enzymatic cycling method in most cases, indicating that GSH is the predominant form. The only exception was static cells under HG + AA whose GSH content was significantly smaller than GSH + GSSG content, probably because of oxidation to GSSG.

De novo synthesis of BH₄ and GSH requires GTPCH-1 and GCL, respectively, as the rate-limiting enzymes. Consistently with the changes of BH₄ and GSH + GSSG content, LSS increased GTPCH-1 and GCLm at the protein and mRNA levels, whereas β-actin or GAPDH expression was not altered (Fig. 4). The expression level of eNOS was upregulated by LSS as expected, but GCLc level appeared to be less sensitive to LSS, implying that GCLm rather than GCLc may play a regulatory role in the biosynthesis of GSH in response to LSS.

**Fig. 3.** Laminar shear stress (LSS) prevents the lipid peroxidation induced by HG + AA and increases the cellular content of BH₄ and GSH. HUVECs were exposed to LSS at 12 dyn/cm² for 24 h in an NG (5.0 mM glucose) or HG + AA medium (30 mM glucose + 40 μM AA). Cell images were captured under a microscope (original magnification, ×400; A). Lipid peroxidation was measured by a spectrophotometric TBARS assay (B). TBA-MDA adducts (C), BH₄ content (D), and GSH content (F) were determined by an HPLC method, and total GSH content (GSH + GSSG) was determined by an enzymatic cycling assay method (E). Data represent means ± SE (n = 3–5). Bars not sharing the same letter are significantly different from each other (P < 0.05).
The study further examined if the LSS effect preventing lipid peroxidation requires GSH synthesis. Treatment with BSO, a specific inhibitor of GCL enzyme, resulted in a significant reduction of the antioxidant effect of LSS (Fig. 5A). BSO effect depleting GSH was verified (data not shown).

To further address the essential role of GSH synthesis for the antioxidant effect of LSS, GCLm expression was knocked down with siRNA targeted to the human GCLm gene. LSS at 12 dyn/cm² prevented the lipid peroxidation induced by HG + AA significantly in control duplex-treated cells, but the effect was much weaker in GCLm siRNA-treated cells (Fig. 5B). GCLm knockdown lowered GSH/GSSG content significantly (Fig. 5C). Specific knockdown of GCLm gene expression was confirmed at the protein and mRNA levels (Fig. 5D).

**DISCUSSION**

The mechanisms in which HG and AA induce oxidative stress in endothelial cells are of importance because these risk factors in combination are closely associated with the development of diabetic complications (11). Although free fatty acids are generally kept at low micromolar concentrations in plasma (20), their concentration increases up to millimolar range in diabetic patients (29). Excessive influx of acetyl-CoA, both derived from glycolysis of glucose and β-oxidation of free fatty acids, in the tricarboxylic acid cycle can generate an accumulation of mitochondrial NADH in excess of electron transport capacity (3, 21). This condition may lead to an overproduction of ROS at the mitochondrial level, which in turn stimulates NAD(P)H oxidase and other enzymes associated with amplification of oxidative stress and inflammation (15, 26). Indeed, metabolic overload-induced oxidative stress appears to be an important common mechanism of diabetes and cardiovascular disease (3).

The data of the present study suggest that uncoupled eNOS might contribute to the lipid peroxidation induced by HG plus AA (Fig. 1E) and that the lipid peroxidation could be mitigated by exogenous antioxidants (Fig. 1F). The present study further demonstrated the novel effect of LSS inhibiting the lipid peroxidation induced by HG plus AA (Fig. 3).

It is of interest to see that NAC was more effective than GSH for the inhibition of lipid peroxidation (Fig. 1F). Because NAC is assumed to be the biosynthetic precursor of GSH, one would have expected comparable antioxidant effects of these two compounds. It is known that NAC is readily taken up by cells and deacetylated, providing L-cysteine, a limiting amino acid for the synthesis of GSH (9). In contrast, extracellular GSH cannot enter cells directly but should be broken down into its constituent amino acids (9). Therefore, external addition of GSH may not lead to rapid increase of intracellular GSH in certain cellular contexts. In this sense, different antioxidant activity of NAC and GSH may be attributed to their different efficiency in increasing intracellular GSH.

The LSS effect inhibiting the lipid peroxidation induced by HG plus AA was partly attributable to the increased biosynthesis of BH4. As an essential cofactor, BH4 may be in greater demand under HG + AA to maintain eNOS activity. The lowered content of BH4 under HG + AA (Fig. 3D) and the inhibitory effect of externally added BH4 on the lipid peroxidation (Fig. 1E) conforms to this notion. A recent study reported that LSS increased BH4 content by activating GT-
PCH-1 through posttranslational modifications (34). In the current study, LSS was observed to increase the expression of GTPCH-1 at the protein and mRNA levels as well, suggesting that LSS regulates BH4 biosynthesis at multiple stages.

Although LSS induced GTPCH-1 expression to a similar extent in both NG and HG/AA media (Fig. 4A), BH4 contents were very different from each other (Fig. 3D). This discrepancy, however, could be attributed to the oxidative loss of BH4 under HG/AA conditions. Indeed, BH4 is very prone to oxidative degradation (19), and similar discrepancy between BH4 content and GTPCH-1 expression has been observed in the cells treated with hydrogen peroxide (17).

The current study provided multiple lines of evidence that GSH biosynthesis underlies the LSS effect preventing the oxidative stress induced by metabolic disturbance. First, LSS increased GSH/GSSG contents by approximately twofold in both the NG and HG/AA media (Fig. 3). GSH/GSSG contents varied from ~20 nmol/mg protein in the static cells in an NG medium to ~60 nmol/mg protein in the LSS-exposed cells under HG/AA (Fig. 3). The overall variation of GSH + GSSG contents might seem to be very big considering a product inhibition mechanism of GSH synthesis, but similar fold changes have been previously observed in endothelial cells treated with an NO donor (24).

The present study also demonstrated an LSS effect increasing GCLm expression at both the protein and mRNA levels (Fig. 4). Because GCLm increases catalytic activity of GCLc by lowering the Michaelis constant for the substrate L-glutamylcysteine and decreasing the inhibition by GSH, its expression level could have an influence on GSH biosynthesis. In agreement with this notion, the changes of GCLm expression level due to LSS and HG/AA (Fig. 4) were very comparable to those of intracellular GSH (GSH + GSSG) content (Fig. 3).

LSS has been shown to stimulate the antioxidant response element (ARE), a cis-acting regulatory element that regulates genes encoding enzymes involved in phase II metabolism of xenobiotics and antioxidant defense, in a different manner to oscillatory shear stress (4, 14). GCL is among the enzymes whose expression is controlled by ARE, and previous studies have shown that LSS increased GCLm, but not GCLc, expression at the mRNA level (4, 33). Our data shown in Fig. 4 are in agreement with those studies.

The association of GSH synthesis with the antioxidant effect of LSS was further demonstrated by intervention of GCLm activity and expression (Fig. 5). The antioxidant effect of LSS was significantly reduced by pharmacological inhibition of GCL activity with BSO and siRNA-mediated knockdown of GCLm (Fig. 5).
Of interest, the siRNA-dependent knockdown of GCLm itself increased lipid peroxidation and enhanced the lipid peroxidation by HG + AA (Fig. 5B). BSO treatment also resulted in an increase of lipid peroxidation under HG + AA condition although its effect under the NG condition was insignificant (Fig. 5A). The data suggest that GSH depletion and HG + AA can cause oxidative stress additively or synergistically. Minor difference between BSO and GCLm siRNA effects may be attributed to a different degree and/or duration of GSH depletion.

GCLm siRNA effectively depleted GCLm mRNA and significantly inhibited the LSS-dependent increase of GCLm protein under HG + AA conditions (Fig. 5D). A relatively smaller change of the GCLm protein level than its mRNA level may be an indication of a slow turnover rate of GCLm protein. Nonetheless, the inhibition of GCLm expression by an siRNA approach resulted in a significant decline of intracellular GSH content (Fig. 5C).

The LSS effect inhibiting lipid peroxidation due to HG + AA was significantly attenuated but not completely abolished by BSO or GCLm siRNA treatments. This may be due to incomplete depletion of intracellular GSH or the presence of other defense mechanisms such as superoxide dismutase and peroxiredoxins (16, 25). Although our data indicated that GSH plays a critical role in antioxidant defense by LSS, association of other antioxidants such as l-ascorbic acid and α-tocopherol cannot be excluded. Further studies are needed to examine if the antioxidant effect of LSS involves these antioxidants.

Even though data presented in this study were obtained by using HUVECs that were chosen because such cells are readily available, many of them could be reproduced in other endothelial cells. For example, HG and AA synergistically decreased cell viability and increased lipid peroxidation in bovine aortic endothelial cells (data not shown). However, considering heterogeneity in the vascular endothelium, further studies using different cell types are warranted to generalize the findings of the present study as a common physiology of endothelial cells. This study suggested that certain strategies to enhance LSS may be potentially useful for the prevention of oxidative stress in endothelial cells. In this regard, frequent exercise would be one of the best choices because it can provide repeated episodes of elevated shear stress. A recent study demonstrated a correlation between brachial artery shear stress and intensity of walking exercise (27). Additionally, pharmacological approaches that can mimic or enhance the LSS effect would be helpful to reduce oxidative stress.

In conclusion, the present study demonstrated a beneficial effect of LSS attenuating oxidative stress due to HG and AA, theimportant cardiovascular risk factors. To the best of our knowledge, this is the first direct demonstration of the antioxidant effect of LSS inhibiting lipid peroxidation. The antioxidant effect of LSS could be attributed to increased biosynthesis of BH₄ and GSH. BH₄ could prevent eNOS from the production of ROS, which triggers lipid peroxidation, and GSH could be used for the removal of lipid peroxide by GSH peroxidases. The unique effect of sustained LSS enhancing antioxidant capacity would help understand the key roles of LSS in maintaining endothelial function and vascular health, especially under pathological conditions.

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