Novel Effect of Oxidized Low-Density Lipoprotein
Cellular ATP Depletion via Downregulation of Glyceraldehyde-3-Phosphate Dehydrogenase

Sergiy Sukhanov, Yusuke Higashi, Shaw-Yung Shai, Hiroyuki Itabe, Koichi Ono, Sampath Parthasarathy, Patrick Delafontaine

Abstract—Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is a classical glycolytic enzyme that is involved in cellular energy production and has important housekeeping functions. We used the natural prooxidant and proatherogenic molecule oxidized low-density lipoprotein (OxLDL) to determine a potential link between OxLDL-promoted oxidative stress, GAPDH expression, and smooth muscle cell energy metabolism. OxLDL but not native LDL (nLDL) produced a 60% to 100% dose- and time-dependent reduction of GAPDH protein. OxLDL increased reactive oxygen species (ROS) formation, including rapid elevation of H₂O₂ levels. OxLDL decreased intracellular catalase expression, likely contributing to the increase in H₂O₂. Antioxidants, anti-CD36 receptor antibody, NADPH oxidase, or lipoxygenase blockers decreased OxLDL-specific ROS and prevented GAPDH downregulation. 12/15-Lipoxygenase or p47phox deficiency resulted in attenuation of GAPDH downregulation, but 5-lipoxygenase suppression had no effect. OxLDL or exogenous H₂O₂ oxidized GAPDH thios, decreasing GAPDH protein half-life and increasing GAPDH sensitivity to proteasome-mediated protein degradation in vitro. OxLDL or small interfering RNA–specific downregulation of GAPDH resulted in 65% reduction in glycolysis rate and 82% decrease in ATP levels. In conclusion, our data demonstrate that OxLDL downregulated GAPDH via a H₂O₂-dependent decrease in protein stability. GAPDH protein damage resulted in marked depletion of cellular ATP levels. Our data have important implications for understanding the metabolic effect of OxLDL on the vessel wall and mechanism of atherogenesis.

Key Words: reactive oxygen species ■ low-density lipoprotein ■ energy metabolism ■ vascular smooth muscle

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (EC 1.2.1.12) is a glycolytic enzyme involved in energy production and is one of the most abundant cellular proteins. GAPDH is constitutively expressed in different cells and is viewed as an important housekeeping molecule. However, it has become clear that the role of GAPDH is not restricted to its pivotal glycolytic function. Indeed, GAPDH regulates multiple other processes, including membrane fusion, nuclear RNA transport, and DNA replication and repair.¹

Increasing evidence suggests that GAPDH is a preferential target for oxidative stress. Nitric oxide (NO) and reactive oxygen species (ROS)-mediated oxidation of GAPDH thios (S-thiolation) and subsequent inhibition of enzyme activity have been described in colon epithelial cells during inflammatory bowel disease.² Wentzel et al³ demonstrated that diabetic embryopathy in the rat is associated with the inhibition of GAPDH activity and a decrease in GAPDH mRNA and that this effect is blunted by the antioxidant N-acetylcysteine. Taken together, these observations indicate a high sensitivity of GAPDH to cell redox status and potential involvement of this enzyme in oxidative stress-promoted events.

Oxidized low-density lipoprotein (OxLDL) is a powerful natural prooxidant derived in vivo from nLDL by cell-mediated oxidation.⁴ OxLDL is a key proatherogenic molecule⁵ that induces, as we have shown, an increase in ROS formation, specific alterations in gene/protein expression, and apoptosis of vascular smooth muscle cells.⁶⁻⁹ We designed this study to determine a potential link between OxLDL-specific increase in ROS, GAPDH expression, and human aortic smooth muscle cell (HASM) energy metabolism. Our findings indicate dramatic OxLDL-induced downregulation of GAPDH via a redox-dependent signal pathway, resulting in depletion of cellular energy stores. These results provide a potential mechanism for understanding metabolic alterations of the vasculature during the process of atherogenesis.

Materials and Methods

Cell culture, isolation, oxidative modification of LDL, and immuno blotting analysis protocols have been described previously.⁶ For-
OxLDL Downregulates GAPDH Protein

Confluent (80% to 90%) HASMC were treated with 60 μg/mL OxLDL or native LDL (nLDL) (control) for 0 to 24 hours or incubated in serum-free media (SFM). No change in GAPDH expression was found after cell treatment with nLDL or SFM; in contrast, cell incubation with OxLDL time-dependently downregulated GAPDH protein (Figure 1A). GAPDH protein level was reduced in OxLDL-treated cells to 68±15% and 33±10% compared with control at 9 and 15 hours, respectively, and GAPDH expression at 24 hours was virtually not detectable.

HASMC treatment by LDLS for 15 hours was selected for generation of dose-response curves and to assess effects of antioxidants. For dose-response experiments, HASMC were treated with 0 to 100 μg/mL OxLDL or nLDL and GAPDH expression was analyzed by immunoblotting. Sixty micromolars per milliliter OxLDL decreased GAPDH levels to 38±11%, and 80 μg/mL OxLDL downregulated GAPDH to 25±3%. OxLDL (20 to 100 μg/mL) produced no changes in protein levels of other housekeeping proteins β-actin and β-tubulin (Figure 1B). However, we noted that treatment of HASMC with highly OxLDL (>50 nmol malondialdehyde per milligram protein) slightly decreased β-actin levels (15±5% compared with nLDL-treated cells), as evidenced by immunoblotting, together with marked downregulation of GAPDH protein levels.

Immunoblotting data obtained with mouse monoclonal anti-GAPDH antibody were further confirmed with rabbit polyclonal anti-GAPDH antibody (data not shown). We also examined the efficiency of the recognition of potentially oxidized antigen (GAPDH) by cognitive antibody (mouse anti-GAPDH) in a routine Western analysis. Purified chicken muscle GAPDH (0.1 mg/mL; Sigma) was directly oxidized by 1.5 mmol/L H2O2, and an aliquot of oxidized and untreated GAPDH (control) were analyzed by immunoblotting. No differences were found between band density of oxidized GAPDH and control (data not shown). Although the possibility remains that there is a loss of immunoreactivity independent of reduced expression of the GAPDH protein. This appears unlikely in view of our results comparing immunoreactivity of oxidized and nonoxidized GAPDH using 2 different epitope-specific anti-GAPDH antibodies.

GAPDH gene expression was analyzed by Northern blotting. In time-course experiments, GAPDH protein was decreased after 9 hours of OxLDL treatment, and for this period (0 to 9 hours), GAPDH mRNA levels were not changed significantly compared with nLDL (Figure 1C). However, longer incubation with OxLDL (15 to 24 hours) produced a slight decrease of GAPDH mRNA (18±5% at 24 hours).

Antioxidants Suppress OxLDL-Specific Peroxides, Decrease Superoxides, and Prevent Downregulation of GAPDH

Because OxLDL signaling can involve ROS,10 we assessed formation of intracellular peroxides and superoxides in LDL-treated HASMC with the peroxide-sensitive dye CDC-H2F and the superoxide-specific dye hydroethidine (HE). Both oxidized and native LDL (60 μg/mL) increased cell peroxides time dependently up to 9 hours of treatment. For 2 to 6 hours of incubation, OxLDL produced 40% to 70% more peroxides than nLDL, but longer treatments resulted in no difference between peroxide levels induced by OxLDL and control (see supplemental Figure IA). Unlike peroxide formation, superoxide level was significantly higher in cells treated with OxLDL versus nLDL for all time points up to 30 hours. OxLDL increased peroxides dose dependently (20 to 80 μg/mL), with a peak formation at 3 to 4 hours. OxLDL-
stimulated by 60

CD36 Scavenger Receptor, 12/15-Lipoxygenase, and NADPH Oxidase Mediate OxLDL-Induced ROS Increase and GAPDH Downregulation

The CD36 scavenger receptor mediates the uptake of OxLDL, allowing OxLDL-specific intracellular signaling and development of atherosclerosis. Using real-time PCR we confirmed the presence of CD36 mRNA in HASMC and one unique protein band immunogenic to CD36 antibody (MW=90±7 kDa, n=3) was detected in cell lysates by immunoblotting (data not shown). To verify the potential involvement of CD36 receptor in OxLDL-specific ROS increase and GAPDH downregulation we used mouse FA6–152 anti-CD36 blocking antibody (Immunotech, Cedex, France), a specific inhibitor of CD36-dependent OxLDL uptake. We found that anti-CD36 antibody (20 μg/mL) decreased OxLDL-specific increase in peroxide and superoxide formation, whereas pretreatment with nonspecific antibody (20 μg/mL normal mouse IgG) had no effect (see supplemental Figure II). Antibody-specific inhibition of CD36 scavenger receptor resulted in complete inhibition of OxLDL-induced GAPDH decrease (Figure 3A).

We used allopurinol, the xanthine oxidase inhibitor; NS-398, the specific blocker of cyclooxygenase-2; indomethacin, the nonspecific blocker of cyclooxygenase-1 and cyclooxygenase-2; and rotenone, the inhibitor of mitochondrial complex I, to study potential involvement of these ROS producing systems in OxLDL-dependent increase in HASMC peroxides and superoxides. Preincubation of HASMC with 100 μmol/L allopurinol, 10 μmol/L NS-398, or 10 μmol/L indomethacin did not block OxLDL-specific peroxide or superoxide formation or downregulation of GAPDH, and rotenone was toxic at concentrations of 10 and 100 μmol/L (data not shown).

Lipoxygenases (LOXs), such as 5-LOX, which is a key enzyme in leukotriene production, and 12/15-LOX are a family of lipid peroxidizing enzymes that are another source of ROS in the vasculature. We verified the presence of 5- and 12/15-LOX proteins and corresponding enzyme activities in HASMC (data not shown). We found that the LOX inhibitor AA-861 (5 μmol/L) markedly reduced OxLDL-induced ROS production (supplemental Figure II). The same dose of AA-861 completely blocked OxLDL downregulation of GAPDH (Figure 3B). To identify the LOX form that mediates OxLDL-induced ROS production and GAPDH downregulation, we used 5-LOX-specific and nonspecific (control) siRNA-treated HASMC and vascular smooth muscle cells (SMC) obtained from 12/15-LOX-deficient mice and wild-type mice (control). All methods involving animals were approved by the Institutional Animal Care and Use Committee (IACUC) of Tulane University Health Sciences Center. siRNA-mediated 5-LOX protein decrease was 65% as measured by immunoblotting analysis (Figure 3D), and there was a similar decrease in 5-LOX mRNA levels as measured by real-time PCR (data not shown). We found that 12/15-LOX deficiency resulted in a 62±7% reduction of OxLDL-induced peroxides; however, 5-LOX suppression had no effect (supplemental Figure III). OxLDL-induced downregulation of GAPDH was significantly blunted in 12/15-LOX−/− vascular SMC (54±14% inhibition compared with wild-type), whereas 5-LOX suppression had no blocking effect on OxLDL-induced GAPDH downregulation (Figure 3E).

The membrane-bound NADPH oxidase is a predominant source of ROS in multiple pathophysiological conditions in vascular smooth muscle. We found that apocynin (50 μmol/L), a potent and selective inhibitor of NADPH oxidase, and the structurally unrelated NADPH oxidase blocker AEBSF (1 mmol/L) were less efficient in suppression of OxLDL-specific ROS compared with AA-861 (supplemental Figure II). NADPH oxidase blockers only partially
suppressed downregulation of GAPDH (Figure 3C). p47phox is a critical subunit of the NADPH oxidase, and a null mutation of this gene resulted in attenuation of superoxide production in SMC and suppression of atherosclerosis. To verify our data obtained with NADPH oxidase inhibitors, we measured peroxides and GAPDH protein levels in vascular SMC from p47phox−/− or wild-type (control) mice exposed to nLDL or OxLDL. We found that p47phox−/− cells produce 82% less peroxides in response to OxLDL compared with wild type (supplemental Figure III). p47phox deficiency completely blocked OxLDL-induced GAPDH downregulation (Figure 3F).

These data suggest involvement of the CD36 receptor, 12/15-LOX, and NADPH oxidase in ROS formation and OxLDL-specific GAPDH downregulation.

**H₂O₂ Scavengers Completely Block OxLDL-Specific GAPDH Downregulation, and Exogenous H₂O₂ Downregulates GAPDH**

H₂O₂ is a major oxidative component of OxLDL-induced ROS and, therefore, could be involved in ROS-mediated GAPDH downregulation. We measured specific H₂O₂ release from LDL-exposed cells with/without pretreatment with Trolox (0.25 mmol/L), specific H₂O₂ scavengers catalase (10 kU/mL), or polyethylene glycol–coupled catalase (PEG-catalase) (1 kU/mL) using the Amplex Red assay. OxLDL (60 µg/mL, 3 hours) significantly increased H₂O₂ levels compared with nLDL or SFM (Figure 4A). Pretreatment with antioxidant or catalase efficiently suppressed OxLDL-induced H₂O₂ formation to below basal levels. After calibration of the Amplex Red signal with standards (H₂O₂, 0 to 1000 µmol/L), we estimated that the OxLDL-specific increase in cellular H₂O₂ level corresponds to 350 to 450 µmol/L H₂O₂ (data not shown). This is consistent with reports showing that oxidative stress enhanced cellular H₂O₂ concentration in vivo to the submillimolar range. Therefore, we examined GAPDH expression in response to similar doses of exogenous H₂O₂. We found that HASMC incubation with H₂O₂ (225 to 450 µmol/L, 16 hours) resulted in a downregulation of GAPDH and that this effect was dose dependent (Figure 4B).

Based on these data, we hypothesized that OxLDL-specific increase in H₂O₂ is critical for subsequent GAPDH protein suppression and verified this hypothesis using H₂O₂ scavengers (catalase and PEG-catalase) and superoxide scavengers (superoxide dismutase [SOD] and PEG-SOD). Only catalases completely blocked OxLDL-induced downregulation of GAPDH (Figure 4C and 4D). The blocking effect of SOD was partial: GAPDH levels still decreased in OxLDL-treated cells compared with nLDL (67 ± 7% of control, n=3, P<0.05) after preincubation with this superoxide scavenger. PEG-SOD at 50 U/mL even potentiated GAPDH downregulation and was toxic at higher concentrations. This data suggested that OxLDL-specific increase in H₂O₂ is responsible for GAPDH downregulation.

**OxLDL Rapidly Decreases Catalase Activity in HASMC and Does Not Alter SOD Activity**

Two major antioxidant enzyme systems protect cells from oxidative stress by maintaining safe levels of ROS: SODs,
enzymes that catalyze conversion of superoxides to H$_2$O$_2$ including intracellular (Mn-SOD, Cu/Zn-SOD) and extracellular (EC-SOD) forms and catalase. To clarify the potential mechanism of OxLDL-induced H$_2$O$_2$ elevation, we measured the activity and protein levels of SODs and catalase in HASMC treated by OxLDL. Immunoblotting analysis showed that the relative amount of catalase was decreased by 58% by OxLDL treatment compared with nLDL, but Mn-SOD protein level was not changed (Figure 5A and 5B). Similarly, the functional activity of catalase was significantly reduced (Figure 5C). However, activity of intracellular SOD, as assessed by Cayman Chemical assay and in situ SOD activity detection, was not different between OxLDL- and nLDL-treated cells (data not shown). Additionally, Western blotting revealed that OxLDL did not alter expression of Cu/Zn-SOD (data not shown). We detected activity of an EC-SOD–like protein in conditioned media from LDL-treated cells using in situ assay and found that this activity also was unregulated by OxLDL (data not shown). These data suggest that the reduction in catalase activity and the maintenance of SOD activity could contribute to the increase in intracellular H$_2$O$_2$ in response to OxLDL.

**OxLDL or H$_2$O$_2$ Oxidize GAPDH Thiols and Decrease Protein Stability**

The GAPDH molecule has 3 easily oxidized SH groups and 2 of them (Cys149 and Cys153) are essential for GAPDH catalytic activity. To clarify the potential mechanism of OxLDL-induced H$_2$O$_2$ elevation, we measured the activity and protein levels of SODs and catalase in HASMC treated by OxLDL.

Immunoblotting analysis showed that the relative amount of catalase was decreased by 58±5% by OxLDL treatment compared with nLDL, but Mn-SOD protein level was not changed (Figure 5A and 5B). Similarly, the functional activity of catalase was significantly reduced (Figure 5C). However, activity of intracellular SOD, as assessed by Cayman Chemical assay and in situ SOD activity detection, was not different between OxLDL- and nLDL-treated cells (data not shown). Additionally, Western blotting revealed that OxLDL did not alter expression of Cu/Zn-SOD (data not shown). We detected activity of an EC-SOD–like protein in conditioned media from LDL-treated cells using in situ assay and found that this activity also was unregulated by OxLDL (data not shown). These data suggest that the reduction in catalase activity and the maintenance of SOD activity could contribute to the increase in intracellular H$_2$O$_2$ in response to OxLDL.
efficiency with the thiol-sensitive dye fluorescein-5-maleimide was dramatically decreased following treatment with OxLDL (60 μg/mL, 3 hours) or, similarly, after incubation with H₂O₂ (400 μmol/L, 3 hours) without alteration of GAPDH protein levels (Figure 6).

In theory, GAPDH thiol (R-S-H) modification by an oxidant can include: (1) formation of inter- or intramolecular disulphide bands (R-S-S-R); (2) formation of mixed disulphides with a small intracellular thiol, glutathione (R-S-S-R₂); or (3) direct oxidation of the Cys thiol groups to sulfenic acid (R-S-O⁻). To address the first possibility, we used nonreducing gel electrophoresis to examine whether formation of an intramolecular disulphide bond or a bond between 2 molecules of GAPDH resulted in altered protein migration by nonreducing SDS-PAGE. Neither H₂O₂ nor OxLDL treatment of cells resulted in altered GAPDH electrophoretic mobility (data not shown). To address the second possibility, we examined GAPDH thiol oxidation by H₂O₂ in cell-free conditions and examined the potential protein glutathionylation by immunoblotting. We found no significant difference in GAPDH glutathionylation level between oxidant-treated cells and controls (data not shown). Moreover, GAPDH oxidized in glutathione-free conditions (Figure 6, top) has an identical thiol pattern to GAPDH oxidized by cell treatment with oxidant (Figure 6, bottom). Little and O’Brien clearly demonstrated that H₂O₂ oxidizes GAPDH thios via formation of sulfenic acid, and our experiments are consistent with formation of -S-O⁻ groups as the most likely mechanism of GAPDH thiol oxidation by OxLDL.

In view of the lack of the effect of OxLDL on GAPDH mRNA levels, we performed pulse-chase labeling experiments to assess the potential effect of OxLDL or H₂O₂ on GAPDH protein stability. As shown in Figure 7A and 7B, either OxLDL or H₂O₂ markedly decreased GAPDH protein half-life (to 18±2 and 4±1 hours, respectively, compared with >24 hours in controls).

Proteasome Inhibitors MG-132 and Lactacystin Do Not Inhibit GAPDH Downregulation In Vivo, but Oxidized GAPDH Has Increased Sensitivity to Proteasome-Mediated Degradation In Vitro

Because it has been shown that H₂O₂-mediated protein oxidation “marks” proteins for preferential utilization in protein-degradation pathways, we compared the sensitivity of the oxidized and nonoxidized GAPDH to proteasome-mediated degradation in vitro. GAPDH purified from OxLDL-treated HASMC was completely degraded after 2 hours of incubation in a proteasome-protein-degradation assay; however, protein level of GAPDH from control cells was not changed. Similarly, H₂O₂-oxidized GAPDH or GAPDH purified from H₂O₂-exposed cells was degraded faster than control protein (Figure 7C).

However, interestingly, pretreatment of cells with the proteasome inhibitor MG-132 (10 μmol/L) or lactacystin (10 μmol/L) did not inhibit OxLDL-induced downregulation of GAPDH (Figure 7D), suggesting that alternative non–proteasome-dependent pathway(s) are primarily responsible for OxLDL-induced degradation of GAPDH protein in vivo.

OxLDL-Specific GAPDH Downregulation Decreases Cell Glycolysis Rate and ATP Stores

We assessed energy metabolism in LDL-treated cells by measurement of glycolysis rates and total ATP levels. Glucose usage was inhibited by 65±7% in HASMC treated with OxLDL (60 μg/mL, 16 hours) compared with cells incubated in lipid-free media, whereas incubation with nonoxidized lipoprotein slightly stimulated cell glycolysis (Figure 8A).

To determine whether downregulation of GAPDH expression sufficed to reduce glycolysis rates, we used GAPDH-specific siRNA to downregulate GAPDH. As shown in Figure 8D, 50 nmol/L anti-GAPDH siRNA markedly reduced HASMC glycolysis rate. In addition, total ATP levels were reduced by 82±4% in OxLDL-treated cells (Figure 8B) and by 89±6% in siRNA-treated cells (Figure 8E) compared with control. These data indicate that GAPDH downregulation results in dramatic reduction of cell glycolysis rates and ATP depletion.

Discussion

GAPDH plays a key role in energy metabolism by conversion of glyceraldehyde-3-phosphate to 1, 3-biphospho-glycerate in the glycolytic pathway. GAPDH has been referred to as a housekeeping protein based on the classical view that GAPDH expression remains relatively constant under changing cellular conditions. However, GAPDH is now known to contribute to a number of cellular functions unrelated to glycolysis that include nuclear RNA export and DNA replication and repair, exocytotic membrane fusion, cytoskeletal organization and phosphotransferase activity, apoptosis, and viral pathogenesis.

In the present study, we explored the potential effect of oxidative stress on GAPDH expression using a model with
significant physiological relevance, namely, the effects of OxLDL, a critically important proatherogenic agent^4–6,9,22 on one of its main cellular targets, namely vascular smooth muscle. We have previously shown that OxLDL is localized to vascular smooth muscle in both early and advanced human atherosclerotic plaques^22 and that OxLDL downregulates expression of insulin-like growth factor-1 (IGF-1) and its receptor in vascular smooth muscle.6,7,8 Here, we report that OxLDL markedly time- and dose-dependently downregulates GAPDH expression in HASMC via a redox-dependent mechanism, leading to a reduction in protein stability. Under the same experimental conditions, protein levels of other housekeeping molecules, namely β-actin and β-tubulin (data not shown) were not changed.

To identify mechanisms mediating OxLDL downregulation of GAPDH, we measured ROS formation in LDL-treated HASMC and demonstrated OxLDL-specific increases in total peroxides, superoxides, and H2O2. Antioxidants (Trolox, Tiron), LOX inhibitor (AA-861), and anti-CD36 receptor antibody suppressed both OxLDL-specific increase in ROS and downregulation of GAPDH and vice versa: inhibitors with no effect on ROS (allopurinol, indomethacin, NS-398) failed to prevent GAPDH downregulation. These results strongly suggest that OxLDL-generated ROS are critically important in mediating the downregulation of GAPDH.

We have recently shown, using a proteomic screen, that the CD36 receptor is markedly upregulated in HASMC exposed to OxLDL.7 This receptor is responsible for the uptake of approximately 80% of modified LDL by smooth muscle cells.23 In the present study, we report that the CD36 receptor is essential for both OxLDL-induced ROS formation and GAPDH downregulation. Our data suggest that OxLDL...
binding to CD36 results in enhanced ROS generation by LOX/NADPH oxidase, consistent with a novel functional relationship between CD36 and oxidases to generate ROS in HASMC.

ROS may be generated by different intracellular sources. The membrane-bound NADPH oxidase is a predominant source of ROS in multiple pathophysiological conditions in vascular smooth muscle. For instance, NADPH oxidase plays an essential role in angiotensin II–specific ROS formation in vascular smooth muscle. 5- and 12/15-LOX also contribute to generation of ROS in SMC and 5-LOX or 12/15-LOX deficiency suppressed atherosclerosis in apolipoprotein E–deficient mice. We demonstrated that specific inhibitors of NADPH oxidase assembly decreased OxLDL-specific ROS and partially prevented downregulation of GAPDH and that GAPDH downregulation was completely blocked in p47phox−/− vascular SMC. The LOX inhibitor AA-861 and 12/15-LOX deficiency also reduced OxLDL-induced ROS generation and GAPDH downregulation. These data strongly suggest that OxLDL uses at least 2 different oxidases (NADPH oxidase and 12/15-LOX) for generation of ROS and downregulation of GAPDH in response to OxLDL in vascular SMC.

Both total peroxide measurements with CDC-H2F and specific H2O2 detection by Amplex Red show that OxLDL quickly enhances cellular H2O2 level. Our previous observation that OxLDL compounds, oxidized fatty acids, could also increase intracellular levels of H2O2 in smooth muscle cells, mediating its cytotoxic effects, is consistent with these data. We report here that H2O2 scavengers, but not scavenging of superoxides, blocked the OxLDL-specific decrease in GAPDH. Furthermore, exogenous H2O2 mimics the effect of OxLDL and dose-dependently downregulates GAPDH. Taken together, these data indicate a major contribution of H2O2 in ROS-mediated GAPDH suppression.

We found that SODs have differential effects on OxLDL-specific GAPDH suppression: SOD acts as a partial blocker, but PEG-SOD is able to slightly potentiate GAPDH suppression. Dual functional roles of SOD (superoxide scavenger/H2O2 generator) could explain the difference between these effects. PEG-SOD, an enzyme with preferential intracellular distribution, decreases superoxides but also increases H2O2, which may reach toxic levels in some cellular compartments. In contrast, SOD-dependent H2O2 increase in the extracellular media may be less critical, because of dilution and/or antioxidant activity in conditioned medium. Our data suggest that one mechanism that could contribute to OxLDL-induced H2O2 elevation is the conversion of high levels of superoxides in OxLDL-treated cells into “extra” H2O2 by different active SODs present in HASMC. Our finding that OxLDL suppressed activity of the H2O2 scavenger, catalase, could be an additional mechanism contributing to the increase in cellular H2O2 levels.

Our data indicate that the primary mechanism for OxLDL downregulation of GAPDH is a reduction in protein stability, as shown by pulse-chase labeling experiments. OxLDL-induced GAPDH oxidation, and namely S-thiolation, was likely mediated via an increase in intracellular H2O2 (consistent with previous reports that GAPDH may be oxidized by

Figure 8. OxLDL- or siRNA-specific GAPDH suppression reduces cell glucose usage (A and D) and decreases cellular ATP levels (B and E). A and D, GAPDH suppression reduces cell glucose usage. LDL-treated cells (60 μg/mL, 16 hours) (A) or GAPDH siRNA– or negative control siRNA–transfected cells (50 nmol/L, 48 hours after transfection) (D) were incubated with 0.5 μCi/mL radioactive glucose, and aliquots of media were collected at time points indicated. H2O2 was separated from radioactive glucose by the vapor-phase equilibration method, and the radioactivity was analyzed. C, GAPDH siRNA–treated HASMC have decreased GAPDH protein level. Cells were resuspended in 100 μL of electroporation Amaxa buffer with 20 to 100 nmol/L Silencer 6-carboxyfluorescein (FAM)-labeled GAPDH siRNA or Silencer FAM-labeled negative control siRNA and pulse electroverted in Amaxa Nucleofector. After 48 hours, GAPDH protein levels were measured by immunoblotting. B and E, GAPDH suppression reduces ATP levels. LDL-treated cells (60 μg/mL, 16 hours) (B) or cells 48 hours after transfection with GAPDH siRNA or negative control siRNA (E) were used for ATP concentration measurement with luciferin/luciferase bioluminescent assay.
exogenous H$_2$O$_2$ in endothelial cells)$^{19,29}$) and resulted in a major decrease in GAPDH protein half-life. Thus, oxidized GAPDH protein was less stable than nonoxidized protein, as evidenced by the increased sensitivity of OxLDL- or H$_2$O$_2$-oxidized GAPDH to proteasome system-mediated degradation. These data strongly suggest that OxLDL-induced H$_2$O$_2$-dependent GAPDH oxidation makes this protein a preferential substrate for cellular protein degradation.

We found that the proteasome inhibitor MG-132 efficiently blocks oxidized GAPDH degradation in vitro (Figure 7C, top); however, this inhibitor as well as lactacystin failed to prevent OxLDL-specific GAPDH downregulation in HASMC (Figure 7D). As reported recently, 4-hydroxy-2-nonenal-induced GAPDH degradation in a leukemia cell line was also resistant to pretreatment with MG-132, suggesting that a system other than the ubiquitin–proteasome pathway was responsible for GAPDH degradation in vivo. Because a variety of protein-degradation systems mediate removal of oxidized proteins from the cell, including the proteasome system, a complex process of chaperone-mediated autophagy (lysosomes) and endoplasmic reticulum-associated degradation, identification of specific system(s) that mediate oxidized GAPDH degradation in vivo is an important area for further investigation.

Great variability has been reported for ATP levels within the atherosclerotic arterial wall depending on lesion stage, development of necrosis, the presence of lipoproteins, oxygen availability, and local levels of glucose. Notably, it has been demonstrated that ATP levels were strongly decreased in atherosclerotic lesions that developed in combination with hypercholesterolemia. Consistent with this in vivo study, we report here that there is marked suppression of cell glucose usage and a reduction in levels of ATP in vascular smooth muscle cells exposed to a major proatherogenic molecule, OxLDL. Furthermore, downregulation of GAPDH using siRNA produced equivalent reductions in glycolysis rate and ATP levels. Our data strongly suggest that OxLDL-specific GAPDH downregulation results in inhibition of glycolysis, which, in turn, probably, mediates general ATP depletion. We have also found that OxLDL decreases levels of 1 of the major glycolysis products, 1-lactate (S. Sukhanov, unpublished data, 2005). Our findings suggest that alterations in arterial wall energy homeostasis, related to OxLDL downregulation of GAPDH, may play an important role in mechanisms of atherogenesis.

Acknowledgments

We thank Dr T. Peng (Baylor College of Medicine, Houston, Tex) for the excellent assistance in preparation of LDL-treated cell lysates. We thank Dr R. Natarajan (Beckman Research Institute of the City of Hope, Calif) for kindly providing wild-type and 12/15-L0X-deficient cells and Drs M. Runge and N. Madamanchi (University of North Carolina at Chapel Hill, NC) for SMC derived from p47phox$^{-/-}$ mice.

Source of Funding

This study was supported by NIH grant HL70241.

Disclosures

None.

References


23. Sukhanov STJ, Serrano C, Bowers C, Delafontaine P. Anti-atherogenic effects of growth hormone releasing peptide-2 in vitro potential implications in prevention of atherosclerosis. American Heart Association...


Novel Effect of Oxidized Low-Density Lipoprotein: Cellular ATP Depletion via Downregulation of Glyceraldehyde-3-Phosphate Dehydrogenase

Sergiy Sukhanov, Yusuke Higashi, Shaw-Yung Shai, Hiroyuki Itabe, Koichi Ono, Sampath Parthasarathy and Patrick Delafontaine

_Circ Res._ 2006;99:191-200; originally published online June 15, 2006; doi: 10.1161/01.RES.0000232319.02303.8c

_Circulation Research_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2006 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/99/2/191

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2006/06/15/01.RES.0000232319.02303.8c.DC1

**Permissions:** Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Circulation Research_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

**Reprints:** Information about reprints can be found online at:
http://www.lww.com/reprints

**Subscriptions:** Information about subscribing to _Circulation Research_ is online at:
http://circres.ahajournals.org/subscriptions/
Online Data Supplements

Materials and Methods

Cell culture, isolation, oxidative modification of LDL and immunoblotting analysis protocols were described earlier\(^1\). Wild-type and 12/15-lipoxygenase-deficient vascular SMC were kindly provided by Dr. R. Natarajan (Beckman Research Institute of the City of Hope, CA). SMC derived from p47phox\(^{-/-}\) mice were obtained from Drs. M. Runge and N. Madamanchi (University of North Carolina at Chapel Hill, NC).

Materials

Trolox ((\pm) - 6-hydroxyl - 2, 5, 7, 8-tetramethylchromane-2-carboxylic acid) was purchased from Acros Organics (Geel, Belgium). Tiron (4, 5-dihidroxy-1, 3-benzene disulfonic acid), hydrogen peroxide, bovine liver catalase, polyethylene glycol-catalase (PEG-catalase) and polyethylene glycol-superoxide dismutase (PEG-SOD) were purchased from Sigma (St. Louis, MO) and bovine erythrocyte superoxide dismutase (SOD) - from ICN Biomedicals Inc. (Irvine, CA). NDGA, \textit{Larrea divaricata} (nordihydroguaiaretic acid), NS-398 (N-(2-cyclohexyloxy-4-nitrophenyl) methanesulfonamide), indomethacin (1-(p-chlorobenzoyl)-2-methoxy-3-methyl-1-H-indole-3-acetic acid), AEBSF (aminoethyl benzenesulfonofluoride) and apocynin (4-hydroxy-3-methoxyacetophenone) were obtained from Calbiochem (San Diego, CA).
ROS Measurements

Formation of intracellular ROS were assessed with the peroxide-sensitive dye 6-carboxy-2',7'-dichlorodihydro-fluorescein diacetate (CDC-H₂F, Molecular Probes)\(^2\), which also is sensitive to H\(_2\)O\(_2\) \(^3\), \(^4\) and the superoxide-specific dye hydroethidine (HE, Molecular Probes) \(^5\). Amplex Red kit (Molecular Probes), highly sensitive and specific hydrogen peroxide assay \(^6\) \(^7\), was used to measure the H\(_2\)O\(_2\) release from LDL-treated cells.

Confluent (80-90%) HASMC in 96-well plate (Nalge Nunc, Rochester, NY), were incubated for 1h at 37\(^\circ\)C with 10 µg/ml CDC-H₂F or 20 µmoll/L HE. HASMC were incubated with LDLs or SFM for 3h (CDC-H₂F-loaded cells) or for 20h (HE-loaded cells) and fluorescence was measured in Synergy HT multi-detection microplate reader (Bio-Tek Instruments, Winooski, VE) set at an excitation wavelength of 485±20 and an emission wavelength of 535±20 for CDC-H₂F and an excitation wavelength of 485±20 and an emission wavelength of 590±35 for HE. For some experiments dye was added after the treatment with OxLDL. Results obtained were essentially similar to those obtained in the standard protocol.

Specificity and linearity of ROS formation measurements were verified using superoxide donor paraquat (0-0.5 mmol/L) with/without scavenger of superoxides (SOD, 1 kU/ml) for HE assay and using H\(_2\)O\(_2\) (0-10 mmol/L) with/without scavenger of H\(_2\)O\(_2\) (catalase, 5 kU/ml) for CDC-H₂F and Amplex Red assays. We obtained a dose-dependent linear correlation between radical detector signal and
concentration of paraquat or H$_2$O$_2$ and significant suppression of signal when specific scavenger was present.

The potential presence of H$_2$O$_2$ in lipoprotein preparations was examined using Amplex Red assay. We found no detectable amount of hydrogen peroxide in LDLs (data not shown).

**Northern Blot Analysis**

Total cellular RNA was isolated using TriPure Isolation Reagent (Roche Diagnostic Corp., Indianapolis, IN) and additionally purified with RNeasy mini-kit (Qiagen) in accordance with manufacturer’s protocol. 10-15 µg of RNA was electrophoresed and transferred onto Hybond nylon membranes (Amersham) using standard molecular biological techniques. Membranes were washed, baked and hybridized to GAPDH cDNA probes in QuikHyb Hybridization Solution (Stratagene, La Jolla, CA) for 16h at 55°C. After washing and film exposing membranes were stripped and reprobed with the cDNA for β-actin to correct for the amount of RNA loaded. GAPDH and β-actin probes were $^{32}$P random prime-labeled (Invitrogen) to a specific activity of 1-3 × 10$^9$ cpm/µg and added to the hybridization solution to a concentration of 1 × 10$^5$ cpm/ml because of the abundance of GAPDH mRNA.

**Catalase Activity Assay**

Peroxidatic activity of catalase was measured with Catalase Assay Kit (Cayman Chemical). Briefly, cell lysates from LDL-treated cells (0.2-0.6 mg/ml), positive
control (bovine liver catalase) and standards (0-75 µmol/L formaldehyde) were mixed with methanol in assay buffer and reaction was initiated by adding H$_2$O$_2$ (4.15 mmol/L final concentrations). After 20 min incubation reaction was terminated by potassium hydroxide and formaldehyde amount was measured spectrophotometrically ($A_{540}$) with 4-amino-3-hydrazino-5-mercapto-1, 2, 4,-triazole. One unit of catalase activity was defined as the amount of enzyme that will cause the formation of 1.0 nmol/L formaldehyde per min at 25°C.

**Metabolic Labeling of HASMC and Immunoprecipitation**

Cells were pulse-labeled overnight (100 µCi/ml EXPRE$^{35}$S$^{35}$S Protein Labeling Mix, Perkin Elmer) and chased for 0, 6, 12 and 24h in SFM with/without OxLDL or nLDL (60 µg/ml) or H$_2$O$_2$ (1 mmol/L). At the end of each chase period, the cells were harvested in cold RIPA buffer$^1$ and after adjustment of protein concentration (BCA method, Pierce) extracts were subjected to immunoprecipitation with 4 µg mouse anti-GAPDH (Chemicon) or mouse anti-β-actin (Sigma) antibody. Immunocomplexes were analyzed after electrophoresis in 5/12% SDS-PAGE following by gel drying and scanning with phosphoimager. The major radioactive band (42 kDa in β-actin immunoprecipitate or 36 kDa in GAPDH precipitate) was clearly detected in each sample, quantitated by densitometry and ratio GAPDH/β-actin was determined for each time-point.

**GAPDH Purification**
GAPDH was purified by immunoprecipitation. After immunocomplex formation, the bound proteins were solubilized from agarose beads by the addition of low pH ImmunoPure IgG Elution Buffer (Pierce) and then neutralized with 1 mol/L Tris-HCl pH 7.5. Actual concentration of GAPDH in each sample was measured by plotting GAPDH band density from samples against known amounts of chicken muscle GAPDH.

**GAPDH Free Thiol Status**

Chicken muscle GAPDH (0.5 mg/ml, Sigma) directly oxidized in vitro by incubation with H$_2$O$_2$ or lyzate from cells treated by 0-2 mM H$_2$O$_2$ (3h) or from cells incubated with OxLDL or nLDL (60 µg/ml) for 0-9h or control (intact chicken muscle GAPDH) was labeled with 2 µmol/L thiol-sensitive fluorescent dye fluorescein-5-maleimide (F5M, Molecular Probes), separated on the 5/12% SDS-PAGE and F5M-labeled protein bands were visualized under UV-light (GelDoc System, Amersham). To identify GAPDH protein band and measure GAPDH protein level, the gel was immediately subjected for immunoblotting analysis with GAPDH antibody. Since F5M labeling efficiency is strictly dependent on availability of Cys thiol groups $^9$, $^{10}$, GAPDH-associated F5M signal intensity per GAPDH protein amount was used for qualitative analysis of GAPDH free thiol status.

**Proteasome Protein Degradation Assay**
GAPDH protein degradation rate was measured *in vitro* essentially by the method of Hernandez-Pigeon et al. 11. Briefly, equal amounts of GAPDH purified from HASMC treated by OxLDL (60 µg/ml, 16h) or nLDL (control) and from cells incubated with H₂O₂ (0.5 mmol/L, 16h) or SFM (control) was added to pre-warmed degradation mix (100 µg rabbit reticulocyte proteasome fraction, 2 mmol/L ATP, 1 mmol/L creatine phosphate, 25 U/ml creatine phosphokinase and 0.1 mg/ml ubiquitin). Aliquots were taken at time-points indicated and analyzed by immunoblotting with anti-GAPDH antibody. To verify the specificity of this degradation assay, the complete reaction mix was pre-incubated with proteasome inhibitor (MG-132, 50 µmol/L) and no subsequent degradation of oxidized GAPDH was detected. The specific activity of the degradation assay and the linearity of proteolytic degradation were verified by monitoring of the degradation of 250 µmol/L sLLVT-AMC (Calbiochem) into fluorogenic substrate AMC for 0-4h.

**Using Small Interfering RNA (siRNA)**

To suppress GAPDH or 5-lipoxygenase expression in HASMC, siRNA technology was employed. Cells (0.5-1.0x10⁶) were harvested, resuspended in 100 µl electroporation buffer (Amaya Biosystems, Cologne, Germany) with 20-100 nmol/L *Silencer* FAM-labeled GAPDH siRNA or 100 nmol/L *SiGENOME* SMART duplex siRNA generated against human 5-lipoxygenase (Dharmacon Inc., Chicago, IL) or *Silencer* FAM-labeled Negative Control siRNA (Ambion, Austin, TX) and pulse-electroporated in Amaya Nucleofector apparatus using the
U-025 program. We found that this protocol provided high efficiency (>80%) HASMC transfection as was measured by visualization of GFP fluorescence after cell electroporation with 2 µg of pmaxGFP vector (Amaxa). All cells plated after electroporation were viable as was verified with calcein-AM staining.

**Metabolic Studies**

The glycolytic flux was estimated by the rate of detritiation of [3-³H] glucose\(^{12}\). Briefly, HASMC were incubated with/without 60 µg/ml OxLDL or nLDL in SFM overnight or 48h after HASMC transfection with GAPDH siRNA or negative control siRNA. After washing, cells were incubated in SFM for 0-4h with tracer amount of radioactive glucose (0.5 µCi/ml, Amersham) and aliquots of media were collected at time-points indicated. \(^{3}\text{H}_2\text{O}\) was separated from radioactive glucose by vapor-phase equilibration method \(^{13}\) and the radioactivity was analyzed using a Beckman counter.

ATP concentrations were measured by luciferase/luciferine luminescent assay (Sigma) in according with manufacturer’s instructions.

**Supplemental Figures Legends**

**Supplemental Figure 1S.** LDLs activate intracellular peroxide (A, C, E) and superoxide (B, D, F) formation in HASMC. Cells loaded by peroxide-sensitive dye CDC-H\(_2\)F (A, B, E) or superoxide-specific dye HE (C, D, F) were stimulated by OxLDL (filled circles) or nLDL (open circles) and ROS formation was measured at time points and doses indicated. A, C – Treatment with 60 µg/ml
LDLs was used. LDL signal was adjusted to basal (SFM) level of peroxides (or superoxides) at each time point. B, D – Cells were treated by 20, 40, 60 and 80 µg/ml LDLs and OxLDL-specific “extra” peroxides (or superoxides) was measured by subtraction the nLDL-treated cells fluorescence signal from signal derived under treatment with OxLDL for each time point. *P<0.05 vs. nLDL. E, F - Fluorescence imaging of dye-loaded cells was performed after 4h (E) or 20h (F) of treatment by 60 µg/ml LDLs.

Supplemental Figure 2S. Block of CD36 scavenger receptor or lipoxygenase or NADPH oxidase decreases peroxide (A) and superoxide (B) formation. CDC-H2F–loaded (A) or HE-loaded (B) HASMC were stimulated by 60 µg/ml LDLs with or without pre-treatment (1 h) with 20 µg/ml anti-CD36 blocking antibody or normal mouse IgG (mlG) or 50 µmol/L NADPH oxidase blocker apocynin or 1 mmol/L AEBSF or 5 µmol/L lipoxygenase inhibitor AA-861 and fluorescence signal was measured after 4h (A) or 20h (B) of LDL treatment. *P<0.05, ‡P>0.05 vs.OxLDL no inhibitor.

Supplemental Figure 3S. 12/15-Lipoxygenase (B) or p47phox (C) deficiency decreases OxLDL-induced peroxide formation, however downregulation of 5-lipoxygenase has no effect (A). CDC-H2F–loaded wild-type or 12/15-lipoxygenase- or p47phox-deficient mouse vascular SMC were stimulated by 60 µg/ml LDLs and fluorescence signal was measured after 4h of LDL treatment (B, C). Human aortic SMC were resuspended in 100 µl electroporation Amaxa buffer with 100 nmol/L siGENOME SMART 5-lipoxygenase siRNA or Silencer FAM-
labeled negative control siRNA and pulse-electroporated in Amaxa Nucleofector and after 48h used for peroxide measurement in response to nLDL or OxLDL (4h). Showing is the increase in signal in response to OxLDL compared with nLDL (B). *P<0.05, ‡P>0.05 vs. control (nLDL).

References


