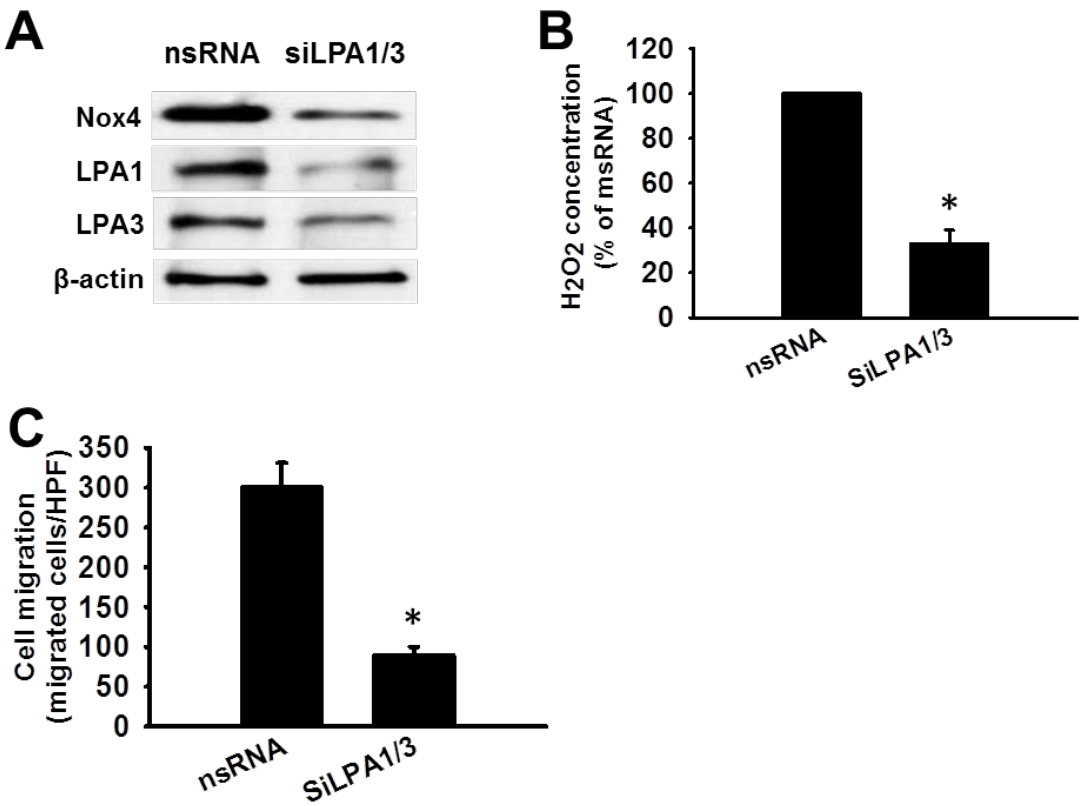
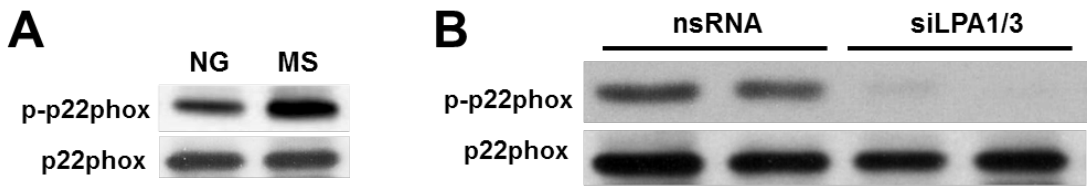
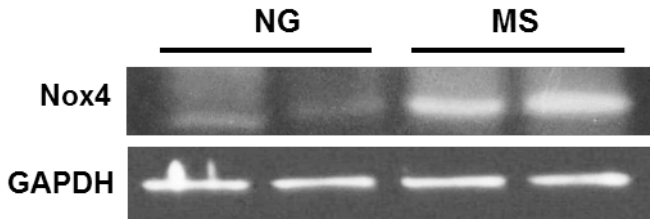
Online Figures I – VII and legends



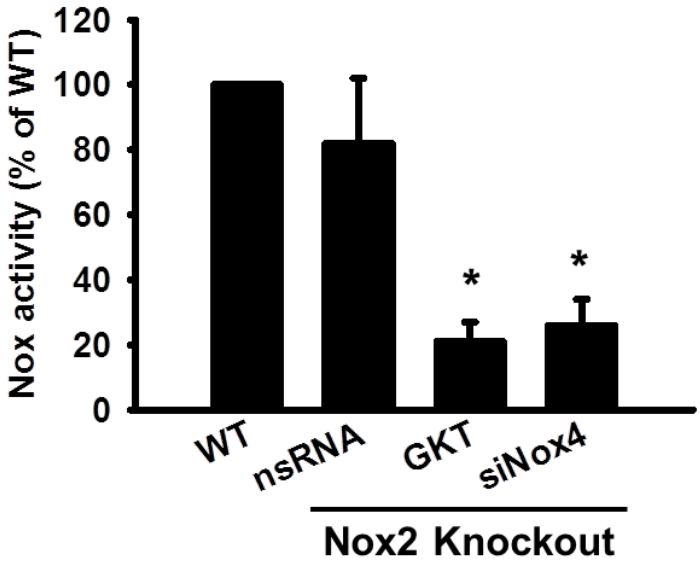
Online Figure I. Knockdown of LPA receptor expression with siRNA suppresses the amplification of Nox4 expression and ROS production otherwise observed in macrophages incubated ex vivo with high concentrations of glucose and low density lipoprotein. Peritoneal macrophages isolated from C57BL/6J mice were 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 H_2O_2 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).



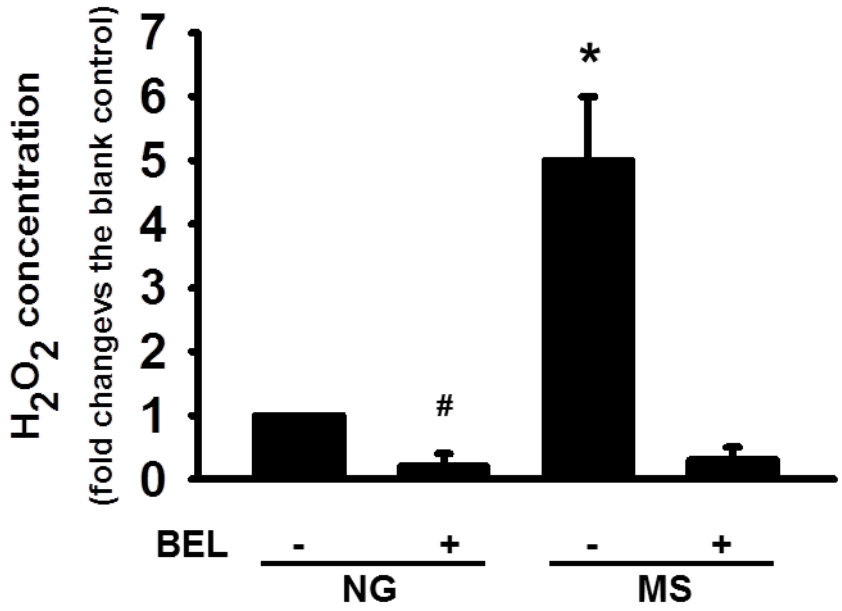
Online Figure II. Phosphorylation of p22phox increases in macrophages incubated under conditions of metabolic stress, and this is suppressed by LPA receptor knockdown with siRNA. Peritoneal macrophages isolated from C57BL/6J mice were pre-incubated without ("nsRNA") or with a mixture of siRNAs (designated "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.



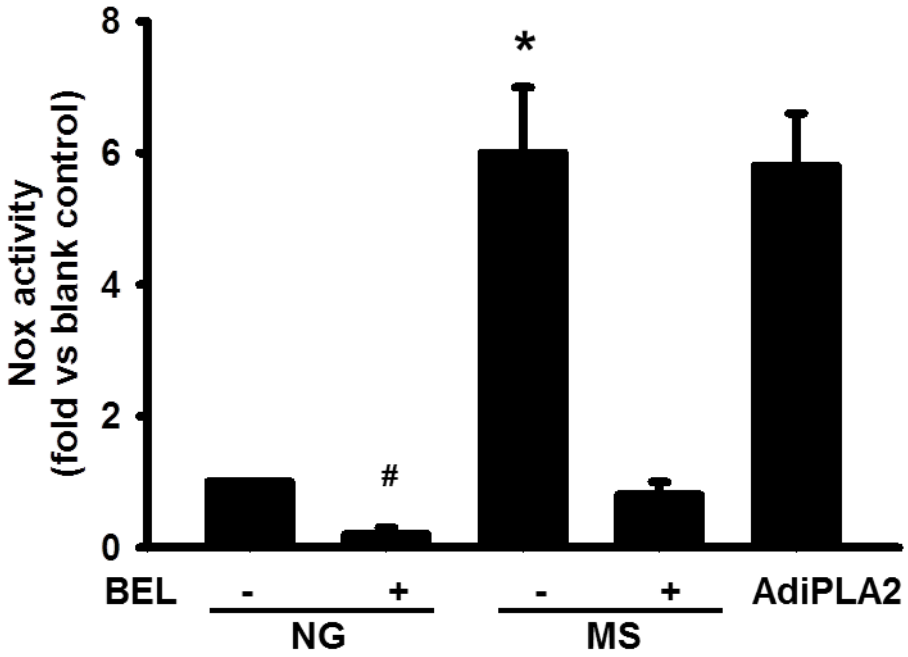
Online Figure III. Incubation of mouse peritoneal macrophages *ex vivo* under conditions of metabolic stress results in increased expression of Nox4 mRNA Peritoneal macrophages isolated from C57BL/6J mice were incubated (24 hr) in medium containing 5 mM D-glucose ("NG") or 30 mM D-glucose plus 100 µg/mL 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).



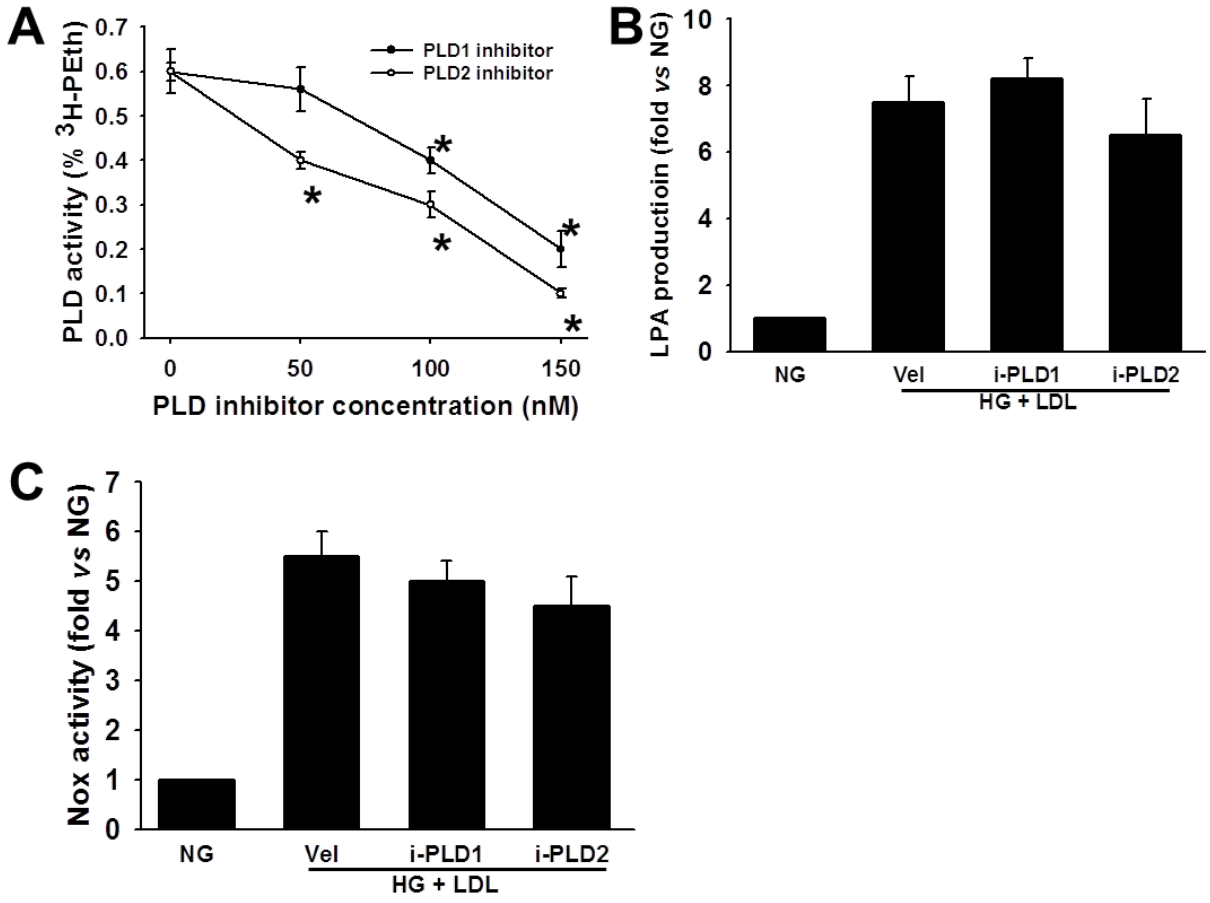
Online Figure IV. Nox activity in Nox2-deficient macrophages. Peritoneal macrophages isolated from wild type (WT) or Nox2 knockout mice (age 8 wk males, Jackson Laboratories) were pre-incubated (overnight) with vehicle control, with non-silencing RNA (nsRNA), with the LPA receptor antagonist GKT137831 (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_2O_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_2O_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 iPLA$_2$β 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.