Decreased Superoxide Production in Macrophages of Long-lived p66Shc Knock-out Mice*

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A decrease in reactive oxygen species (ROS) production has been associated with extended life span in animal models of longevity. Mice deficient in the p66Shc gene are long-lived, and their cells are both resistant to oxidative stress and produce less ROS. Our microarray analysis of p66Shc (−/-) mouse tissues showed alterations in transcripts involved in heme and superoxide production and insulin signaling. Thus, we carried out analysis of ROS production by NADPH oxidase (PHOX) in macrophages of control and p66Shc knock-out mice. p66Shc (−/-) mice had a 40% reduction in PHOX-dependent superoxide production. To confirm whether the defect in superoxide production was a direct consequence of p66Shc deficiency, p66Shc was knocked down with siRNA in the macrophage cell line RAW264, and a 30% defect in superoxide generation was observed. The pathway of PHOX-dependent superoxide generation was investigated. PHOX protein levels were not decreased in mutant macrophages; however, the rate and extent of phosphorylation of p47phox was decreased in mutants, as was membrane translocation of the complex. Consistently, phosphorylation of protein kinase C, Akt, and ERK (the kinases responsible for phosphorylation of p47phox) was decreased. Thus, p66Shc deficiency causes a defect in activation of the PHOX complex that results in decreased superoxide production. p66Shc-deficient mice have recently been observed to be resistant to atherosclerosis and to oxidative injury in kidney and brain. Because phagocyte-derived superoxide is often a component of oxidative injury and inflammation, we suggest that the decreased superoxide production by PHOX in p66Shc-deficient mice could contribute significantly to their relative protection from oxidative injury and consequent longevity.

The free radical theory of aging predicts that oxygen-derived free radicals produced throughout life cause progressive damage and inflammation, ultimately leading to death (1). In the long-lived p66Shc-deficient mouse, embryonic fibroblasts produce less ROS and are more resistant to stressors, including hydrogen peroxide, and signal less through ROS-dependent pathways (2, 3). p66Shc-deficient mice produce less mitochondrial ROS following CCl4 stimulation (4). p66Shc KO mice also have reduced systemic and tissue oxidative stress (5); are resistant to atherosclerosis (6), oxidant-related endothelial dysfunction (7), kidney oxidant injury (8); and are protected from high fat diet-induced obesity (3).

We carried out a microarray study that indicated alterations in transcripts related to heme and NADPH oxidase superoxide production and thus investigated the impact of the p66Shc deficiency on ROS-generating activity of macrophages.

EXPERIMENTAL PROCEDURES

Animals—p66Shc (−/-) mice have been described previously (2). Mice were kept pathogen-free through the study at a barrier facility at the University of California (Davis, CA). All experimental procedures were approved by the Institutional Animal Care and Use Committee and were performed in compliance with local, state, and federal regulations. Mice used for this study were 2–6 months old and were age-matched for each experiment.

Antibodies and Reagents—Diphenyleneiodonium (DPI) and gliboxin were purchased from Axonra LLC (San Diego, CA), phorbol 12-myristate 13-acetate (PMA) was from Enzo Life Sciences International Inc. (Plymouth Meeting, PA); N-formyl-Met-Leu-Phe (fMLP) was from Tocris Bioscience (Ellisville, MO); arachidonic acid (AA) was from Acros Organics (Morris Plains, NJ); OxyBURST Green H₂FF-BSA dye was purchased from Molecular Probes, Inc. (Eugene, OR); goat anti-p22phox antibody was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); rabbit anti-p40phox polyclonal and rabbit anti-p67phox polyclonal antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); rabbit anti-p40phox polyclonal and rabbit anti-p67phox polyclonal antibodies were from Upstate Cell Signaling Solutions (Lake Placid, NY); rabbit anti-phospho-p40phox polyclonal antibody was from Cell Signaling Technology Inc. (Danvers, MA); goat anti-p47phox polyclonal, goat anti-Rac2 polyclonal, mouse anti-PKCδ polyclonal, and rabbit anti-phospho-PKCδ monoclonal antibody were from Abcam Inc. (Cambridge, MA); rabbit anti-Shc polyclonal antibodies and mouse anti-gp91phox antibody were from BD Biosciences; Rac/Cdc42

‡1 The abbreviations used are: DPI, diphenyleneiodonium; MAPK, mitogen-activated protein kinase; PI3K, phosphoinositide 3-kinase; PM, phorbol 12-myristate 13-acetate; fMLP, N-formyl-Met-Leu-Phe; AA, arachidonic acid; PM, peritoneal macrophage(s); PBS, phosphate-buffered saline; FBS, fetal bovine serum; SOD, superoxide dismutase; siRNA, small interfering RNA; CHAPS, 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonic acid; GTPyS, guanosine 5’-O-(thiotriphosphate); PHOX, NADPH oxidase; PKC, protein kinase C; ERK, extracellular signal-regulated kinase; KO, knock-out; PAK, p21-activated kinase; GPCR, G protein-coupled receptor; PIP2, phosphatidylinositol 3’-4’,5’-trisphosphate; MIEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; H₂FF, dihydro2, 5,6,7,7’-hexafluorofluoroscein; BSA, bovine serum albumin; IR, infrared.

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 assay reagent PAK-1 p21-binding domain-agarose conjugate and mouse monoclonal anti-Rac1 antibody were from Millipore Inc. (Temecula, CA); and goat anti-rabbit monoclonal antibody labeled with infrared (IR) dye 700CW, donkey anti-mouse monoclonal antibody labeled with IR dye 800CW, and donkey anti-goat polyclonal antibodies conjugated with IR dye 800CW were from Li-Cor Biosciences (Lincoln, NE). Primers for quantification of p66Shc forward (5′-gaggtggggctgacct-3′) and reverse (5′-gaccattgtgctgcccttc-3′), actin β forward (5′-ttggcctggagcagcagttg-3′) and reverse (5′-gatggttttgaggttggacct-3′), and p66Shc-specific siRNA (9) were synthesized by Integrated DNA Technology (Corvalis, IA), and non-target control siRNA-AllStar was purchased from Qiagen (Valencia, CA). RAW264.7 cells were from ATCC (Manassas, VA), BioLyte 5/8 and Bio-Lyte 3/10 amphotoles were from Bio-Rad.

*Peritoneal Macrophages (PM)—PM were harvested 4 days after thioglycollate injection of the peritoneal cavity. Cells were washed with chilled PBS, red blood cells were hypotonically lysed, and macrophages were resuspended in RPMI 1640 containing 15% FBS, 50 μg/ml penicillin, and 50 μg/ml streptomycin and plated on 100-mm round Petri dishes. After a 2-h incubation at 37 °C, 5% CO2 non-adherent cells were removed, and the remaining adherent cells were cultured in RPMI 1640 containing 15% FBS, 50 μg/ml penicillin, and 50 μg/ml streptomycin for no more than 48 h before functional assays.

*Tissue Culture—RAW264.7 cells were cultured in RPMI 1640 containing 15% FBS, 50 μg/ml penicillin, and 50 μg/ml streptomycin and subcultured twice a week.

RNA Isolation and Semiquantitative Reverse Transcription-PCR—Total RNA was extracted by direct lysis of the cells on the tissue culture plate using an RNeasy minikit (Qiagen) according to the manufacturer’s instructions. Equal RNA amounts were added to Superscript II First Strand reverse transcriptase reaction mixture (Invitrogen) with oligo(dT) primer. The resulting templates were subject to SYBR Green-based quantitative PCR using specific primers, listed above. The cycling conditions were 94 °C for 3 min as initial denaturation followed by 35 cycles of 94 °C for 15 s, 60 °C for 20 s, and 72 °C for 15 s and finished by melting curve by gradual heating until 95 °C. Only experiments with a single melting peak were considered for analysis. Reaction qualities have being verified by gel electrophoresis. PCR was carried out using a LightCycler480 real time PCR instrument and LightCycler480 analysis software (Roche Applied Science).

Blood Heme Measurement—Blood heme measurement was performed on heart blood samples. Blood was collected in capillary tubes containing electrolyte-balanced heparin at 70 IU/ml of blood. Following collection, blood samples were immediately analyzed for hemoglobin levels on a Radiometer OSM3 hemoximeter (Copenhagen, Denmark).

Superoxide Production by RAW264.7 Cells—Cells were cultivated and transfected on 6-well plates (Nunc) started at 200,000 cells/well. Transfection was performed as described, and after 48 h, cells were washed with PBS, pH 7.4, at 37 °C, and 3 ml of KRP, which contains PBS, pH 7.4, 1 mM CaCl2, 1.5 mM MgCl2, 5.5 mM glucose, and 10 μg/ml OxyBURST Green H2HFF-BSA dye, was added. Cells were incubated at 37 °C in the dark for 2 min, and readings of fluorescence at 530 nm excited at 480 nm were taken for 12 min using CytoFluor Multi-Well Plate Reader (PerSeptive Biosystems). Then PMA until 3 μg/ml was added to experimental wells, and readings were continued for the next 30 min, and then DMSO (solvent for PMA) was added to control cells, used as base line. After the assay, one-half of the cells from each well were taken to the cell count and trypan blue-based viability assay, and the number of cells in each well was calculated. Fluorescence readings were normalized to exact viable cell number in each well. Another half of the cells were used for either protein extraction for Western blots or total RNA extraction for semiquantitative reverse transcription-PCR. For the gliotoxin or DPI inhibition of NAD(P)H-oxidase, gliotoxin or DPI was directly added to the tissue culture medium until a final concentration of 10 μg/ml for gliotoxin or 10 μM for DPI, and cells were preincubated for 10 min at 37 °C prior to measurements of superoxide production.

Superoxide Production by Mouse PM—For the H2HFF-based assay, cells were cultured at 37 °C and 5% CO2 on 100-mm tissue culture dishes for 24–48 h after harvesting. Medium was RPMI 1640 containing 15% FBS, 50 μg/ml penicillin, 50 μg/ml streptomycin. Cells were washed with ice-cold PBS, pH 7.4, and resuspended in ice-cold KRP. Aliquots were taken for the cell counting and viability assay, and the exact number of cells was calculated. The indicated number of cells was taken into a warm 96-well plate with fresh KRP supplemented with OxyBURST Green H2HFF-BSA dye until 10 μl/ml, and the final volume was 190 μl. After a 2-min incubation in the dark, readings were started at emission of 530 nm and excitation of 480 nm. As a stimulus, PMA at 3 μg/ml, fMLP at 3 μM, or AA at 15 μM was added at minute 12, and readings were continued during the next 30 min. A 10-s mixing step was used in each reading cycle of the instrument to prevent the cells from sedimentation. Gliotoxin or DPI inhibition was done similarly, as described above.

For the SOD-inhibitable cytochrome c reduction-based assay, PM were harvested from the peritoneal cavity 4 days after thioglycollate injection by lavage with ice-cold PBS. Contaminating erythrocytes were hypotonically lysed, and PM were resuspended in KRP, pH 7.4, at 5 × 106 cells/ml and kept on ice until use. The reaction mixture was 250 μl and contained 50 μM cytochrome c and 6.25 × 105 PM in KRP. The superoxide release was induced with 4 μg of PMA, and readings of absorbance at 550 nm were taken for 10 min using a Tecan Spectra Rainbow 96-well plate spectrophotometer (Tecan). The same assay was carried out in the presence of SOD at 2.5 μg/reaction in order to evaluate the superoxide-independent change in absorbance. A sample without PMA was used as a negative control.

siRNA Transfection—siRNA Transfection of RAW264.7 cells was carried out on 6-well plates (Nunc). 200,000 cells/well were plated in antibiotic-free RPMI 1640, 15% FBS and after settlement washed with PBS. The transfection mixture was prepared using 70 nM siRNA specific for p66Shc or nonspecific AllStar siRNA (Qiagen), Opti-MEM medium (Invitrogen) and Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. After 6 h of transfection in 600 μl of transfection mixture/well, cells were fed with fresh RPMI 1640 supplemented with 15% FBS. After 48 h of cultivation, cells were used.
in NAD(P)H-oxidase measurement assays followed by cell counting and RNA or protein extraction as described above. Microarray Analysis of Differently Regulated Transcripts—Total RNA was extracted from the following tissues of individual age-matched mice: liver, spleen, lungs, epididymal fat, and PM at 3 months of age and liver, retroperitoneal fat, and spleen at 12 months of age. TRIzol reagent was used according to the manufacturer’s instructions (Invitrogen), and then RNA was purified using RNeasy Mini Kit (Qiagen). Two mutant and two control mice were used for each experiment. In total, 16 samples from mutant mice and 16 samples from control mice tissues were used. First and second strand of cDNA were generated using the One-Cycle cDNA Synthesis Kit (Affymetrix), and labeled cRNAs were synthesized using the Gene Chip IVT Labeling System (Affymetrix), fragmented, and hybridized to the Mouse Genome 430 2.0 arrays (Affymetrix) according to the manufacturer’s instructions. Resulting CEL files were analyzed for each group of tissue individually using dChip (DNA chip analyzer) software (10). Updated annotations were obtained from the NetAffx data base, and multisample analysis was performed by combining the dChip lists using Excel. Probesets with a pCall of >20% and p < 0.05 were considered significantly altered. An additional set of CEL files was obtained. Liver, spleen, and lungs samples from 3-month-old p66Shc(−/−) and 3-month-old control mice were hybridized in a similar way to the Affymetrix Mouse Genome U74Av2 chips. RNA samples of each tissue from three animals were pulled together. Two chips for each RNA sample of control and two chips for each RNA sample of mutant mice were used. The CEL files were analyzed with dChip in our laboratory and incorporated into our express data table using a >90% probeset match between two different chip formats, Mouse Genome 430 2.0 and U74Av2. Probesets were then resorted by number of times they were significantly changed through different experiments. Lists of the top 250 up- and down-regulated genes were categorized using the Onto-Express Pathway analysis tool, and lists of the 100 top up- and down-regulated genes were categorized using the Onto-Express bioprocess analysis tool (11). For filtering OntoExpress results, we used a cut-off value for p of 0.05. Lists of significantly altered pathways (which included 27 pathways) and significantly altered bioprocesses (18 bioprocesses) were sorted by impact factor and p value, respectively, and the four top up- and down-regulated pathways and bioprocesses were tabulated.

Western Blotting—Total protein was isolated by direct lysis of adherent cells or isolated from cell pellets washed with ice-cold PBS, pH 7.4, using cell lysis buffer (Cell Signaling Technologies), containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na3EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na2VO4, 1 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride and supplemented with Complete miniblotterese inhibitor mixture and PhosStop phosphatase inhibitor mixture (Roche Applied Science). 40 μg of protein/line, as determined by a Bradford assay (Bio-Rad), were resolved by SDS-PAGE, transferred to a nitrocellulose membrane, blocked with Odyssey Blocking Buffer (Li-Cor Biosciences), and hybridized with the indicated primary antibodies, followed by development with infrared IR dye 700CW- and/or 800CW-labeled secondary antibodies (Li-Cor Corp.). Blots were scanned on an Odyssey infrared imaging instrument and quantified using Odyssey 2.1 software. Use of different IR dye-labeled secondary antibodies allowed us to measure the level of housekeeping proteins at the same time as the proteins of interest on the same membrane and improved the accuracy of quantification and normalization.

Two-dimensional Electrophoresis—Macroprobes were induced with DMSO (mock treatment) or PM for 5 min, as indicated; reactions were stopped with 5 volumes of ice-cold PBS; cells were collected by quick centrifugation at 0 °C and lysed by sonication with First Dimension Buffer, containing 8.0 mM urea, 2.0% Triton X-100, 5% β-mercaptoethanol, 1.6% Bio-Lyte 5/7 and 0.4% Bio-Lyte 3/10 ampholytes, and 1.5% CHAPS. Samples were isoelectrofocused on 4% PAGE (T = 25%, C = 3%), supplemented with 8 mM urea, 1.5% CHAPS at a pH range of 4–9. Isolelectrofocusing gels were then transferred on top of 4–15% gradient SDS-PAGE for the second dimension. Blots were probed with goat anti-p47phox antibody. For the second hybridization, blots were probed with donkey anti-goat monoclonal antibodies (Li-Cor), labeled with IR dye 800CW. The membrane was scanned with an Odyssey infrared scanner and analyzed with Odyssey 2.1 software.

Subfractionation of Protein Extracts—Total protein was isolated from control and p66Shc(−/−) PM induced with PMA for 5 min or mock-treated as described above. Cells were collected and lysed by 20 strokes in CHAPS lysis buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 10% glycerol, 0.5% CHAPS and supplemented with Complete Mini protease inhibitor mixture and PhosStop phosphatase inhibitor mixture (Roche Applied Science). Unbroken cells were collected by brief centrifugation at 400 × g, and protein concentrations were determined. 100 μg of total protein were adjusted to 60 μl with CHAPS lysis buffer and centrifuged at 100,000 × g for 30 min at +4 °C. Supernatant was called the cytosolic fraction, and pellet was washed with CHAPS lysis buffer and contained the membrane fraction of proteins. The cytosolic fraction was supplemented until 1 × with Laemmli sample buffer, and the membrane fraction was resuspended in 1 × Laemmli sample buffer. Fractions were boiled until they dissolved completely and were loaded on SDS-PAGE. Western blots with the indicated antibody were performed as described above.

Rac Activation Assay—Macroprobes were isolated from 3-month-old mice as described above, and 7 × 104 cells were resuspended in 6 ml of KRP medium. 3 ml of cell suspension were induced with stimulus: 3 μg/ml PMA for 5 min or 5 μM fMLP for 60 s, as indicated; the other half of the cell suspension was treated with an equal amount of DMSO (mock treatment). For fMLP induction, cells were pretreated with 10 μM cytochalasin b for 10 min. Reactions were stopped by a 5-volume dilution of ice-cold PBS, and cells were collected by rapid centrifugation and lysed in ice-cold lysis buffer: 25 mM HEPES, pH 7.5, 150 mM NaCl, 1% Igepal CA 630, 10% glycerol, 25 mM NaF, 10 mM MgCl2, 1 mM EDTA, 1 mM sodium orthovanadate, and Complete Mini proteinase inhibitor mixture (Roche Applied Science). Lysates were rapidly clarified by low speed centrifugation at +4 °C and immediately frozen in
Each data set was analyzed individually, transcripts of the hybridization with Affymetrix oligo-microarray chips, mutants and 19 age/sex-matched control animals were used for analysis. Tool were truncated to the most significant four in each category and are presented below.

### Genomic effects of p66Shc knock-out

| Pathways                              | Impact factor | p value | Bioprocesses                              | No. of altered genes | p value |
|---------------------------------------|---------------|---------|-------------------------------------------|-----------------------|---------|
| Phosphatidylinositol signaling system | 35            | 3.2E-14 | Negative regulation of programmed cell death | 6                     | 3.3E-03 |
| Antigen processing and presentation   | 28            | 1.4E-11 | Anti-apoptosis                             | 5                     | 1.2E-03 |
| Chronic myeloid leukemia              | 10            | 6.7E-04 | Heme biosynthetic process                  | 3                     | 1.7E-04 |
| Adipokytokine signaling pathway       | 8             | 2.4E-03 | Porphrin biosynthetic process              | 3                     | 3.8E-04 |
| ECM-receptor interaction              | 9             | 8.4E-04 | Actin filament-based process               | 8                     | 3.8E-03 |
| Insulin signaling pathway             | 9             | 1.1E-03 | Cell-matrix adhesion                       | 4                     | 3.1E-03 |
| GLP1 receptor interaction             | 6             | 1.2E-02 | Cell-substrate adhesion                    | 4                     | 3.7E-03 |

Up-regulated

| Pathways                              | Impact factor | p value | Bioprocesses                              | No. of altered genes | p value |
|---------------------------------------|---------------|---------|-------------------------------------------|-----------------------|---------|
| Glioma                                | 10            | 7.6E-04 | Actin cytoskeleton organization and biogenesis | 8                     | 3.8E-03 |
| Natural killer cell-mediated cytotoxicity | 9             | 8.4E-04 | Actin filament-based process               | 8                     | 4.4E-03 |
| ECM-receptor interaction              | 9             | 1.1E-03 | Cell-matrix adhesion                       | 4                     | 3.1E-03 |
| Insulin signaling pathway             | 6             | 1.2E-02 | Cell-substrate adhesion                    | 4                     | 3.7E-03 |

RESULTS

**p66Shc-deficient Mice Have Transcriptional Alterations in Phosphatidylinositol and Heme Pathways**—To identify transcripts altered in long-lived p66Shc knock-out mice, we microarrayed RNA from liver, spleen, lungs, fat, and peritoneal macrophages of 3-month-old males and 12-month-old males and females of p66Shc(−/−) and control mice. A total of 19 mutants and 19 age/matched control animals were used for the hybridization with Affymetrix oligo-microarray chips, followed by analysis of gene expression data using dChip (10). Each data set was analyzed individually, transcripts of p < 0.05 were organized in a megatable, and the top 250 up- and down-regulated genes were entered into OntoExpress (12) as described under “Experimental Procedures.” The top four significantly altered pathways are presented. Also, lists of top 100 up- and down-regulated genes were analyzed with OntoExpress for biological process (11). The top four regulated bioprocesses are shown in Table 1.

PI3K and antigen processing were significantly altered, and heme transcripts were preferentially affected. Some of the genes underlying these down-regulated processes were related to NADPH oxidase activity. Given the involvement of heme in NADPH oxidase-dependent ROS production, this activity was specifically measured.

**p66Shc(−/−) Macrophages Have a Defect in NAD(P)H Oxidase-dependent ROS Generation**—NADPH oxidase (PHOX) is the basis of the respiratory burst. We measured the respiratory burst of p66Shc(−/−) peritoneal macrophages by two different methods: the conventional SOD-inhibitable reduction of cytochrome c assay and a more sensitive, fluorescence-based H2HFF-oxidation assay (Fig. 1). Both H2HFF dye oxidation and cytochrome c reduction were inhibitable with DPI or gliotoxin, specific PHOX inhibitors (13–16). As determined by the cytochrome c assay, the mean value of SOD-inhibitable superoxide production by 600,000 PMA-induced control macrophages was 151 pmol of superoxide/min, whereas p66Shc(−/−) macrophages produce 104 pmol of superoxide/min. Thus, mutant macrophages have 69% of the NAD(P)H-oxidase activity of control. The difference in means was significant, p < 0.000003 (Fig. 2A). Similarly, the H2HFF oxidation method demonstrated an about 40% defect in superoxide production by mutant macrophages, p = 0.01084 (Fig. 2B). Similar results were obtained for fMLP- and AA-stimulated macrophages (Fig.
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A. Superoxide generation by PMA-induced peritoneal macrophages from control and p66Shc(-/-) mice, as indicated, was assayed using the SOD-inhibitable cytochrome c reduction method. Bars, mean amount of superoxide (pmol/min/600,000 cells). Error bars, S.D. (n = 12). B. Superoxide generation by PMA-induced peritoneal macrophages from p66Shc(-/-) and control mice, as indicated, was assayed using the H2HFF fluorescent dye oxidation method. Bars, means of slopes of fluorescence increase (excitation 480 nm, emission 530 nm) after PMA induction of macrophages. Non-induced cells were used as the base line. C. Superoxide generation by fMLP-induced peritoneal macrophages from p66Shc(-/-) and control mice, as indicated, was assayed using the H2HFF fluorescent dye oxidation method. D. Superoxide generation by AA-induced peritoneal macrophages from p66Shc(-/-) and control mice, as indicated, was assayed using the H2HFF fluorescent dye oxidation method. Error bars, S.D. For the H2HFF dye oxidation method, n = 4.

p66Shc-null Mice Have No Defects in Expression Levels of NOX2 Subunits—There are at least three explanations for a p66Shc-dependent defect in PMA-stimulated NAD(P)H-oxidase activity (i.e. a defect in expression of a PHOX component, in the heme necessary for superoxide production, or the rate of activation of the complex). First, we investigated the expression of the components of the NOX2 complex (i.e. Rac1/2, p40phox, p47phox, p67phox, p22phox, and gp91phox), using Western blots of protein extracts from peritoneal macrophages of p66Shc(-/-) and control mice (Fig. 3) (data not shown). There was no significant decrease in the protein levels of any tested NOX2 subunit in mutant macrophages versus control. Thus, the defect in PHOX activity in mutant p66Shc(-/-) cells could not be explained by a defect in NOX2 subunit levels.

Heme Levels Are Unchanged in Mutant Mouse Blood—Heme deficiency causes delayed maturation and degradation of gp91phox, resulting in reduced gp91phox protein level in mammalian cells (17, 18). Microarray results suggested a heme defect in p66Shc mice. However, because there was no difference between gp91phox abundance in mutant and control macrophages, it was unlikely that the heme level would be different in p66Shc(-/-) mice. We investigated total blood heme levels in p66Shc(-/-) mice versus littermate controls. No significant difference was observed (Fig. 4). p66Shc(-/-) Cells Have Reduced Phosphorylation of p47phox—Because there was no deficiency in expression of cytosolic NOX2 subunits in the p66Shc(-/-) mice, no deficiency in membrane subunit gp91phox, and no heme deficiency, the remaining explanations for the decreased level of PHOX activity included a reduction in phosphorylation of the main regulatory subunit p47phox or reduced Rac activation. After induction of macrophages with PMA or fMLP, p47phox rapidly becomes phosphorylated. The autoinhibitory domain (19) of p47phox contains four clusters of serine residues. Each cluster contains two or three Ser residues, separated by few amino acids (10 serines in total) (20). This phosphorylation induces the ultimate translocation of p47phox and p67phox, existing as a complex with p40phox in cytosol, to the heme-containing catalytic subunit gp91phox. The phosphorylation rate of p47phox is one of the limiting steps for the assembly and activation of PHOX (21). The last phosphorylation step occurs only after p47phox translocates and associates with p22phox (22). At the same time, GDP-GTP exchange factors Rac1 and Rac2 activate and translocate to cytochrome b558.

We measured phosphorylation rates of p47phox in PMA-induced macrophages from p66Shc(-/-) and control macrophages by two-dimensional electrophoresis, as described under “Experimental Procedures,” followed by Western blot with anti-p47phox antibody (Fig. 5A). The isoelectric point for non-phosphorylated p47phox is 9.07. Upon phosphorylation, the isoelectric point of the protein shifts toward acidic pH due to the increasing number of PO4− residues attached to the protein.

Upon mock treatment without PMA, we observed a spot at molecular mass 47 kDa and pH about 9, corresponding to p47phox and labeled with state “0” of phosphorylation. Upon PMA treatment for 5 min, four spots appeared at molecular mass 47 kDa with a more acidic isoelectric point, corresponding to higher phosphorylation states of p47phox. Although only 22% of p47phox from wild type PM remained unphosphorylated after a 5-min treatment with PMA, 55% from mutant PM was unphosphorylated (Fig. 5B). Consistently, mutants had an about 40% decrease in the phosphorylated isoforms of p47phox. Of the phosphorylated forms, p66Shc mutants had a
higher fraction of p47phox in states 1 and 2 (30%) than controls that had a higher fraction of p47phox in phosphorylation states 3 and 4. Because the final phosphorylation step of p47phox occurs only after PHOX is fully assembled, we suggest that assembly of NAD(P)H-oxidase is reduced in p66Shc(-/-) cells. The 40% reduction in phosphorylation rate is sufficient to explain the 40% reduced rate of ROS production from p66Shc-deficient mice.

**p66Shc(-/-) Macrophages Have Reduced Translocation of p47phox**—We investigated PMA-inducible translocation of p47phox to plasma membrane using ultracentrifugation to separate total protein from mutant and control macrophages on membrane and cytosolic fractions in five experiments. Cytoplasmic and membrane fractions were then resolved on SDS-PAGE, and a very slight Mn-SOD signal was observed in membrane fraction of PM protein extracts from both mutant and control mice, suggesting that only very slight contamination (<1%) of membrane fraction by proteins from cytosolic fraction occurred. β-Actin bands (loading control) were present in all fractions. P47phox was about 35% less abundant in membrane fractions of PMA-stimulated mutant macrophages versus controls. Mock-treated PM had similar levels of p47phox in the membrane fraction. In some experiments, membrane fractions were sonicated with SDS prior to SDS-PAGE analysis; under these conditions, membrane p47phox fraction migrated at the same molecular weight as cytosolic p47phox, demonstrating the PMA-dependent shift in the molecular weight of p47phox (Fig. 6) (data not shown).

The proteins for the experiment were isolated under mild lysis conditions with the agent CHAPS at a concentration of 0.5%, as described under “Experimental Procedures” (Fig. 6). After centrifugation of cleared lysates at 100,000 × g, precipitates of membrane-associated proteins were tight and hard to dissolve. On the blot presented, the shift in membrane fraction p47phox mobility on SDS-PAGE is large; also, p47phox runs as a doublet. We suggest that this was the result of incomplete penetration of loading buffer components into the gel and loaded on the gel. The shift in mobility of p47phox was less apparent under these conditions, and membrane-associated p47phox was running at its expected mass as single band. We present the blot where membrane-associated p47phox ran more slowly because we suggest that this shift in mobility provides one more internal control for contamination of membrane fraction by cytosolic fraction. The doublet bands corresponding to membrane-associated p47phox were added and normalized to β-actin levels to correct for loading. We suggest that reduced translocation of p47phox in mutant cells is the result of reduced phosphorylation of p47phox in p66Shc(-/-) mice and could explain reduced superoxide generation by PM from p66Shc(-/-) mice.

**siRNA-mediated p66Shc Knockdown Decreases NADPH Oxidase-dependent Superoxide Generation**—To address whether the defect in NADPH oxidase-dependent superoxide production was a direct consequence of p66Shc, we knocked down p66Shc in the mouse macrophage cell line RAW264. In 25 independent transfections, we observed a mean reduction of p66Shc of 40 and 60% at the mRNA and protein levels, respectively (Fig. 7). p66Shc siRNA did not affect expression of NADPH oxidase subunits or other p52 and p46 Shc isoforms at the protein level (Fig. 7) (data not shown). The p66Shc-silenced RAW264.7 cells had an about 30% reduction in PMA-inducible H2O2 dye oxidation, which was inhibitable with DPI or glitoxin. We conclude that p66Shc deficiency is sufficient for partial defect in activation of NAD(P)H-oxidase.

**p66Shc(-/-) Macrophages Do Not Have a Big Defect in Rac Activation**—Another component of NADPH oxidase activation in phagocytic cells is GTPase Rac (23, 24). Correct positioning of p67phox with gp91phox depends mostly on two reg-
ulatory subunits: p47phox and Rac1 or Rac2 (21). A defect in activation of either one will lead to a defect in NADPH oxidase activity. In our experiments, we observed a decrease in activation and translocation of p47phox; however, if Rac activation was reduced as a consequence of altered Shc, it would lead to a decrease in NADPH oxidase activation.

We tested activation of both Rac1 and Rac2 by fMLP and PMA. The maximal rate of Rac activation by PMA is 5 min and 1 min of fMLP treatment (25). We found no difference in PMA-stimulated Rac1 activation between p66Shc(-/-) and wild type macrophages. A very small (1.18 times) but significant (\( p < 0.023346 \)) reduction in Rac1 activation was found in fMLP-stimulated mutant macrophages (Fig. 8). Because p66Shc was reported to signal specifically to Rac1, and Rac1 is 4 times more abundant in peritoneal macrophages than Rac2 (26), we present blots measuring GTP-Rac1. GTP-Rac2 also precipitated with the PAK-1 p21-binding domain-agarose conjugate, and we tested GTP-Rac2 levels as well. Rac2 activation by PMA and fMLP was not different from Rac1 activation (data not shown). GTP-Rac bands have similar intensities between p66Shc(-/-) and wild type agonist-stimulated macrophages. A slight difference with fMLP stimulation was revealed only after densitometry and normalization to the loading control bands. Statistical analysis of four independent experiments with two technical replicates each (eight gels total) showed that p66Shc(-/-) macrophages have very slightly less fMLP-induced Rac1 activation, whereas PMA stimulation results in no statistically different activation of Rac in mutants and wild type macrophages.

The decrease in respiratory burst between mutant and wild type macrophages was 30–35% and was observed with fMLP, PMA, and arachidonic acid stimulation. We conclude that difference in Rac activation could not explain the difference in oxidative burst between mutant and control macrophages.

**p66Shc(-/-) Cells Have Reduced Phosphorylation of PrkCδ—**

PMA through translocation to the plasma membrane drives phosphorylation of multiple protein kinases C (PKCs) and their targets. PKCδ is thought to play a central role in activation of PHOXs by phorbol esters (27). We measured the activation of PKCδ in mutant and control macrophages induced with PMA. A Western blot, representing 18 similar experiments, is shown (Fig. 9A). The primary antibodies for

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**FIGURE 5.** p66Shc(-/-) cells have reduced phosphorylation of p47phox. A, total protein was isolated from control or p66Shc(-/-) macrophages induced with DMSO (mock treatment) or PMA for 5 min, as indicated. Samples were isoelectrofocused at a pH range of 4–9. Isoelectrofocusing gels were transferred on top of SDS-PAGE for the second dimension. Blots were probed with goat anti-p47phox antibody. Phosphorylated (1, 2, 3, and 4) and non-phosphorylated (0) forms of p47phox are indicated. B, bars represent densitometry results for the levels of phosphorylated p47phox expressed as part of the total amount of p47phox (n = 2).

**FIGURE 6.** p66Shc(-/-) macrophages have a defect in translocation of p47phox to membrane upon PMA induction. A, total protein was isolated from control or p66Shc(-/-) macrophages induced with DMSO (mock treatment) or PMA for 5 min, as indicated. Samples were cleaned from unbroken cells by centrifugation and then were separated on membrane and cytosolic fractions by ultracentrifugation at 100,000 \( \times g \), as described under “Experimental Procedures.” Cytosolic and membrane fractions were then resolved on SDS-PAGE and transferred to nitrocellulose membrane. Membrane was cut by a 35 kDa marker band, and the upper part was probed with goat anti-p47phox polyclonal antibody and mouse anti-actin \( \beta \) primary antibody. A Western blot image of one of five experiments is presented. B, bars in the graph represent means of fluorescence intensities of p47phox bands from membrane fraction divided by fluorescence levels of actin \( \beta \) bands. Error bars, S.D. (n = 5).
Decreased Superoxide Production in p66Shc Knock-out Mouse

FIGURE 7. Mouse macrophage cell line RAW 264 with siRNA-mediated p66Shc knockdown have a defect in NAD(P)H-oxidase-dependent superoxide generation. A, isoform-specific siRNA-mediated knockdown of p66Shc in RAW264 cells. Total protein was extracted 48 h after transfection with p66Shc siRNA or control siRNA, as indicated. The blot was probed with rabbit polyclonal anti-ShcA antibody. B, bars represent the amount of p66Shc as a percentage of all three Shc isoforms. C, quantitative reverse transcription-PCR results of p66Shc expression measured normalized to β-actin levels (n = 25). D, NAD(P)H-oxidase activity in p66Shc knockdown and control RAW264 cells. Bars, means of slopes of fluorescence increase of H2HFF dye after PMA induction. Non-PMA-induced cells were used as base line. Error bars, S.D. (n = 25).

total PKCδ were from mice, and primary antibodies for the phospho-PKCδ were from rabbit host species. We used secondary antibody labeled with different infrared dyes as described under “Experimental Procedures.” This creates the possibility to run total PKCδ (loading control) and phospho-PKCδ quantification on the same membrane. We measured band intensities for phospho-PKCδ and total PKCδ on our Western blots. Bars on the graph presented in Fig. 9B show intensities of phospho-PKCδ divided by total PKCδ. The error bars show S.D. of 18 experiments. On the Western blot (Fig. 9A), the p66Shc(−/−) PMA-induced phospho-PKCδ band looks lighter than the control band. As shown on the bar graph, PMA-stimulated PKCδ phosphorylation in mutant macrophages was significantly reduced by about 30% compared with control macrophages ones (p = 0.017). In mock-treated macrophages, no significant difference in phospho-PKCδ abundance in mutant macrophages versus control was observed (p = 0.91) (Fig. 9B). Because the difference in ROS production is only 30–40%, we did not expect a bigger defect in phosphorylation of PKCδ. Thus, the defect in NADPH oxidase activation in p66Shc(−/−) cells can be explained by a decrease in phosphorylation rate of PKCδ and, as a result, decrease in activation of p47phox.

p66Shc(−/−) Macrophages Have Reduced Activation of Akt and ERK by fMLP—A number of kinases have been proposed to participate in p47phox phosphorylation events, among which Akt and ERK have been reported (28–30). We tested Akt and ERK activation after 1 min of fMLP treatment in mutant and control macrophages using anti-phospho Akt, anti-total Akt, anti-phospho-ERK, and anti-total ERK antibody. The antibodies were from different species, and it was possible to analyze phospho-specific and total antigen specific bands on the same membrane at the same time. The activation of Akt and ERK was lower in mutant macrophages (Fig. 10) (p = 0.029532 and p = 0.046206, respectively). The difference in PKC activation between p66Shc(−/−) and wild type cells in our experiments was about 30%. The difference in Akt and ERK activation was about 30% also. We suggest that because Akt and ERKs have been reported to be activators of p47phox, decreased phosphorylation of Akt/ERK in p66Shc-deficient mice contributes to decreased activation of p47phox.
DISCUSSION

Long-lived 66Shc-deficient Mice Produce Less Superoxide through Macrophage NADPH Oxidase—p66Shc-deficient mice have an extended life span, and their cells produce less ROS and are resistant to oxidative stress. We sought to identify the basis of the extended life span. We observe that the NADPH-dependent production of superoxide is deficient in p66Shc KO mice. The defect does not appear to result from any deficiency in PHOX protein expression but rather through a Shc-dependent defect in the signaling and phosphorylation and assembly of the PHOX complex. Resistances to oxidative stress, altered insulin sensitivity, and reduced body mass have been reported for multiple mouse longevity models (31). We suggest that reduced NADPH oxidase-dependent superoxide production could be the basis for reduced inflammatory and oxidative disease in these long-lived mutants and could contribute to their increased life span.

Activation of p47phox and Kinases Implicated in p47phox Phosphorylation, Are Lower in p66Shc(−/−) Mice—The deficiency in NADPH oxidase-mediated superoxide production in mutant mice could have been the result of several p66Shc-dependent causes: deficiency in PHOX subunits or heme. However, we observed no evidence of deficiency of PHOX-related proteins or heme. By contrast, the phosphorylation rate of p47phox was clearly and significantly lower in mutants compared with controls.

A number of kinases have been proposed to participate in p47phox phosphorylation events, in particular PKC, MAPKs (20), ERK1/2 (28), p21-activated kinase (PAK) (32, 33), and protein kinase B/Akt (29, 30). Because we did not observe a big difference between p66Shc KO and wild type macrophages in activation of Rac, we do not expect activation of PAK, which is downstream of Rac. fMLP treatment of phagocytic cells leads to activation of Src-related kinase Lyn (34) and Syk. Shc proteins are downstream and implicated in signaling to Akt and ERK through binding to Grb2, SOS, and activation of Ras in phagocytic cells (35, 36). Major signal conductors were reported to be p52Shc and p66Shc (37). However, this is not the only way for activation of ERK and MAPK in hematopoietic cells (38). In the p66Shc KO background, changes in Shc expression could affect activation of PI3K, Akt, and ERK.

In our experiments, we observed that level of activation of PKCδ was lower in mutants. PKCδ is critical for phosphorylation of p47phox (27, 39). It has been shown that Shc directly interacts, co-localizes, and co-translocates with PKCδ in different types of cells, including mouse embryonic fibroblasts (40), hematopoietic cells (41), pancreatic cells (42), and COS cells (43). Upon stress, p52Shc and especially p66Shc (43) get phosphorylated by PKCδ; p46Shc has been shown to be similarly phosphorylated by PKCδ as p66Shc. Thus, there is clear connection of Shc proteins to PKC signaling, and changes in expression of Shcs are expected to affect PKC activation rates, as we observe in p66Shc(−/−) cells.

Akt and ERK kinases are downstream of Shc proteins in signaling cascades. Thus, a defect in expression of Shc would lead to a defect in signaling to Akt and ERK through the Shc/Grb2/SOS/Ras/PI3K pathway. In our experiments, we observed such defects as decreased activation of Akt and ERK for about 30%. We propose...
that a defect in PKCδ, Akt, and ERK activation contributes to the defect in p47phox phosphorylation and ultimately to the defect in PHOX activation and superoxide production.

**Rac Activation Is Similar in p66Shc(-/-) Macrophages**—Another component crucially important for efficiency of NADPH oxidase activation in phagocytic cells is GTPase Rac (23, 24). Studies have shown that p66Shc redirects the signaling of Shc through SOS toward Rac1 activation and consequently leads to ROS production in mouse embryonic fibroblasts in response to stress (44). Activated Rac, in turn, has been reported to increase phosphorylation, reduce ubiquitination, and stabilize p66Shc protein (45). P67phox was reported to be an effector of both Rac1 and Rac2. In macrophages, mostly Rac1 is responsible for NADPH oxidase activation (46). Moreover, binding of Rac- or Cdc42-GTP leads to PAK autophosphorylation and activation of the ability to phosphorylate exogenous substrates on serine and/or threonine residues. Substrates for PAK in human neutrophils may include the p47phox and p67phox-NADPH oxidase components (32, 47). Thus, a defect in Rac/PAK-1 activation would lead to a defect in p47phox phosphorylation. Also, a defect in Rac activation itself would lead to a defect in p67phox assembly with gp91phox.

In our experiments, GTP-Rac levels in p66Shc(-/-) and wild type agonist-stimulated macrophages were similar. A very slight decrease with fMLP stimulation was observed. PMA stimulation resulted in no significant difference in the activation of Rac in mutants and wild type macrophages. However, the 30–40% decrease in respiratory burst in mutant macrophages was observed with all stimulators: fMLP, PMA, and arachidonic acid. We conclude that the difference in Rac activation cannot explain the difference in oxidative burst between mutant and control macrophages.

It has been shown that p66Shc signals to Rac1 and leads to ROS production in some cell types. In our study, we saw only a tiny effect of p66Shc deletion on Rac activation in peritoneal macrophages. The p66Shc level in wild type macrophages is very small and is very similar to the Shc levels in RAW cells (Fig. 7), <1% compared with the other isoforms p52 and p46Shc. However mouse embryonic fibroblasts have very high p66Shc levels, such that p66 = p52 = p46 (45) (data not shown). This is a potential explanation for the different results obtained in the current study on macrophages and a former study employing mouse embryonic fibroblasts (44).

**G Protein-coupled Receptors, Shc, and NADPH Oxidase Activity**—Agonists activate G protein-coupled receptors (GPCRs), the G family of heterotrimeric G proteins in hematopoietic cells, which are composed of Go, Gβ, and Gγ subunits. Ligand binding to receptors catalyzes the GDP for GTP exchange on the Gα subunit, liberating it from the Gβγ complex. Free Gβγ and Gγ subunits directly bind to downstream effectors (48) and direct functions, including chemotaxis and superoxide production (49). Thus, GPCR signals to PI3Kγ (50) with subsequent synthesis of phosphatidylinositol 3',4',5'-tris-
phosphate (PIP$_3$), followed by PIP$_3$-activated kinase and Akt activation. Akt is one of the activators of p47phox (28, 30). PIP$_3$ required for the activation of GTP exchange factor-DOCK-2 and for the anchoring of p47phox and p40phox on the membrane during NADPH oxidase assembly (51–55). Activation of PI3K has being reported to require active Ras, which is downstream of Shcs. Shcs are activated by GPCRs through tyrosine kinases Lyn and Syk (34, 35, 56) and cause GRB2/SOS/Ras/Raf/MEK-dependent ERK activation. ERKs are activators of p47phox (28).

Active G$\beta$$\gamma$s also activate phosphoinositide-specific phospholipase-$\beta$ (57, 58), which, in turn, leads to generation of inositol trisphosphate, with the consequent mobilization of Ca$^{2+}$ from intracellular stores, and diacylglycerol, with the consequent activation of various PKC isoforms, including diacylglycerol-sensitive calcium-dependent kinase-PKC$\delta$. It has been demonstrated that PKC$\delta$, on one hand, directly interacts with Shc (40–43); also, PKC$\delta$ is an activator of p47phox (27, 39, 59, 60).

Thus, activations of PKC$\delta$, Akt, and ERKs are dependent on the activation and expression of Shcs. On another arm of the pathway, GPCR signals to GTP exchange factor-Vav through the Src family kinases Hck and Fgr and causes GDP-GTP exchange and activation of Rac GTPase, leading to Rac translocation and participation in NADPH oxidase assembly (58). Also, activated Rac signals to PAK-1, which has been reported to be a p47phox (an NADPH oxidase-regulatory subunit) activator (32, 33). One more way in which GPCR activates Rac is through the direct activation of GTP exchange factor-P-Rex by the free G$\beta$$\gamma$ (61); however, this requires PIP$_3$. Thus, Rac activation has the potential to bypass the Shc-dependent pathways (Fig. 11).

Thus, we observe an Shc-dependent defect in activation of PKC$\delta$, Akt, and ERK with a consequent effect on activation of p47phox, consistent with defective activation of the right and center arms of the pathway descending from $G_i$ stimulation.

We only observe a very slight decrease in GDP-Rac binding (the left arm and non-Shc-dependent part of the pathway), and this defect is not sufficient to explain the 30–40% decrease in NADPH oxidase-dependent superoxide production.

Potential Consequences of NADPH Oxidase Deficiency in p66Shc(−−) Mice—Reduced ROS production and resistance to oxidative stress has also been reported to extend life span in several invertebrate model systems (62–67). Resistance to oxidative stress, altered insulin sensitivity, and reduced body mass have been reported for multiple mouse longevity models (31). However, the support for longevity as a result of increased protection from oxidative stress in rodents is mixed. Although CuZn-SOD and Mn-SOD deficiency cause increased ROS in vivo and shortened life span (68, 69), overexpression of CuZn-SOD and Mn-SOD does not appear to extend mouse life span (70–72). However, increased mitochondrial catalase expression has been reported to increase life span and health span in mice (73, 74), and increased CuZn-SOD in rats increases life span.

p66Shc-deficient mice have been observed to be resistant to several types of age-related pathology associated with oxidative stress and/or inflammation (which can be caused by phagocytic cells), including vascular disease (5), age-related endothelial dysfunction (75), oxidative inflammatory kidney damage (8, 76), hyperglycemia-induced endothelial dysfunction and oxidative stress (7), and brain oxidative stress (77). This resistance could be the result of decreased oxidative stress or decreased inflammatory response. Two major sources of cellular ROS production are PHOX and mitochondria. A small decrease in CCL4-dependent mitochondrial ROS generation was shown previously for p66Shc KO mice (4); however, PHOX-dependent superoxide production has not been investigated.

We observe that the NADPH-dependent production of superoxide is deficient in p66Shc mice. The defect does not appear to result from any deficiency in PHOX protein expression but rather through a Shc-dependent defect in the signaling and phosphorylation and assembly of the PHOX complex. This defect in PHOX-dependent superoxide generation has the potential to explain the reported resistance of p66Shc knock-out mice to oxidative pathologies in endothelial cells, kidney, and brain, and could also underlie the relative sensitivity of p66Shc mice to insulin and their longevity. Further studies are necessary to clarify the role of reduced oxidative stress in longevity.

In the present work, we demonstrate that ROS production by NADPH oxidase is deficient in p66Shc knock-outs. We suggest that reduced NADPH oxidase-dependent superoxide production could be the basis for reduced inflammatory and oxidative disease in these long-lived mutants. Activation of NADPH oxidase and inflammation is perhaps the most clear example of antagonistic pleiotropy (78) (i.e. an increase in ROS production is protective at a young age but causes multiple age-related pathologies, including atherosclerosis, hypertension, and nephropathy rheumatoid arthritis) (79). An increase in several markers of inflammation with age is observed in
many species, including humans, and is thought to cause life span-limiting pathology, in inflammatory hypotheses of aging (80–83) with special emphasis on phagocytic cells (84). Multiple anti-inflammatory changes occur in long-lived Ames dwarf mice (85). Dietary restriction appears to decrease inflammation, and it is thought that this is one mechanism by which it increases life span (86). Anti-inflammatory drugs can block age-related pathology and neurodegeneration and extend life span in several species, including mice (87–90) and Drosophila (91). We suggest that reduced NADPH oxidase activity could contribute to reduced inflammation in p66Shc KO mice and underlie their increased life span.

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