FORUM ORIGINAL RESEARCH COMMUNICATION

Nox2-Mediated PI3K and Cofilin Activation Confers Alternate Redox Control of Macrophage Pinocytosis

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Abstract

Aims: Internalization of extracellular fluid and its solute by macropinocytosis requires dynamic reorganization of actin cytoskeleton, membrane ruffling, and formation of large endocytic vacuolar compartments, called macropinosomes, inside the cell. Although instigators of macropinocytosis, such as growth factors and phorbol esters, stimulate NADPH oxidase (Nox) activation and signal transduction mediators upstream of Nox assembly, including Rac1 and protein kinase C (PKC), are involved in macropinocytosis, the role of Nox enzymes in macropinocytosis has never been investigated. This study was designed to examine the role of Nox2 and the potential downstream redox signaling involved in macropinocytosis.

Results: Phorbol myristate acetate activation of human and murine macrophages stimulated membrane ruffling, macropinosome formation, and subsequent uptake of macromolecules by macropinocytosis. Mechanistically, we found that pharmacological blockade of PKC, transcriptional knockdown of Nox2, and scavenging of intracellular superoxide anion abolished phorbol ester-induced macropinocytosis. We observed that Nox2-derived reactive oxygen species *via* inhibition of phosphatase and tensin homolog and activation of the phosphoinositide-3-kinase (PI3K)/Akt pathway lead to activation of actin-binding protein cofilin, membrane ruffling, and macropinocytosis. Similarly, activation of macropinocytosis by macrophage colony-stimulating factor involves Nox2-mediated cofilin activation. Furthermore, peritoneal chimera experiments indicate that macropinocytotic uptake of lipids in hypercholesterolemic ApoE^{-/-} mice was attenuated in Nox2^{y/-} macrophages compared with wild-type controls.

Innovation and Conclusion: In summary, these findings demonstrate a novel Nox2-mediated mechanism of solute uptake *via* macropinocytosis, with broad implications for both general cellular physiology and pathological processes. The redox mechanism described here may also identify new targets in atherosclerosis and other disease conditions involving macropinocytosis. *Antioxid. Redox Signal.* 26, 902–916.

Keywords: macropinocytosis, NADPH oxidase, macrophage, cofilin, atherosclerosis

Introduction

EUKARYOTIC CELLS TRANSPORT extracellular nutrients and macromolecules, such as proteins, lipids, and extracellular ligands, across the cell membrane *via* energy-dependent processes called endocytosis (21). Endocytosis can be classified into three major categories: phagocytosis, receptor-mediated endocytosis, and pinocytosis (6). Fluid-phase endocytosis or macropinocytosis is distinct in many ways from the better characterized receptor-mediated endocytosis and phagocytosis. Macropinocytosis, unlike other cellular uptake mecha-

nisms, is initiated by protrusions of the plasma membrane that form cup-shaped circular ruffles or fold onto the nonextended plasma membrane, leading to generation of fluid-filled, membrane-derived vacuoles, also known as macropinosomes, which are larger (0.2–5 μ m) than other endocytic vesicles (12). After scission from the plasma membrane, macropinosomes undergo maturation, interact with endocytic compartments, and release their engulfed content into the cytosol. Although macropinocytosis is a low-grade constitutive process under control conditions (53), extensive and enduring macropinocytosis has been reported in response to growth factors,

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Innovation

The findings of the current study demonstrate that Nox2 signaling *via* inactivation of phosphatase and tensin homolog and activation of the PI3K/Akt pathway leads to membrane ruffling and macropinocytosis. Activation of this novel signaling pathway stimulates macrophage lipid internalization, leading to foam cell formation in hypercholesterolemic conditions *in vivo*. The redox mechanism described here may identify new targets in vascular inflammatory disease and other pathological conditions involving macropinocytosis.

macrophage colony-stimulating factor (M-CSF) (60), and phorbol esters (9, 55, 59). Macropinocytosis is important in a range of physiological and pathological processes. For instance, antigen-presenting cells use macropinocytosis to sample their environment for antigens and load-processed immunogenic peptides onto major histocompatibility complex II (MHCII) molecules for presentation to naïve T cells (53). Viruses and bacteria activate growth factor receptors or mimic phosphatidylserine-containing membranes of apoptotic cells to invade host cells *via* macropinocytosis (37, 42). In addition, macropinocytosis regulates cell motility, cancer cell proliferation, lipid uptake, and glomerular filtration rate, and it provides an effective platform for drug delivery into cells (12, 14, 32, 37). Although a number of signaling molecules, including Rac1, Cdc42, and phosphoinositides (PIs), have been implicated in macropinocytosis (6), the discrete mechanisms responsible for initiation of macropinocytosis and the sequence of how the different components are coordinated during macropinocytosis remain elusive.

The NADPH oxidase (Nox) of “professional” phagocytic cells (a.k.a. phagocyte Nox, Nox2, or *CYBB*) is the prototype isoform of a family of Nox enzymes (4). Phagocytes, in a process called *oxidative burst*, increase their oxygen consumption to provide the molecular oxygen (O_2) necessary for Nox2-derived superoxide anion ($O_2^{\cdot-}$) generation and microbicidal activity (54). Nox2 consists of flavocytochrome b558, an integral membrane heterodimer comprising gp91^{phox} and p22^{phox}, and four cytoplasmic protein subunits: p47^{phox}, p67^{phox}, p40^{phox}, and the regulatory low-molecular-weight GTPase Rac (4). Nox2 is dormant in resting cells and becomes rapidly activated on stimulation by growth factors, cytokines, and phorbol esters (25, 34). For instance, the 4 β stereoisomer of phorbol 12-myristate 13-acetate (4 β -PMA) stimulates phosphorylation of p47^{phox}, followed by translocation of cytosolic subunits to the catalytic core of Nox2 and generation of $O_2^{\cdot-}$, the precursor free radical of multiple reactive oxygen species (ROS). Nox2 is a target in numerous pathological conditions, as excessive Nox2-derived ROS has been shown to be involved in atherosclerosis, diabetes mellitus, cancer, Alzheimer’s disease, and allergic airway inflammation (4).

Interestingly, signal transduction mediators upstream of Nox2 activation, such as Rac1 and protein kinase C (PKC), have been reported to play a role in membrane ruffling and initiation of macropinocytosis (6). In addition, activators of macropinocytosis share the ability to stimulate Nox2 activity and to regulate downstream redox signaling pathways (25, 34). Despite this information, no prior studies have investigated the role of Nox2 in macropinocytosis or elucidated the

pathophysiological consequences of Nox-regulated macropinocytosis in a disease setting *in vivo*.

This study was designed to test the hypothesis that Nox2-derived ROS stimulate macropinocytosis, leading to internalization of macromolecules in phagocytes. The results of the present study provide seminal mechanistic insight into how increased Nox2 activity *via* inactivation of phosphatase and tensin homolog (PTEN) and activation of the phosphoinositide 3-kinase (PI3K)/Akt pathway leads to cofilin dephosphorylation, membrane ruffling, and macropinocytosis. Moreover, the present study suggests the role of Nox2 in macropinocytotic uptake of lipids by macrophages in an atherosclerotic mouse model *in vivo*. These findings are the first to demonstrate redox control of macropinocytosis through Nox2 activation.

Results*Stereoisomer 4 β -PMA stimulates macropinocytosis*

The signaling pathway initiating orchestration of actin reorganization in the submembranous region, leading to membrane ruffling and macropinocytosis is poorly defined. As shown in Figure 1A, high-resolution differential interference contrast (DIC) microscopy indicated that treatment of RAW 264.7 macrophages with 4 β -PMA (1 μ M, 30 min) induced extensive membrane ruffling, followed by formation of U- and O-shaped ruffles, cup closure, and internalization of macropinosomes, consistent with the ability of phorbol esters to stimulate macropinocytosis (55). As internalization of extracellular fluid and its solute is characteristic of macropinocytosis, we investigated whether 4 β -PMA stimulates uptake of native low-density lipoprotein (nLDL) in macrophages. Cells were incubated with nLDL (250 μ g/ml), treated with vehicle or 4 β -PMA (1 μ M, 24 h), fixed; intracellular lipids were stained with the fluorescent lipophilic dye Nile Red; and lipid accumulation was analyzed by confocal laser scanning microscopy and fluorescence-activated cell sorting (FACS). As shown in Figure 1B and Table 1, 4 β -PMA induced significant lipid accumulation in macrophages after treatment with nLDL. These results were confirmed by internalization of fluorescently labeled dextran (FITC-dextran, 70,000 MW) after 4 β -PMA treatment (Supplementary Fig. S1; Supplementary Data are available online at www.liebertpub.com/ars). Importantly, 70,000 MW FITC-dextran falls within the classically defined size range of 0.2–5 μ m macropinosomes (14).

Next, we interrogated whether 4 β -PMA stimulates internalization of nLDL *via* macropinocytosis or other endocytic processes. Macropinocytosis is often characterized by its sensitivity to PI3K inhibitors (32), actin perturbants (1), and Na^+/H^+ blockers (30). Preincubation of macrophages with the PI3K inhibitor LY294002 (1 μ M, 30 min), actin perturbant latrunculin A (1 μ M, 30 min), and Na^+/H^+ blocker amiloride (1 μ M, 30 min) significantly attenuated 4 β -PMA-induced lipid accumulation after nLDL treatment (Table 1). In contrast, inhibitors of clathrin- [chlorpromazine, (10 μ M, 60 min)] (5) and caveolin-mediated endocytosis [nystatin, (10 μ M, 60 min)] (61) had no effect on 4 β -PMA-induced lipid internalization (Table 1). To complement the pharmacological approach, we silenced the clathrin heavy chain (CLTC) by using siRNA in human macrophages, which did not inhibit FITC-dextran internalization (Fig. 1C). Validation of effective mRNA silencing is shown in Supplementary Figure S2. Finally, Supplementary Figure S3 demonstrating accumulation of 1 μ m red (580/605)

FIG. 1. 4β -PMA facilitates macropinocytosis via stimulating intracellular superoxide anion generation. “PMA” in figures means treatment with 4β -PMA, unless otherwise indicated. (A) RAW 264.7 macrophages pretreated with 4β -PMA ($1\ \mu\text{M}$) for 30 min were visualized using time-lapse DIC microscopy. Red arrows indicate membrane ruffles that dynamically changed to U- (green arrow) and O-shaped ruffles (yellow arrow), followed by cup closure (orange arrow), and internalization of macrophinosomes inside the cell (white arrows). DIC images are representative of three independent experiments. Scale bar: $5\ \mu\text{m}$. (B) RAW 264.7 macrophages were treated with nLDL ($250\ \mu\text{g}/\text{ml}$) and incubated with vehicle or 4β -PMA ($1\ \mu\text{M}$) for 24 h. Cells were fixed in 2% PFA and lipids stained by Nile Red (red). Images were taken with a Zeiss 780 confocal microscope ($63\times$). Similar results have been observed in three independent experiments. (C) THP-1 macrophages were transfected with control (Ctrl) or *CLTC* siRNA (48 h), incubated with FITC-dextran ($100\ \mu\text{g}/\text{ml}$) in the presence or absence of 4β -PMA ($1\ \mu\text{M}$) for 4 h, and FACS was performed. Bar graphs show the mean fold change of FITC fluorescence intensity ($n=3$). (D) WT and *CD36*^{-/-} macrophages were treated with nLDL ($250\ \mu\text{g}/\text{ml}$), incubated with vehicle or 4β -PMA ($1\ \mu\text{M}$, 24 h), fixed, and stained with Nile Red, and FACS analysis was performed ($n=4$). (E) RAW 264.7 macrophages were treated with vehicle, SOD ($150\ \text{U}/\text{ml}$, 1 h), or polyethylene glycol-conjugated SOD (PEG-SOD; $50\ \text{U}/\text{ml}$, 2 h), incubated with nLDL, and treated with 4β -PMA ($1\ \mu\text{M}$, 24 h). Nile Red fluorescence was analyzed by FACS ($n=3$). (F) RAW 264.7 macrophages were treated with vehicle or SOD ($150\ \text{U}/\text{ml}$, 1 h) and incubated with 4β -PMA ($1\ \mu\text{M}$, 24 h). Cells were fixed in 2% PFA and nuclei stained with Hoechst (blue); F-actin was labeled with 488 phalloidin (green); and lipids were visualized using Nile Red (red). Images were taken with a Zeiss 780 confocal microscope ($63\times$). Similar results have been observed in three independent experiments. Scale bar: $10\ \mu\text{m}$. (G, H) RAW 264.7 macrophages were preincubated with DHE ($10\ \mu\text{M}$, 30 min) and treated with vehicle or 4β -PMA ($1\ \mu\text{M}$) for 30 min. 2-hydroxyethidium (2-OH-E⁺) was quantified in vehicle- and 4β -PMA-treated macrophages by using HPLC ($n=3$). Confocal images were captured by following the same treatment protocol ($63\times$). Data represent the mean $\pm$ SEM. * $p < 0.05$ versus vehicle, # $p < 0.05$ versus ctrl PMA. CLTC, clathrin heavy chain; DHE, dihydroethidium; DIC, differential interference contrast; PMA, phorbol-12-myristate-13-acetate; nLDL, native low-density lipoprotein; SOD, superoxide dismutase.

TABLE 1. INHIBITION OF LIPID ACCUMULATION (%) IN 4 β -PHORBOL 12-MYRISTATE 13-ACETATE-TREATED MACROPHAGES AFTER INCUBATION WITH NATIVE LOW-DENSITY LIPOPROTEIN

Treatment	Inhibition (%)	p
Amiloride	56 $\pm$ 11.9	<0.05
Latrunculin A	99.1 $\pm$ 0.6	<0.05
LY294002	89 $\pm$ 1.7	<0.05
Chlorpromazine	No inhibition	
Nystatin	No inhibition	

FluoSpheres after treatment with 4 β -PMA is also consistent with 4 β -PMA inducing macropinocytosis, as the upper size limits for particles undergoing clathrin- and caveolin-mediated endocytosis are 200 nm and 500 nm in diameter, respectively (52). In summary, these data demonstrate that 4 β -PMA stimulates membrane ruffling, macropinosome formation, and macropinocytotic uptake of macromolecules in macrophages.

4 β -PMA promotes macropinocytosis via stimulating intracellular superoxide anion generation

4 β -PMA mimics endogenously produced diacylglycerol (DAG) and stimulates PKC activation in both macrophages and nonphagocytic cells (29). Interestingly, previous studies demonstrated that phorbol ester-induced macropinocytosis is completely blocked by PKC inhibitors and macropinocytotic uptake of macromolecules is stimulated by activators of PKC (31). As direct stimulation of PKC by 4 β -PMA phosphorylates the Nox organizer subunit p47^{phox} in macrophages, leading to enzymatic assembly of Nox2 and subsequent O₂^{•-} generation, we speculated that (i) O₂^{•-} acts as a signaling molecule in phorbol ester-induced macropinocytosis and (ii) 4 β -PMA-induced macropinocytosis involves activation of Nox2.

4 β -PMA stimulates extracellular O₂^{•-} generation that may lead to LDL oxidation in the media of cultured macrophages, subsequently stimulating its scavenger receptor (e.g., CD36)-mediated uptake. Consequently, we first tested the role of scavenger receptors in 4 β -PMA-induced lipid accumulation. As shown in Figure 1D, 4 β -PMA significantly stimulated lipid accumulation in CD36^{-/-} macrophages after nLDL treatment. These data demonstrate that 4 β -PMA induces lipid accumulation in macrophages lacking one of the major scavenger receptors, CD36. To confirm these results, we scavenged extracellular O₂^{•-} in the media by the cell-impermeant superoxide dismutase (SOD, 150 U/ml, 1 h), which did not inhibit phorbol ester-induced lipid accumulation in macrophages (Fig. 1E, F). Interestingly, scavenging of intracellular O₂^{•-} generation by the cell-permeant polyethylene glycol-conjugated SOD (PEG-SOD; 50 U/ml, 2 h) significantly attenuated 4 β -PMA-induced lipid internalization, suggesting a role for intracellular O₂^{•-} in phorbol ester-induced macropinocytosis (Fig. 1E). To support these findings, HPLC quantification of 2-hydroxyethidium (2-OH-E⁺), the O₂^{•-} specific oxidation product of dihydroethidium (DHE), indicated that intracellular O₂^{•-} generation was significantly increased in macrophages after 4 β -PMA treatment (1 μ M, 30 min) (Fig. 1G). Confocal images demonstrating increased DHE fluorescence after 4 β -PMA treatment are also shown in Figure 1H. Finally, Electron Paramagnetic Resonance (EPR) using the cell-permeant hydroxyl-

amine spin probe CMH confirmed that 4 β -PMA stimulates O₂^{•-} production in macrophages (Supplementary Fig. S4). Taken together, these findings suggest that 4 β -PMA stimulates macropinocytosis *via* a yet undefined mechanism, which involves intracellular O₂^{•-} as a signaling molecule.

Nox2 plays a pivotal role in 4 β -PMA-induced macropinocytosis

To investigate the role of the DAG-PKC pathway in phorbol ester-induced macropinocytosis, additional experiments were performed. First, the effect of 1,2-dioctanoyl-sn-glycerol (DOG, 100 μ M), a cell-permeant analog of DAG and an activator of PKC (58), was tested on internalization of FITC-dextran. As shown in Figure 2A, DOG significantly stimulated uptake of FITC-dextran in macrophages. Second, the inactive stereoisomer 4 α -PMA, which does not activate PKC (44), did not stimulate O₂^{•-} generation and macropinocytosis of FITC-dextran in macrophages (Fig. 2B, C). Third, calphostin c, an inhibitor of conventional and novel isoforms of PKC (33), completely blocked 4 β -PMA-induced accumulation of FITC-dextran (Fig. 2D). These data demonstrate that activation of the DAG-PKC pathway is involved in 4 β -PMA-induced macropinocytosis.

Because PKC, an upstream activator of Nox2 in macrophages (7), is involved in 4 β -PMA-stimulated macropinocytosis, we investigated whether 4 β -PMA stimulates macropinocytosis *via* Nox2 activation. Diphenyleneiodonium (DPI, 10 μ M), a widely used inhibitor of flavin-containing oxidases, significantly ameliorated 4 β -PMA-induced lipid uptake (Fig. 2E, F), suggesting a possible role of Nox in macropinocytosis. Though multiple isoforms of Nox are expressed in phagocytes, Nox2 is the most highly expressed isoform, and its activation by 4 β -PMA leads to a robust generation of O₂^{•-} (Supplementary Fig. S5). Consequently, we silenced Nox2 in THP-1 macrophages using siRNA and tested whether 4 β -PMA-induced macropinocytosis is attenuated. A significant decrease of FITC-dextran internalization was observed in macrophages that were transfected with Nox2 siRNA compared with scrambled siRNA-treated cells (Fig. 2G). qPCR data indicated that levels of Nox2 mRNA were decreased by 80% in Nox2 siRNA- *versus* scrambled siRNA-treated cells (Supplementary Fig. S6). In contrast, we observed no change in Nox1 and Nox5 mRNA levels in Nox2 gene-silenced cells (data not shown). Nox3 mRNA levels are undetectable in macrophages (35) and Nox4 is constitutively active (16). In separate experiments, peritoneal macrophages isolated from respective wild type (WT) and Nox2^{y/-} mice were treated with vehicle or 4 β -PMA, and macropinocytosis was investigated. As shown in Figure 2H, 4 β -PMA-induced internalization of FITC-dextran was completely blocked in Nox2^{y/-} macrophages compared with WT controls. These results are the first to demonstrate that 4 β -PMA stimulates macropinocytosis *via* Nox2. A distinct role for Nox1 in matricellular protein- and CD47-mediated macropinocytosis adds to the complex role of the Nox in this process and is described in detail elsewhere in this issue.

Nox2 activation is essential for 4 β -PMA-induced membrane ruffle formation

The next experiments were designed to investigate which step(s) during 4 β -PMA-induced macropinocytosis require(s) Nox2 activation. Scanning Electron Microscopy (SEM)

FIG. 2. Nox2 plays a pivotal role in 4β-PMA-induced macropinocytosis.

(A) RAW 264.7 macrophages incubated with FITC-dextran were treated with vehicle, 4β-PMA (1 μM), or DOG (100 μM), and FACS was performed (*n* = 6). (B) Cells were treated with vehicle, 4β-PMA (1 μM), or 4α-PMA (1 μM) and O₂^{•-} production was monitored by using L-012 chemiluminescence. The inset shows O₂^{•-} generation during the first 8 min of stimulation (*n* = 4). (C) FITC-dextran uptake after treatment as indicated in (B) (*n* = 6). (D) Cells were pretreated with calphostin c (1 μM, 60 min), treated with FITC-dextran, and incubated with vehicle or 4β-PMA. FITC fluorescence was analyzed by FACS (*n* = 6). (E, F) Cells were treated with vehicle or preincubated with diphenyleneiodonium (DPI, 5 μM, 10 min), treated with FITC-dextran, and challenged with 4β-PMA. FITC fluorescence was analyzed by FACS (*n* = 6). (G) Control (Ctrl) and Nox2 siRNA-transfected macrophages were incubated with FITC-dextran, treated with vehicle or 4β-PMA (1 μM, 4 h) and FACS was performed. The upper panels show representative FACS histograms. Lower panel indicates averaged FITC fluorescence (*n* = 3). (H) WT and Nox2^{yl-} peritoneal macrophages were treated as in (G), and FACS was performed (*n* = 3). Data represent the mean ± SEM. **p* < 0.05 versus vehicle, #*p* < 0.05 versus PMA, &*p* < 0.05 versus Ctrl siRNA + 4β-PMA or WT + 4β-PMA. FACS, fluorescence-activated cell sorting; WT, wild type. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

images demonstrated that 4β-PMA stimulates membrane ruffle formation on the dorsal surface of macrophages (Fig. 3A). Preincubation of cells with calphostin c, DPI, and EUK 134 inhibited 4β-PMA-induced membrane ruffling, demonstrating that PKC and O₂^{•-} derived from a DPI-inhibitable enzymatic source are involved in 4β-PMA-induced membrane ruffle formation (Fig. 3A). Next, live cell DIC microscopy was used to investigate the dynamics of 4β-PMA-induced plasma membrane activities (Supplemental Movie S1). 4β-PMA-stimulated plasma membrane exten-

sions dynamically changed their morphology, leading to ruffle bending, cup formation, and closure. DIC imaging confirmed that DPI and EUK 134 block 4β-PMA-induced membrane ruffle formation (Fig. 3B). To investigate whether 4β-PMA stimulates membrane ruffling *via* Nox2, we silenced Nox2 in macrophages, challenged them with 4β-PMA, and imaged the cell surface by using SEM. As shown in Figure 3C, 4β-PMA stimulated membrane ruffle formation in scrambled but not in Nox2 siRNA-treated cells, demonstrating the role of Nox2 in 4β-PMA-induced membrane ruffling.

FIG. 3. Nox2 activation is essential for 4 β -PMA-induced membrane ruffle formation. (A) RAW 264.7 macrophages were treated with vehicle, 4 β -PMA (1 μ M, 30 min), or pretreated with calphostin c (Calph.c, 1 μ M), DPI (10 μ M), or EUK 134 (10 μ M), and challenged with 4 β -PMA (1 μ M, 30 min). Cells were processed for SEM. Red arrows indicate membrane ruffle formation. Scale bar: 5 μ m. Bar graph shows the number of membrane ruffles in vehicle- and 4 β -PMA-treated macrophages normalized to total cell number ($n=3$). (B) DIC images after treatment as indicated in (A). Red arrows indicate membrane ruffle formation. Scale bar: 5 μ m. Representative of three independent experiments. (C) SEM images of control and Nox2 gene-silenced THP-1 macrophages after treatment with vehicle or 4 β -PMA (1 μ M, 30 min). Bar graph shows the number of membrane ruffles normalized to total cell number ($n=3$). Data represent the mean $\pm$ SEM. * $p<0.05$ versus vehicle, # $p<0.05$ versus Ctrl 4 β -PMA, & $p<0.05$ versus Control siRNA + 4 β -PMA. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

Nox2 induces membrane ruffle formation via dephosphorylation of cofilin

Previous studies demonstrated that binding of epidermal growth factor (EGF) to EGF receptor (EGFR) promotes circular dorsal ruffle formation in epithelial and mesenchymal cells (47). As Nox signaling mediates transactivation of EGFR (13), we tested whether the EGFR tyrosine kinase inhibitor erlotinib inhibits 4 β -PMA-induced macropinocytosis. As shown in Supplementary Figure S7, erlotinib did not inhibit macropinocytosis, suggesting that EGFR transactivation is not involved in phorbol ester-induced membrane ruffle formation.

Next, we postulated that actin-binding proteins that regulate rapid actin polymerization are associated with Nox2-mediated membrane ruffling. Accumulated evidence suggests that cofilin, through its actin-severing activity, creates new actin barbed ends for Arp2/3 to rapidly rebuild the actin network near the plasma membrane (19). As the severing activity of cofilin is increased on its dephosphorylation at Ser-3, we first tested whether 4 β -PMA dephosphorylates cofilin at Ser-3 in macrophages. Western blot data demonstrated that 4 β -PMA activates

cofilin within 15 min of incubation (Fig. 4A). This effect was completely inhibited in Nox2 gene-silenced macrophages (Fig. 4B). Time-course experiments indicated that 4 β -PMA starts stimulating ruffle formation within the first 15 min of incubation (Fig. 4C). Activated PI3K and its phosphorylation product PI(3,4,5)P₃ promote cofilin activation (45); whereas PTEN negatively regulates PI3K/Akt signaling by dephosphorylating PI(3,4,5)P₃ and it inactivates cofilin (45). As phosphorylation of PTEN at the serine-threonine cluster (Ser380, Thr382, and Thr383) in the C-terminal tail suppresses its phosphatase activity, we investigated whether 4 β -PMA phosphorylates PTEN at residues Ser380, Thr382, and Thr383 and phosphorylates Akt at Ser473. These data demonstrate decreased phosphatase activity of PTEN and increased PI3K/Akt signaling after phorbol ester stimulation. Consistent with the role of Nox2 in this process, 4 β -PMA did not phosphorylate PTEN in Nox2^{-/-} macrophages (Supplementary Fig. S8). To investigate the role of PI3K in 4 β -PMA-induced cofilin dephosphorylation, we preincubated macrophages with the PI3K inhibitor LY294002, treated cells with 4 β -PMA, and

FIG. 4. 4β-PMA phosphorylates PTEN and stimulates cofilin activation. (A) RAW 264.7 macrophages were treated with vehicle or 4β-PMA (1 μM; 15, 30, or 60 min). Cells were lysed and subjected to Western blot analysis for phospho- or total cofilin, Akt, and PTEN expression. Representative Western blot images are shown. Bar graphs represent averaged optical-density data that are expressed as a ratio of phospho- to total proteins ($n=3$). (B) Control and Nox2 siRNA-silenced macrophages were incubated with vehicle or 4β-PMA. Cells were processed for Western blot analysis for phospho- and total cofilin expression ($n=3$). (C) SEM data indicating the number of ruffle formation after treatment with 4β-PMA at the indicated time-points ($n=3$). Data represent the mean $\pm$ SEM. * $p<0.05$ versus vehicle. PTEN, phosphatase and tensin homolog. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

assessed the phosphorylation status of cofilin at Ser-3 *via* Western blotting. Figure 5A indicates that LY294002 inhibited 4β-PMA-induced cofilin dephosphorylation in macrophages. To provide functional evidence that cofilin is involved in 4β-PMA-stimulated macropinocytosis, we silenced cofilin using siRNA, treated the cells with vehicle or 4β-PMA, and analyzed FITC-dextran uptake using FACS. Figure 5B demonstrates that cofilin siRNA significantly attenuated 4β-PMA-induced FITC-

dextran internalization compared with scrambled siRNA-transfected controls. Validation of effective mRNA silencing is shown in Supplementary Figure S9. Finally, SEM data indicated that 4β-PMA-stimulated membrane ruffling was abrogated in cofilin gene-silenced macrophages (Fig. 5C). Taken together, these data demonstrate that 4β-PMA-induced membrane ruffling occurs through Nox2-mediated inactivation of PTEN, stimulation of PI3K/Akt signaling, and cofilin dephosphorylation.

Physiological stimulation of macropinocytosis by M-CSF involves Nox2 activation

To test the physiological importance of this pathway, additional experiments were performed by using M-CSF, a physiologically relevant stimulator of macropinocytosis (60). Importantly, previous data demonstrated that M-CSF stimulates Nox activity in leukocytes (56); however, the role of Nox or ROS in M-CSF-induced macropinocytosis has not been investigated. FACS data indicated that M-CSF treatment (100 ng/ml, 4 h) significantly stimulated internalization of FITC-dextran in WT macrophages (Fig. 6A). The amiloride derivative 5-(N-Ethyl-N-isopropyl)amiloride (EIPA) (10 μM, 30 min) abolished M-CSF-induced FITC-dextran internalization, consistent with previous findings that M-CSF induces macropinocytosis (51). Pretreatment with the cell-permeant antioxidant EUK 134 (10 μM, 1 h) and DPI (10 μM, 1 h) inhibited M-CSF-stimulated macropinocytosis (Fig. 6A). These results suggest that M-CSF-induced macropinocytosis involves $O_2^{\cdot-}$ production that is generated by a DPI-inhibitable enzymatic source. Next, WT and Nox2^{-/-} macrophages were treated with vehicle or M-CSF and macropinocytosis was investigated. As shown in Figure 6B, internalization of FITC-dextran was significantly decreased in Nox2^{-/-} macrophages compared with WT controls.

Western blot data indicated that M-CSF rapidly dephosphorylated cofilin at Ser-3 in WT macrophages (Fig. 6C). EUK 134 and DPI inhibited M-CSF-induced cofilin activation (Fig. 6C and Supplementary Fig. S10). The effect of M-CSF on cofilin dephosphorylation was attenuated in macrophages derived from Nox2^{-/-} mice (Fig. 6D). As shown in Figure 6C, M-CSF phosphorylated PTEN and Akt in WT macrophages, demonstrating decreased phosphatase activity of PTEN and increased PI3K/Akt signaling. Incidentally, when cells were pretreated with EUK 134 or DPI, M-CSF-induced PTEN phosphorylation and Akt activation were attenuated. Finally, M-CSF did not phosphorylate PTEN in Nox2^{-/-} cells (Fig. 6D).

Role of Nox2-mediated macropinocytosis in an animal model of atherosclerosis

To determine the role of Nox2-mediated macropinocytosis *in vivo*, we evaluated the ability of macrophages derived from WT or Nox2^{-/-} mice (donors) to accumulate cholesterol after their adoptive transfer into the peritoneal cavities of high-fat diet-fed ApoE^{-/-} mice (recipients). This approach was validated by previous studies and demonstrated to provide useful information about the role of specific genes in the regulation of foam cell formation *in vivo* (36). Donor macrophages were fluorescently labeled by using the cell tracker dye CFDA, incubated with vehicle or treated with 4β-PMA (1 μM, 30 min) to stimulate membrane ruffling, and transferred into the peritoneal cavities of ApoE^{-/-} mice (Fig. 7A). Twenty

hours later, peritoneal macrophages were isolated, visualized under a fluorescent microscope (Fig. 7A), fixed; lipids accumulated from the peritoneal cavity stained with Nile Red; and CFDA⁺ (donor) macrophages were selected for FACS analysis (Fig. 7B). 4 β -PMA stimulated lipid accumulation in WT-CFDA⁺ macrophages by approximately four-fold compared with vehicle treatment (Fig. 7D). In contrast, measurement of Nile Red fluorescence by FACS indicated that 4 β -PMA-induced lipid accumulation was attenuated in Nox2^{-/-}CFDA⁺ macrophages (Fig. 7C, D). These data suggest that Nox2-mediated membrane ruffling and subsequent macropinocytosis of cholesterol may lead to significant lipid accumulation in macrophages in hypercholesterolemic conditions.

Discussion

To our knowledge, this is the first study that seeks to examine the role of Nox2 in macropinocytosis. Although our knowledge of the regulatory mechanisms in macropinocytosis has greatly increased in recent years (3, 39, 60), the precise signaling pathway responsible for the initiation of macropinocytosis remains unknown. Results here demonstrate that Nox2 signaling *via* inactivation of PTEN and stimulation of the PI3K/Akt pathway dephosphorylates cofilin, leading to membrane ruffle formation and macropinocytotic solute internalization. As such, these results may contribute to a better understanding of the underlying mechanisms, regulating membrane ruffling and macropinocytosis.

Although macropinocytosis and phagocytosis share many common features, including a large vacuole size, transient activation of actin cytoskeleton, and other downstream regulatory components, they are fundamentally different processes (42). Phagocytosis is a strictly particle-driven process whereas macropinocytosis is nonspecific internalization of large amounts of fluid and solutes. During phagocytosis, actin modifications remain localized around the particle, whereas macropinocytosis involves global activation of the actin cytoskeleton, resulting in extensive plasma membrane ruffling and fluid internalization. Macropinocytosis is also distinct from receptor-mediated endocytic processes, as it does not require receptor-ligand interaction for internalization. As such, the

FIG. 5. Pivotal role of cofilin in 4 β -PMA-induced membrane ruffle formation. (A) RAW 264.7 macrophages were treated with vehicle or 4 β -PMA (1 μ M, 30 min), or they were preincubated with LY294002 (LY, 10 μ M, 60 min) and stimulated with 4 β -PMA. Cells were processed for Western blot analysis for phospho- and total cofilin expression. Representative Western blot images are shown. Bar graphs represent averaged optical-density data that are expressed as a ratio of phospho- to total cofilin (n=3). (B) Control (Ctrl) and cofilin siRNA-transfected macrophages were incubated with FITC-dextran, treated with vehicle or 4 β -PMA (1 μ M, 4 h), and FACS was performed. (C) Cells were treated as indicated in (B), and SEM experiments were performed. The upper panels show representative SEM images. The lower panel indicates the number of membrane ruffles normalized to cell number (n=3). Data represent the mean $\pm$ SEM. * p < 0.05 versus vehicle, # p < 0.05 versus Control siRNA + 4 β -PMA. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

FIG. 6. Physiological stimulation of macropinocytosis by M-CSF involves Nox2 activation. (A) WT macrophages were pretreated for 1 h with EIPA (10 μ M), DPI (10 μ M), or EUK 134 (10 μ M), and challenged with M-CSF (100 ng/ml, 4 h) in the presence of FITC-dextran. FITC fluorescence was analyzed by FACS. (B) WT and Nox2^{-/-} macrophages were treated with vehicle and M-CSF, and FITC-dextran incorporation was analyzed by FACS. Three independent experiments were executed. Data represent the mean $\pm$ SEM. * $p \leq 0.05$ versus vehicle, & $p \leq 0.05$ versus WT M-CSF or Ctrl M-CSF. (C) WT macrophages were either treated with M-CSF alone or pretreated with inhibitors mentioned in (A). Lysates were subjected to Western blot analysis for phospho- or total cofilin, Akt, and PTEN proteins. Representative Western blot images are shown ($n = 3$). (D) Nox2^{-/-} macrophages were incubated with vehicle, or M-CSF. Cell lysates were processed for Western blot analysis for phospho- and total cofilin and PTEN expression ($n = 3$). M-CSF, macrophage colony-stimulating factor. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

signaling mechanisms involved in membrane ruffling and subsequent macropinosome formation are different from the mechanisms initiating local activation of actin cytoskeleton during phagocytosis or receptor-mediated endocytosis.

The Nox of “professional” phagocytic cells is a multi-component enzyme that consists of the membrane-bound Nox2 and p22^{phox} and several cytosolic adaptor proteins (4). The electron transport by phagocyte Nox requires translocation of the cytosolic subunits, p47^{phox}, p67^{phox}, and Rac1, to Nox2 and p22^{phox} (54). In dormant phagocytes, the tandem Src homology 3 (SH3) domain of p47^{phox} is masked by the auto-inhibitory region (AIR) to keep p47^{phox} in its inactive conformation (41). Phosphorylation of serine residues within the AIR by DAG-mediated PKC activation exposes the SH3 domain for p22^{phox} binding and reveals its binding sites for interaction with phosphatidylinositols, such as PI(3,4)P2 (11). During the next steps of Nox assembly, p47^{phox} brings p67^{phox} in contact with Nox2, whereas Rac1 induces conformational change of p67^{phox}, promoting its interaction with the catalytic core of the enzyme, Nox activation, and subsequent O₂⁻ generation (43). In phagocytes, Nox2 localizes to both intracellular and plasma membranes. This provides the basis for the concept of compartmentalization in ROS-mediated signaling and function (4, 8). ROS generation from intracellular com-

partments leads to activation of redox-sensitive cell signaling pathways (28), whereas plasma-membrane-bound Nox contributes to phagosomal ROS generation, pathogen killing, and paracrine/autocrine ROS-mediated signaling (54).

Although previous studies provided hints for a possible mechanistic interaction between Nox2 activation and macropinocytosis, this has never been tested experimentally. For example, the classical activators of macropinocytosis, including phorbol esters, growth factors, and M-CSF (3, 32, 51, 59), stimulate Nox activation and ROS generation (24, 34, 56). In addition, the following reports demonstrated that signal transduction mediators upstream of Nox2 activation, such as Rac1, PKC, and phosphatidylinositols, are involved in membrane ruffling and macropinocytosis: (i) Photoactivation of PA-Rac1 in macrophages resulted in plasma membrane ruffling in 1–5 min and subsequent macropinosome formation (23); (ii) M-CSF via stimulation of PI3K and DAG generation leads to PKC activation and cup closure (60); and (iii) sequential dephosphorylation of PI(3,4,5)P3 $\rightarrow$ PI(3,4)P2 $\rightarrow$ PI(3)P $\rightarrow$ PI is required for EGF-induced macropinocytosis (39).

Previous studies showed that reconstitution of Nox activity in cell-free systems by phorbol esters or anionic amphiphiles is almost instantaneous, peaking within the first 1–2 min of stimulation (15, 43). Results of our O₂⁻ measurements are

FIG. 7. Role of Nox2 in macrophage lipid uptake in an atherosclerotic mouse model *in vivo*. (A) Flowchart style representation of experimental procedure. (B) Representative flow cytometry data illustrating the gating strategy for CFDA⁺ (donor) cells. (C, D) Representative Nile Red fluorescence histograms indicating lipid accumulation in vehicle- and 4β-PMA-treated CFDA⁺ WT and *Nox2*^{-/-} macrophages. Bar graph indicates averaged Nile Red fluorescence in CFDA⁺ WT and *Nox2*^{-/-} macrophages after incubation with vehicle or 4β-PMA (*n* = 3). (E) A scheme summarizing the mechanism by which 4β-PMA *via* Nox2 stimulates membrane ruffling and macrophinocytosis.

consistent with previous reports showing that 4β-PMA stimulates O₂^{•-} generation in 4–5 min in intact cells (24). These data together with our SEM time-course experiments suggest that 4β-PMA-stimulated O₂^{•-} generation precedes or occurs concomitantly with the earliest membrane ruffles in macrophages. To test whether O₂^{•-} is functionally involved in 4β-PMA-induced macrophinocytosis, we pretreated macrophages with the cell-impermeant SOD, cell-permeant PEG-SOD, or the synthetic SOD/catalase mimetic EUK 134. FACS data indicated that the cell-permeant O₂^{•-} scavengers, but not the cell-impermeant SOD, attenuated 4β-PMA-stimulated macrophinocytosis, suggesting the role of intracellular O₂^{•-} in macrophinocytosis. Imaging studies utilizing DHE staining and quantification of the 2-OH-E⁺ after DHE treatment confirmed that 4β-PMA stimulates intracellular O₂^{•-} generation. These results suggest that 4β-PMA stimulates macrophinocytosis *via* a redox-mediated mechanism, which involves intracellular O₂^{•-} as a signaling molecule.

Earlier studies showed that phorbol ester-stimulated macrophinocytosis is inhibited by PKC inhibitors and macrophinocytotic uptake of macromolecules is stimulated by activators of PKC (31). We have confirmed this by using the PKC inhibitor calphostin c and demonstrated that the inactive stereoisomer 4α-PMA, which does not activate PKC (44), is unable to stimulate O₂^{•-} generation or induce macrophinocytosis. Because analogs of DAG stimulate Nox activity in phagocytes (22), we tested whether incubation of macrophages with the DAG analog DOG

stimulates macrophinocytosis. DOG increased uptake of FITC-dextran by macrophages, which is consistent with the role of the DAG-PKC pathway in macrophinocytosis.

Since Nox2 is the most highly expressed Nox isoform in macrophages and phorbol esters activate Nox2 *via* the DAG-PKC pathway (35, 50), we tested whether Nox2 is involved in 4β-PMA-induced macrophinocytosis. DPI, a small-molecule inhibitor of flavin-containing oxidases, significantly attenuated 4β-PMA-induced macrophinocytosis. Although DPI has been widely used to inhibit the oxidative burst by phagocyte Nox, it inhibits all Nox isoforms (26). In addition, as DPI accepts an electron from FAD moieties, followed by a covalent reaction with the flavoenzyme, it inhibits not only Nox enzymes but also xanthine oxidase, mitochondrial complex I, and cytochrome P-450 reductase (4). To further extend our findings and to use a more specific approach, we isolated peritoneal macrophages from *Nox2*^{-/-} mice, challenged them with 4β-PMA, and investigated whether macrophinocytosis is ameliorated. Our data demonstrated that 4β-PMA-induced internalization of fluorescently labeled dextran was significantly blocked in *Nox2*^{-/-} macrophages compared with WT controls. As compensatory changes in *Nox2*^{-/-} macrophages in response to the loss of Nox2 function may occur, we silenced Nox2 in macrophages using siRNA and investigated whether it inhibits 4β-PMA-induced macrophinocytosis. FACS results indicate that internalization of FITC-dextran was inhibited in Nox2-silenced macrophages after 4β-PMA

treatment compared with control siRNA-transfected cells. qPCR demonstrated that Nox2 mRNA levels were decreased in Nox2 siRNA-treated cells, whereas we observed no compensatory changes in Nox1 and Nox5 mRNA levels [Nox3 is undetectable in macrophages (35)]. Importantly, extensive inquiry is required to investigate whether other Nox isoforms under various conditions modulate macropinocytosis in phagocytes and other cell types. Indeed, in an associated paper in this issue, we demonstrate that dynamic Nox1 activation by matrix protein thrombospondin-1 and its cognate receptor CD47 mediates macropinocytosis (15a). On the other hand, as previous studies have reported that Nox4 is constitutively active and it is regulated at the transcriptional level (46), it is likely that Nox4 is not involved in macropinocytosis under acute conditions. However, it is possible that factors that induce Nox4 expression, such as TGF β 1 and HIF-1 α (2, 20), may stimulate macropinocytosis under chronic conditions.

The plasma membrane activities leading to macropinosome formation consist of multiple morphologically distinct steps that are each associated with a corresponding sequence of signal transduction mediators. We next used time-lapse DIC and SEM to investigate which morphological step during macropinocytosis is mediated by Nox2. Calphostin c, DPI, and EUK 134 blocked 4 β -PMA-induced membrane ruffling, demonstrating that PKC and O $_2^{\bullet -}$ derived from a DPI-inhibitable flavoenzyme are required for the initiation of membrane ruffle formation. In addition, gene silencing Nox2 inhibited 4 β -PMA-induced membrane ruffling. Taken together, these findings suggest that phorbol ester PMA stimulates membrane ruffling *via* Nox2-derived O $_2^{\bullet -}$ generation. In contrast to our observations, a previous study utilizing pharmacological approaches suggested that DAG and PKC are involved in cup closure but not in earlier stages of macropinosome formation (60). In addition, there are inconsistencies between previous results, as some (23), but not all (57), studies demonstrated that Rac is required for membrane ruffling. It is important to note that using different means to activate macropinocytosis, utilizing different cell types that may express different Rac or PKC isoforms, or differences in protein levels of PKC and Nox enzymes may all contribute to these differences.

Cofilin is a key regulator of actin remodeling, lamellipodia formation, and cellular motility (19). The severing and depolymerization activity of cofilin is increased by its dephosphorylation on Ser3, which stimulates its actin-binding activity. Western blot data indicated that 4 β -PMA dephosphorylates cofilin at Ser 3 in 15 min, which was inhibited in Nox2-gene silenced as well as in Nox2 $^{y/-}$ macrophages. PTEN, a tumor-suppressor protein, inactivates cofilin by antagonizing PI3K signaling and decreasing intracellular levels of PI(3,4,5)P3 (45). On the contrary, PI3K and PI(3,4,5)P3 promote cofilin dephosphorylation and activation. We demonstrated that 4 β -PMA decreases the phosphatase activity of PTEN *via* Nox2 and stimulates Akt signaling. Interestingly, a previous study reported that slingshot phosphatase (SSH) is activated downstream of PI3K/Akt, leading to cofilin dephosphorylation and insulin-induced membrane protrusions (45). Consistent with this study, our data demonstrated that pharmacological inhibition of PI3K inhibits 4 β -PMA-induced cofilin dephosphorylation. In addition, we demonstrated that gene silencing cofilin using siRNA inhibited ruffle formation and macropinocytosis after 4 β -PMA treatment. When taken together, these data motivate

the speculation that redox inactivation of PTEN by Nox-derived ROS leads to activation of PI3K/Akt signaling, cofilin dephosphorylation, and initiation of membrane ruffle formation. Importantly, this signaling pathway was also demonstrated in M-CSF-induced macropinocytosis, demonstrating the physiological relevance of our study.

A large body of literature supports macrophage lipid accumulation as the key initiating event in atherosclerosis (38). After deposition of nLDL in the subendothelial matrix of arteries, monocytes home to the vessel wall, differentiate into macrophages, and imbibe excessive amounts of cholesterol. It is generally accepted that local biological responses to retained nLDL particles lead to their modification, that is, oxidation, and subsequent internalization by macrophage scavenger receptors, leading to foam cell formation and atherosclerosis. In addition to this "classical" mode of lipid uptake, it has been demonstrated *in vitro* that macropinocytosis may contribute to lipid internalization by macrophages (32). The relative contribution of macrophage nLDL macropinocytosis to atherogenesis is currently unknown, as no pharmacological or genetic tools are available to selectively target this process.

Shifting to an alternative approach, we performed a peritoneal macrophage chimera study to investigate macropinocytic uptake of lipids from the peritoneal cavity of ApoE $^{-/-}$ mice. In this experiment, WT and Nox2 $^{y/-}$ macrophages were pre-incubated with 4 β -PMA to stimulate membrane ruffling and injected into the peritoneal cavity of ApoE $^{-/-}$ mice. Consistent with a role of Nox2 in macropinocytosis, our results demonstrated that 4 β -PMA stimulated lipid accumulation in WT, but not in Nox2 $^{y/-}$, macrophages. These *in vivo* data demonstrate for the first time that Nox2-mediated membrane ruffling and subsequent macropinocytosis of cholesterol may lead to significant lipid accumulation in macrophages in hypercholesterolemic conditions. Importantly, recent studies have also demonstrated that macropinocytosis is involved in other pathological processes. For instance, cancer cells utilize macropinocytosis to internalize nutrients and support their metabolic needs, which is critical for their proliferation and tumor growth (14). Macropinocytosis is also a major mechanism by which renal tubular cells endocytose calcium oxalate crystals, leading to kidney stone formation (27). *Escherichia coli* infection stimulates Shiga toxin 1 macropinocytosis, leading to intestinal and systemic pathologies (40). Thus, a better understanding of the signaling mechanisms regulating macropinocytosis may lead to new pharmacological concepts to target pathophysiological conditions involving macropinocytosis.

To our knowledge, no direct link has ever been made between Nox2 signaling and macrophage macropinocytosis. Importantly, redox regulation of macropinocytosis downstream of Nox2, or any Nox for that matter, had not been previously demonstrated in any cell types or disease model. In addition, the results of the current study provide seminal mechanistic insight into how Nox2 signaling *via* inactivation of PTEN and activation of the PI3K/Akt pathway leads to cofilin dephosphorylation, extensive membrane ruffling, and macropinocytosis. Peritoneal chimera studies presented are the first that implicate Nox2-mediated macropinocytosis in macrophage lipid accumulation *in vivo*. These data are expected to reveal an intriguing new line of study in the field of endocytosis, with broad implications for cellular physiology and pathological conditions involving macropinocytosis.

Materials and Methods

Reagents

Cytochrome c, SOD, PEG-SOD, DPI, 4 α - and 4 β -PMA, Nile Red, LY294002, calphostin c, amiloride, EIPA, latrunculin A, chlorpromazine, nystatin, FITC-Dextran, and thioglycollate medium were purchased from Sigma-Aldrich. DOG and EUK-134 were obtained from Cayman Chemical. Vybrant[®] CFDA Cell Tracer Kit was purchased from Thermo Fisher. FluoSpheres[®] were obtained from Life Technologies. Human nLDL was purchased from Kalen Biomedical, LLC. M-CSF was purchased from Miltenyi Biotec, Inc.

Cell culture

RAW 264.7 macrophages (ATCC) were maintained in Dulbecco's modified Eagle's medium (DMEM; Mediatech, Inc.) that was supplemented with 100 IU/ml of penicillin G, 100 μ g/ml streptomycin, and 10% (vol/vol) heat-inactivated FBS in a humidified incubator at 37°C and 5% CO₂. Thioglycollate-elicited peritoneal macrophages and bone marrow-derived monocytes were cultured in RPMI-1640 medium (without 2-mercaptoethanol) containing 10% FBS. Bone marrow-derived monocytes were differentiated into macrophages using murine M-CSF (20 ng/ml, 6 days) as previously reported (49). THP-1 monocytes (ATCC) were maintained in cell-free conditioned media of RAW 264.7 macrophages and differentiated into macrophages using human M-CSF (20 ng/ml, 9 days). The cells were serum starved (1% FBS, 4 h) before experiments.

Animals

All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC), Augusta University, Augusta, the United States. Male C57BL/6 (wild type), ApoE^{-/-}, and Nox2^{y/-} mice were purchased from The Jackson Laboratory. CD36^{-/-} mice were kindly provided by Dr. Roy Silverstein (Medical College of Wisconsin).

Imaging experiments

DIC microscopy. DIC microscopy was performed as previously described (48) by using a Zeiss 780 inverted microscope. Cells were imaged by using a 63 $\times$ (Alpha Plan-Apo, NA: 1.46) objective. Live images were collected at every second for 2 min. At least four representative fields were chosen for each dish, and the experiment was performed in triplicate.

Confocal microscopy. Macrophages were fixed in 2% paraformaldehyde (PFA), permeabilized with 0.1% Triton X-100, and stained with Alexa Fluor 488[®] phalloidin, Hoechst 33342 (Life Technologies), and Nile Red (50 ng/ml; 10 min). Images were taken with a Zeiss 780 inverted confocal microscope.

Scanning electron microscopy. Macrophages were fixed (4% paraformaldehyde, 2% glutaraldehyde in 0.1 M sodium cacodylate solution) overnight at 4°C. Then, the cells were dehydrated through a graded ethanol series (25%–100%) and washed with 100% ethanol before critical point drying (Tousimis Samdri-790). Coverslips were mounted onto aluminum stubs and sputter coated with 3.5 nm of gold/palladium (Anatek USA-Hummer). Cells were imaged at 20 KV by using a Philips XL30 scanning electron microscope (FEI).

Gene silencing

THP-1 macrophages were transfected with siRNA against CLTC, Nox2, and cofilin (OriGene Technologies) by using the transfection reagent TransIT-TKO[®] (Mirus Bio LLC). To control for possible nonspecific effects of siRNA, a Silencer[®] negative control siRNA was used. Gene silencing by siRNA was confirmed by qPCR. Cells were used 48 h post transfection.

Real-time PCR analysis

Total RNA was extracted from macrophages by using an RNA extraction kit (IBI Scientific). cDNA was generated by using the TaqMan[®] Reverse Transcriptase kit (Applied Biosystems). Real-time PCR was carried out by using the SYBR Green Super mix (Applied Biosystems, Inc.). All amplifications were performed in triplicate, and GAPDH was used as the internal control. The primers used for real-time PCR are as follows:

Cofilin: F-TTCAACGACATGAAGGTGCGT, R-TCCTC CAGGATGATGTTCTTCT,

CLTC: F-TGATCGCCATTCTAGCCTTGC, R-CTCCC ACCACACGATTTTGCT,

Nox2: F-GTCACACCCTTCGCATCCATTCTCAAGTC AGT, R-CTGAGACTCATCCCAGCCAGTGAGGTAG,

GAPDH: F-CATGTTTCGTCATGGGTGTGAACCA, R-AGTGATGGCATGGACTGTGGTCAT.

Flow cytometry

FACS experiments were performed by using the Becton Dickinson FACSCalibur flow cytometer using a standard protocol. Briefly, macrophages were incubated with nLDL (250 μ g/ml) and treated with vehicle or 4 β -PMA (1 μ M, 24 h). Cells were fixed in 2% PFA and stained with Nile Red (25 ng/ml) for 10 min. In separate experiments, macrophages were incubated with FITC-dextran (70,000 MW, 150 ng/ml) and treated with vehicle, 4 β -PMA (1 μ M), or M-CSF (100 ng/ml) for 4 h. Fluorescence intensity was measured by using the FL3 channel for Nile Red (Ex: 488 nm, Em: 670 nm) and FL1 for FITC (Ex: 488 nm, Em: 530 nm). The gating strategy for flow cytometry analysis is shown in Supplementary Figure S11.

Measurement of ROS

Quantification of 2-OH-E⁺ using HPLC. 2-hydroxyethidium (2-OH-E⁺), the O₂^{•-}-specific oxidation product of DHE, was quantified in RAW 264.7 macrophages as previously described (62). Briefly, an ether-linked phenyl column (100 $\times$ 4.6 mm; Phenomenex) was employed by using two mobile phases (Solution A: 50 mM phosphate buffer in 90% water and 10% acetonitrile and Solution B: 50 mM phosphate buffer in 40% water and 60% acetonitrile), and a gradient elution to increase the acetonitrile concentration from 25% to 60% over 10 min at a flow rate of 0.75 ml/min. Forty microliters of sample was injected into the HPLC system (ESA, Inc., with a CoulArray electrochemical detection array system). The areas of the corresponding peaks were measured by using the software provided by ESA, Inc. The limit of detection for this assay was determined from a standard curve

and established at 20 fmoles. The concentration of 2-OH-E[±] was calculated by using a standard curve and normalized to total protein of the cell lysate.

L-012 chemiluminescence. L-012 chemiluminescence to measure O₂^{•−} generation was previously described (10). Cells (50,000/well) were plated in 96-well plates in sterile phosphate-buffered saline (PBS) containing L-012 (400 μM; Wako Chemicals) and treated with vehicle, 4β-PMA (1 μM), or 4α-PMA (1 μM). Chemiluminescence was measured at 37°C by using a Lumistar Galaxy (BMG) luminometer.

Electron paramagnetic resonance. The spin probe 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine hydrochloride (CMH; Alexis Corp.) was used to examine O₂^{•−} production in macrophages by using a Bruker eScan table-top EPR spectrometer (Bruker Biospin). Superoxide production was measured in oxidase assay buffer (65 mM sodium phosphate buffer, pH 7.0, 1 mM EGTA, 10 μM FAD, 1 mM MgCl₂, and 2 mM NaN₃) that was supplemented with 50 μM CMH as previously reported (17).

Western blot

Western blotting was performed as previously described (18). Briefly, protein lysates were prepared by incubating cells with RIPA lysis buffer (Pierce Biotechnology). The cell lysates were sonicated by using the Sonic Dismembrator, Model 100 (Fisher Scientific) for 2 × 10 s at setting 2 and centrifuged at 14,000 *g* for 15 min at 4°C to pellet the cell debris. The protein concentration of supernatant was estimated by using the bicinchoninic acid (BCA) protein assay (Pierce Biotechnology), according to the manufacturer's instructions. Equal amounts of protein (30 μg) were heated in Laemmli sample buffer (BioRad Laboratories, Inc.) at 95°C for 5 min, separated on SDS-PAGE gels, transferred onto nitrocellulose membranes (Li-Cor Biosciences), and probed with cofilin, Akt, total PTEN (Cell Signaling), and p-PTEN (Abcam) antibodies. The IRDye-conjugated secondary antibodies (Li-Cor Biosciences) were used to detect the primary antibodies. The internal control β-actin was used to normalize the amount of protein transferred onto the membranes.

Adoptive transfer of macrophages into ApoE^{−/−} mice

The adoptive transfer was performed as previously described, with slight modifications (49). Bone marrow-derived macrophages from WT and Nox2^{−/−} mice (donors) were resuspended in sterile PBS at a concentration of 2 × 10⁶ cells per ml and labeled with 10 μM carboxy fluorescein diacetate-succinimidyl ester (CFDA, Molecular Probes) as per the manufacturer's instructions. Cells were treated with vehicle or 4β-PMA (1 μM, 30 min) and injected into recipient ApoE^{−/−} mice (i.p., 2 × 10⁶ cells/mouse). ApoE^{−/−} mice were fed a high-fat diet for 8 weeks before experiments. Twenty hours later, peritoneal macrophages were isolated, stained with Nile Red, and processed for FACS analysis.

Statistical analysis

The data shown are the means ± SEM. Significance of the differences were assessed by Student's *t*-test followed by analyzing the confidence interval at 95% or one- or two-way

ANOVA, as appropriate for the particular experiment and treatment groups. *p* values less than 0.05 were considered significant.

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Author Disclosure Statement

No competing financial interests exist.

References

- Aleksandrowicz P, Marzi A, Biedenkopf N, Beimforde N, Becker S, Hoenen T, Feldmann H, and Schnittler HJ. Ebola virus enters host cells by macropinocytosis and clathrin-mediated endocytosis. *J Infect Dis* 204 Suppl 3: S957–S967, 2011.
- Amara N, Goven D, Prost F, Muloway R, Crestani B, and Boczkowski J. NOX4/NADPH oxidase expression is increased in pulmonary fibroblasts from patients with idiopathic pulmonary fibrosis and mediates TGFβ1-induced fibroblast differentiation into myofibroblasts. *Thorax* 65: 733–738, 2010.
- Anzinger JJ, Chang J, Xu Q, Buono C, Li Y, Leyva FJ, Park BC, Greene LE, and Kruth HS. Native low-density lipoprotein uptake by macrophage colony-stimulating factor-differentiated human macrophages is mediated by macropinocytosis and micropinocytosis. *Arterioscler Thromb Vasc Biol* 30: 2022–2031, 2010.
- Bedard K and Krause KH. The NOX family of ROS-generating NADPH oxidases: physiology and pathophysiology. *Physiol Rev* 87: 245–313, 2007.
- Blanchard E, Belouzard S, Goueslain L, Wakita T, Dubuisson J, Wychowski C, and Rouille Y. Hepatitis C virus entry depends on clathrin-mediated endocytosis. *J Virol* 80: 6964–6972, 2006.
- Bohdanowicz M and Grinstein S. Role of phospholipids in endocytosis, phagocytosis, and macropinocytosis. *Physiol Rev* 93: 69–106, 2013.
- Brandes RP and Kreuzer J. Vascular NADPH oxidases: molecular mechanisms of activation. *Cardiovasc Res* 65: 16–27, 2005.
- Brown DI and Griendling KK. Regulation of signal transduction by reactive oxygen species in the cardiovascular system. *Circ Res* 116: 531–549, 2015.
- Bryant DM, Kerr MC, Hammond LA, Joseph SR, Mostov KE, Teasdale RD, and Stow JL. EGF induces macropinocytosis and SNX1-modulated recycling of E-cadherin. *J Cell Sci* 120(Pt 10): 1818–1828, 2007.
- Chen F, Pandey D, Chadli A, Catravas JD, Chen T, and Fulton DJ. Hsp90 regulates NADPH oxidase activity and is necessary for superoxide but not hydrogen peroxide production. *Antioxid Redox Signal* 14: 2107–2119, 2011.
- Cheng N, He R, Tian J, Dinanuer MC, and Ye RD. A critical role of protein kinase C delta activation loop phosphorylation in formyl-methionyl-leucyl-phenylalanine-induced phosphorylation of p47(phox) and rapid activation of nicotinamide adenine dinucleotide phosphate oxidase. *J Immunol* 179: 7720–7728, 2007.

12. Chung JJ, Huber TB, Godel M, Jarad G, Hartleben B, Kwok C, Keil A, Karpitskiy A, Hu J, Huh CJ, Cella M, Gross RW, Miner JH, and Shaw AS. Albumin-associated free fatty acids induce macropinocytosis in podocytes. *J Clin Invest* 125: 2307–2316, 2015.
13. Clemens RE and Griendling KK. Reactive oxygen species signaling in vascular smooth muscle cells. *Cardiovasc Res* 71: 216–225, 2006.
14. Comisso C, Flinn RJ, and Bar-Sagi D. Determining the macropinocytic index of cells through a quantitative image-based assay. *Nat Protoc* 9: 182–192, 2014.
15. Cox JA, Jeng AY, Sharkey NA, Blumberg PM, and Tauber AI. Activation of the human neutrophil nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase by protein kinase C. *J Clin Invest* 76: 1932–1938, 1985.
- 15a. Csányi G, Feck DM, Ghoshal P, Singla B, Lin H, Nagarajan S, Meijles DN, Ghoulah IA, Cantu-Medellin N, Kelley EE, Mateuszuk L, Isenberg JS, Watkins S, and Pagano PJ. CD47 and Nox1 mediate dynamic fluid-phase macropinocytosis of native LDL. *Antioxid Redox Signal* 26: 886–901, 2017.
16. Csányi G and Pagano PJ. Strategies Aimed at Nox4 Oxidase Inhibition Employing Peptides from Nox4 B-Loop and C-Terminus and p22 (phox) N-Terminus: an Elusive Target. *Int J Hypertens* 2013: 842827, 2013.
17. Csányi G, Cifuentes-Pagano E, Al Ghoulah I, Ranayhossaini DJ, Egana L, Lopes LR, Jackson HM, Kelley EE, and Pagano PJ. Nox2 B-loop peptide, Nox2ds, specifically inhibits the NADPH oxidase Nox2. *Free Radic Biol Med* 51: 1116–1125, 2011.
18. Csányi G, Yao M, Rodriguez AI, Al Ghoulah I, Sharifi-Sanjani M, Frazziano G, Huang X, Kelley EE, Isenberg JS, and Pagano PJ. Thrombospondin-1 regulates blood flow via CD47 receptor-mediated activation of NADPH oxidase 1. *Arterioscler Thromb Vasc Biol* 32: 2966–2973, 2012.
19. DesMarais V, Ghosh M, Eddy R, and Condeelis J. Cofilin takes the lead. *J Cell Sci* 118(Pt 1): 19–26, 2005.
20. Diebold I, Petry A, Hess J, and Grolach A. The NADPH oxidase subunit NOX4 is a new target gene of the hypoxia-inducible factor-1. *Mol Biol Cell* 21: 2087–2096, 2010.
21. Doherty GJ and McMahon HT. Mechanisms of endocytosis. *Ann Rev Biochem* 78: 857–902, 2009.
22. Erickson RW, Langel-Peveri P, Traynor-Kaplan AE, Heyworth PG, and Curnutte JT. Activation of human neutrophil NADPH oxidase by phosphatidic acid or diacylglycerol in a cell-free system. Activity of diacylglycerol is dependent on its conversion to phosphatidic acid. *J Biol Chem* 274: 22243–22250, 1999.
23. Fujii M, Kawai K, Egami Y, and Araki N. Dissecting the roles of Rac1 activation and deactivation in macropinocytosis using microscopic photo-manipulation. *Sci Rep* 3: 2385, 2013.
24. Inanami O, Johnson JL, and Babior BM. The leukocyte NADPH oxidase subunit p47PHOX: the role of the cysteine residues. *Arch Biochem Biophys* 350: 36–40, 1998.
25. Inanami O, Johnson JL, McAdara JK, Benna JE, Faust LR, Newburger PE, and Babior BM. Activation of the leukocyte NADPH oxidase by phorbol ester requires the phosphorylation of p47PHOX on serine 303 or 304. *J Biol Chem* 273: 9539–9543, 1998.
26. Jaquet V, Scapozza L, Clark RA, Krause KH, and Lambeth JD. Small-molecule NOX inhibitors: ROS-generating NADPH oxidases as therapeutic targets. *Antioxid Redox Signal* 11: 2535–2552, 2009.
27. Kanlaya R, Sintiprungrat K, Chaiyapit S, and Thongboonkerd V. Macropinocytosis is the major mechanism for endocytosis of calcium oxalate crystals into renal tubular cells. *Cell Biochem Biophys* 67: 1171–1179, 2013.
28. Karlsson A and Dahlgren C. Assembly and activation of the neutrophil NADPH oxidase in granule membranes. *Antioxid Redox Signal* 4: 49–60, 2002.
29. Kazanietz MG, Caloca MJ, Eroles P, Fujii T, Garcia-Bermejo ML, Reilly M, and Wang H. Pharmacology of the receptors for the phorbol ester tumor promoters: multiple receptors with different biochemical properties. *Biochem Pharmacol* 60: 1417–1424, 2000.
30. Koivusalo M, Welch C, Hayashi H, Scott CC, Kim M, Alexander T, Touret N, Hahn KM, and Grinstein S. Amiloride inhibits macropinocytosis by lowering sub-membranous pH and preventing Rac1 and Cdc42 signaling. *J Cell Biol* 188: 547–563, 2010.
31. Kruth HS, Huang W, Ishii I, and Zhang WY. Macrophage foam cell formation with native low density lipoprotein. *J Biol Chem* 277: 34573–34580, 2002.
32. Kruth HS, Jones NL, Huang W, Zhao B, Ishii I, Chang J, Combs CA, Malide D, and Zhang WY. Macropinocytosis is the endocytic pathway that mediates macrophage foam cell formation with native low density lipoprotein. *J Biol Chem* 280: 2352–2360, 2005.
33. Larsen EC, DiGennaro JA, Saito N, Mehta S, Loegering DJ, Mazurkiewicz JE, and Lennartz MR. Differential requirement for classic and novel PKC isoforms in respiratory burst and phagocytosis in RAW 264.7 cells. *J Immunol* 165: 2809–2817, 2000.
34. Lavigne MC, Malech HL, Holland SM, and Leto TL. Genetic demonstration of p47phox-dependent superoxide anion production in murine vascular smooth muscle cells. *Circulation* 104: 79–84, 2001.
35. Lee NK, Choi YG, Baik JY, Han SY, Jeong DW, Bae YS, Kim N, and Lee SY. A crucial role for reactive oxygen species in RANKL-induced osteoclast differentiation. *Blood* 106: 852–859, 2005.
36. Li AC, Binder CJ, Gutierrez A, Brown KK, Plotkin CR, Pattison JW, Valledor AF, Davis RA, Willson TM, Witztum JL, Palinski W, and Glass CK. Differential inhibition of macrophage foam-cell formation and atherosclerosis in mice by PPARalpha, beta/delta, and gamma. *J Clin Invest* 114: 1564–1576, 2004.
37. Lim JP and Gleeson PA. Macropinocytosis: an endocytic pathway for internalising large gulps. *Immunol Cell Biol* 89: 836–843, 2011.
38. Lusis AJ. Atherosclerosis. *Nature* 407: 233–241, 2000.
39. Maekawa M, Terasaka S, Mochizuki Y, Kawai K, Ikeda Y, Araki N, Skolnik EY, Taguchi T, and Arai H. Sequential breakdown of 3-phosphorylated phosphoinositides is essential for the completion of macropinocytosis. *Proc Natl Acad Sci U S A* 111: E978–E987, 2014.
40. Malyukova I, Murray KF, Zhu C, Boedeker E, Kane A, Patterson K, Peterson JR, Donowitz M, and Kovbasnjuk O. Macropinocytosis in Shiga toxin 1 uptake by human intestinal epithelial cells and transcellular transcytosis. *Am J Physiol Gastrointest Liver Physiol* 296: G78–G92, 2009.
41. Meijles DN, Fan LM, Howlin BJ, and Li JM. Molecular insights of p47phox phosphorylation dynamics in the regulation of NADPH oxidase activation and superoxide production. *J Biol Chem* 289: 22759–22770, 2014.
42. Mercer J and Helenius A. Virus entry by macropinocytosis. *Nat Cell Biol* 11: 510–520, 2009.

43. Molshanski-Mor S, Mizrahi A, Ugolev Y, Dahan I, Berdichevsky Y, and Pick E. Cell-free assays: the reductionist approach to the study of NADPH oxidase assembly, or “all you wanted to know about cell-free assays but did not dare to ask”. *Methods Mol Biol* 412: 385–428, 2007.
44. Murray KT, Hu NN, Daw JR, Shin HG, Watson MT, Mashburn AB, and George AL, Jr. Functional effects of protein kinase C activation on the human cardiac Na⁺ channel. *Circ Res* 80: 370–376, 1997.
45. Nishita M, Wang Y, Tomizawa C, Suzuki A, Niwa R, Uemura T, and Mizuno K. Phosphoinositide 3-kinase-mediated activation of cofilin phosphatase Slingshot and its role for insulin-induced membrane protrusion. *J Biol Chem* 279: 7193–7198, 2004.
46. Nisimoto Y, Jackson HM, Ogawa H, Kawahara T, and Lambeth JD. Constitutive NADPH-dependent electron transferase activity of the Nox4 dehydrogenase domain. *Biochemistry* 49: 2433–2442, 2010.
47. Orth JD, Krueger EW, Weller SG, and McNiven MA. A novel endocytic mechanism of epidermal growth factor receptor sequestration and internalization. *Cancer Res* 66: 3603–3610, 2006.
48. Patel PC and Harrison RE. Membrane ruffles capture C3bi-opsonized particles in activated macrophages. *Mol Biol Cell* 19: 4628–4639, 2008.
49. Potts BE, Hart ML, Snyder LL, Boyle D, Mosier DA, and Chapes SK. Differentiation of C2D macrophage cells after adoptive transfer. *Clin Vaccine Immunol* 15: 243–252, 2008.
50. Raad H, Paclet MH, Boussetta T, Kroviarski Y, Morel F, Quinn MT, Gougerot-Pocidalo MA, Dang PM, and El-Benna J. Regulation of the phagocyte NADPH oxidase activity: phosphorylation of gp91phox/NOX2 by protein kinase C enhances its diaphorase activity and binding to Rac2, p67phox, and p47phox. *FASEB J* 23: 1011–1022, 2009.
51. Racoosin EL and Swanson JA. M-CSF-induced macropinocytosis increases solute endocytosis but not receptor-mediated endocytosis in mouse macrophages. *J Cell Sci* 102(Pt 4): 867–880, 1992.
52. Rejman J, Oberle V, Zuhorn IS, and Hoekstra D. Size-dependent internalization of particles via the pathways of clathrin- and caveolae-mediated endocytosis. *Biochem J* 377(Pt 1): 159–169, 2004.
53. Sallusto F, Cella M, Danieli C, and Lanzavecchia A. Dendritic cells use macropinocytosis and the mannose receptor to concentrate macromolecules in the major histocompatibility complex class II compartment: downregulation by cytokines and bacterial products. *J Exp Med* 182: 389–400, 1995.
54. Segal AW. The function of the NADPH oxidase of phagocytes and its relationship to other NOXs in plants, invertebrates, and mammals. *Int J Biochem Cell Biol* 40: 604–618, 2008.
55. Swanson JA. Phorbol esters stimulate macropinocytosis and solute flow through macrophages. *J Cell Sci* 94(Pt 1): 135–142, 1989.
56. Wang Y, Zeigler MM, Lam GK, Hunter MG, Eubank TD, Khramtsov VV, Tridandapani S, Sen CK, and Marsh CB. The role of the NADPH oxidase complex, p38 MAPK, and Akt in regulating human monocyte/macrophage survival. *Am J Respir Cell Mol Biol* 36: 68–77, 2007.
57. West MA, Prescott AR, Eskelinen EL, Ridley AJ, and Watts C. Rac is required for constitutive macropinocytosis by dendritic cells but does not control its downregulation. *Curr Biol* 10: 839–848, 2000.
58. Yamamura K, Steenbergen C, and Murphy E. Protein kinase C and preconditioning: role of the sarcoplasmic reticulum. *Am J Physiol Heart Circ Physiol* 289: H2484–H2490, 2005.
59. Yoshida S, Pacitto R, Yao Y, Inoki K, and Swanson JA. Growth factor signaling to mTORC1 by amino acid-laden macropinosomes. *J Cell Biol* 211: 159–172, 2015.
60. Yoshida S, Gaeta I, Pacitto R, Krienke L, Alge O, Gregorka B, and Swanson JA. Differential signaling during macropinocytosis in response to M-CSF and PMA in macrophages. *Front Physiol* 6: 8, 2015.
61. Zhu XD, Zhuang Y, Ben JJ, Qian LL, Huang HP, Bai H, Sha JH, He ZG, and Chen Q. Caveolae-dependent endocytosis is required for class A macrophage scavenger receptor-mediated apoptosis in macrophages. *J Biol Chem* 286: 8231–8239, 2011.
62. Zielonka J, Vasquez-Vivar J, and Kalyanaraman B. Detection of 2-hydroxyethidium in cellular systems: a unique marker product of superoxide and hydroethidine. *Nat Protoc* 3: 8–21, 2008.

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Abbreviations Used

AIR = autoinhibitory region
 AKT = protein kinase B
 CLTC = clathrin heavy chain
 DAG = diacylglycerol
 DHE = dihydroethidium
 DIC = differential interference contrast microscopy
 DOG = 1,2-dioctanoyl-sn-glycerol
 DPI = diphenyleneiodonium
 EGF = epidermal growth factor
 EGFR = EGF receptor
 EIPA = 5-(N-Ethyl-N-isopropyl)amiloride
 EPR = electron paramagnetic resonance
 FACS = fluorescence-activated cell sorting
 M-CSF = macrophage colony-stimulating factor
 nLDL = native low-density lipoprotein
 Nox = NADPH oxidase
 O₂^{•−} = superoxide
 PI3K = phosphatidylinositol 3-kinases
 PKC = protein kinase C
 PMA = phorbol-12-myristate-13-acetate
 PTEN = phosphatase and tensin homolog
 ROS = reactive oxygen species
 SEM = scanning electron microscopy
 SOD = superoxide dismutase
 WT = wild type