Control of Hepatic Nuclear Superoxide Production by Glucose 6-Phosphate Dehydrogenase and NADPH Oxidase-4*§

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Redox-regulated signal transduction is coordinated by spatially controlled production of reactive oxygen species within subcellular compartments. The nucleus has long been known to produce superoxide (O$_2^-$), however, the mechanisms that control this function remain largely unknown. We have characterized molecular features of a nuclear superoxide-producing system in the mouse liver. Using electron paramagnetic resonance, we investigated whether several NADPH oxidases (NOX1, 2, and 4) and known activators of NOX (Rac1, Rac2, p22phox, and p47phox) contribute to nuclear O$_2^-$ production in isolated hepatic nuclei. Our findings demonstrate that NOX4 most significantly contributes to hepatic nuclear O$_2^-$ production that utilizes NADPH as an electron donor. Although NOX4 protein immunolocalized to both nuclear membranes and intranuclear inclusions, fluorescent detection of NADPH-dependent nuclear O$_2^-$ predominantly localized to the perinuclear space. Interestingly, NADP$^+$ and G6P also induced nuclear O$_2^-$ production, suggesting that intranuclear glucose-6-phosphate dehydrogenase (G6PD) can control NOX4 activity through nuclear NADPH production. Using G6PD mutant mice and G6PD shRNA, we confirmed that reductions in nuclear G6PD enzyme decrease the ability of hepatic nuclei to generate O$_2^-$ in response to NADP$^+$ and G6P. NOX4 and G6PD protein were also observed in overlapping microdomains within the nucleus. These findings provide new insights on the metabolic pathways for substrate regulation of nuclear O$_2^-$ production by NOX4.

Reactive oxygen species (ROS)$^2$ such as superoxide (O$_2^-$) and H$_2$O$_2$ play important roles in cellular oxidative stress as well as in the regulation of cellular signal transduction in the healthy state. Understanding the regulatory pathways that control cellular ROS at specific sites in the cell is vital to determining their function in cell signaling (1). Important for the regulation of redox signaling is the controlled production of ROS at specific intracellular sites such as mitochondria and endosomes. ROS production and transport at these locations are tightly regulated and linked to effector redox signals (2–6). Another, much less studied, site of ROS production is the nucleus. Despite the fact that NADPH-dependent O$_2^-$ production by the nucleus was discovered over three decades ago (7–10), the regulation and function of nuclear O$_2^-$ remain largely uncharacterized.

Over the years, several enzymes including cytochrome P450 and many others have been suggested as candidate sources of nuclear O$_2^-$. More recently, endothelial nuclei disrupted by sonication (11), and endothelial nuclear protein extracts (12) have both been shown to produce ROS that is, at least in part, NOX4-dependent. NOX4 has been localized in nuclei of vascular smooth muscle cells, but its subnuclear localization (such as within specific nuclear membranes) remains unclear (13). Nuclear NOX4 has also been implicated in DNA damage resulting from both hemangiendothelioma formation (14) and hepatitis C infection (15). However, NOX4 is not the only NOX localized to nuclei, because nuclear NOX1 and NOX2 have also been described (16, 17). The γ-isofor of the p47phox homolog NOXO1 (utilized by NOX1) has been shown to localize strongly in nuclei (18). A great deal remains to be learned about which NOX isoforms and associated subunits participate in nuclear ROS production. Nuclei of distinct cell types may utilize different NOX isoforms or splice isoforms (19) and associated subunits to produce nuclear ROS.

The NOXs (NOX1, 2, 3, 4, and 5 and Duox 1 and 2) are transmembrane proteins that produce superoxide and/or hydrogen peroxide. NOX1–4 appear to require association with p22phox for processing and activity, whereas NOX1–3 require additional cytosolic proteins such as p47phox, p67phox, Rac1 or 2, NOXO1, and/or NOXAI for activation (13, 20, 21). Although regulatory components of most of the NOXs are less well understood than those for the phagocytic NOX2, there is

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‡¶The abbreviations used are: ROS, reactive oxygen species; NOX, NADPH oxidase; G6PD, glucose-6-phosphate dehydrogenase; DMPO, 5,5-dimethyl-1-pyrroline N-oxide; DHE, dihydroethidium; TEM, transmission electron microscopy; SOD1, superoxide dismutase-1; BMPO, 5-tert-butoxycarbonyl-5-methyl-1-pyrroline N-oxide; MT, mutant; ANOVA, analysis of variance; ER, endoplasmic reticulum.
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general agreement that Rac1, rather than Rac2, is the primary Rac used by nonphagocytic NOXs that are dependent on Rac. Some studies suggest that NOX4 uses p47phox, whereas others suggest it is independent of this subunit (21–24); there is similar confusion about the requirement of NOX4 for Rac1 (21, 24, 25). Although NOX4 is inducible at the transcriptional level (21, 26), it is accepted as being constitutively active once it is synthesized (27, 28). Thus, NOX4 recruitment of subunits may not depend on the same kinds of stimuli as required in the case of other NOXs. Although unexplored to date, NOX4 may also be regulated by the local availability of its substrate NADPH. Other unresolved questions include whether NOX4 utilizes NADPH or NADH as a substrate to produce O2 (11, 26, 29) and whether it primarily produces superoxide or hydrogen peroxide (11, 21, 24, 30).

All of these as yet unanswered questions have resulted in numerous interesting, although often apparently contradictory, findings about NOX4 regulation in the nucleus. In the present study, we evaluated the biologic components required for NADPH-dependent O2 production by murine hepatic nuclei. Hepatic nuclei demonstrated an absolute requirement for NADPH, not NADH, as an electron donor for the production of O2. Although these findings differ from previous studies demonstrating NADPH dependence of NOX4 in endothelial nuclei (11), we demonstrate, using in vivo NOX4-shRNA-mediated knockdown, that NOX4 is indeed the primary NADPH-dependent O2 producing enzyme in hepatic nuclei. NOX4-dependent hepatic nuclear O2 appears to be produced directionally into the perinuclear space of the nuclear envelope. Using mutant mice and shRNA knockdown, we also demonstrate that nuclear glucose 6-phosphate dehydrogenase (G6PD) serves as a major source of NADPH utilized by hepatic nuclear NOX4. These novel findings define a new metabolic pathway for regulating nuclear NOX4 through availability of its substrate.

EXPERIMENTAL PROCEDURES

Mice—All of the animal studies were performed in accordance with University of Iowa policies and Institutional Animal Care and Use Committee guidelines. For all experiments not involving genetically defined strains, B6SJLF1/j female mice were used at the age of 6–10 weeks. Several genetically defined strains of mice were also used, including: G6PD mutant mice (G6pd−1MNeu) (31), NOX1 KO mice (32), NOX2 KO mice (33), NOX1/2 double KO mice, p47−/− mutant mice (34), p22phox mutant mice (35), Rac1fl/fl conditional KO mice (36), Rac2 KO mice (37), and AlbCre-Rac1fl/fl/Rac2 KO mice (which are deleted for Rac2 in all cells and also for Rac1 in hepatocytes). Because G6PD null mutations are embryonically lethal (38), G6PD mutant mice were used in these studies. These mice have a mutation in the 5′-UTR of the G6PD gene (which is on the X chromosome) that leads to an ~80% reduction in G6PD protein levels (39). Rac1fl/fl conditional KO mice, Rac2 KO mice, and AlbCre-Rac1fl/fl/Rac2 KO mice, all on a BL6 background, were a kind gift from Dr. David A. Williams. NOX1, NOX1/2, and AlbCre-Rac1fl/fl/Rac2 KO mice and p22phox mutant mice were bred onto a BL6 background for at least eight generations. NOX2 KO mice and p47−/− mutant mice were inbred on a BL6 background for at least 12 generations by the Jackson Laboratories. As controls for all knock-out and mutant mice, age-matched wild-type BL6 mice or littermates were used.

Isolation of Marine Hepatic Nuclei—The mice were euthanized in accordance with Institutional Animal Care and Use Committee-approved standard protocols by carbon dioxide gas. The liver was immediately dissected, and hepatic nuclei were purified using a sucrose gradient protocol described previously by our laboratory (40). The liver was placed in 2 ml of ice-cold nuclear homogenization buffer (300 mM sucrose, 10 mM HEPES, pH 7.6, 10 mM KCl, 0.74 mM spermidine, 0.15 mM spermine, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF) and homogenized in a Wheaton 7-ml Dounce tissue grinder. Cushion buffer (the same as homogenization buffer, except containing 2.2 mM sucrose) was added to the homogenized tissue to a final volume of 11 ml. This mixture was gently layered over 1 ml of cushion buffer in a 12-ml ultracentrifuge tube, and then the tube was topped off with homogenization buffer. The sample was centrifuged at 104,000 × g for 2 h at 4 °C. The nuclear pellet was gently resuspended in 0.1 ml of nuclear storage buffer (composed of 25 mM HEPES, pH 7.6, 25% glycerol, 3 mM MgCl2, 0.1 mM EDTA, 0.5 mM PMSF, 1 mM DTT). Isolated nuclei were either used for experiments immediately or snap-frozen in liquid nitrogen and stored at −80 °C. In the latter case, the nuclei were thawed rapidly at 37 °C just before experiments and then kept on ice until use. The nuclei were quantitated by A260 and/or protein concentration to normalize quantities used in experiments.

Electron Paramagnetic Resonance—EPR was performed in the University of Iowa EPR Core Facility using a Bruker EPR spectrometer. Spin trapping experiments were done using 50 mM 5,5-dimethyl-1-pyrroline N-oxide (DMPO) at 25 °C unless otherwise specified. All of the EPR experiments were done in PBS, pH 7.4, with the metal chelator diethylenetriaminepenta-acetic acid (final concentration, 0.1 mM). When NADPH or NADH was added to nuclei, the samples were incubated for exactly 5 min at 22 °C prior to starting the EPR scans, unless otherwise indicated in the text. EPR parameters were as follows: center field, 3480 G; sweep width, 80 G; frequency, 10.78 GHz; power, 20.3 milliwatt; receiver gain, 5.02 × 102; modulation frequency, 100 kHz; modulation amplitude, 1 G; conversion time, 20.48 ms; time constant, 81.92 ms; sweep time, 20,972 s; number of scans, 15; resolution, 1024 points. EPR spectra were quantitated by measurement of peak-to-peak height of the second DMPO-OH line based on double integration and comparison with double-integrated 3-carboxyl proxyl standard spectra (41).

shRNA Viruses—For knockdown of G6PD, we produced five recombinant adenoviral vectors that expressed five independent shRNAs against mouse G6PD. The design of these shRNA sequences (Table 1) followed the principles described previously by Boudreau et al. (42). Adenoviruses expressing these shRNAs were produced and amplified using standard procedures (43). After screening each of the shRNAs in TIB-73 cells (an immortalized mouse hepatocyte cell line) for knockdown of G6PD protein, we selected viruses 4 and 5 (named Ad.shG6PD4 and Ad.shG6PD5) for further in vivo experiments. For knock-
down of NOX4, we used a previously characterized recombinant adenoviral vector expressing shRNA against mouse NOX4 (Ad.shNOX4) (44). We used an adenovirus (Ad.shGFP) that expresses shRNA against GFP as a control (45). The viruses were intravenously administered into the tails of mice by bolus injection (200 μl in phosphate-buffered saline) of ~10^11 particles/mouse of Ad.shNOX4, Ad.shG6PD, or Ad.shGFP.

**Western Blotting**—For Western blotting experiments of isolated pure nuclei, the nuclei were treated with DNase in 0.1% Triton X-100 or sonicated, or both, to decrease viscosity caused by high DNA concentrations. For Western blotting of whole cells in culture, the lysates were prepared in ice-cold radioimmune precipitation assay buffer (0.15 M NaCl, 50 mM Tris, pH 7.4, 1% deoxycholic acid, 1% Triton X-100, 0.1% SDS), supplemented with EDTA-free protease inhibitor mixture (Roche Applied Science). SDS-PAGE gels were transferred to nitrocellulose and Western blotted using standard protocols. All of the secondary antibodies were conjugated to infrared dyes, and images were performed using an Odyssey infrared detection system. Primary rabbit antibody against NOX4 was generated and characterized as reported previously (46) and was used at a 1:2000 dilution. Glucose-6-phosphate dehydrogenase antibody was from Bethyl Laboratories (catalog number A300-404A) and was used at a 1:1000 dilution. Antibodies against mtHSP70 (mitochondrial marker, ab53098) and GRP78Bip (lumenal endoplasmic reticulum marker, ab53068) were purchased from Abcam and were used at 1:300 dilution.

**Production of Chicken Anti-NOX4 Antibody**—To facilitate colocalization of NOX4 with G6PD in hepatic nuclei, it was necessary to generate an anti-NOX4 antibody in a species other than rabbit. We generated a rabbit anti-mouse NOX4 antibody using an epitope that spans amino acids 214–274 of mouse NOX4. This region of NOX4 has no homology to other mouse NOX proteins. This sequence was cloned into Invitrogen pET151 vector to produce an amino-terminal His-V5-tagged fusion protein. We purified the fusion protein using a nickel FPLC column (GE Healthcare) followed by a second tagging on a cobalt spin column (Thermo). This purified nickel FPLC column (GE Healthcare) was used (affinity-purified chicken anti-NOX4 at 1:10; rabbit anti-G6PD at 1:50) in blocking buffer and applied to slides for 1 h. This was followed by three 5-min washes in blocking buffer. Secondary antibodies (AlexaFluor 488-conjugated goat anti-chicken antibody and AlexaFluor 568-conjugated donkey anti-rabbit antibody) were diluted 1:200 in blocking buffer and applied to the specimens for 1 h in the dark. This was followed by three 5-min washes in PBS, one wash in 0.1% Triton X-100, and one rinse in H2O. DAPI-containing antifadent (Vectorshield H-1200; Vector Labs) was applied, and the slides were coverslipped immediately. The slides were imaged on a Leica spinning disk microscope.

Fluorescent detection of O_2^- production by isolated nuclei was performed using dihydroethidium (DHE), which reacts specifically with O_2^- to form 2-hydroxyethidium (excitation/emission = 530/579 nm) and nonspecifically with numerous reactive oxygen species to form ethidium (excitation/emission = 510/580 nm) (47–49). Intact, unfixed nuclei suspended in PBS containing DHE and NADPH were quickly pipetted directly onto slides and were imaged with a Bio-Rad Radiance 2100MP confocal multiphoton microscope 10 min after initiating the reaction for detection of 2-hydroxyethidium or with a Texas Red filter in a Leica spinning disk microscope for detection of ethidium.

**Transmission Electron Microscopy (TEM)**—Isolated nuclei were fixed in a solution of 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer. The samples were post-fixed with 1% osmium tetroxide for 30 min and washed. They were dehydrated through a graded series of ethanol concentrations, embedded in resin, and sectioned. The sections were evaluated using a Jeol JEM-1230 transmission electron microscope. Immunogold labeling for NOX4 was performed on nuclei cytoplasm onto glass slides and preincubated with rabbit anti-NOX4 antibody, FITC-conjugated secondary antibody, and 10 nm-gold labeled anti-FITC tertiary antibody. This protocol allowed for immunofluorescence microscopy to be performed prior to TEM sample preparation. The nuclei were then directly embedded into Epon on the glass slide and sectioned for TEM.

**RESULTS**

**Production of Superoxide by Isolated Hepatic Nuclei**—Using a sucrose gradient ultracentrifugation protocol, we isolated highly pure mouse liver nuclei that retained both membrane layers of the nuclear envelope (Fig. 1A). These preparations
were devoid of mitochondrial contamination, as evidenced both by electron microscopy (Fig. 1A) and by Western blotting for the mitochondrial-specific marker mtHSP70 (Fig. 1B). Similar studies were performed using the lumenal endoplasmic reticulum (ER) marker GRP78Bip. As anticipated, because the ER lumen is contiguous with the lumen of the nuclear envelope, GRP78Bip was observed in isolated nuclei (Fig. 1B). Nuclei treated with NADPH (0.1 mM) produced significant ROS, as determined by EPR using DMPO spin trapping (Fig. 1C). The nuclei were incubated with DMPO with no electron donor, with NADPH (0.1 mM), with NADH (0.1 mM), or with both NADPH (0.1 mM) and SOD (3000 units/ml) for 5 min prior to EPR analysis. The error bars represent the mean [DMPO-OH] ± S.E. (n = 3). The asterisk indicates p < 0.05 compared with all other bars, as determined by one-way ANOVA and Bonferroni’s multiple comparison test. E, membrane-permeable scavengers of O$_2^\cdot$ halt DMPO-OH production in hepatic nuclei. Top spectrum, nuclei were incubated with NADPH, and EPR analysis was performed. Middle spectrum, tiron (10 mM) was added to nuclei prior to adding NADPH. Bottom spectrum, MnTBAP (0.15 mM) was added to nuclei prior to adding NADPH.

FIGURE 1. Nuclei produce O$_2^\cdot$ in an NADPH-dependent manner. A, top panel, transmission electron micrographs of isolated nuclei confirming their integrity. Bottom panel, higher magnification of the intact nuclear envelope membranes. B, Western blots (WB) for the mitochondrial marker mtHSP70 (top panel) or ER marker GRP78Bip (bottom panel). In both panels, lane 1 contains molecular mass standards, lane 2 contains whole liver lysate (10 μg of protein), and lane 3 contains pure isolated nuclei (10 μg of protein). C, hepatic nuclei use NADPH as a substrate to produce ROS. Isolated nuclei were incubated with DMPO (50 mM) with or without NADPH (0.1 mM) at 22 °C for 5 min, and EPR spectra were immediately obtained. Upper spectrum, nuclei without NADPH treatment. Lower spectrum, nuclei treated with NADPH. Control samples without nuclei (not shown) demonstrated no ROS. Asterisks indicate the spectral peak used for quantitation in all of the following figures. D, hepatic nuclei require NADPH, not NADH, as a substrate to produce O$_2^\cdot$. The nuclei were incubated with DMPO with no electron donor, with NADPH (0.1 mM), with NADH (0.1 mM), or with both NADPH (0.1 mM) and SOD (3000 units/ml) for 5 min prior to EPR analysis. The error bars represent the mean [DMPO-OH] ± S.E. (n = 3). The asterisk indicates p < 0.05 compared with all other bars, as determined by one-way ANOVA and Bonferroni’s multiple comparison test. E, membrane-permeable scavengers of O$_2^\cdot$ halt DMPO-OH production in hepatic nuclei. Top spectrum, nuclei were incubated with NADPH, and EPR analysis was performed. Middle spectrum, tiron (10 mM) was added to nuclei prior to adding NADPH. Bottom spectrum, MnTBAP (0.15 mM) was added to nuclei prior to adding NADPH.
ical sample that likely contains peroxidases and other unknown enzymatic systems (data not shown).

Identification of the Enzymatic Source of Nuclear O₂⁻—Numerous possible enzymatic sources for nuclear O₂⁻ have been suggested over the past three decades, including cytochrome P450 enzymes (8–10), P450 reductases (52), flavin-containing monooxygenases (53), cyclooxygenases (54), mitochondria-like electron transport chains located in the nuclear envelope (55, 56), and, more recently, NOX enzymes (11, 15–17). We initially tested several of these possibilities using various enzyme inhibitors delivered in the diet of mice (supplemental Table S1). Nuclei from livers of wild-type mice fed diets including grapefruit juice, erythromycin, cimetidine, quinidine, itraconazole, or flucloxacillin (all cytochrome P450 inhibitors), or aspirin or indomethacin (both cyclooxygenase inhibitors) singly or in combination were compared with nuclei from livers of mice fed standard diets for their ability to produce NADPH-dependent O₂⁻ detectable by EPR. However, no differences in nuclear O₂⁻ production among these groups were seen (data not shown). Additional experiments were undertaken in which isolated nuclei were incubated with several of the above compounds or additionally with rotenone (an electron transport chain inhibitor), thioridazine (another P450 inhibitor), and diphenyleneiodonium (a widely used inhibitor of flavoenzymes including NOXs) either singly or in combination to inhibit potential O₂⁻-producing enzymes (supplemental Table S1). Only treatment of nuclei with diphenyleneiodonium inhibited their NADPH-dependent production of O₂⁻ (data not shown but summarized in supplemental Table S1).

Cumulatively, the above experiments suggested it was unlikely that cytochrome P450, cyclooxygenases, or electron transport chain enzymes were primarily responsible for hepatic nuclear NADPH-dependent O₂⁻ production. However, because other kinds of nuclei have been reported to contain NOX1, 2, or electron transport chain enzymes located in the nuclear envelope (55, 56), and, more recently, NOX enzymes (11, 15–17). We initially tested several of these possibilities using various enzyme inhibitors delivered in the diet of mice (supplemental Table S1). Nuclei from livers of wild-type mice fed diets including grapefruit juice, erythromycin, cimetidine, quinidine, itraconazole, or flucloxacillin (all cytochrome P450 inhibitors), or aspirin or indomethacin (both cyclooxygenase inhibitors) singly or in combination were compared with nuclei from livers of mice fed standard diets for their ability to produce NADPH-dependent O₂⁻ detectable by EPR. However, no differences in nuclear O₂⁻ production among these groups were seen (data not shown). Additional experiments were undertaken in which isolated nuclei were incubated with several of the above compounds or additionally with rotenone (an electron transport chain inhibitor), thioridazine (another P450 inhibitor), and diphenyleneiodonium (a widely used inhibitor of flavoenzymes including NOXs) either singly or in combination to inhibit potential O₂⁻-producing enzymes (supplemental Table S1). Only treatment of nuclei with diphenyleneiodonium inhibited their NADPH-dependent production of O₂⁻ (data not shown but summarized in supplemental Table S1).

Because many mutant and knock-out mouse lines exist for the NOX genes and/or NOX regulators, we tested this hypothesis using a genetic approach. Nuclei isolated from livers of NOX1/2 double KO mice, p47<sup>phox</sup> mutant mice, p22<sup>phox</sup> mutant mice, and Alb-Cre Rac1<sup>fox/fox</sup>/Rac2 KO mice demonstrated no significant changes in NADPH-dependent O₂⁻ as compared with nuclei from wild-type controls (Fig. 2A and supplemental Fig. S1). Additionally, hepatic nuclei from single knock-out mice, including NOX1 or NOX2 KO mice, Alb-Cre Rac1<sup>fox/fox</sup> mice, and Rac2 KO mice, did not differ from wild-type controls (not shown).

To investigate NOX4 as a candidate for hepatic nuclear O₂⁻ production, we used an shRNA-mediated genetic knockdown approach. We found that infection of the liver with a recombinant adenovirus expressing shRNA against NOX4 substantially reduced nuclear NOX4 protein levels and NADPH-dependent O₂⁻ production by day 2 following infection (Fig. 2, B and C). Importantly, no change in nuclear O₂⁻ levels was observed in mice infected with control (shGFP expressing) virus (Fig. 2C).

Nuclear Localization of NADPH-dependent Superoxide Production—The fact that SOD1 was unable to degrade all O₂⁻ produced by isolated nuclei (Fig. 1D), in contrast to the membrane-permeable O₂⁻ scavengers Tiron and MnTBAP (Fig. 1E), suggested that nuclear NOX4 produces O₂⁻ in a compartment inaccessible to diffusion of SOD1. Given that NOX4 is an integral membrane protein, the most obvious membrane-bound intranuclear compartment where O₂⁻ might be produced is the perinuclear space of the nuclear envelope (Fig. 3A). In this context, NOX4 could reside in the outer and/or inner membrane of the nuclear envelope. To investigate this possibility, we exam-
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We investigated the location of nuclear O$_2^-$ production using fluorescence microscopy of DHE-treated nuclei. In the presence of DHE, NADPH-treated nuclei fluoresced more brightly than nuclei not treated with NADPH (Fig. 3, D and E). We observed high level fluorescence of 2-hydroxyethidine (specific for O$_2^-$) at the periphery of the nuclei, a region consistent with the perinuclear space of the nuclear envelope (Fig. 3, D and E). Fluorescent detection of ethidium (nonspecific ROS detection) demonstrated bright staining both in the region of the nuclear envelope, and inside nuclei, as would be expected following intercalation into the DNA (data not shown). Thus, the superoxide-specific product of DHE appeared to form primarily within the perinuclear space. The production of O$_2^-$ in this membrane-bound compartment is in agreement with the inability of SOD1 protein to degrade all NADPH-dependent O$_2^-$ produced by isolated nuclei (Fig. 1D).

To gain insight into whether nuclear NOX4 requires intact nuclear membranes for its function, we investigated whether nuclear membrane disruption affected the ability of isolated nuclei to generate O$_2^-$ in an NADPH-dependent manner. Detergents dramatically reduced the level of O$_2^-$ produced (Fig. 3F). Thus, nuclear membrane organization is required for NOX4 function. In conjunction with our data on ROS production by both the inner and outer nuclear membranes (Fig. 3B), these findings strongly suggest that NOX4 is an integral membrane protein that produces ROS in the nuclear envelope.

Nuclear G6PD Can Serve as a Source of the NADPH Used in Nuclear NOX4-dependent Superoxide Production—The compartmentalized localization of NADPH-dependent nuclear O$_2^-$
to the interior of the nuclear envelope, together with NOX4 localization and function in the inner and outer nuclear membranes, suggested that the NADPH-binding domains of NOX4 exist on both the cytoplasmic and nucleoplasmic sides of the nuclear envelope. The localization of NOX4 to the inner nuclear membrane raises questions as to how its substrate, NADPH, is delivered to the NOX4 complex. Because several NADPH-producing enzymes exist in the cytosol and the mitochondria, diffusion of NADPH into the nucleus is one potential mechanism. However, such diffusion might be limited by cytosolic enzyme competition for this substrate. Alternatively, because G6PD-type activity has been demonstrated at low levels in cardiac myocyte nuclei (58), we hypothesized that this enzyme might be present in hepatic nuclei and thus might supply NADPH directly to nuclear NOX4 (Fig. 4A).

To test this hypothesis, we first sought to evaluate whether G6PD was present in isolated hepatic nuclei using Western blotting. Indeed, Western blotting demonstrated low levels of G6PD protein in the nucleus (Fig. 4B). By contrast, nuclear G6PD levels were significantly decreased in G6PD mutant mice (Fig. 4B) known to have an ~80% reduction in G6PD protein levels (39), confirming the specificity of immunoreactive protein. To confirm functionally that nuclear G6PD activity can produce sufficient NADPH to serve as a substrate for NOX4, we tested whether the addition of the substrates for G6PD (glucose 6-phosphate [G6P] and NADP$^+$) but not G6P or NADP$^+$ alone, increased nuclear superoxide production. These data are representative of three independent experiments.

G6PD-deficient (MT) mice liver nuclei (n = 15) or liver nuclei from WT control mice produced from siblings of the same colony (n = 12) were isolated. The nuclei were treated with NADP$^+$ + G6P (D) or with NADPH (E). Pure nuclei were incubated at 37 °C for 5 min with G6P (100 μM) and NADP$^+$ (100 μM) or at 22 °C for 5 min with NADPH (100 μM). EPR was immediately performed. The results depict the mean ± S.E. F, graph depicting the (G6P + NADP$^+$ /NADPH) ratio of [DMPO-OH] signal. The ratios were calculated as follows. The individual [DMPO-OH] data point produced in response to NADP$^+$ + G6P from each mouse (from D) was divided by the individual [DMPO-OH] data point produced in response to NADPH from the same mouse (from E). These individual ratios from all of the mice in the experiment were averaged in F. Statistical significance in D–F is indicated with an asterisk, where p < 0.05 was determined by one-tailed Student’s t test.

FIGURE 4. G6PD localized to hepatic nuclei serves as a substrate source for NOX4-mediated O$_2^-$ production. A, schematic of the chemical reaction of NADPH production by G6PD from NADP$^+$ and G6P. B, Western blotting (20 μg of protein/lane) of purified mouse liver nuclei with rabbit anti-G6PD antibody (lower panel) or with Lamin B1 antibody (upper panel) as loading control. The two left lanes are nuclei from two different WT mouse livers. The two right lanes are nuclei from two different homozygous G6PD-MT mouse livers. These data are representative of three independent experiments. C, EPR demonstrates that isolated nuclei produce O$_2^-$ when exposed to the combination of G6P and NADP$^+$ but not G6P or NADP$^+$ alone. Inset, WT nuclei were incubated for 20 min at 37 °C with 0.2 mM NADP$^+$ (top spectrum), 0.2 mM G6P (middle spectrum), or NADP$^+$ + G6P (bottom spectrum), in the presence of DMPO and then analyzed by EPR. The graph depicts the quantification of these experiments showing the mean [DMPO-OH] ± S.E., n = 3. D–F, G6PD deficiency decreases superoxide production from NADP$^+$ + G6P, but not from NADPH. G6PD homozygous-deficient (MT) mice liver nuclei (n = 15) or liver nuclei from WT control mice produced from siblings of the same colony (n = 12) were isolated. The nuclei were treated with NADP$^+$ + G6P (D) or with NADPH (E). Pure nuclei were incubated at 37 °C for 5 min with G6P (100 μM) and NADP$^+$ (100 μM) or at 22 °C for 5 min with NADPH (100 μM). EPR was immediately performed. The results depict the mean ± S.E. F, graph depicting the (G6P + NADP$^+$ /NADPH) ratio of [DMPO-OH] signal. The ratios were calculated as follows. The individual [DMPO-OH] data point produced in response to NADP$^+$ + G6P from each mouse (from D) was divided by the individual [DMPO-OH] data point produced in response to NADPH from the same mouse (from E). These individual ratios from all of the mice in the experiment were averaged in F. Statistical significance in D–F is indicated with an asterisk, where p < 0.05 was determined by one-tailed Student’s t test.
termates (Fig. 4D). However, both G6PD-MT and G6PD-WT nuclei produced an indistinguishable amount of O$_2^\cdot$ when exposed to NADPH (Fig. 4E). Comparison of the ratio of O$_2^\cdot$ production by NADP$/H_2O_2$ and G6P with that of NADPH demonstrated a statistically significant 2-fold reduction in G6PD-MT as compared with G6PD-WT mice. These findings strongly suggest that G6PD can serve as a substrate source for NOX4 and that G6PD deficiency does not alter the ability of NOX4 to produce O$_2^\cdot$ in response to NADPH.

To substantiate these observations found in G6PD-MT mice, we generated five recombinant adenoviral vectors encoding different candidate shRNAs against mouse G6PD. We screened these G6PD-shRNAs in TIB-73 cells (an immortalized mouse hepatocyte cell line) by infection with increasing concentration of virus followed by Western blotting for G6PD protein (Fig. 5, A–F). Of the five adenoviruses, two demonstrated a clear dose response in knockdown of G6PD protein (Ad.shG6PD4 and Ad.shG6PD5) that was not observed in control virus (Ad.shGFP) infections. These two G6PD-shRNAs were chosen for further in vivo experiments. B6SJLF1 WT mice were infected with Ad.shGFP (control virus) (G), Ad.shG6PD4 (H), or Ad.shG6PD5 (I). Every day for 3 days after infection, the livers were harvested, and the nuclei were isolated. EPR was performed, and the ratios were calculated as described for Fig. 4F. The error bars represent S.E., n = 3–4 mice/experimental point. The asterisks indicate statistical significance (p < 0.05) as determined by ANOVA and Bonferroni’s multiple comparison test.

**FIGURE 5.** shRNA knockdown of G6PD decreases nuclear ROS in response to NADP$^+$ + G6P. A–F, characterization and screening of recombinant adenoviruses expressing shRNA to G6PD. Five shRNA-G6PD adenoviral vectors were produced, each expressing a different shRNA sequence to mouse G6PD. TIB-73 cells (immortalized mouse hepatocytes) were infected at 75% confluence with the indicated multiplicities of infection (MOI, defined as number of virus particles/cell) of control adenovirus (Ad.shGFP) or the indicated Ad.shG6PD viruses. Three days after infection, the cellular lysates were analyzed by Western blotting (WB) for G6PD. Actin was used as loading control. Based on the Western blotting results, adenoviruses 4 and 5 (Ad.shG6PD4 and Ad.shG6PD5, E and F) were used in in vivo experiments. G–I, B6SJLF1 WT mice were infected with Ad.shGFP (control virus) (G), Ad.shG6PD4 (H), or Ad.shG6PD5 (I). Every day for 3 days after infection, the livers were harvested, and the nuclei were isolated. EPR was performed, and the ratios were calculated as described for Fig. 4F. The error bars represent S.E., n = 3–4 mice/experimental point. The asterisks indicate statistical significance (p < 0.05) as determined by ANOVA and Bonferroni’s multiple comparison test.

**FIGURE 6.** shRNA-G6PD-mediated knockdown in G6PD-deficient mice further reduces nuclear superoxide production in response to NADP$^+$ + G6P. G6PD homozygous-deficient (MT) mice were infected with Ad.shG6PD4 (black bars) or with Ad.shGFP (white bars). The liver nuclei were isolated 1, 2, or 3 days after infection. The nuclei were treated with NADP$^+$ + G6P or with NADPH. EPR was performed, and the ratios of [DMPO-OH] signal with each substrate are plotted as described for Fig. 4F. The results depict the mean ± S.E. for n = 3–5 mice for each experimental point. Statistical significance was tested using ANOVA and Bonferroni’s multiple comparison test. The symbols indicating statistical significance (p < 0.05) are as follows: ‡, day 1 Ad.shG6PD4 versus day 1 Ad.shGFP. #, day 2 Ad.shG6PD4 versus day 2 Ad.shGFP. *, day 3 Ad.shG6PD4 versus day 3 Ad.shGFP. next sought to combine these two approaches (i.e. Ad.shG6PD4 infection of G6PD-MT mice) to further enhance the knockdown of G6PD. As shown in Fig. 6, Ad.shG6PD4 infection of G6PD-MT mice led to a further 2-fold significant reduction in the NADP$^+$ /G6P-responsive O$_2^\cdot$ production as compared with Ad.shGFP-infected controls.
Regulation of Nuclear Superoxide Production in Mouse Liver

NOX4 and G6PD Localize to Partially Overlapping Microdomains within the Nucleus—The ability of nuclear G6PD to generate the NADPH substrate for NOX4 suggested that G6PD and NOX4 might occupy similar microdomains in the nucleus. To approach this question we sought to colocalize G6PD and NOX4 within isolated nuclei by immunofluorescence. To this end, it was necessary generate a new chicken anti-NOX4 antibody compatible for colocalization with an available rabbit anti-G6PD antibody. Immunofluorescent localization of G6PD and NOX4 demonstrated a punctate pattern of staining in isolated hepatic nuclei (Fig. 7A). No signal was observed with secondary antibodies alone (Fig. 7A, right panel). Using Metamorph image analysis software, we quantified the extent of colocalization between NOX4 and G6PD signal and found 20 ± 3% of NOX4 signal colocalized with G6PD signal. Conversely, 11 ± 2% of the G6PD signal colocalized with NOX4 signal. These findings suggest that a fraction of nuclear NOX4 colocalizes with G6PD.

Because this was the first report of NOX4 localization using this newly generated chicken antibody, we performed several controls to demonstrate the specificity of immunoreactivity. First, using a characterized rabbit anti-NOX4 antibody, a similar punctate pattern of staining was observed in nuclei (supplemental Fig. S2). Second, we performed similar colocalization experiments on nuclei harvested from Ad.shNOX4- and Ad.shGFP-infected livers of WT mice. As expected, shNOX4-expressing nuclei demonstrated a statistically significant 5-fold lower level of NOX4 staining than control nuclei (Fig. 7B and C). Staining using rabbit anti-NOX4 antibody also showed a similar decrease in NOX4 expression level in nuclei from Ad.shNOX4-infected mice compared with Ad.shGFP-infected mice (supplemental Fig. S2). Interestingly, G6PD levels showed a downward trend in nuclei that were knocked down for NOX4, but this did not reach statistical significance (Fig. 7C). It should also be noted that it was necessary to use Triton X-100 permeabilization of cytoplasm nuclei to detect Nox4 immunostaining with both anti-Nox4 antibodies; this required technique may have prevented clear localization to the nuclear membranes as might be expected from immunogold NOX4 localization in ultrathin sections by TEM. Cumulatively, these findings demonstrate that a subset of G6PD indeed localizes to nuclear microdomains that contain NOX4 and hence suggest the potential for physiologic involvement in the regulation of the NOX4 complex.

DISCUSSION

The ability of hepatic nuclei to generate NADPH-dependent O$_2^\cdot$ has been known for over 30 years. Several groups have demonstrated the presence of NOX4 in the nucleus of intact cells, as well as in endoplasmic reticulum and other organelles (6, 11, 12, 14, 15, 59), leaving an unclear picture of whether NOX4 is indeed responsible for nuclear O$_2^\cdot$ production. A recent study has suggested that TGF-$\beta$ enhances hepatic nuclear O$_2^\cdot$ production in the context of hepatitis C viral infection, and this likely involves induction of NOX4 mRNA (15), giving some functional insight into pathophysiological involvement of nuclear ROS. However, despite these studies, little is known about the regulation and subcellular sites of NOX4-dependent nuclear O$_2^\cdot$ production by the liver. In the present study, we have clarified several unanswered questions about the regulation of NOX4-dependent nuclear O$_2^\cdot$ production in the normal liver.

Although previous studies have implicated NOX4 in nuclear ROS production based on its localization to the nucleus in intact cells and cellular changes in ROS production that occur when NOX4 is up-regulated (11, 12, 14, 15), definitive genetic proof that NOX4 regulates nuclear O$_2^\cdot$ has been lacking. In part,
this is due to the complex origins of ROS when working with intact cells. Our studies are the first to demonstrate that genetic knockdown of NOX4 clearly reduces the ability of hepatic nuclei to produce O$_2^-$ in response to NADPH (Fig. 2C). Our studies have also ruled out several other NOX family members (NOX1 and NOX2) as major sources of NADPH-dependent nuclear O$_2^-$ in the liver. Given the abundance of NOX isoforms within non-nuclear organelles, resolution of these questions using intact isolated nuclei was very important.

Another extremely important unanswered question about NOX4 function in the nucleus is the topologic location of the complex and its O$_2^-$ product. Most studies localizing NOX4 protein to the nucleus demonstrate diffuse staining throughout the nucleus (13, 15, 60). Because NOX4 is an integral membrane protein, this staining pattern is inconsistent with the known membrane-associated functions of all NOX family members. Indeed, our functional studies on isolated nuclei in the presence of ionic detergents suggest that intact nuclear membrane structure is imperative for function of nuclear O$_2^-$ production (Fig. 3F). Topologically, NOX4 appears to produce O$_2^-$ within the perinuclear space of the nuclear envelope (Fig. 3, D and E). The relatively tight staining pattern of positively charged 2-hydroxyethidine (Fig. 3E) to the perinuclear space is consistent with the known topology of other transmembrane NOX proteins, which produce O$_2^-$ in the intravesicular side of organelles and vesicles. The production of O$_2^-$ within the perinuclear space is also supported by the finding that only membrane-permeable O$_2^-$ scavengers such as MnTBAP, but not SOD1 protein, can remove all O$_2^-$ from intact nuclei (Fig. 1). Additionally, high resolution localization of NOX4 by TEM places this complex in the inner and outer nuclear membranes, a finding supported by biochemical fractionation of inner and outer nuclear membranes followed by NADPH-dependent O$_2^-$ measurements (Fig. 3B). Together, these findings for the first time place NOX4 in both the inner and outer nuclear membranes and its NADPH-dependent O$_2^-$ product in the perinuclear space.

It is important to recognize that the perinuclear space is contiguous with the ER lumen, because the outer nuclear membrane joins the ER membranes. Because NOX4 activity has previously been observed in the ER (1, 6), functional NOX4 in the outer nuclear membrane is perhaps not surprising. Furthermore, the demonstration that NOX4 also exists in the inner nuclear membrane suggests the potential for specific nuclear functions controlled by NOX4-dependent ROS production in the perinuclear space of the nuclear envelope. For example, ROS produced inside the nuclear envelope may be directionally transported across the inner nuclear membrane, thus impacting nuclear functions such as specific redox-regulated transcriptional activities. In this regard, it is interesting that SOD1 protein could quench only ~50% of nuclear O$_2^-$ despite the fact that we could observe no diffusion into the nucleus of SOD1 or similarly sized FITC-dextran molecules (data not shown). Anion channels have been demonstrated to pass O$_2^-$ through plasma membranes (61), mitochondrial membranes (62), and endosomal membranes (63), and such channels may also control O$_2^-$ flux across hepatocyte nuclear membranes as suggested by the ability of SOD1 to partially quench nuclear O$_2^-$ Additionally, NOX4 has been proposed to generate membrane-permeable hydrogen peroxide directly in some systems, and this feature could also locally influence nuclear functions.

The finding that G6P and NADP$^+$ can together activate nuclear O$_2^-$ production by NOX4 is very intriguing (Fig. 4C). Our studies in G6PD mutant mice and/or shRNA knockdown of G6PD clearly demonstrate that G6PD can regulate hepatic nuclear O$_2^-$ production (Figs. 5 and 6). These studies implicate G6PD in substrate regulation of NOX4 function, a concept supported by overlap in nuclear G6PD and NOX4 localization to microdomains in the nucleus. It is currently unclear why only 20% of NOX4 colocalizes with G6PD in the nucleus, but it is reasonable to hypothesize, based on known mechanisms of NOX complex activation by coactivators, that recruitment of these two proteins to similar microdomains could control NOX4 activation. G6PD has traditionally been thought to be a cytosolic protein, with the exception of a report describing nuclear G6PD activity in myocytes (58). Although the fraction of cellular G6PD in the nucleus is quite small, our studies suggest new potentially important functions for nuclear G6PD that should be re-examined in other systems.

NOX4 has traditionally been thought to be a constitutively active complex that is primarily regulated by transcriptional changes in the NOX4 gene (24, 27). The concept that NOX4 may be regulated by substrate availability through G6PD adds a new level of acute regulation that is more similar to that of NOX1 and NOX2. Additionally, substrate availability of G6P and NADP$^+$ may also influence regulation of NOX4 in the nucleus. NADP$^+$ is not membrane-permeable (64). G6P must be transported across membranes by a transporter enzyme (65). Thus, the acute regulation of nuclear ROS for signaling may be extremely complex, whereas its long term regulation may be mostly dependent on the level of NOX4 gene transcription.

In summary, our findings have suggested that hepatic nuclei have unique pathways for generating nuclear ROS. In the baseline state, NOX4 is the major source of nuclear ROS. Additionally, G6PD has the capacity to activate NOX4 by serving as a substrate source within the nucleus. Because the expression pattern of these two proteins shows overlap in the nucleus, their regulation may be coordinated by a macromolecular complex at the inner nuclear membrane. These findings have begun to clarify metabolic pathways that may rapidly regulate nuclear ROS-mediated signaling in hepatocytes.

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