Group VIA Phospholipase A₂ Mediates Enhanced Macrophage Migration in Diabetes Mellitus by Increasing Expression of Nicotinamide Adenine Dinucleotide Phosphate Oxidase 4

Chunyan Tan, Robert Day, Shunzhong Bao, John Turk, Qingwei David Zhao

Objective—We previously demonstrated that nicotinamide adenine dinucleotide phosphate oxidase 4 (Nox4) mediates increased monocyte priming and chemotaxis under conditions of diabetic metabolic stress, and emerging data indicate that group VIA phospholipase A₂ (iPLA₂β) also participates in regulating monocyte chemotaxis. Here, we examined relationships between iPLA₂β expression and Nox4 action in mouse peritoneal macrophages subjected to diabetic metabolic stress.

Approach and Results—Increased iPLA₂β expression and activity were observed in macrophages from low-density lipoprotein receptor knockout mice that were fed a high-fat diet, and this was associated with time-dependent increases in atherosclerotic lesion size and macrophage content. Incubating macrophages with 30 mmol/L D-glucose, 100 μg/mL low-density lipoprotein, or both (D-glucose+low-density lipoprotein) induced a robust increase in iPLA₂β expression and activity and in cell migration in response to monocyte chemotactant protein-1. The increases in iPLA₂β activity and cell migration were prevented by a bromoenol lactone iPLA₂β suicide inhibitor or an iPLA₂β antisense oligonucleotide. Incubating macrophages under conditions that mimic diabetic metabolic stress ex vivo resulted in increased Nox4 expression and activity and hydrogen peroxide generation compared with controls. Bromoenol lactone prevented those effects without affecting Nox2 expression. Nox4 inhibition eliminated diabetic metabolic stress–induced acceleration of macrophage migration. Lysophosphatidic acid restored Nox4 expression, hydrogen peroxide generation, and migration to bromoenol lactone–treated cells, and a lysophosphatidic acid receptor antagonist abrogated iPLA₂β-mediated increases in Nox4 expression.

Conclusions—Taken together, these observations identify iPLA₂β and lysophosphatidic acid derived from its action as critical in regulating macrophage Nox4 activity and migration in the diabetic state in vivo and under similar conditions ex vivo. (Arterioscler Thromb Vasc Biol. 2014;34:768-778.)

Key Words: diabetes mellitus ■ macrophages ■ NADPH oxidase ■ phospholipases A₂

Diabetes mellitus is associated with an increased risk of cardiovascular disease,1 and individuals with diabetes mellitus have an absolute risk of cardiovascular disease, 1 and individuals with diabetes mellitus have an absolute risk of major coronary events similar to that of those without diabetes mellitus who have established coronary artery disease.2 In the evolution of type 2 diabetes mellitus, insulin resistance eventuates in β-cell failure that results in conditions that we have designated diabetic metabolic stress (DMS) characterized by hyperglycemia, hyperlipidemia, and hyperinsulinemia. Although molecular mechanisms that link diabetes mellitus to atherosclerosis and coronary artery disease are incompletely understood, enhanced recruitment of macrophages into inflamed vascular sites, such as coronary arteries, occurs in DM, and increased cellular oxidative stress contributes to this phenomenon.3-6

Reactive oxygen species (ROS) can be generated by mitochondrial metabolism or as byproducts of oxygenases such as cytochrome P450 monooxygenases, arachidonate dioxygenases, and the uncoupling of nitric oxide synthases, inter alia. In addition, nicotinamide adenine dinucleotide phosphate oxidases (Nox) are now recognized to be enzymes that produce ROS as a primary product rather than as a byproduct,7 and 7 human and animal Nox variants are now recognized. These include Nox1 to Nox5, Duox1, and Duox2, and they share the ability to transport electrons across the plasma membrane and to generate O₂⁻, which is then dismutated to yield hydrogen peroxide (H₂O₂). The Nox2 isoform was originally designated phagocytic Nox gp91phox and was believed to be the predominant ROS producer in phagocytes, and its role in the respiratory burst of neutrophils and macrophages has been studied extensively.8,9

We recently demonstrated that Nox4 is also a major inducible source of ROS in human and mouse macrophages but that...
Nonstandard Abbreviations and Acronyms

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<th>Abbreviation</th>
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<tr>
<td>BEL</td>
<td>bromoenol lactone</td>
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<tr>
<td>DMS</td>
<td>diabetic metabolic stress</td>
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<tr>
<td>$H_2O_2$</td>
<td>hydrogen peroxide</td>
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<tr>
<td>HFD</td>
<td>high-fat diet</td>
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<tr>
<td>HG</td>
<td>high glucose</td>
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<tr>
<td>iPLA$_2$</td>
<td>Ca$^{2+}$-independent phospholipase A$_2$</td>
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<td>LDL</td>
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<td>LPAR</td>
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<td>MCP-1</td>
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<tr>
<td>Nox</td>
<td>nicotinamide adenine dinucleotide phosphate oxidase</td>
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<td>PLD</td>
<td>phospholipase D</td>
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<td>ROS</td>
<td>reactive oxygen species</td>
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<td>siRNA</td>
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Nox isoforms other than Nox2 and Nox4 are barely detectable in these cells. Our most recent data demonstrated that Nox4, but not Nox2, contributes to increases in monocyte chemotaxis and macrophage recruitment under conditions of DMS in vivo and similar conditions ex vivo, but little is known about the relationship between expression and activity of iPLA$_2$ that produced by HG, and the combination of HG and LDL specific enzymatic activity that was similar in magnitude to the resultant lesions exhibit increased content of macrophages including monocyte chemoattractant protein-1 (MCP-1). Metabolites of arachidonic acid (eicosanoids, eg, prostaglandins and leukotrienes) and of lysophospholipids (eg, platelet-activating factor and endocannabinoids) serve as important inflammatory mediators, and both arachidonic acid and lysophospholipids, including LPA, also have intrinsic mediator functions. iPLA$_2$ has been reported to be an important participant in macrophage chemotactic responses to chemoattractants, including monocyte chemoattractant protein-1 (MCP-1). We demonstrated previously that transgenic mice that over-express iPLA$_2$ specifically in vascular smooth muscle cells develop exaggerated neointima formation in a carotid artery ligation model of vascular injury and atherogenesis and that the resultant lesions exhibit increased content of macrophages and inflammatory cytokines compared with those of control mice. In addition, emerging data reveal increased iPLA$_2$ expression in the tissues of humans and animals with diabetes mellitus, but the mechanism underlying this phenomenon or whether it involves Nox4 has not previously been examined to our knowledge. To address this issue, we fed a Western high-fat diet (HFD) to mice rendered deficient in the low-density lipoprotein (LDL) receptor by homologous recombination and compared them with mice fed a standard chow maintenance diet. We also studied isolated mouse peritoneal macrophages ex vivo that were incubated in medium containing a high glucose (HG) concentration, added LDL, or both in experiments that examined the following: (1) iPLA$_2$ expression and activity under conditions that mimic DMS; (2) any relationship between expression and activity of iPLA$_2$ and Nox4; and (3) effects of the potential iPLA$_2$ products arachidonic acid and LPA on Nox4 expression and activity.

Materials and Methods

Materials and Methods are available in the online-only Supplement.

Results

Increased iPLA$_2$ Expression and Activity in Macrophages From Mice Subjected to Metabolic Stress

We fed LDL receptor knockout mice a Western HFD for 8, 16, or 24 weeks to induce metabolic stress characterized by hyperglycemia and hyperlipidemia. Age-matched mice fed a standard chow maintenance diet served as controls. As summarized in the Table, consumption of the HFD resulted in a time-dependent increase in body weight, white fat pad mass, and concentrations of blood glucose, plasma total cholesterol, plasma triglycerides, and plasma insulin. Atherosclerotic lesion area and macrophage content also increased in a time-dependent manner in the HFD-fed mice (Table). Figure 1 illustrates that peritoneal macrophages from HFD-fed mice exhibited a time-dependent increase in iPLA$_2$ enzymatic activity (Figure 1A) and iPLA$_2$ enzymatic activity (Figure 1B). In addition, the HFD-fed mice exhibited a time-dependent increase in aortic sinus atherosclerotic lesion area and macrophage content, and both parameters exhibited positive linear correlations with peritoneal macrophage iPLA$_2$-specific enzymatic activity (Figure 1C and 1D). Similar correlations were observed between iPLA$_2$-specific activity and atherosclerotic lesion in aortic arch (data not shown). These findings suggest that increased iPLA$_2$ expression in macrophages of mice with diabetes mellitus may promote their migration into the subendothelial space of inflamed arteries to accelerate atherogenesis.

Metabolic Stress Enhances Expression and Activity of iPLA$_2$ in Macrophages Ex Vivo

To determine whether the changes in macrophage iPLA$_2$ expression that occurred in vivo in mice with diet-induced diabetes mellitus could be mimicked by exposure of macrophages to high concentrations of glucose and lipids ex vivo, peritoneal macrophages were isolated from C57BL/6J mice and cultured (24 hours) in medium containing normal level glucose (5.5 mmol/L), HG (30 mmol/L), LDL (100 μg/mL), or both (HG+LDL). The nonmetabolized L-glucose enantiomer was used as an osmotic control. As illustrated in Figure 2, incubation of macrophages with HG induced a 10-fold rise in content of immunoreactive iPLA$_2$ protein (Figure 2A) and an 8-fold rise in iPLA$_2$-specific enzymatic activity that was sensitive to inhibition by bromoenol lactone (BEL) and suppressed by an iPLA$_2$ antisense oligonucleotide (Figure 2B). The increased expression of iPLA$_2$ occurred as early as 6 hours after initiating exposure to HG and persisted at the same level for ≥8 hours (Figure 2A). Addition of LDL to the incubation medium also induced a rise in macrophage iPLA$_2$-specific enzymatic activity that was similar in magnitude to that produced by HG, and the combination of HG and LDL in the incubation medium induced an additive rise in iPLA$_2$. Thus, exposure ex vivo to incubation medium containing high
concentrations of glucose and lipoprotein induces a rise in macrophage iPLA\textsubscript{2}\textsubscript{β} expression and activity that is similar to that which occurs in vivo in the setting of hyperglycemia and hyperlipidemia that characterize diabetes mellitus. Notably, macrophage cell death was not observed on incubation in medium with HG and BEL (25 \text{μmol/L}) under the conditions of these experiments (Figure 2D).

**Metabolic Stress–Induced Enhancement of Macrophage Migration in Response to MCP-1 Requires Participation of iPLA\textsubscript{2}\textsubscript{β}**

To determine whether iPLA\textsubscript{2}\textsubscript{β} participates in the acceleration of the macrophage migratory response that occurs under conditions of DMS, the migration of isolated mouse peritoneal macrophages was measured in a Boyden chamber chemotaxis assay after 24 hours of incubation under various test conditions. Macrophages exposed to medium containing HG exhibited about a 3-fold increase in migration in response to MCP-1 compared with control macrophages incubated in medium with normal glucose concentration (Figure 3). Macrophages incubated in normal glucose medium supplemented with LDL exhibited migratory responses to MCP-1 similar to those of macrophages incubated with HG alone (Figure 3A). Incubation in HG medium supplemented with LDL resulted in macrophage migratory responses to MCP-1 that were additive (6-fold over control) compared with those that resulted from incubation with either HG or LDL alone (Figure 3A). Either pharmacological inhibition of iPLA\textsubscript{2}\textsubscript{β} activity with BEL or...
macrophages cultured with HG and without or with LDL and assayed without or with BEL. * Either a

obtide had no effect (Figure 4A). A time course of cell viability of peritoneal macrophages, as assessed by trypan blue exclusion, on incubation in medium supplemented with HG plus LDL (100 μmol/L) without or with BEL (25 μmol/L). No cell death was detectable within 48 hours of incubation under these conditions, and viability exceeded 90% at 72 hours without a significant difference between groups (n=3).

suppression of iPLAβ expression with antisense oligonucleotides prevented the effects of supplementing the incubation medium with HG, LDL, or both to stimulate macrophage migratory responses to MCP-1, although a sense oligonucleotide had no effect (Figure 3A). Restoration of iPLAβ expression to BEL-treated macrophages with adenoviral vectors reversed the inhibition of MCP-1–induced migration of macrophages that had been incubated in medium supplemented with both HG and LDL (Figure 3B). Moreover, peritoneal macrophages isolated from iPLAβ-knockout mice prepared by homologous recombination exhibited a much diminished enhancement of MCP-1–induced migration after incubation in medium supplemented with both HG and LDL compared with wild-type macrophages (Figure 3C). These observations demonstrate that the enhanced MCP-1–induced migration that occurs with macrophages incubated with high concentrations of glucose and LDL requires the participation of iPLAβ.

**Nox4 Expression and Activity Increase in Macrophages Incubated Under Conditions of Metabolic Stress, and This Requires the Participation of iPLAβ**

Accumulating evidence suggests that increased cellular ROS generation occurs in diabetes mellitus, and we have previously demonstrated that increased Nox4 activity and the resultant ROS generation contribute to the enhancement of macrophage migration and accumulation at inflammatory sites that occur under conditions of metabolic stress.11 To determine whether iPLAβ participates in these processes, ROS production in DCFH-DA–loaded macrophages was measured.

**Figure 2.** Expression and activity of group VIA phospholipase A2 (iPLAβ) increase in peritoneal macrophages from cultured in the presence of high concentrations of D-glucose or lipoproteins. iPLAβ immunoreactive protein expression and specific enzymatic activity were determined as in Figure 1 for mouse peritoneal macrophages cultured in medium supplemented with 5 mmol/L D-glucose (NG) or 30 mmol/L D-glucose (HG) without or with 100 μg/mL low-density lipoprotein (LDL) for the indicated intervals. The entanriometer L-glucose (LG) is not metabolized by mammalian cells and was used as an osmotic control at final concentration of 30 mmol/L. A, Quantitative immunoblotting of expression of iPLAβ protein by macrophages cultured under conditions described above. *P<0.01 for the difference between the indicated condition compared with NG (n=3). B, iPLAβ–specific enzymatic activity in macrophages cultured with HG or HG and assayed without or with the iPLAβ inhibitor bromoeno lactone (BEL; 25 μmol/L) or an iPLAβ antisense (AS) or sense (S) oligonucleotide. *P<0.01 for the difference between the indicated condition compared with HG (n=3). C, iPLAβ–specific enzymatic activity in macrophages cultured with NG or HG and assayed without or with BEL. *P<0.01 for the difference between the indicated condition compared with NG (n=3). D, A time course of cell viability of peritoneal macrophages, as assessed by trypan blue exclusion, on incubation in medium supplemented with HG plus LDL (100 μmol/L) without or with BEL (25 μmol/L). No cell death was detectable within 48 hours of incubation under these conditions, and viability exceeded 90% at 72 hours without a significant difference between groups (n=3).

**Figure 3.** Culture of peritoneal macrophages ex vivo in medium supplemented with high concentrations of glucose or lipoproteins results in an enhanced migratory response to monocyte chemoattractant protein-1 (MCP-1) in which group VIA phospholipase A2 (iPLAβ) participates. Mouse peritoneal macrophages were incubated (24 hours) in medium supplemented with 5 mmol/L D-glucose (control) or 30 mmol/L D-glucose (HG) without or with 100 μg/mL low-density lipoprotein (LDL). The HG+LDL condition is designated MS for metabolic stress. Macrophage migratory responses to MCP-1 were then assayed in a Boyden chamber in the absence or presence of bromoeno lactone (BEL) and iPLAβ antisense (AS) or sense (S) oligonucleotides. A, Culture with HG and LDL increases macrophage migration, and this is suppressed by BEL or AS. *P<0.01 vs the control condition. #No statistically significant difference between the indicated condition and the HG+LDL+S condition. B, Adenoviral vector–driven overexpression of iPLAβ (AdiPLAβ), but not scrambled control (scr) restores an enhanced migratory response to MCP-1 to BEL-treated macrophages. * Either a P<0.01 for the indicated condition compared with the other BEL-treated groups or a lack of a statistically significant difference compared with the MS condition. C, MCP-1–induced migration of peritoneal macrophages isolated from wild-type (WT) or iPLAβ-knockout (KO) mice after incubation in NG or MS medium. *P<0.01 compared with WT. #P<0.01 vs WT+MS (n=3).
by monitoring DCF fluorescence by fluorescence-activated cell sorter and was found to rise markedly in macrophages incubated in medium supplemented with HG and LDL compared with control macrophages incubated in normal glucose medium (Figure 4A). Moreover, this response was suppressed by the iPLA2β BEL, which also inhibited basal ROS production. We found that intracellular H2O2 levels were 6-fold higher in macrophages incubated in medium supplemented with HG and LDL compared with macrophages incubated in normal glucose medium and that both basal and HG+LDL-stimulated H2O2 levels were greatly reduced in BEL-treated macrophages (Figure 4B). Similar results were obtained when H2O2 was measured by an Amplex Red assay (Figure V in the online-only Data Supplement). Consistent with reports that Nox4 is the predominant source of macrophage H2O2 generation,21–23 we found that incubation in medium supplemented with HG and LDL resulted in a rise in immunoreactive Nox4 and iPLA2β protein levels, and we also found that BEL blocked the rise in Nox4 protein but did not affect the rise in iPLA2β protein (Figure 4C). The reverse transcription polymerase chain reaction analyses revealed that Nox4 mRNA levels rose in parallel with Nox4 immunoreactive protein in macrophages incubated in medium supplemented with HG and LDL, as illustrated in Figure III in the online-only Data Supplement, and Nox4 enzymatic activity also rose under these conditions (Figure 4D). The latter effect was blocked by BEL but restored to BEL-pretreated macrophages by infection with an adenoviral vector that drives expression of iPLA2β (Figure 4D). Because cytosolic superoxide dismutase (SOD) could affect measurements of Nox activity in whole cell homogenates, Nox activity was also measured in isolated total membrane fractions by the lucigenin method, and the results of these experiments were concordant with those from whole cell homogenates, as illustrated in Figure VI in the online-only Data Supplement. These observations suggest that increased Nox4 expression and the resultant ROS production in macrophages incubated in medium supplemented with HG and LDL require signals derived from iPLA2β. Consistent with this interpretation, we found that adenoviral vector–driven overexpression of iPLA2β in macrophages results in increased expression of Nox4 protein (Figure 4E). Incubation of macrophages in medium supplemented with HG and LDL also resulted in increased expression of Nox2 but not Nox1 protein, but BEL did not affect the Nox2 response (Figure 4F). Moreover, adenoviral vector–driven overexpression of iPLA2β did not affect macrophage Nox2 expression levels (Figure 4G). We also observed Nox activity in macrophages isolated from Nox2 knockout mice. On incubation in medium supplemented with HG and LDL, total Nox activity in Nox2-deficient macrophages was lower than that in wild-type macrophages infected with AdiPLA2β and in the presence of BEL, then loaded with DCFH-DA (10 μmol/L, 30 minutes), and intracellular ROS production reflected by DCF fluorescence was determined by fluorescence-activated cell sorter. *P<0.01 for the difference between the indicated condition compared with the other groups. #P<0.01 compared with the blank control. B, Intracellular H2O2 production was measured with an Abnova Hydrogen Peroxide Assay kit (catalog No. KA0801). †P<0.01 for the difference between the indicated condition compared with the other groups. #P<0.01 vs the blank control. C, Immunoblots of Nox4, iPLA2β, and actin in macrophages incubated under control or MS conditions without or with BEL. D, Nox enzymatic activity assays for macrophages incubated under control or MS conditions without or with BEL and effect of adenoviral vector–driven iPLA2β overexpression (AdiPLA2β) to restore increased Nox4 activity to BEL-pretreated cells. *P<0.01 vs the control or MS+BEL conditions. †P<0.01 vs the blank control. E, Immunoblot of Nox4 and iPLA2β in macrophages infected with AdiPLA2β or uninfected control macrophages. F, Immunoblot of Nox4 and iPLA2β in macrophages infected with AdiPLA2β or uninfected control macrophages, or in the presence of BEL. G, Immunoblot of Nox2, iPLA2β, and actin in macrophages infected with AdiPLA2β, uninfected control macrophages, or in the presence of BEL.
macrophages incubated under the same conditions, but this effect did not achieve statistical significance. In contrast, total Nox activity was reduced by $>70\%$ by adding either the Nox4 inhibitor GKT13783 or Nox4 small interfering RNA (siRNA; Figure IV in the online-only Data Supplement). These observations indicate that the increased ROS production observed in macrophages incubated in medium supplemented with HG and LDL is predominantly attributable to increased expression of Nox4 protein rather than to another Nox isozyme and that signals derived from the increased expression of iPLA$_β$ under these conditions are required for the effects on macrophage Nox4 expression and ROS production.

**Nox4 Activity Regulates Macrophage Migratory Responses to MCP-1**

We recently reported that Nox4 is the major source of ROS in macrophages and that Nox4-derived H$_2$O$_2$ participates regulating macrophage migration and in the signaling events that lead to monocyte dysfunction and a hyperchemotactic, proatherogenic macrophage phenotype.$^{10,11}$ Here, we used the pharmacological agent GKT137831 (Genkyotex, Geneva, Switzerland), which is a potent inhibitor of Nox4 and Nox1 activity, and Nox4 siRNA to examine Nox4 involvement in the macrophage migratory response to MCP-1 (Figure 5). We found that GKT137831 greatly attenuated (by $75\%$) the enhancement in MCP-1–induced migration that occurred with macrophages that had been incubated in medium supplemented with HG and LDL, that the migratory response was restored by adenoviral vector–driven overexpression of Nox4, and that Nox4 siRNA also suppressed the enhancement of migration with macrophages incubated with HG and LDL (Figure 5A). These findings indicate that Nox4 is a critical component of the machinery that produces enhanced MCP-1–induced migration of macrophages subjected to metabolic stress. To examine further the effect of Nox4-derived H$_2$O$_2$ on cell migration, we performed experiments in the presence of PEG-catalase and PEG-SOD and measured H$_2$O$_2$ production with an Amplex Red Assay. PEG-catalase pretreatment was found to suppress the enhancement of MCP-1–induced macrophage migration otherwise observed in macrophages that had been incubated in medium supplemented with HG and LDL, and this was associated with a reduction in measured H$_2$O$_2$ levels. This is consistent with catalase-catalyzed decomposition of H$_2$O$_2$ and with the hypothesis that H$_2$O$_2$ is involved in producing the enhanced migratory response. No similar effect of PEG-SOD was observed on cell migration and H$_2$O$_2$ production, which suggests that endogenous SOD activity is sufficient to support maximal H$_2$O$_2$ production so that no additional effect of exogenous SOD is observed (Figure 5B and 5C).

**LPA Derived From iPLA$_β$ Action is Required for the Increased Nox4 Expression and ROS Production That Occurs in Macrophages Subjected to Metabolic Stress**

We next examined the possibility that products of iPLA$_β$ action are responsible for the increased Nox4 expression and ROS production by macrophages subjected to metabolic stress. Such products include a free fatty acid, for example, arachidonic acid, and a 2-lysophospholipid, for example, 2-lyso-phosphatidylcholine generated from phosphatidylcholine or LPA generated directly from phosphatidic acid or indirectly by the action of autotaxin on 2-lysophosphatidylcholine.$^{24}$

Generation of LPA and 2-lysophosphatidylcholine is known to be severely impaired in some circumstances in iPLA$_β$-knockout mice.$^{25}$ In addition, arachidonic acid can be further metabolized to biologically active eicosanoids via lipooxygenases, cyclooxygenases, and cytochrome P450–dependent monoxygenases that produce a variety of mediators that affect inflammation.$^{26}$ Incubation of isolated mouse peritoneal macrophages in medium supplemented with arachidonic acid without or with inhibitors of cyclooxygenases (indomethacin), lipooxygenases (nordihydroguaiaretic acid), or cytochrome P450–dependent monoxygenases (17-octadecynoic acid) under conditions described in the Materials and Methods in the online-only Supplement did not affect macrophage Nox4 expression or activity or MCP-1–induced migration (not shown). In contrast, adenoviral vector–driven overexpression of iPLA$_β$ or incubation of macrophages in medium

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**Figure 5.** The nicotinamide adenine dinucleotide phosphate oxidase 4 (Nox4) inhibitor GKT137831 and catalase reduce monocyte chemoattractant protein-1 (MCP-1)–induced macrophage migration. **A**, GKT137831 reduces MCP-1–induced migration of both control macrophages and those subjected to metabolic stress (MS) by incubation with high concentrations of glucose and low-density lipoprotein (LDL). Mouse peritoneal macrophages were incubated (16 hours) in medium containing 5 mmol/L D-glucose (control condition) or in medium supplemented with 30 mmol/L D-glucose and 100 μg/mL LDL (MS condition). MCP-1–induced migration was measured in a Boyden chamber assay in the presence or absence of the Nox4 inhibitor GKT137831 (10 μmol/L) for macrophages infected with an adenoviral vector driving overexpression of Nox4 (AdNox4), control uninfected cells, or cells treated with Nox4 small interfering RNA (siRNA). **B** and **C**, Pretreatment with PEG-catalase (500 U/mL) but not with PEG-superoxide dismutase (SOD; 500 U/mL) reduces the effect of MS to enhance MCP-1–induced macrophage migration and hydrogen peroxide (H$_2$O$_2$) production, as measured with an Amplex Red assay kit (Invitrogen, catalog No. A22188). $^*P<0.01$ for the difference between the indicated condition compared with PEG alone (n=3).
supplemented with HG and LDL resulted in a several-fold increase in LPA production, and the response to both conditions together was additive (Figure 6A). Moreover, BEL prevented a rise in LPA production in response to either or both conditions (Figure 6A). That this increased LPA production has functional significance was suggested by the fact that the LPA receptor (LPAR) antagonist VPC32183 prevented the increase in macrophage Nox4 expression (Figure 6B), \( \text{H}_{2}\text{O}_{2} \) production (Figure 6C), and MCP-1–induced migration (Figure 6D) that otherwise occurred after incubation in medium supplemented with HG and LDL. Moreover, exogenous LPA reversed the BEL–induced suppression of the rises in Nox4 expression (Figure 7A), \( \text{H}_{2}\text{O}_{2} \) production (Figure 7B), and MCP-1–induced migration (Figure 7C) that occurred with macrophages incubated in medium supplemented with HG and LDL. To complement the findings with the LPAR antagonist, we examined effects of reducing LPAR expression with siRNAs directed at LPA1 and LPA3 receptors. Knockdown of LPA1 and LPA3 was found to attenuate metabolic stress–induced increases in Nox4 expression, ROS production, and cell migration, and these findings are concordant with those obtained with the LPAR antagonist VPC32183 (Figure I in the online-only Data Supplement). These findings indicate that LPA derived from the action of iPLA\(_2\) participates in the increased expression of Nox4, production of ROS, and enhanced MCP-1–induced migration that occur in macrophages subjected to metabolic stress ex vivo.

LPA derived from iPLA\(_2\) action could arise from direct action on phosphatidic acid or indirectly via initial action on phosphatidylcholine to produce lysophosphatidylcholine, followed by conversion of lysophosphatidylcholine to LPA by the action of a phospholipase D (PLD). To examine these possibilities further, we measured PLD activity and performed immunoblotting experiments to determine the phosphorylation state of PLD1 and PLD2 in macrophages incubated under control conditions or in medium supplemented with HG and LDL. Neither an increase in PLD activity nor an increase in phosphorylation of PLD1 or PLD2 was observed in these experiments (data not shown). These observations are consistent with a direct action of iPLA\(_2\) on PA to produce LPA but do not definitively exclude a contribution of PLD. To address whether inhibition of PLD would block the production and effects of LPA, we measured LPA production and Nox activity in metabolic stress–challenged macrophages in the presence of PLD1 and PLD2 inhibitors. The results of these experiments are illustrated in Figure VII in the online-only Data Supplement and demonstrated that the PLD2 inhibitor, but not the PLD1 inhibitor, resulted in slight suppression of LPA production and Nox activity, but these effects did achieve statistical significance relative to control cell incubations without inhibitors. These data suggest that PLD activity is not required for the effects or production of the LPA that arises as a consequence of iPLA\(_2\) action.

Nox4 activity might be increased by promoting phosphorylation of its binding partner p22phox, and we therefore conducted immunoblotting experiments to examine the phosphorylation state of p22phox under the conditions of our experiments. Knockdown of LPARs with siRNA was observed to reduce phosphorylation of p22phox, as illustrated in Figure II in the online-only Data Supplement. This suggests that the LPA/LPAR signaling axis may act to increase p22phox phosphorylation, which could represent one means by which Nox4 activation is achieved.

**Figure 6.** Macrophage production of lysophosphatidic acid (LPA) derived from group VIA phospholipase A\(_2\) (iPLA\(_2\)) action increases in cells subjected to metabolic stress (MS) by incubation with high concentrations of glucose and lipoprotein, and LPA drives increased nicotinamide adenine dinucleotide phosphate oxidase 4 (Nox4) expression, hydrogen peroxide \( (\text{H}_{2}\text{O}_{2}) \) production, and enhanced monocyte chemoattractant protein-1 (MCP-1)–induced migration. Peritoneal macrophages isolated from C57BL/6J mice were incubated in medium containing 5 mmol/L D-glucose (control condition) or supplemented with 30 mmol/L D-glucose and 100 \( \mu \)g/mL low-density lipoprotein (MS condition) without or with bromoenol lactone (BEL) and infection with an adenovirus driving iPLA\(_2\) overexpression (AdiPLA\(_2\)). **A,** Macrophage LPA production. **B** to **D,** Effects of pretreating macrophages with the LPA1/3 receptor antagonist VPC32183 (5 nmol/L, 30 minutes) before exposing them to MS conditions (24 hours) were examined, and VPC3218 pretreatment was found to suppress MS–induced increases in macrophage immunoreactive Nox4 expression in Western blotting experiments, **C** prevent MS–induced increases in macrophage \( \text{H}_{2}\text{O}_{2} \) production, and **D** prevent MS–induced enhancement of migration in response to MCP-1. \( P<0.01 \) for the difference between the indicated condition compared with the rest of the groups (n=3).
that expression of the enzyme iPLA₂β increases markedly in macrophages subjected to metabolic stress in vivo in mice with diabetes mellitus or ex vivo by incubation in medium supplemented with HG and LDL and that this results in increased production of LPA via iPLA₂ action. LPA then triggers a signaling cascade that leads to increased Nox4 expression and activity, increased ROS production, and enhanced MCP-1–induced macrophage migration under conditions of DMS.

Of 7 recognized Nox isoenzymes, Nox2 and Nox4 are the predominant ROS producers in macrophages. Nox activity in phagocytic cells is known to be regulated phosphorylation/dephosphorylation reactions. Phosphorylation of the p47phox subunit stimulates interaction with p67phox, and the resultant complex associates with Nox2 in the plasma membrane to assemble the active form of the oxidase. It has been reported that p41 (NoxO1) and p51 (NoxA1), which are homologues of p47phox and p67phox, respectively, are required for Nox activation in nonphagocytic cells. In contrast, Nox4 is constitutively active and is primarily regulated at the transcriptional level, although recent evidence suggests that post-translational regulation also occurs. Although Nox2 localizes primarily in the plasma membrane, Nox4 is distributed in intracellular membranous loci that include mitochondria, endoplasmic reticulum, and nuclear membranes. There is little information about how Nox4 expression and activity are regulated under physiological or pathological conditions, although a recent report indicates that Poldip2 associates with p22phox to activate Nox4, which regulates focal adhesion turnover and migration of vascular smooth muscle cell and thereby links ROS production and cytoskeletal remodeling. Our studies here are the first of which we are aware to demonstrate relationships among macrophage iPLA₂β activity, its product LPA, expression of Nox4, ROS production, and migratory responses to MCP-1.

Formerly, Nox2 was thought to be the predominant source of macrophage ROS, and its roles in the respiratory burst of phagocytes have been extensively examined. Nonetheless, participation of Nox2 in macrophage migration and accumulation at inflammatory sites in diabetes mellitus and atherosclerosis had not been demonstrated, although a recent report indicates that Nox2 participates in the migration of bone marrow–derived macrophages toward a gradient of increasing colony-stimulating factor–1 concentrations. Despite this, transfer of Nox2-deficient bone marrow cells into apoE-deficient mice had no significant effect on atherosclerotic lesion area at 24 weeks even though Nox2-deficient macrophages exhibited a profound reduction in basal and PMA-induced superoxide production. This suggests that Nox2 is not essential for monocyte recruitment to sites of vascular inflammation in vivo. We have previously demonstrated that knockdown of Nox4 protected monocytes from metabolic priming, although Nox2 expression was unaffected, and this also argues against a significant role for Nox2 in metabolic stress–induced monocyte dysfunction. Here, we demonstrate that incubation of macrophages in medium supplemented with HG and LDL does result in increased Nox2 expression, but neither iPLA₂β inhibition with BEL nor adenoviral vector–driven overexpression of iPLA₂β affected Nox2 expression, although those interventions had profound effects on macrophage Nox4 expression, ROS production, and MCP-1–induced migration. Together, these observations indicate Nox4 expression is regulated by iPLA₂β activity and that Nox4–derived ROS play an essential role in accelerated migration of macrophages subjected to DMS but that Nox2 does not play a similar role in these processes.

The profile ROS derived from Nox4 transfected is characterized by a greater proportion of H₂O₂ relative to O₂•− than is the case for Nox1, 2, or 3 or for Nox5. The mechanism underlying the preferential production of H₂O₂ versus O₂•− by Nox4 is proposed to be a highly conserved histidine residue in the E-loop that promotes the rapid dismutation of O₂•− before it is released from the enzyme. Here, we demonstrate that increased iPLA₂β expression in macrophages incubated with high concentrations of glucose and LDL results in production of ROS with a prominent H₂O₂ component and that LPA derived from iPLA₂β action participates in upregulating macrophage Nox4 expression.

Oxidation of cellular proteins via regulated redox systems is among the post-translational modifications that can modulate enzyme action in physiological and pathological conditions, and ionizable cysteine thiol groups react readily with H₂O₂. For example, reversible S-glutathionylation of actin at Cys promotes actin depolymerization, and prevention of...
this modification by site-directed mutagenesis of Cys^274 to alanine inhibits cell spreading.\textsuperscript{44} Nox4 colocalizes with \(\alpha\)-actin in smooth muscle cells and is found in actin-rich invadopodia of cancer cells, suggesting that Nox enzymes might be involved in the S-glutathionylation and redox regulation of actin. Continual treadmillng of F-actin filaments via polymerization–depolymerization is the process that underlies formation of retractable pseudopods in mobile macrophages. We have reported previously that Nox4 is recruited to and colocalizes with F-actin filaments in macrophages subjected to DMS or in which Nox4 overexpression is driven by an adenoviral vector and that this results in increased actin S-glutathionylation and accelerated F-actin turnover.\textsuperscript{11} Moreover, in preliminary experiments (not shown), we have observed that Nox4 activation affects F-actin assembly by promoting S-glutathionylation and consequent dephosphorylation of cofilin, which is an important actin depolymerizing factor. These observations suggest that Nox4-derived ROS may regulate S-glutathionylation of actin and of other redox regulated proteins, for example, cofilin. Other signaling complexes essential for macrophage migration might be similarly affected, although the molecular mechanisms by which Nox4 translocates to various intracellular loci and by which Nox4-derived H$_2$O$_2$ selectivity modifies target proteins remain the subjects of ongoing investigation and are incompletely understood at present.

We searched for but failed to find complexes of iPLA$_2^{\beta}$ and Nox4 in immunoprecipitation and immunoblotting experiments (data not shown), which argues against the possibility that iPLA$_2^{\beta}$ regulates Nox4 activity via a physical interaction. This suggests that an iPLA$_2^{\beta}$ reaction product might produce the downstream effects that result from increased iPLA$_2^{\beta}$ expression. One such product is a free fatty acid, such as arachidonic acid. This polyunsaturated fatty acid is distributed primarily in the sn-2 position of phospholipids, which is the site of action of iPLA$_2^{\beta}$. Arachidonic acid has essential mediator functions that regulate the activities of signaling enzymes, such as phospholipase C and protein kinase C isoforms (PLC-\(\gamma\), PLC-\(\delta\), and PKC-\(\alpha\), -\(\beta\), and -\(\gamma\)). In addition, arachidonic acid is a key inflammatory intermediate in metabolic cascades that result in production of prostaglandins, thromboxane, leukotrienes, epoxysisosteres, eicosanoids, and a variety of other eicosanoids with signaling functions.\textsuperscript{26} We have previously demonstrated that transgenic overexpression of iPLA$_2^{\beta}$ in vascular smooth muscle cells promotes production of inflammatory cytokines, macrophages infiltration, and proliferation of vascular smooth muscle cells in response to arterial injuries and that these responses are dependent on production of arachidonic acid metabolites. Here, we failed to observe any effects of adding exogenous arachidonic acid without or with various inhibitors of arachidonic acid oxygenases on macrophage Nox4 expression, which argues against the possibility that arachidonic acid or its metabolites represent the product(s) of iPLA$_2^{\beta}$ action that regulates macrophage Nox4 expression and downstream events.

In addition to free fatty acids, such as arachidonic acid, another product of iPLA$_2^{\beta}$ action is a 2-lyso phospholipid, such as LPA, and LPA production is greatly reduced in iPLA$_2^{\beta}$-knockout mice in some circumstances.\textsuperscript{25} LPA has diverse biological actions that affect Ca$^{2+}$ mobilization, cAMP accumulation, PLD activity,\textsuperscript{45} changes in cell shape, cell motility, actin rearrangement, and proliferation in various cells.\textsuperscript{46,47} Extracellular LPA has also been implicated in the pathogenesis of atherosclerosis and cancer.\textsuperscript{48,49} LPA can be generated in 2 potential pathways that involve iPLA$_2^{\beta}$, including direct hydrolysis of phosphatidic acid to yield LPA or via an indirect route that involves initial action on phosphatidylcholine to yield lysophosphatidylcholine followed by the action of the PLD autotaxin to yield LPA. Here, we have demonstrated that LPA derived from iPLA$_2^{\beta}$ action is required for increased Nox4 expression, H$_2$O$_2$ generation, and MCP-1–induced migration that occurs in macrophages subjected to DMS. Our data suggest that LPA derived from the action of iPLA$_2^{\beta}$ acts through LPARs to increase Nox4 activity and that the mechanism of this effect may involve phosphorylation of p22phox, which is a critical component of superoxide-generating NADH/nicotinamide adenine dinucleotide phosphate oxidase.

In summary, this study identifies iPLA$_2^{\beta}$ as a regulator of macrophage Nox4 expression and activity by the pathway depicted in Figure 8. Subjecting macrophages to metabolic stress in the diabetic state in vivo or by incubation ex vivo in medium supplemented with high concentrations of glucose and LDL results in increased expression of iPLA$_2^{\beta}$, and LPA derived from its action causes increased macrophage expression of Nox4 and production of H$_2$O$_2$ that leads to enhanced migration of macrophages into sites of vascular inflammation in vivo and in response to stimulation with MCP-1 ex vivo. Inhibition of iPLA$_2^{\beta}$ with BEL,

![Figure 8. Model for participation of group VIA phospholipase A2 (iPLA$_2^{\beta}$) in the signaling cascade induced by subjecting macrophages to metabolic stress that leads to increased nicotinamide adenine dinucleotide phosphate oxidase 4 (Nox4) expression and enhanced migration. Under conditions of diabetes mellitus in vivo or on exposure of macrophages to high concentrations of glucose and lipoproteins ex vivo, iPLA$_2^{\beta}$ expression and activity rise, and lysophosphatidic acid (LPA) production increases directly from hydrolysis of PA or indirectly by hydrolysis of phosphatidylcholine to yield 2-lyso phosphatidylcholine that is then metabolized to LPA. Interaction of LPA with its receptor(s) initiates a signaling cascade that results in increased Nox4 expression and activity, which in turn leads to increased production of reactive oxygen species (ROS) and hydrogen peroxide (H$_2$O$_2$) that enhance the migratory responses of macrophages to signals in vivo that lead to their accumulation at sites of vascular inflammation and to monocyte chemoattractant protein-1 ex vivo. This sequence can be interrupted by inhibiting iPLA$_2^{\beta}$ (with bromoenol lactone [BEL] or antisense oligonucleotides or in iPLA$_2^{\beta}$-knockout mice), by blocking LPA receptors (LPA-R; with the competitive antagonist VPC32183), or by inhibiting Nox4 (with GKT137831). In the diagram, arrows indicate activation or production, and T-lines indicate inhibition.](http://atvb.ahajournals.org/)

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blockade of LPARs with VPC32183, or inhibition of Nox4 with GTKI137831 interrupts this cascade and the downstream events of increased macrophage H2O2 production and enhanced migration. These findings suggest that iPLAβ may be the long-sought link between diabetes mellitus and accelerated atherosclerosis (and perhaps other complications of diabetes mellitus) that results from increased inflammation and that iPLAβ might thus be a suitable target for therapeutically beneficial interventions on these processes. A combinatorial approach directed at inhibiting iPLAβ, blocking LPARs, and inhibiting Nox4 might be particularly useful in that regard.

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Disclosures
None.

References


**Significance**

Diabetes mellitus is associated with increased inflammation and macrophage infiltration into inflamed tissues. We previously demonstrated that NADPH oxidase 4 is a major source of macrophage reactive oxygen species and participates in enhanced macrophage chemotaxis in diabetes mellitus, but how NADPH oxidase 4 is regulated is incompletely understood. Here, we have identified lysophosphatidic acid derived from the action of group VIA phospholipase A2 as a critical macrophage NADPH oxidase 4 regulator. Our findings suggest that group VIA phospholipase A2 may represent a long-sought link between diabetes mellitus and accelerated atherosclerosis that results from increased inflammation and that group VIA phospholipase A2 might thus be a suitable target for therapeutically beneficial interventions in these processes. A combinatorial approach directed at inhibiting group VIA phospholipase A2, blocking lysophosphatidic acid receptors, and inhibiting NADPH oxidase 4 might be particularly useful in that regard.
Group VIA Phospholipase A₂ Mediates Enhanced Macrophage Migration in Diabetes Mellitus by Increasing Expression of Nicotinamide Adenine Dinucleotide Phosphate Oxidase 4

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Online Figure I. Knockdown of LPA receptor expression with siRNA suppresses the amplification of Nox4 expression and ROS production otherwise observed in macrophages incubated ex vivo with high concentrations of glucose and low density lipoprotein. Peritoneal macrophages isolated from C57BL/6J mice were pre-incubated without (“nsRNA”) or with a mixture of siRNAs (designated “siLPA1/3”) directed against the receptors LPA1 and LPA3 and were then incubated (24 hr) in medium containing D-glucose (30 mM) and LDL (100 µg/mL). Preincubtion with the siLPA1/3 mixture was found to result in: A. Suppression of the increase in immunoreactive Nox4 expression otherwise observed in macrophages incubated with 30 mM glucose and 100 µg/mL LDL (“metabolic stress” condition); B. Prevention of metabolic stress-induced increases in macrophage H₂O₂ production, as measured by an Amplex Red assay; and C. Prevention of metabolic stress-induced enhancement of migration in response to MCP-1. nsRNA denotes non-silencing RNA and siLPA1/3 a mixture of siRNA species directed at the receptors LPA1 and LPA3. An asterisk (*) denotes a p value < 0.01 (n = 3).
Phosphorylation of p22phox increases in macrophages incubated under conditions of metabolic stress, and this is suppressed by LPA receptor knockdown with siRNA. Peritoneal macrophages isolated from C57BL/6J mice were pre-incubated without ("nsRNA") or with a mixture of siRNAs (designated “siLPA1/3”) directed against the receptors LPA1 and LPA3 and were then incubated (24 hr) in medium containing 5 mM D-glucose (“NG”) or 30 mM D-glucose plus 100 µg/mL LDL (“MS”). A, Macrophages subjected to metabolic stress (“MS”) by incubation with HG+LDL exhibit increased phosphorylation of p22phox compared to macrophages incubated in NG medium. B, Pre-incubation with the siLPA1/3 siRNA mixture was found to result in suppression of the metabolic stress-induced increase in macrophage p22phox phosphorylation (n = 4). The designations nsRNA and siLPA1/3 are as in Online Figure I.
Online Figure III. Incubation of mouse peritoneal macrophages ex vivo under conditions of metabolic stress results in increased expression of Nox4 mRNA. Peritoneal macrophages isolated from C57BL/6J mice were incubated (24 hr) in medium containing 5 mM D-glucose (“NG”) or 30 mM D-glucose plus 100 µg/mL LDL (“MS”). Representative images from RT-PCR analysis reflect increased Nox4 mRNA expression in macrophages incubated under conditions of metabolic stress. GAPDH was used as internal control (n = 4).
Online Figure IV. Nox activity in Nox2-deficient macrophages. Peritoneal macrophages isolated from wild type (WT) or Nox2 knockout mice (age 8 wk males, Jackson Laboratories) were pre-incubated (overnight) with vehicle control, with non-silencing RNA (nsRNA), with the LPA receptor antagonist GKT137831 (10 µM, “GKT”), or with Nox4 siRNA (“siNox4”) and were then incubated (24 hr) in medium supplemented with D-glucose (30 mM) plus LDL (100 µg/mL). Nox activity was then measured with a lucigenin chemiluminescence assay and normalized to the WT value. An asterisk (*) denotes a p value < 0.01 (n = 3).
Online Figure V. Macrophage H$_2$O$_2$ production as measured by the Amplex Red assay. Peritoneal macrophages isolated from C57BL/6J mice were incubated (24 hr) in medium containing 5 mM D-glucose ("NG") or 30 mM D-glucose plus 100 µg/mL LDL ("MS") with or without BEL (25 µM) pretreatment. Macrophage H$_2$O$_2$ production was then measured with an Amplex Red Hydrogen Peroxide Kit (Invitrogen). An asterisk (*) denotes a p value < 0.01 for the difference between the indicated condition compared to the other groups, and a hashtag symbol (#) denotes a p value < 0.05 vs. the blank control. (n = 3). Other conditions are as in Figure 4B.
Online Figure VI. Nox activity in macrophage total mixed membrane preparations. Conditions were as described in Figure 4D. Total mixed membrane fractions were separated using an ABCAM Membrane Fractionation Kit (#ab139409). Nox activity in the mixed membrane preparation was measured using a lucigenin chemiluminescence assay, as described in Experimental Procedures. An asterisk (*) denotes a p value < 0.01 for the difference between the indicated condition compared to the other groups, and a hashtag symbol (#) denotes a p value < 0.05 vs. the blank control (n = 3).
Online Figure VII. PLD activity is not for the production or effects of LPA generated as a result of iPLA₂β action. Mouse peritoneal macrophages in medium containing 5 mM D-glucose (“NG”) or 30 mM D-glucose plus 100 µg/mL LDL (HG+LDL) were incubated (16 hr) with the PLD1-specific inhibitor VU0359595 (50 nm) or the PLD2-specific inhibitor VU0285655-1 (50 nM) (Sigma-Aldrich) and then assayed for: A, PLD activity, which was measured in macrophages treated with HG plus LDL as described in Materials and Methods; B and C, LPA production and Nox activity, respectively, in macrophages incubated with NG or HG plus LDL, which were measured in the presence of PLD inhibitors (“i-PLD1” or “i-PLD2”) or vehicle. Macrophages incubated in medium containing 5 mM glucose were used as controls. Nox activity was measured with a lucigenin chemiluminescence assay. No significant difference was found among the groups of HG and LDL treated cells (n = 6). An asterisk (*) denotes a p value < 0.01 (n = 3) in Panel A.
MATERIALS AND METHODS

Sources of genetically modified mice and description of dietary intervention studies. All animal studies were performed with the approval of the UTHSCSA Institutional Animal Care and Use Committee. LDLR\(^{-/-}\) mice were obtained from Jackson Laboratories (Bar Harbor, ME). iPLA\(_{2}\)\(^{-/-}\) mice (iPLA\(_{2}\)-KO), and their wild type littermates were generated by mating heterozygotes and maintained as previously described.\(^{1-3}\) For dietary intervention studies, male LDLR\(^{-/-}\) mice 8 wk of age were housed in colony cages and maintained on a 12-h light/12-h dark cycle. After one week on a standard chow maintenance diet (MD, 7% soybean oil, AIN-93G, F3156, BioServ), mice were randomized into two groups and fed either MD (control mice) or a Western-style high-fat diet (HFD; 21% milk fat and 0.15% cholesterol, AIN-76A, F5540, BioServ) for 8, 16, or 24 weeks. Evening body weights and blood glucose levels were monitored biweekly and at the end of the study using a Contour® meter (Bayer). Plasma was prepared from whole blood drawn by cardiac puncture at the time of animal sacrifice. Plasma cholesterol and triglyceride levels were determined using enzymatic assay kits (Wako Chemicals). Epididymal fat pads were isolated and weighed. HFD-fed LDLR\(^{-/-}\) mice eventually developed hyperglycemia and hyperlipidemia (Table).

Quantitative morphometric analyses of atherosclerotic lesions. Heart and aortic arch were processed as described previously to prepare aortic sinus sections for staining and histological analyses.\(^{4-7}\) Tissues were frozen in OCT compound (Tissue- Tek) and cut into sections (10 µm thickness) that were stained with Oil-Red O to visualize neutral lipids and counterstained with hematoxylin. Atherosclerotic lesion areas were determined in 10 serial sections at 80 µm intervals that represented the entire length of each aortic sinus and quantified with Image-Pro Plus 6.0 software as previously described.\(^{3, 5, 8, 9}\) Macrophages in atherosclerotic lesions were identified by immunohistochemical staining with a primary antibody against CD68 and quantified with Image-Pro Plus 6.0 software.

Adenoviral vectors to drive overexpression of iPLA\(_{2}\)\(^\beta\) and Nox4. An adenoviral construct encoding iPLA\(_{2}\)\(^\beta\) was prepared with a ViraPower adenovirus expression system (Invitrogen) according to the manufacturer’s instructions.\(^{10}\) Briefly, cDNA that encodes the 84-kDa iPLA\(_{2}\)\(^\beta\) was subcloned into the pENTR directional TOPO cloning vector.\(^{11}\) After sequence verification, the iPLA\(_{2}\)\(^\beta\) cDNA was transferred into pAd/CMV/V5-DEST vector with the Gateway system using LR clonase (Invitrogen). Positive clones were confirmed by sequencing. The clones were linearized using PacI (New England Biolabs) and then transfected into 293A cells with Lipofectamine 2000 using Opti-MEM medium. Virus was prepared and amplified with the ViraPower adenovirus expression system (Invitrogen), and viral titers were determined by plaque-forming assays with 293A cells. An aliquot of viral suspension was used to infect mouse peritoneal macrophages, and iPLA\(_{2}\) activity was assayed 3 days after infection. As a control, pAd/CMV/V5-GW/lacZ vector (Invitrogen) was transfected into 293A cells to produce lacZ-bearing adenovirus that did not contain the iPLA\(_{2}\)\(^\beta\) coding sequence. Adenovirus encoding wild type Nox4 (AdWTNox4) was generously provided by Dr. B. J. Goldstein (Merck, Sharp, & Dohme Corp.) Experiments ex vivo to examine whether overexpression of iPLA\(_{2}\)\(^\beta\) or Nox4 reversed effects of the inhibitors BEL and GKT137831, respectively, involved preincubating macrophages with the appropriate inhibitor, followed by removal of the medium and replacing it with fresh medium without inhibitor, rinsing the cells once with PBS (37°C), and then transfection with the adenoviral vectors described above.

Isolation and culture of mouse peritoneal macrophages. Resident peritoneal macrophages from male LDLR\(^{-/-}\) mice fed MD or HFD, and from male C57BL/6J mice (age 16 wk) fed standard chow were harvested by lavage\(^{4, 5}\) and cultured (45 min) in medium supplemented with 15% fetal bovine serum (FBS) in petri dishes that had been coated with heat-inactivated FBS by overnight incubation. Non-adherent cells were removed by washing,
and macrophages, which remained attached, were cultured overnight in medium containing 15% FBS. Adherent cells were detached by incubation in Ca\(^{2+}\) and Mg\(^{2+}\)-free PBS containing 0.02% EDTA. Peritoneal macrophage recoveries of 90% were routinely obtained, and all recovered cells were viable, as demonstrated by trypan blue exclusion. Ex vivo experiments with the isolated macrophages were performed at a measured cell density of 2 x 10\(^5\) cells/mL in RPMI 1640 medium containing 5 mM D-glucose (normal glucose, NG) or 30 mM D-glucose (high glucose, HG) without or with human LDL (100 µg/mL) for 8, 16, or 24 hr as specified in the figure legends. The designation “MS” denotes culture in medium that contained both 30 mM D-glucose and 100 µg/mL LDL.

**LDL isolation.** LDL was purchased from Biomedical Technologies (Stoughton, MA) or prepared from pooled plasma from healthy blood donors by KBr-gradient ultracentrifugation and gel-filtration chromatography, followed by sterilization by filtration, and characterized by described procedures.\(^4\)

**Extraction and quantification of macrophage 2-lysophosphatidic acid (LPA).** LPA was extracted from macrophages essentially as described with minor modifications.\(^12\) In brief, isolated macrophages were homogenized in normal saline (0.9% NaCl, 200 µl, ice-cold) containing o-vanadate (100 mM) and EDTA (1 mM). Homogenates were placed in glass tubes (13 x 100 mm), mixed with acetone (1 mL), vortex-mixed, and centrifuged (1300 x g, 5 min). The resultant pellet was washed (0.5 mL acetone, twice), and concentrated to dryness under nitrogen. The residue was reconstituted in chloroform (0.1 mL), methanol (0.2 mL), and water (0.08 mL), and the mixture was centrifuged (1300 x g, 5 min). Supernatant was mixed with chloroform (0.2 mL), aqueous KCl (5%, 0.2 mL), and aqueous ammonia (28%, 0.001 mL), and the mixture was again centrifuged (1300 x g, 5 min). The supernatant was washed with chloroform/methanol (17:3, v/v, 0.4 mL, four times), and then monoisotopic [\(^{68}\text{Zn}\)]\(^2+\)-Phos-tag (10 nmol) and chloroform/methanol (17:3, v/v, 0.4 mL) were added to the supernatant (that consisted of the water/methanol phase). The mixture was vortex-mixed and centrifuged, and the lower (chloroform) phase was collected. The residual water/methanol phase was extracted again, and the combined chloroform phases were concentrated to dryness under nitrogen. The residue was reconstituted (50 µL, methanol containing 0.1% aqueous ammonia) and stored at -20°C until the time of analysis. Quantification of LPA content was performed with an Echelon LPA competitive ELISA kit (K-2800S, Echelon, Salt Lake City, UT) according to the manufacturer’s instructions.

**iPLA\(_2\) enzymatic activity assay.** iPLA\(_2\) enzymatic activity was measured by a commercial iPLA\(_2\) assay kit (Cayman, Ann Arbor, MI) under Ca\(^{2+}\)-free conditions as described.\(^13\)-\(^15\) Briefly, at the end of the incubation intervals under conditions described in the figure legends, macrophages were detached from the plates with a cell scraper in buffer (500 µL, 50 mM HEPES, pH 7.4, 1 mM EDTA) and disrupted by sonication (on ice, Branson Sonifier 450, duty cycle 30%, speed 30 s, interval 1 min, 6–10 times). The resultant homogenates were centrifuged (10,000 x g, 15 min, 4°C), and the protein concentration of the supernatant was determined. iPLA\(_2\) activity was measured by incubating (1 hr, 24°C) an aliquot of supernatant with substrate (arachidonoyl thio-phosphatidylcholine) in Ca\(^{2+}\)-free buffer (300 mM NaCl, 0.5% Triton X-100, 60% glycerol, 4 mM EGTA, 10 mM HEPES, pH 7.4, with 2 mg/ml bovine serum albumin). Reactions were terminated by adding 5,5′-dithiobis(nitrobenzoic acid. After 5 min, absorbance was determined (414 nm) with a standard plate reader and expressed per mg of protein as a measure of iPLA\(_2\) activity. As described,\(^8\),\(^16\) a BEL-insensitive background iPLA\(_2\)-independent component of basal lipase activity was determined in control samples when specific iPLA\(_2\) activity was inhibited by BEL pretreatment (25 µM, 30 min, 37 °C). This background activity was subtracted to achieve a measure of BEL-sensitive iPLA\(_2\) activity.
Measurements of ROS and H$_2$O$_2$. Intracellular ROS (O$_2^-$ and H$_2$O$_2$) production was measured in cells loaded DCFH-DA (Invitrogen, 10 μM, 1 hr), with a redox-sensitive indicator that preferentially reacts with H$_2$O$_2$ and other ROS. Intracellular ROS production (reflected by DCF fluorescence) was measured by fluorescence-activated cell sorting (FACS Calibur System, Becton Dickinson). Measurement of intracellular H$_2$O$_2$ was performed with Abnova Hydrogen Peroxide Assay kits (KA0801) or the Amplex Red assay kit from Invitrogen (A22188) according to the manufacturer’s instructions.

Measurement of NADPH oxidase activity. Macrophages were washed in PBS (ice-cold, five times), detached from the plates, and centrifuged (800 rpm, 10 min, 4°C). Cellular pellets were resuspended in lysis buffer [20 mmol/l KH$_2$PO$_4$, pH 7.0, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml aprotinin, 0.5 μg/ml leupeptin], and the suspensions were homogenized (300 strokes, Dounce apparatus, on ice). Assays were initiated by adding aliquots (20 μg protein) of cellular homogenates to phosphate buffer (50 nM, pH 7.0, 1 mmol/l EGTA, 150 mmol/l sucrose, 5 μmol/l lucigenin) containing NADPH (100 μM). Photon emission was measured (20 to 30 s intervals, 10 min) in a luminometer and expressed as relative light units (RLU). There was no measurable activity in the absence of NADPH. A buffer blank was measured and subtracted from each reading. Blank values represented less than 5% of signal obtained from cellular activity. Superoxide production was expressed as RLU/(mg protein)/min. Protein content was measured using a Bio-Rad protein assay reagent.

Macrophage migration in response to Monocyte Chemoattractant Protein-1 (MCP-1). Isolated mouse peritoneal macrophages were preincubated in medium supplemented with 5 mM D-glucose (NG) or 30 mM D-glucose (HG) without or with LDL (100 μg/mL), and medium was then removed and replaced with fresh medium without or with various test agents for the intervals indicated in the figure legends. At the end of the incubation intervals, the cells were placed in the upper wells of a 48-well modified Boyden chamber (NeuroProbe). Medium in the lower wells contained MCP-1 (2 nM, R&D Systems). A polyvinylpyrrolidone-free polycarbonate filter membrane with 5 μm pores was placed between the upper and lower chambers, and incubations were performed (90 min, 37°C, 5% CO$_2$). The membrane was then washed, and cells that had transmigrated through the filter to reach the lower side of the membrane were stained (Diff-Quik® Set, Dade Behring, Newark, DE) and counted (five separate high power fields, 400X magnification, light microscope).

Western blotting analyses. The protein contents of cellular homogenates prepared in RIPA lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1 mM Na$_3$VO$_4$, 1 mM PMSF, 20 μg/mL aprotinin, 20 μg/mL leupeptin, 1% NP-40) were determined by Bio-Rad protein assay, and aliquots were analyzed by SDS-PAGE. Gel protein bands were electroblotted onto polyvinylidene fluoride microporous membranes (Bio-Rad) that were then blocked (5% low-fat milk, Tris-buffered saline) and probed with primary antibodies (1:1000 dilutions) directed against target proteins specified in the figure legends that included a Nox 4 monoclonal antibody (Epitomics) and an iPLA$_2$β polyclonal antibody (Cayman Chemical, Ann Arbor, MI). Appropriate HRP-conjugated secondary antibodies were then added (1:3000), and bands were visualized by enhanced chemiluminescence. Densitometric analysis was performed using NIH Image/ImageJ software.

Knockdown of LPA receptor expression with siRNA. Peritoneal macrophages were transiently transfected with siRNA oligonucleotides by using Lipofectamine 2000 (Invitrogen). Two or three different siRNAs against each target were tested. The most effective siRNAs for LPA1 (GAAAUGAGCGCCACCUUA), LPA3 (CAGCAGGAGTTACCTTGTT), and non-silencing RNA (NS, D-001810-10) were obtained from Dharmaco (Lafayette, CO). The primary rabbit polyclonal IgG antibodies against LPA receptors 1 and 3 were purchased from Millipore.
Phospholipase D activity assay. PLD activity was measured by synthesis of \[^{3}H\]-phosphatidylethanol (PEth). Macrophages were seeded in 6-well-plates. To label phospholipids, cells were incubated (24 hr) in serum-free medium containing \[^{3}H\]-glycerol (1 µCi per mL). Subsequently, the cells were washed and incubated medium containing 5 mM glucose or 30 mM glucose and 100 µg/mL LDL. PLD inhibitors were added 30 min prior to addition of 30 mM glucose and LDL. After incubation (16 hr), phospholipids were extracted from cells and analyzed by thin layer chromatography. Spots corresponding to phosphatidylcholine, phosphatidic acid, and PEth were scraped from the plates separately, and their \[^{3}H\] content was determined by liquid scintillation counting.

Statistical analyses. Data were analyzed using ANOVA (SPSS 17.0) and subjected to parametric or nonparametric post hoc analyses. Multiple comparisons were performed by using the Least Significant Difference method. Linear correlation tests were performed with SigmaPlot version 12. Data are presented as mean ± SEM of at least 3 independent experiments unless otherwise indicated, and results were considered significant for p < 0.05.

REFERENCES


