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# p66Shc Links $\alpha_1$-Adrenergic Receptors to a Reactive Oxygen Species–Dependent AKT-FOXO3A Phosphorylation Pathway in Cardiomyocytes

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## Abstract

p66Shc is an adapter protein that is induced by hypertrophic stimuli and has been implicated as a major regulator of reactive oxygen species (ROS) production and cardiovascular oxidative stress responses. This study implicates p66Shc in an $\alpha_1$-adrenergtic receptor ($\alpha_1$-AR) pathway that requires the cooperative effects of protein kinase (PK)C$\varepsilon$ and PKC$\delta$ and leads to AKT-FOXO3a phosphorylation in cardiomyocytes. $\alpha_1$-ARs promote p66Shc-YY$^{239/240}$ phosphorylation via a ROS-dependent mechanism that is localized to caveolae and requires epidermal growth factor receptor (EGFR) and PKC$\varepsilon$ activity. $\alpha_1$-ARs also increase p66Shc-S$^{36}$ phosphorylation via an EGFR transactivation pathway involving PKC$\delta$. p66Shc links $\alpha_1$-ARs to an AKT signaling pathway that selectively phosphorylates/inactivates FOXO transcription factors and downregulates the ROS-scavenging protein manganese superoxide dismutase (MnSOD); the $\alpha_1$-AR-p66Shc-dependent pathway involving AKT does not regulate GSK3. Additional studies show that RNA interference–mediated downregulation of endogenous p66Shc leads to the derepression of FOXO3a-regulated genes such as MnSOD, p27Kip1, and BIM-1. p66Shc downregulation also increases proliferating cell nuclear antigen expression and induces cardiomyocyte hypertrophy, suggesting that p66Shc exerts an antihypertrophic action in neonatal cardiomyocytes. The novel $\alpha_1$-AR– and ROS-dependent pathway involving p66Shc identified in this study is likely to contribute to cardiomyocyte remodeling and the evolution of heart failure.

### Keywords

p66Shc; $\alpha_1$-adrenergic receptors; protein kinase C; ROS; AKT

Cardiomyocyte hypertrophy in response to mechanical forces or growth factors that activate intracellular signaling pathways. Hypertrophy has been classified as physiological/adaptive or pathological/maladaptive based on whether it progresses to frank cardiac failure. Physiological hypertrophy develops during normal postnatal development, exercise training, or pregnancy. This form of hypertrophy typically is not associated with functional derangements and has been attributed to a phosphatidylinositol 3-kinase/AKT pathway that phosphorylates targets such as GSK3 and FOXO transcription factors.[1] In contrast, hypertension or valvular heart disease induce pathological forms of hypertrophy that progress to frank heart failure in part by



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activating heptahelical receptors, protein kinase (PK)C, and pathways that render the heart susceptible to apoptosis.[1]

This relatively broad classification of hypertrophy as physiological or pathological has been useful from an experimental standpoint. However, it fails to adequately describe most clinically reactive hypertrophies in humans which contain features of both hypertrophic phenotypes. The notion that all heptahelical receptors act in a stereotypical manner to trigger a similar hypertrophic response also is an oversimplification, because individual heptahelical receptors (such as the $\alpha_1$-adrenergic receptor [$\alpha_1$-AR] or protease-activated receptor-1) induce morphologically distinct forms of cardiac hypertrophy.[2] Models that consider AKT as exclusively a mediator of adaptive/physiological hypertrophy also are inadequate, because AKT is activated by G$\alpha$q-coupled receptors that induce pathological hypertrophy and it recruits effectors that contribute to pathological remodeling under some experimental conditions.[3] The effector responses activated by the G$\alpha$q-coupled receptor–dependent AKT signaling pathway remain uncertain.

p66Shc has recently emerged as a master regulator of reactive oxygen species (ROS) production and cardiovascular oxidative stress responses. p66Shc shares similar domain structure with p52Shc and p46Shc, adapter proteins that link receptor tyrosine kinases to growth regulatory pathways. All 3 Shc isoforms contain phospho-protein binding SH2 and PTB domains flanking a central CH1 domain that is the site for $YY^{239/240}$ and $Y^{317}$ phosphorylation (nomenclature based on human p52Shc). p66Shc contains an additional N-terminal extension that contains a phosphorylation site ($S^{36}$) critical for the unique cellular function of p66Shc to amplify basal and stimulus-dependent ROS generation in mitochondria. Recent studies also implicate p66Shc in a redox-dependent pathway that sensitizes cells to proapoptotic stimuli by activating AKT, phosphorylating/inactivating FOXO transcription factors, and preventing the induction of antioxidant/free radical scavenging genes (such as manganese superoxide dismutase [MnSOD]4). This is distinct from the AKT-FOXO inactivation pathway recruited by trophic factors that promote cell survival by preventing the induction of proapoptotic genes (such as Fas ligand and the Bcl-2 family member Bim-1). This study implicates p66Shc in a redox-sensitive $\alpha_1$-AR pathway that couples to AKT-FOXO3a phosphorylation in cardiomyocytes.

## Materials and Methods

Methods to prepare cardiomyocyte cultures from 2-day-old Wistar rats[5,6] and methods to infect cultures with adenoviral constructs that drive expression of WT-PKC$\delta$, KD-PKC$\delta$, WT-PKC$\varepsilon$, or $\beta$-galactosidase ($\beta$-gal)7 have been published. Infections with an Ad-p66ShcRNAi (that drives expression of a 19-mer sequence corresponding to bases 45 to 63 in the p66Shc unique N-terminal CH2 domain8) was performed in a similar manner. The detergent-free caveolae membrane isolation protocol and methods for immunoprecipitation and immunoblot analysis are published.5,6 Intracellular ROS was measured by fluorescence microscopy on cardiomyocytes loaded with dihydroethidium. A detailed description of all methods is provided in the expanded Materials and Methods section in an online data supplement, available at http://circres. ahajournals.org.

## Results

### $\alpha_1$-ARs Promote p66Shc-$YY^{239/240}$ Phosphorylation in Cardiomyocytes

Shc phosphorylation mechanisms were examined in neonatal cardiomyocytes that coexpress p42Shc, p52Shc, and p66Shc.[9] Figure 1A shows that p52Shc is constitutively $YY^{239/240}$-phosphorylated in resting cardiomyocytes, whereas p66Shc- and p46Shc-$YY^{239/240}$ phosphorylation is at the limits of detection under basal conditions. $\alpha_1$-AR activation with

norepinephrine (NE) induces a prominent increase in p66Shc-YY$^{239/240}$ phosphorylation at 5 minutes that is sustained for another 10 to 15 minutes and wanes with continuous stimulation for 60 minutes. Although NE also increases p46Shc/p52Shc-YY$^{239/240}$ phosphorylation with similar kinetics, the magnitude of the NE-dependent increase in p52Shc-YY$^{239/240}$ phosphorylation is modest (and evident only at lighter gel exposures).

NE promotes AKT phosphorylation at T$^{308}$ in the activation loop and S$^{473}$ in the C terminus. NE-dependent AKT and p66Shc phosphorylation display identical time courses. NE also activates extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK), but ERK activation is more sustained (for at least 60 minutes), and JNK activation is more delayed (Figure 1A). NE-dependent Shc tyrosine phosphorylation is via an $\alpha_1$-AR–dependent mechanism that is blocked by prazosin ($\alpha_1$-AR antagonist) and not propranolol ($\beta$-AR antagonist) (Figure 1B). $\beta$-AR activation with isoproterenol does not increase Shc-YY$^{239/240}$ phosphorylation.

Endothelin-1 and prostaglandin F2$\alpha$ mimic the effect of NE to increase Shc (predominantly p66Shc) YY$^{239/240}$ phosphorylation (Figure 1C). p66Shc-YY$^{239/240}$ phosphorylation is increased to an even greater extent by $H_2O_2$. Agonists for other heptahelical receptors such as angiotensin II and thrombin activate ERK without increasing Shc-YY$^{239/240}$ phosphorylation.

## NE-Dependent p66Shc-YY$^{239/240}$ Phosphorylation Is via an Epidermal Growth Factor Receptor–Dependent Pathway

Because Shc proteins play well-recognized roles as adapters for epidermal growth factor receptor (EGFRs), and $\alpha_1$-ARs can transactivate EGFRs, we examined whether EGFRs link $\alpha_1$-ARs to p66Shc-YY$^{239/240}$ phosphorylation. Initial studies examined effects of ligands that directly activate EGFRs, namely EGF (ligand for EGFR/ErbB1) and heregulin (ligand for ErbB3 and ErbB4, receptors that heterodimerize with ErbB2). Figure I in the online data supplement shows that EGF increases YY$^{239/240}$ phosphorylation on all 3 Shc isoforms; EGF also slows p66Shc mobility in SDS-PAGE. Effects of EGF are maximal at 5 minutes and wane by 1 hour; EGF activates ERK and AKT with similar kinetics. In contrast, heregulin increases p46Shc/p52Shc phosphorylation (and induces a robust/sustained increase in ERK and AKT phosphorylation), without increasing p66Shc-YY$^{239/240}$ phosphorylation. Basal and EGF-/heregulin-dependent responses are completely abrogated by AG1478 (EGFR kinase inhibitor); PP1 (inhibitor of SFKs and c-Abl) reduces basal, but not EGF-/heregulin-dependent, Shc-YY$^{239/240}$ phosphorylation. These results implicate p66Shc in the EGFR (but not ErbB2/ErbB4) signaling pathway.

AG1478 induces a pronounced decrease in NE-dependent p66Shc-YY$^{239/240}$ phosphorylation (Figure 2A) and AKT activation (Figure 2B) but only a modest decrease in NE-dependent ERK activation (22±6%, n=5, $P$<0.5). AG1478 does not block the JNK activation pathway. NE and EGF pathways leading to p66Shc-YY$^{239/240}$ phosphorylation are not blocked by PP1 (Figure 2A and supplemental Figure I). Collectively, these results indicate that cardiac $\alpha_1$-ARs increase p66Shc-YY$^{239/240}$ phosphorylation via an AG1478-sensitive mechanism that is presumed to involve the EGFR (which also promotes p66Shc-YY$^{239/240}$ phosphorylation) and not other ErbB family members (which do not). This EGFR transactivation-p66Shc-YY$^{239/240}$ phosphorylation pathway links $\alpha_1$-ARs to AKT, but it plays only a minor role in $\alpha_1$-AR–dependent ERK activation (and it is not required for $\alpha_1$-AR–dependent JNK activation).

## nPKC Isoform Activity Is Required for NE-Dependent p66Shc-YY$^{239/240}$ Phosphorylation

PKC isoforms comprise a family of enzymes that contribute to cardiac remodeling. We used PKC activators (phorbol 12-myristate 13-acetate [PMA]) and inhibitors (GF109203X, Gö6976) as an initial strategy to explore the role of PKCs in the NE-dependent p66Shc-

YY$^{239/240}$ phosphorylation pathway. Figure 3A shows that PMA mimics the effect of NE to increase p66Shc-YY$^{239/240}$ phosphorylation. PMA also decreases p66Shc mobility in SDS-PAGE (Figure 3A). NE- and PMA-dependent p66Shc-YY$^{239/240}$ phosphorylation (and agonist-dependent p66Shc band shifts) are abrogated by GF109203X (a relatively nonspecific inhibitor of PKC isoforms; Figure 3A and 3B). In contrast, the effect of 5 mmol/L $H_2O_2$ to promote Shc-YY$^{239/240}$ phosphorylation (on all 3 isoforms) is preserved in GF109203X-treated cultures, excluding a nonspecific inhibitory effect of GF109203X (Figure 3B). Gö6976 is a selective inhibitor of conventional PKC isoforms and PKD; Gö6976 attenuates PMA-dependent Shc-YY$^{239/240}$ phosphorylation without blocking NE-dependent Shc-YY$^{239/240}$ phosphorylation (Figure 3A). NE-dependent p66Shc-YY$^{239/240}$ phosphorylation is abrogated by a 24-hour pretreatment with PMA (which downregulates phorbol ester-sensitive PKC isoforms); EGFR-dependent Shc-pYY$^{239/240}$ phosphorylation persists under these conditions (Figure 3C). Collectively, these results implicate a novel (n)PKC (presumably PKC$\delta$ or PKC$\varepsilon$) in the $\alpha_1$-AR–dependent p66Shc-YY$^{239/240}$ phosphorylation pathway.

## NE Promotes p66Shc-YY$^{239/240}$ Phosphorylation via a PKC$\varepsilon$-Dependent Mechanism in Caveolae

The observation that NE-dependent p66Shc-YY$^{239/240}$ phosphorylation requires EGFR and PKC activities provided the rationale to consider a role for caveolae; we previously implicated caveolae as signaling microdomains for PKC in cardiomyocytes and EGFRs localize to caveolae in other cell types.[6] Caveolae were isolated using a biochemical fractionation scheme that separates buoyant cholesterol-/glycosphingolipid-enriched caveolae membranes from the bulk of the cellular material (cytosolic proteins, intracellular membranes, and noncaveolae surface membranes) that pellet to heavier gradient fractions (F8-13). Figure 4A shows that all 3 Shc isoforms partition predominantly to F8-13 gradient fractions in resting cardiomyocytes. Nevertheless, a pool of p46Shc/p52Shc and trace amounts of p66Shc are constitutively recovered (with some p52Shc- and p66Shc-YY$^{239/240}$ phosphorylation) in caveolae isolated from resting cardiomyocytes. NE and PMA increase p66Shc-YY$^{239/240}$ phosphorylation exclusively in caveolae (without altering p66Shc recovery in this fraction). Caveolae p66Shc-pYY$^{239/240}$ immunoreactivity also increases in cardiomyocytes treated with diC8 (1,2-dioctanoyl-*sn*-glycerol) (a membrane-permeant analog of the endogenous PKC-activating lipid cofactor DAG; supplemental Figure II). Additional studies show that caveolae contain the bulk of the cellular EGFR protein immunoreactivity (supplemental Figure II) and that NE-/PMA-dependent p66Shc-YY$^{239/240}$ phosphorylation is blocked by GF109203X (Figure 4A). These results are consistent with the notion that NE promotes p66Shc-YY$^{239/240}$ phosphorylation via a mechanism that requires EGFR and PKC activities.

We used adenoviruses that drive expression of wild-type (WT)-PKC$\varepsilon$ or WT-PKC$\delta$, to identify the nPKC isoform that regulates p66Shc-YY$^{239/240}$ phosphorylation. Figure 4B shows that caveolae isolated from resting $\beta$-gal–infected cardiomyocytes contain a low level of PKC$\delta$ but no PKC$\varepsilon$ immunoreactivity; PMA drives PKC$\varepsilon$ and PKC$\delta$ to the caveolae fraction. The WT-PKC$\varepsilon$ transgene is detected at low levels in resting caveolae and in considerably higher amounts in caveolae from PMA-treated cardiomyocytes. WT-PKC$\varepsilon$ overexpression does not alter PKC$\delta$ or p66Shc protein partitioning between caveolae and noncaveolae fractions. Rather, Ad-WT-PKC$\varepsilon$ overexpression increases PMA- and NE-dependent p66Shc-YY$^{239/240}$ phosphorylation in the caveolae fraction (Figure 4B and 4C). Basal and agonist-dependent p66Shc-YY$^{239/240}$ phosphorylation are not influenced by WT-PKC$\delta$ overexpression (data not shown). These results implicate PKC$\varepsilon$ as the nPKC isoform that regulates p66Shc-YY$^{239/240}$ phosphorylation in caveolae.

### NE Promotes p66Shc-YY$^{239/240}$ Phosphorylation by Increasing ROS Generation

Recent studies in vascular models suggest that caveolae regulate growth responses at least in part by increasing ROS generation.[10] Because ROS are implicated in EGFR transactivation pathways, ROS activate certain PKC isoforms, and ROS-dependent oxidative modifications have been implicated in $\alpha_1$-AR signaling in adult rat cardiomyocytes,[11] we considered a role for ROS in the $\alpha_1$-AR-p66Shc-YY$^{239/240}$ phosphorylation pathway in neonatal cardiomyocytes. Intracellular ROS production was monitored with dihydroethidium (DHE), a nonfluorescent membrane-permeant probe that interacts with $O_2^-$, leading to the liberation of membrane-impermeant ethidium cations that fluoresce on intercalating with nuclear DNA. Figure 5A through 5C shows that NE increases nuclear DHE fluorescence; this effect is detected at 2 minutes, is sustained for at least 15 minutes, and is similar to the response to 50 $\mu$mol/L $H_2O_2$. NE- and $H_2O_2$-dependent increases in nuclear DHE fluorescence are prevented by Tiron, a membrane-permeable nonenzymatic superoxide scavenger (Figure 5C). Tiron also abrogates NE-dependent p66Shc-YY$^{239/240}$ and AKT phosphorylation; Tiron does not block NE-dependent ERK activation, indicating that Tiron is not a general nonspecific inhibitor of signaling by $\alpha_1$-ARs (Figure 5D). Tiron also attenuates Shc-YY$^{239/240}$ phosphorylation, but not ERK or AKT activation, in cardiomyocytes treated with a very high $H_2O_2$ concentration (5 mmol/L). These results implicate ROS in the $\alpha_1$-AR signaling pathway involving p66Shc-YY$^{239/240}$ phosphorylation that activates AKT, but not ERK, in cardiomyocytes.

### NE Promotes p66Shc-S$^{36}$ Phosphorylation via a PKC$\delta$-Dependent Mechanism

NE, PMA, and range of $H_2O_2$ concentrations (0.05 to 5 mmol/L) increase p66Shc-S$^{36}$ phosphorylation (Figure 6A). Endothelin-1 and EGF also induce robust increases in p66Shc-S$^{36}$ phosphorylation, whereas the response to heregulin (the ErbB3/4 agonist) is relatively modest in magnitude (Figure 6B and data not shown). Phosphatase inhibition with calyculin A also increases p66Shc-S$^{36}$ phosphorylation and leads to a massive p66Shc mobility shift, far in excess of the mobility shift elicited by other agonists (Figure 6C). This presumably is attributable to phosphorylation at multiple sites, not just S$^{36}$.

Pharmacological studies show that NE- and $H_2O_2$-dependent increases in p66Shc-S$^{36}$ phosphorylation are blocked by AG1478 and U0126 (Figure 6B); U0126 also attenuates p66Shc-S$^{36}$ phosphorylation by PMA (data not shown). These results implicate ERK as the proline-directed kinase that phosphorylates p66Shc at S$^{36}$ (a site with a proline in the +1 position). p66Shc-S$^{36}$ phosphorylation is not blocked by LY294002, a phosphoinositide 3-kinase inhibitor that prevents AKT activation. NE-dependent p66Shc-S$^{36}$ phosphorylation is inhibited by Tiron and GF109203X (Figure 6B); GF109203X also inhibits p66Shc-S$^{36}$ phosphorylation by PMA (data not shown). These results are consistent with recent studies that attribute p66Shc-S$^{36}$ phosphorylation to PKC; although previous studies focused on PKC$\beta$, an additional or alternate role for PKC$\delta$ was not excluded.[12] Figure 6C shows that basal- and agonist-dependent p66Shc-S$^{36}$ phosphorylation is increased by WT-PKC$\delta$, but not WT-PKC$\varepsilon$. Figure 6D shows that NE- and $H_2O_2$-dependent p66Shc-S$^{36}$ phosphorylation is blocked by KD-PKC$\delta$, whereas PMA-dependent p66Shc-S$^{36}$ phosphorylation persists in KD-PKC$\delta$ cultures. These results implicate PKC$\delta$ in $\alpha_1$-AR- and $H_2O_2$-dependent p66Shc-S$^{36}$ phosphorylation pathways.

### The NE-Dependent AKT Signaling Pathway Regulates FOXO3a, but Not GSK3, Phosphorylation

Figure 7A shows that NE activates AKT in a dose-dependent manner and that FOXO3a-T$^{32}$ phosphorylation (a modification that has been attributed to AKT) increases in parallel. As recently reported by Ni et al,[13] T$^{32}$-phosphorylated FOXO3a accumulates in the cytosol of NE-treated cardiomyocytes (data not shown). NE treatment also increases GSK3 phosphorylation (Figure 7B). However, pharmacological studies provided unanticipated

evidence that LY294002 abrogates NE-dependent AKT and FOXO3a-$T^{32}$ phosphorylation, without blocking GSK3 phosphorylation. Additional studies show that PMA also increases GSK3 phosphorylation, without activating AKT. NE- and PMA-dependent GSK3 phosphorylation are blocked by GF109203X. These results indicate that agonist-dependent GSK3 activation is via a PKC-dependent mechanism that does not require phosphatidylinositol 3-kinase or AKT activity. Collectively, these studies expose heretofore unrecognized signaling specificity, showing that the NE-dependent AKT signaling pathway selectively couples to the phosphorylation of FOXO3a, but not GSK3.

### p66Shc Links $\alpha_1$-ARs to the AKT-FOXO3a-$T^{32}$ Phosphorylation Pathway and Regulates Cardiomyocyte Growth

The final set of studies used gene silencing, with an adenovirus that selectively downregulates p66Shc (Ad-p66ShcRNAi) to explore the role of p66Shc in $\alpha_1$-AR signaling responses. Figure 8A and 8B show that Ad-p66ShcRNAi treatment at a multiplicity of infection as low as 10 leads to a profound downregulation of p66Shc, but not p52/p46Shc, expression that persists for at least 48 hours.

Figure 8C shows that NE and physiological low $H_2O_2$ concentrations (10 to 50 $\mu$mol/L) exert similar effects to increase p66Shc-$YY^{239/240}$, AKT-$T^{308}$/$S^{473}$, FOXO-$T^{32}$, and ERK phosphorylation and that the AKT-FOXO3a phosphorylation pathways activated by NE and low $H_2O_2$ concentrations are abrogated by p66Shc downregulation. In contrast, high $H_2O_2$ concentrations (0.1 to 1 mmol/L) also increase p66Shc-$YY^{239/240}$, AKT-$T^{308}$/$S^{473}$, and ERK phosphorylation, but this is via a different mechanism that does not lead to FOXO3a-$T^{32}$ phosphorylation; it is not inhibited by p66Shc downregulation. Agonist-dependent p52Shc-$YY^{239/240}$ and ERK phosphorylation pathways also persist in Ad-p66ShcRNAi cultures. Control experiments show that p66Shc knockdown does not lead to changes in the expression of signaling proteins that might indirectly influence $\alpha_1$-AR signaling responses such as PKC$\delta$, PKC$\varepsilon$, or caveolin-3 (supplemental Figure III). Collectively, these results indicate that p66Shc couples $\alpha_1$-ARs and physiologically low $H_2O_2$ concentrations to the AKT-FOXO3a phosphorylation pathway.

We examined whether the NE-dependent AKT-FOXO3a phosphorylation pathway influences FOXO3a target gene expression. Figure 8D shows that NE induces a modest decrease in the expression of MnSOD (an antioxidant scavenger that limits oxidative stress). Other FOXO3a targets such as the cyclin-dependent kinase (CDK) inhibitor p27Kip1 that regulates cell cycle and the proapoptotic Bcl-2 family member Bim-$1^{14}$ are expressed at very low levels and are not regulated by NE (Figure 8E and 8F). However, p66Shc downregulation prevents the NE-dependent decrease in MnSOD expression and leads to a general derepression of FOXO3a targets such as MnSOD, p27Kip1, and BIM-1; Bcl-2 expression is not affected by p66Shc downregulation (Figure 8D through 8F).

The functional consequences of the Ad-p66ShcRNAi treatment are difficult to predict; a decrease in the direct proapoptotic actions of p66Shc in mitochondria and derepression of cytoprotective FOXO3a-regulated genes such as MnSOD might be cytoprotective, but this could be offset by a decrease in signaling via the AKT pathway and an increase in the proapoptotic actions of BIM-1. Therefore, we examined whether p66Shc modulates the proapoptotic effects of doxorubicin, a chemotherapeutic agent that increases oxidant production and induces cardiomyocyte apoptosis. Figure 8G shows that doxorubicin treatment leads to prominent cleavage of caspase-3 in both vector and Ad-p66ShcRNAi cultures; in each case, this is associated with gross morphological evidence of cell death (data not shown). The lack of protection against doxorubicin-dependent apoptosis in Ad-p66ShcRNAi cultures cannot be attributed to a defect in AKT activation (which typically mitigates the doxorubicin-

dependent proapoptotic response); doxorubicin-dependent AKT activation is not decreased by p66Shc downregulation (supplemental Figure IV).

An effect of p66Shc downregulation to derepress FOXO targets such as p27Kip1 and "atrogenes" (proteins that inhibit hypertrophy) is predicted to limit cardiomyocyte growth.[15, 16] However, Figure 8H provides surprising evidence that p66Shc downregulation markedly increases basal cell size and protein content. Of note, NE increases cell size and protein content in Ad-p66ShcRNAi cultures (to a level that is comparable to the cell size and protein content in Ad-$\beta$-gal cultures). The observation that NE-dependent hypertrophy is preserved in Ad-p66ShcRNAi cultures that exhibit a defect in NE-dependent AKT activation indicates that AKT is not required for $\alpha_1$-AR-dependent cardiac hypertrophy. The mechanism(s) underlying the growth promoting effects of p66Shc downregulation is uncertain. Figure 8I shows that p66Shc downregulation increases proliferating cell nuclear antigen (PCNA) expression. Although this might encourage entry into the cell cycle, an effect of Ad-p66ShcRNAi treatment to induce gross changes in DNA content or cell number was not detected (data not shown).

## Discussion

p66Shc has recently emerged as a master regulator of intracellular ROS production and cardiovascular oxidative stress responses. Recent studies identify a p66Shc electron transfer reaction with cytochrome *c* that enhances basal and stimulus-dependent ROS accumulation and promotes cell death.[12,17–19] Studies reported herein indicate that p66Shc exerts an additional role as a redox-sensitive target of the $\alpha_1$-AR. The results of this study (schematized in supplemental Figure V) show that $\alpha_1$-ARs increase ROS accumulation and promote p66Shc-$YY^{239/240}$ and $S^{36}$ phosphorylation via a ROS-dependent mechanism that requires the cooperative effects of PKC$\varepsilon$ and PKC$\delta$. p66Shc-$YY^{239/240}$ phosphorylation is localized to caveolae and increased by PKC$\varepsilon$ overexpression. The mechanism linking PKC$\varepsilon$ to increased p66Shc-$YY^{239/240}$ phosphorylation is uncertain but could involve the inhibition of a tyrosine phosphatase. $\alpha_1$-ARs also activate a PKC$\delta$-dependent pathway that promotes p66Shc-$S^{36}$ phosphorylation. However, a direct role for PKC$\delta$ as$S^{36}$ kinase is unlikely, because $S^{36}$ lies in mitogen-activated protein kinase (not a PKC) consensus phosphorylation motif and agonist-dependent p66Shc-$S^{36}$ phosphorylation is blocked by a MEK inhibitor. A previous study implicated PKC$\beta$ in an $H_2O_2$-dependent pathway leading to p66Shc-$S^{36}$ phosphorylation. However, it is important to note that there is compelling evidence that PKC$\delta$ mimics PKC$\beta$s effects on mitochondrial calcium/apoptosis responses[12]; previous efforts to determine whether PKC$\delta$ controls p66Shc function were inconclusive, because the studies relied on rottlerin (an ineffective inhibitor of PKC$\delta$20). Our results implicate PKC$\delta$ in the agonist-dependent p66Shc-$S^{36}$ phosphorylation pathway. It is interesting to speculate that $S^{36}$ phosphorylation is confined to the pool of $YY^{239/240}$-phosphorylated p66Shc in caveolae. The role of caveolae in the PKC$\delta$-dependent p66Shc-$S^{36}$ phosphorylation pathway is the focus of ongoing studies. The observation that PKC$\varepsilon$ and PKC$\delta$ cooperate to regulate p66Shc phosphorylation deserves emphasis. Most studies have assigned opposing roles to PKC$\varepsilon$ and PKC$\delta$ in ischemia/reperfusion, showing that PKC$\delta$ promotes oxidative stress, apoptosis, and inflammation (hallmarks of reperfusion injury) and that PKC$\varepsilon$ mimics preconditioning. A simple model that links PKC$\varepsilon$ exclusively to cardioprotection and PKC$\delta$ to reperfusion injury does not accommodate these cooperative actions of PKC$\varepsilon$ and PKC$\delta$ in the control of p66Shc.

Recent studies implicate p66Shc in a ROS-dependent mechanism that inhibits FOXO activity. This study identifies a similar role for p66Shc in a ROS-sensitive $\alpha_1$-AR pathway that increases AKT phosphorylation, inactivates FOXO3a, and decreases MnSOD expression. These results suggest that p66Shc can exert proapoptotic actions both by increasing ROS production (when complexed with cytochrome *c* in mitochondria) and by decreasing ROS detoxification (by activating the AKT-FOXO3a pathway that regulates MnSOD expression). The $\alpha_1$-AR subtype

that activates this ROS-p66Shc-AKT-FOXO3a pathway and the role of this $\alpha_1$-AR pathway in adult cardiomyocytes requires further study. In particular, it is interesting to note that $\alpha_1$-AR activation of the AKT-FOXO3a pathway has not been detected in adult cardiomyocytes that lack p66Shc expression.[9] Because p66Shc expression is induced by hypertrophic stimuli and oxidative stresses,[9] it will be interesting to examine whether $\alpha_1$-ARs recruit the AKT-FOXO3a phosphorylation pathway and repress antioxidant gene expression exclusively in diseased adult cardiomyocytes that express the p66Shc protein.

This study identifies a novel $\alpha_1$-AR-ROS-p66Shc-AKT signaling pathway that selectively couples to the phosphorylation/inactivation of FOXO3a; this mode of AKT activation does not lead to GSK3 phosphorylation. A mechanism that selectively targets AKT to FOXO3a, but not GSK3, phosphorylation has not been reported. Although previous studies in nonmyocytes suggest that AKT signaling specificity can be regulated through phosphorylation at $S^{473}$ in the C terminus (which is required for phosphorylation of FOXO3a, but not GSK321), this mechanism would not account for the signaling specificity identified in our study; AKT is dually phosphorylated at $T^{308}$ and $S^{473}$ in NE-treated cardiomyocytes. Other differences in AKT phosphorylation patterns, AKT interactions with binding partners, and/or AKT localization might impart specificity and should be considered.

This study identifies an $\alpha_1$-AR-ROS-p66Shc-AKT-FOXO3a phosphorylation pathway that decreases MnSOD expression and is predicted to impair ROS detoxification and enhance apoptosis; the deleterious consequences of AKT activation via this $\alpha_1$-AR-dependent pathway would be quite distinct from the cardioprotective actions of AKT when activated by various other growth factors. These stimulus-specific differences in AKT actions may be pertinent to the lingering controversies that cloud the interpretation of overexpression studies, where AKT has been implicated in cardiomyocyte growth, cardiomyocyte survival (without evidence of hypertrophy), or cardiac dysfunction, depending on the level, location, or chronicity of AKT transgene expression. Our studies emphasize that AKT can elicit functionally divergent responses that result in distinct biological outcomes; these cell- or stimulus-specific responses tend to be obscured by overexpression strategies.

Finally, this study implicates p66Shc in the control of FOXO3a-regulated gene products such as MnSOD, p27Kip1, and BIM-1. We also show that p66Shc inhibits the expression of the cell cycle regulatory protein PCNA (which might explain the increase in the number of cycling cardiomyocytes previously detected in $p66Shc^{-/-}$ hearts22). Finally, we identify a role for p66Shc as a negative regulator of cardiomyocyte hypertrophy; this results was not anticipated, because a recent study did not detect a difference in cardiomyocyte size between WT and $p66Shc^{-/-}$ mice.[23] However, an antihypertrophic effect of p66Shc in cardiomyocytes might be analogous to the antimitogenic effect of p66Shc identified in lymphocytes.[24] Our studies also provide surprising evidence that p66Shc downregulation is not cytoprotective in doxorubicin-treated cardiomyocytes. This result runs counter to the current opinion that pharmacological therapies that inhibit p66Shc expression or action act as panaceas for clinical disorders characterized by increased oxidative stress.[25–28] However, our findings resonate with recent evidence that p66Shc can exert pleiotropic effects on a range of seeming unrelated fundamental biological processes involving cellular adhesion, cytoskeletal morphology, and intracellular calcium homeostasis[29,30] and that p66Shc exerts an antiapoptotic effect by activating a Notch-3 pathway that enhances human stem/progenitor cell survival.[31] These more nuanced and cell-specific effects of p66Shc are predicted to be important for stem cell-mediated tissue renewal and tissue remodeling, perhaps explaining why a protein such as p66Shc, that plays such a deleterious role in the pathogenesis of age-related diseases characterized by increased oxidative stress, has been conserved during evolution.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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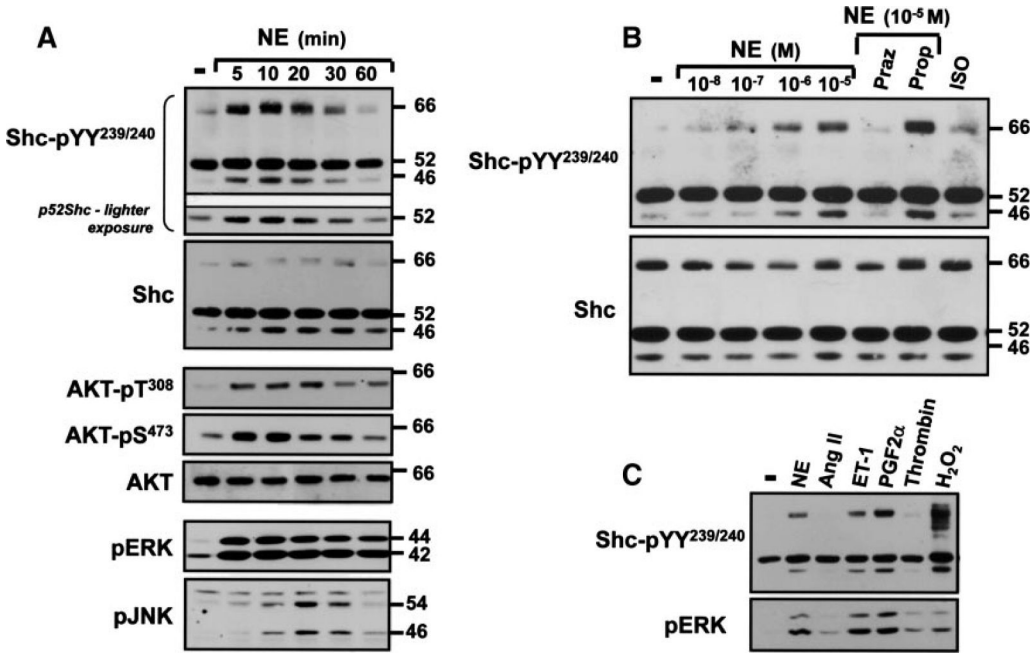


**Figure 1.**

$\alpha_1$-ARs and selected other G protein–coupled receptors promote p66Shc tyrosine phosphorylation in cardiomyocytes. Stimulations were for the indicated intervals in A or 5 minutes in B and C with NE (1 $\mu$mol/L, or the indicated concentrations in B), 1 $\mu$mol/L angiotensin II (Ang II), 0.1 $\mu$mol/L endothelin-1 (ET-1), 1 $\mu$mol/L prostaglandin F2$\alpha$ (PGF2$\alpha$), 1 U/mL thrombin, 5 mmol/L $H_2O_2$, or 0.1 $\mu$mol/L isoproterenol (ISO). Stimulations followed a pretreatment with prazosin (Praz) or propranolol (Prop) (each at 0.1 $\mu$mol/L) as indicated in B. Immunoblotting on cell lysates was with the indicated antibodies, with all results replicated in at least 3 separate culture preparations. Although propranolol induces a modest increase in NE-dependent p66Shc-pYY$^{239/240}$ phosphorylation in B, this was not replicated in other experiments.

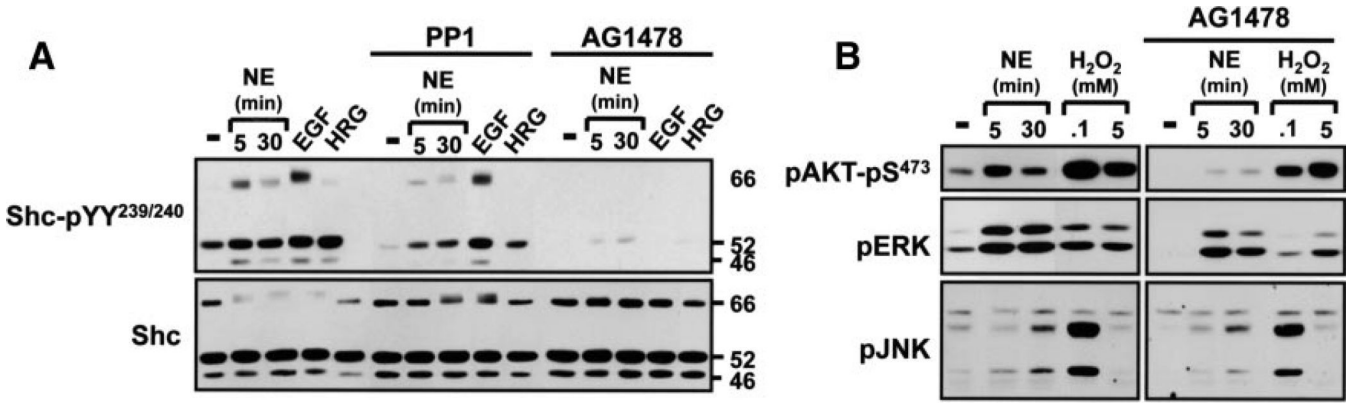


**Figure 2.**

EGFR activity is required for p66Shc-YY$^{239/240}$ phosphorylation. Immunoblotting is on cell lysates from cardiomyocytes pretreated for 30 minutes with vehicle, PP1 (10 $\mu$mol/L), or AG1478 (2 $\mu$mol/L) followed by stimulations for 5 minutes (unless indicated otherwise) with 100 nmol/L EGF, 100 nmol/L heregulin (HRG), 1 $\mu$mol/L NE, or the indicated $H_2O_2$ concentrations. Results were replicated in at least 3 separate culture preparations.

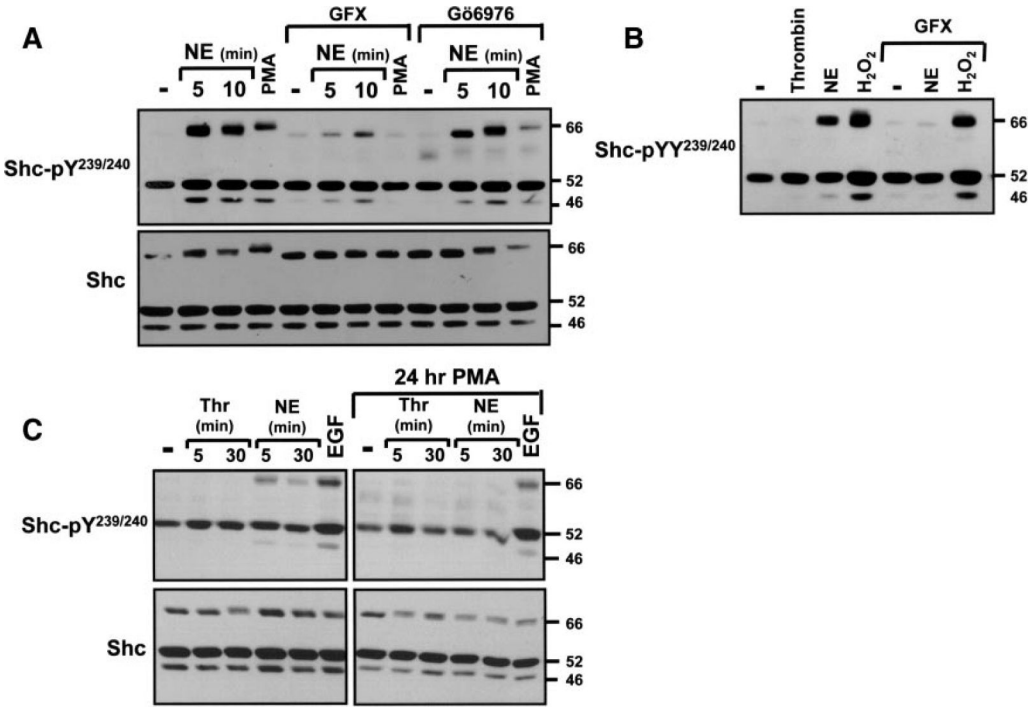


**Figure 3.**

NE promotes p66Shc tyrosine phosphorylation via an nPKC-dependent pathway. A and B, Immunoblotting on lysates from cultures pretreated with GF109203X (GFX) or Gö6976 (each at 5 $\mu$mol/L for 30 minutes) before challenge with vehicle, NE (10 $\mu$mol/L, for 5 minutes unless indicated otherwise), 1 U/mL thrombin, 300 nmol/L PMA, or 5 mmol/L $H_2O_2$. C, Stimulations followed a 24-hour pretreatment with vehicle or PMA. Control studies showing that GFX inhibits PKCs and chronic PMA treatment downregulates phorbol ester-sensitive PKCs are published.[7]

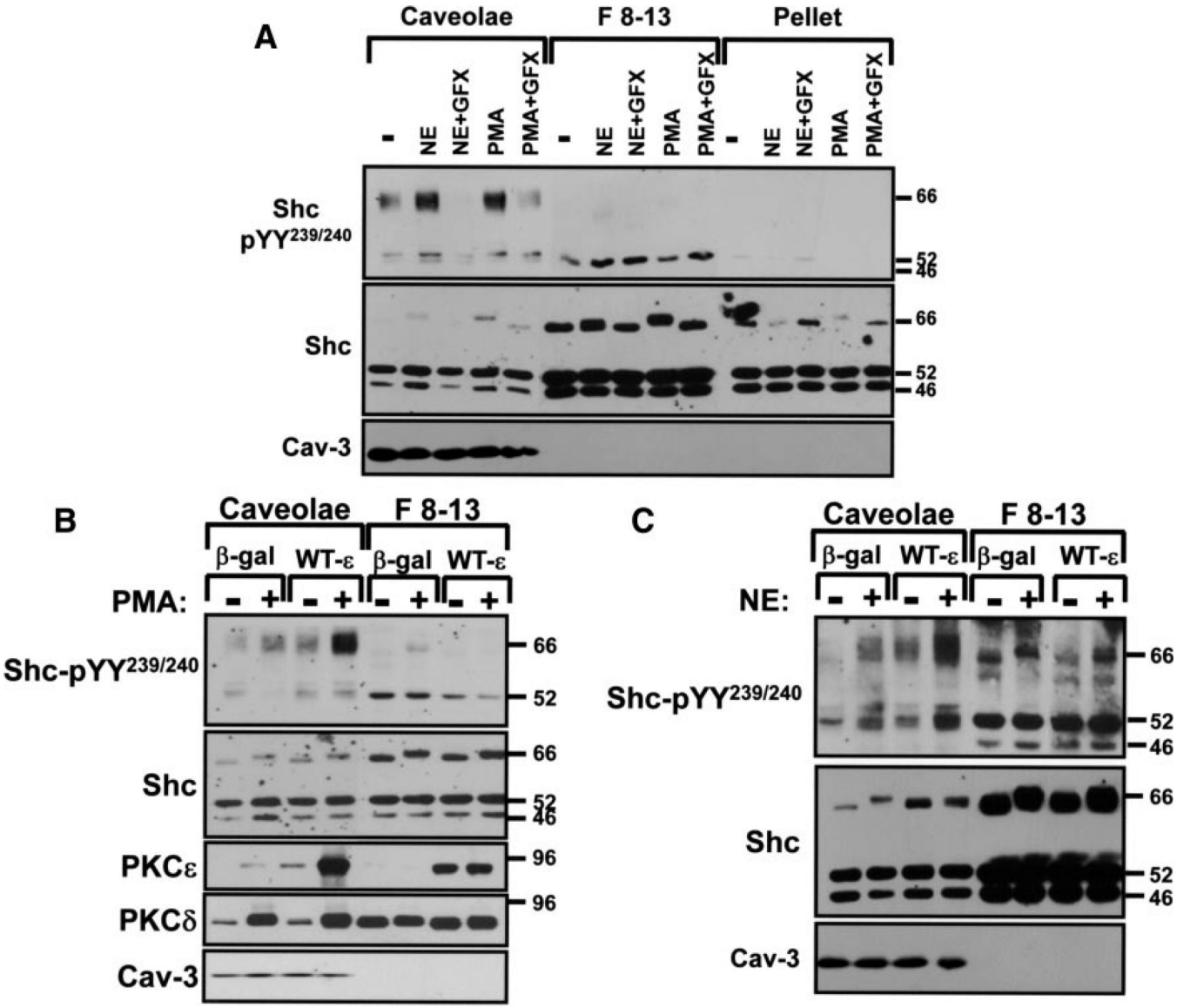


**Figure 4.**

NE and PMA increase p66Shc-YY$^{239/240}$ phosphorylation via a PKC-dependent mechanism in caveolae. Cardiomyocytes pretreated with vehicle or GF109203X (GFX) (5 $\mu$mol/L for 30 minutes) were challenged with 10 $\mu$mol/L NE (5 minutes) or 300 nmol/L PMA (20 minutes). Adenoviral-mediated gene transfer was used to overexpress WT-PKC$\varepsilon$ or $\beta$-gal as a control (multiplicity of infection, 100 pfu/cell) in B and C, with agonist treatments and caveolae isolation performed 48 hours after infections. Immunoblotting on caveolae (Cav), F8-13, and pellet fractions was as described in the expanded Materials and Methods section, with results replicated in 3 separate experiments.

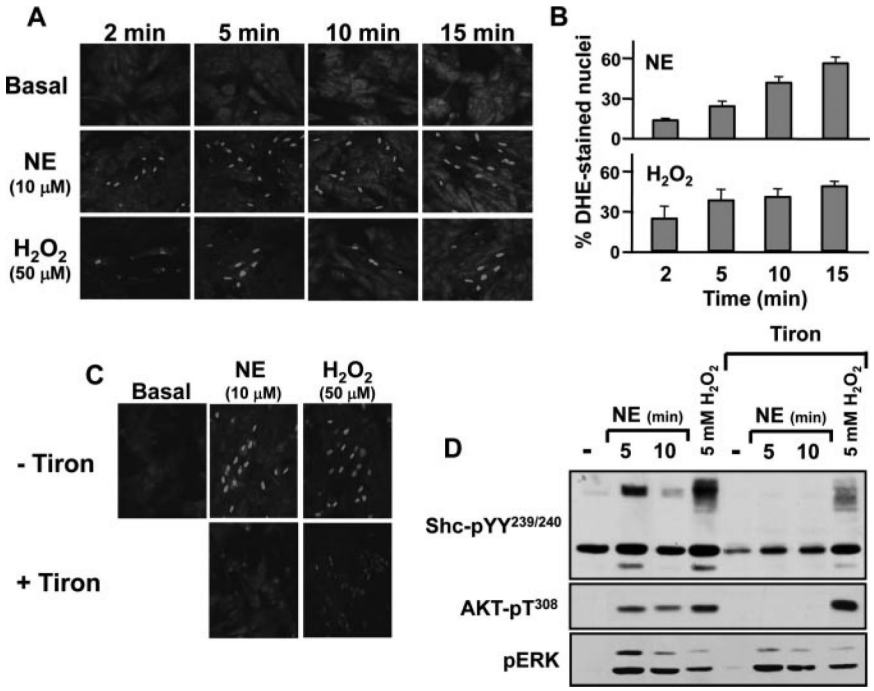


**Figure 5.**

NE increases ROS accumulation, and NE-dependent p66Shc-YY$^{239/240}$ phosphorylation is via a ROS-sensitive mechanism. A through C, Cardiomyocytes were incubated with the redox-sensitive indicator dihydroethidium (5 $\mu$mol/L) in the presence of vehicle or the indicated stimuli (without or with Tiron in C) and then imaged (excitation, 488 nm; emission, 543 nm cutoff) every 3 to 5 minutes. Agonist-dependent increases in the percentage of cells with DHE-stained nuclei are quantified in B (n=250 to 300 cells per assay condition; means±SEM) and was significant at all time points. C, Immunoblots on lysates from cardiomyocytes pretreated for 30 minutes with vehicle or 10 mmol/L Tiron followed by stimulation for 10 minutes with 10 $\mu$mol/L NE or 5 mmol/L $H_2O_2$. Results were replicated in 3 separate experiments.

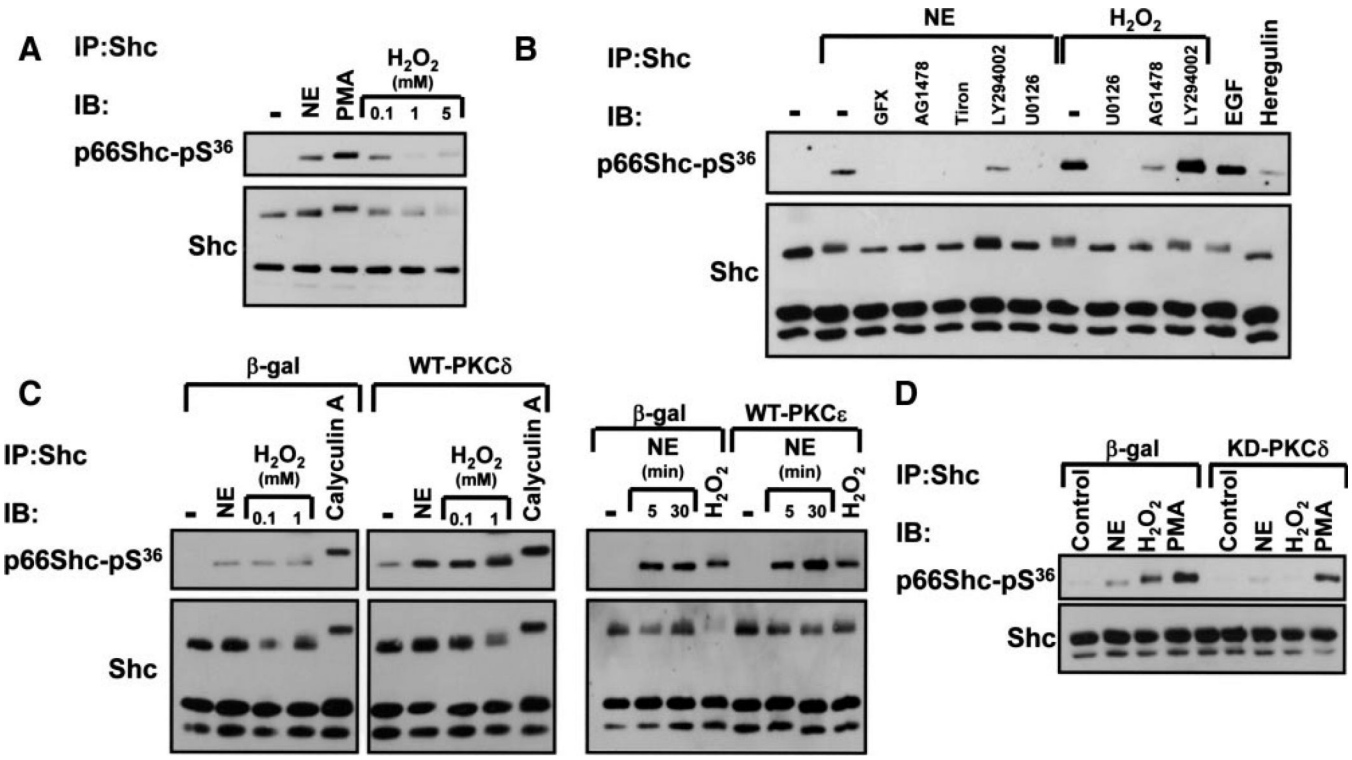


**Figure 6.**

p66Shc-$S^{36}$ phosphorylation is augmented by PKC$\delta$ overexpression. Cardiomyocytes were stimulated with 10 $\mu$mol/L NE, 300 nmol/L PMA, 5 mmol/L $H_2O_2$, or 0.1 $\mu$mol/L calyculin A as indicated. Stimulation followed pretreatment with 5 $\mu$mol/L GFX, 2 $\mu$mol/L AG1478, 10 mmol/L Tiron, 10 $\mu$mol/L LY294002, or 5 $\mu$mol/L U0126 in B or adenoviral-mediated WT-PKC$\delta$, WT-PKC$\varepsilon$, KD-PKC$\delta$, or $\beta$-gal overexpression in C and D. Shc was immunoprecipitated and equal amounts of protein (derived from 600 mg of starting cell extract) was subjected to immunoblotting for p66Shc-$pS^{36}$ and Shc protein immunoreactivity (to verify equal protein recovery and loading). Immunoblotting to track Shc protein recovery was performed on 5-fold less material in D, because of limited amounts of protein. Only the smaller p46/p52Shc isoforms are detected under these conditions. All results were replicated in 3 separate cultures.

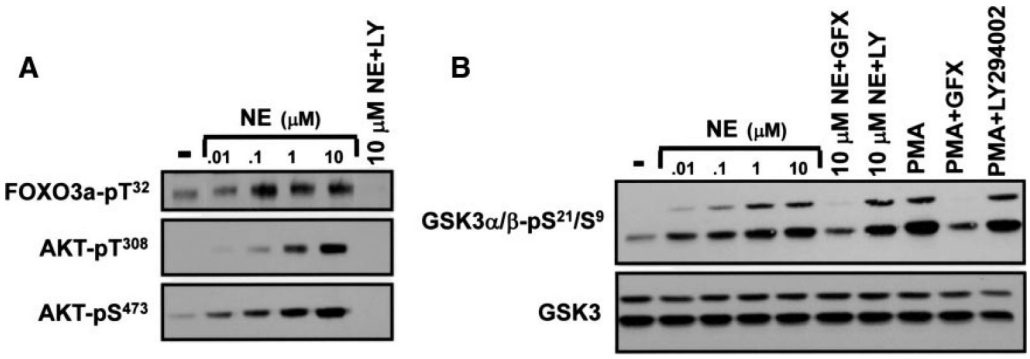


**Figure 7.**

NE-dependent phosphorylation of AKT, GSK3, and FOXO3a. Immunoblotting on lysates from cardiomyocytes stimulated with the indicated concentrations of NE or 300 nmol/L PMA for 10 minutes. Stimulations followed pretreatment with vehicle, LY294002 (LY) or GF109203X (GFX) as indicated. Results were replicated in 3 experiments.

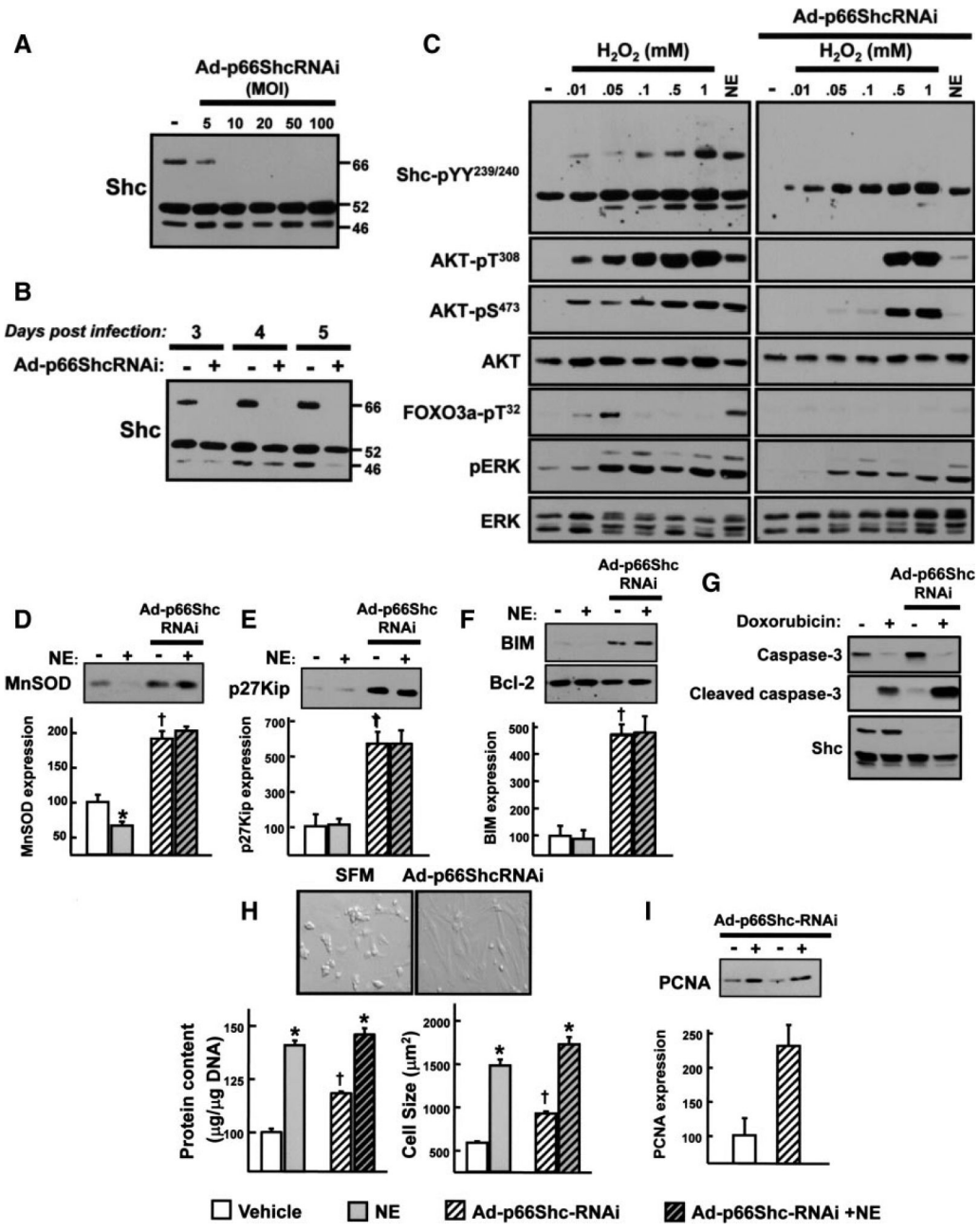


**Figure 8.**

Ad-p66ShcRNAi downregulates p66Shc protein expression and inhibits the NE- and low $H_2O_2$-dependent AKT-FOXO3a phosphorylation pathways. Ad-p66ShcRNAi infection was performed on culture day 1 at the indicated multiplicity of infection (MOI) (A) or a multiplicity of infection of 40 (B through I). C, Stimulation was with the indicated concentrations of $H_2O_2$ or 10 $\mu$mol/L NE (each for 10 minutes) at day 3 following infection with Ad-p66ShcRNAi or empty vector. D through F, H, and I, Treatment was with vehicle or 10 $\mu$mol/L NE for 3 days (starting at culture day 1). Cell lysates were subjected to immunoblotting for MnSOD, p27Kip, BIM, Bcl-2, or PCNA (D through F and I), with a typical experiment depicted on top and the results quantified on the bottom (n=3, *$P$<0.05 NE vs vehicle; †$P$<0.05 Ad-p66ShcRNAi vs empty vector). G, Immunoblotting on cell lysates from cardiomyocytes treated

for 48 hours with vehicle or doxorubicin (1 $\mu$mol/L for 48 hours). H, Measurements of cell size (by phase-contrast microscopy) and protein synthesis were as described in the expanded Materials and Methods section. Results were replicated in 3 experiments.