

Mechanism of Angiotensin II-induced Superoxide Production in Cells Reconstituted with Angiotensin Type 1 Receptor and the Components of NADPH Oxidase*

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The mechanism of angiotensin II (Ang II)-induced superoxide production was investigated with HEK293 or Chinese hamster ovary cells reconstituted with the angiotensin type 1 receptor (AT₁R) and NADPH oxidase (either Nox1 or Nox2) along with a pair of adaptor subunits (either NOXO1 with NOXA1 or p47^{phox} with p67^{phox}). Ang II enhanced the activity of both Nox1 and Nox2 supported by either adaptor pair, with more effective activation of Nox1 in the presence of NOXO1 and NOXA1 and of Nox2 in the presence of p47^{phox} and p67^{phox}. Expression of several AT₁R mutants showed that interaction of the receptor with G proteins but not that with β-arrestin or with other proteins (Jak2, phospholipase C-γ1, SH2 domain-containing phosphatase 2) that bind to the COOH-terminal region of AT₁R, was necessary for Ang II-induced superoxide production. The effects of constitutively active α subunits of G proteins and of various pharmacological agents implicated signaling by a pathway comprising AT₁R, Gα_{q/11}, phospholipase C-β, and protein kinase C as largely, but not exclusively, responsible for Ang II-induced activation of Nox1 and Nox2 in the reconstituted cells. A contribution of Gα_{12/13}, phospholipase D, and phosphatidylinositol 3-kinase to Ang II-induced superoxide generation was also suggested, whereas Src and the epidermal growth factor receptor did not appear to participate in this effect of Ang II. In reconstituted cells stimulated with Ang II, Nox2 exhibited a more sensitive response than Nox1 to the perturbation of protein kinase C, phosphatidylinositol 3-kinase, or the small GTPase Rac1.

Angiotensin II (Ang II)³ achieves its effects in adult mammalian cells by binding directly to the angiotensin type 1 receptor (AT₁R), which belongs to the G protein-coupled receptor family. Engagement of AT₁R elicits a complex network of signaling cascades that result in short-term vascular effects, such as contraction, as well as long-term effects, such as cell growth, migration, and adhesion, that eventually lead to pathophysiological vascular remodeling and cardiac hypertrophy (1, 2). The earliest response to Ang II is Gα_q-mediated activation of phospholipase C (PLC)-β, which is followed by Ca²⁺ mobilization and activation of protein kinase C (PKC) (3). In addition to this pathway, activation of Src family kinases occurs within seconds of Ang II stimulation and appears to be achieved through G protein-dependent and -independent signaling mechanisms (1, 4). Many other signaling molecules, including Janus family kinases (Jaks), the epidermal growth factor receptor (EGFR), mitogen-activated protein kinases, phospholipase D (PLD), and phosphatidylinositol (PI) 3-kinase, are activated subsequent to the activation of PLC-β and Src.

Ang II also induces activation of NADPH oxidase and the consequent production of reactive oxygen species (ROS), such as superoxide and hydrogen peroxide (5), that contribute to many of the pathophysiological effects of this hormone (6). ROS, thus, mediate the Ang II-induced activation of various protein-tyrosine kinases (7), mitogen-activated protein kinases (8), the kinase Akt (8), and redox-sensitive transcriptional factors (9).

The Nox-dependent generation of ROS has been studied extensively in neutrophils and functionally reconstituted non-hematopoietic cells (10–17). Nox of phagocytes is a multicomponent enzyme whose activity requires assembly of the cytosolic subunits p47^{phox} and p67^{phox} and the small GTPase Rac2 with a membrane-associated heterodimer of p22^{phox} and

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³ The abbreviations used are: Ang II, angiotensin II; AT₁R, angiotensin type 1 receptor; PLC, phospholipase C; PLD, phospholipase D; PKC, protein kinase C; Jak, Janus family kinase; EGFR, epidermal growth factor (EGF) receptor; PI, phosphatidylinositol; ROS, reactive oxygen species; ALD, autoinhibitory domain; Nox, NADPH oxidase; NOXO1, Nox organizer 1; NOXA1, Nox activator 1; PX, phox homology; VSMC, vascular smooth muscle cell; PMA, phorbol 12-myristate 13-acetate; SHP2, SH2 domain-containing phosphatase 2; DAG, diacylglycerol; IC₅₀, median inhibitory concentration; PIP₃, PI 3,4,5-trisphosphate; GEF, guanine nucleotide exchange factor; RT, reverse transcription; CL, chemiluminescence; VSMC, vascular smooth muscle cell; CHO, Chinese hamster ovary; HEK cells, human embryonic kidney cells; siRNA, small interfering RNA.

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gp91^{phox}. Among these components, gp91^{phox} mediates electron transfer from NADPH to molecular oxygen to produce superoxide, which is then spontaneously or enzymatically converted to H₂O₂. Activation of gp91^{phox} requires stimulus-induced translocation of p47^{phox}, p67^{phox}, and Rac2 to the plasma membrane. In resting cells, p47^{phox} and p67^{phox} are present in a cytosolic complex, and their membrane translocation is dependent on phosphorylation of multiple serine residues in p47^{phox} by several kinases including PKC and Akt (18, 19). Phosphorylation of p47^{phox} occurs in an autoinhibitory domain (AID) and induces a conformational change that renders the tandem SH3 domains competent to bind a proline-rich region of p22^{phox}, the partner of gp91^{phox} (10, 11, 13, 20). This conformational change also exposes the phox homology (PX) domain of p47^{phox}, which specifically recognizes 3'-phosphorylated phosphoinositides, the products of PI 3-kinase. Cell stimulation also induces the conversion of GDP-bound Rac2 to the GTP-bound form and its resulting recruitment to the NADPH oxidase complex, where it interacts with p67^{phox} and gp91^{phox}.

Multiple homologs of gp91^{phox} (which has been renamed Nox2) have been identified in nonphagocytic cells (13–15). Among the seven members of Nox family, Nox1 is both structurally and functionally similar to Nox2. Rac and p22^{phox} are present in almost all cell types examined, whereas expression of p47^{phox} and p67^{phox} is largely restricted to mature phagocytes. The presence in most cell types of one or more Nox enzymes nevertheless suggested the possibility that homologs of p47^{phox} and p67^{phox} might be expressed in nonphagocytic cells. Such homologs were subsequently identified (20–22). The p47^{phox} homolog is referred to as Nox organizer 1 (NOXO1) and the p67^{phox} homolog as Nox activator 1 (NOXA1). Both NOXO1 and NOXA1 possess domain organizations almost identical to their respective homologs, with the exception that NOXO1 does not contain an AID. NOXO1, thus, interacts with p22^{phox} via its SH3 domains and with NOXA1 via its proline-rich carboxyl-terminal domain, whereas NOXA1 interacts with GTP-bound Rac (20).

Transfection experiments have shown that NOXO1 and NOXA1 reconstitute with Nox1 to produce ROS in a p22^{phox}-dependent manner (20–22). As predicted from the similarities to their phagocyte homologs, NOXO1 and NOXA1 are able to substitute for p47^{phox} and p67^{phox}, respectively, in the activation Nox2. Nevertheless, NOXO1 and NOXA1 activate Nox2 more effectively than they do Nox2, whereas p47^{phox} and p67^{phox} activate Nox2 more effectively (20–23). Moreover, the mechanism of activation by NOXO1 differs in some respects from that mediated by p47^{phox}. Unlike p47^{phox}, NOXO1 is localized at the cell membrane even in the absence of stimulatory signals (24), probably because it lacks an AID, which blocks the SH3 domains of p47^{phox} from interacting with p22^{phox} in the absence of phosphorylation. The phosphoinositide binding specificity of the PX domain of NOXO1 also differs from that of the PX domain of p47^{phox}; the NOXO1 domain binds to mono-phosphorylated forms of PI such as PI 4-phosphate and PI 5-phosphate, which are abundant in nonactivated cells (24). NOXO1 is, thus, capable of activating Nox1 and Nox2 even without cell stimulation when coexpressed with p67^{phox} or NOXA1. NOXO1 also activates Nox3 in a manner independent

of NOXA1 (13). Nox4 appears to be constitutively active in the absence of NOXO1 and NOXA1 (25–28).

Although Nox is implicated as the source of Ang II-induced ROS production (11, 29, 30), it has remained unclear which Nox isoforms are linked to AT₁R. Vascular cells express Nox1, Nox2, and Nox4 (15). There are some hints for the functional coupling between Nox1 and AT₁R; Nox1 is up-regulated upon Ang II stimulation of vascular smooth muscle cells (VSMCs) (31, 32), Ang II fails to induce ROS production in Nox1-deficient VSMCs (31), and Ang II-mediated hypertension and blood pressure are decreased in Nox1-deficient mice (33, 34). Nox2 as an Ang II-responsive enzyme has also been speculated based on the observations that Nox2 components (Nox2, p22^{phox}, p47^{phox}, p67^{phox}, and p40^{phox}) are induced by Ang II in endothelial cells (35–41), that Ang II-dependent cardiac hypertrophy and blood pressure are moderately decreased in Nox2-deficient mice (42–44), and that Ang II-dependent ROS production is severely compromised in endothelial cells (45) and VSMCs (46) derived from mice deficient in p47^{phox}, the Nox subunit that is most effective for the activation of Nox2.

Given that coexpression of Nox1 with NOXO1 and NOXA1 is sufficient for a substantial level of activity in the absence of extracellular stimulation, it is important to determine whether Ang II is able to elicit further activation of this complex. It is also important to know if Ang II is able to activate Nox2. If so, the mechanism responsible for such receptor-mediated activation is also of interest. To address these issues, we have now transfected HEK293 and CHO cells with expression vectors for AT₁R and the various components of the Nox1 and Nox2 system and have characterized the oxidative responses of these reconstituted models in response to Ang II stimulation. Although Nox4 is highly expressed in vascular cells, this distant homolog of Nox1 and Nox2 is not included in this study because its activation neither requires cytosolic subunits nor needs cell stimulation upon heterologous expression (25–28).

EXPERIMENTAL PROCEDURES

Materials—Ang II and wortmannin were obtained from Sigma, GF109203X, LY294002, EGF, and phorbol 12-myristate 13-acetate (PMA) were from Calbiochem, and AG1478 and PP1 were from Biomol Research Laboratories. Rabbit polyclonal antibodies to Gα subunits were obtained from Santa Cruz Biotechnology; mouse monoclonal antibody to β-actin was from Abcam and mouse monoclonal antibody to AU5 was from Covance; sheep polyclonal antibody to EGF receptor and monoclonal antibody to phosphotyrosine and Rac1 were from Upstate. Polyclonal antibody for PLD1 and PLD2 was kindly provided by Sung ho Ryu (Pohang University of Science and Technology) (47). The retroviral vector MFG-S-Nox1 for expression of human Nox1 (23), pcDNA3.1-NOXO1 for expression of human NOXO1 (21), pcDNA3.1-NOXA1 for expression of human NOXA1 (21), pcDNA3-p47^{phox} for expression of human p47^{phox}, and pcDNA3-p67^{phox} for expression of human p67^{phox} were described previously. Given that infection of CHO cells with MFG-S-Nox1 viruses was inefficient, we prepared a pantrophic retroviral vector, pMSCV-Nox1, by subcloning full-length human Nox1 cDNA into the XhoI and EcoRI sites of pMSCV puro (Clontech). The retrovirus of gp91^{phox} was kindly provided by Harry L. Malech (National

Institutes of Health). Expression plasmids derived from pcDNA1/AMP that harbor cDNAs for wild-type rat AT₁R or for one of three rat AT₁R mutants (M17, del 221/222, Y319stop) were described previously (48–50). Plasmids derived from pCEFL that encode AU5-tagged constitutively active forms of human G α subunits (G α_q QL, G α_i QL, G α_{12} QL, G α_s QL) or AU5-tagged forms of human Rac1 (RacN17, RacV12) were kindly provided by J. Silvio Gutkind (NIDCR, National Institutes of Health) (51, 52). The pcDNA3-EGFR vector for expression of EGFR was kindly provided by Yosef Yarden (Weizmann Institute of Science) (53).

Cell Culture and Transfection—HEK293 cells were maintained under an atmosphere of 5% CO₂ in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum and penicillin-streptomycin. CHO cells were maintained under an atmosphere of 5% CO₂ in F12K medium (ATCC) supplemented with 10% fetal bovine serum and penicillin-streptomycin. For establishment of Nox1-expressing cells, HEK293 cells were infected with MFG-S-Nox1 retroviruses as described (23), and for establishment of Nox2-expressing cells, HEK293 cells were transduced using method described previously (54), and CHO cells were infected with pMSCV-Nox1 viruses and then subjected to selection with puromycin (60 μ g/ml). For receptor binding assays, immunoblot analysis, and measurement of superoxide, cells (2×10^6) that had been infected with Nox1 or Nox2 retroviruses were seeded on collagen-coated 100-mm dishes (BD Biosciences) and, after 24 h, subjected to transient transfection with the use of FuGENE 6 (Roche Applied Science) for 48 h with 6 μ g each of pcDNA3.1-NOXO1, pcDNA3.1-NOXA1 or pcDNA3-p47^{phox}, pcDNA3-p67^{phox}, pcDNA1/AMP harboring AT₁R cDNA (wild type, M17, del 221/222, Y319stop), pCEFL plasmids encoding G α subunits (G α_q QL, G α_i QL, G α_{12} QL, G α_s QL), or pcDNA3-EGFR, as needed. For expression of Rac1 mutants (RacN17, RacV12), cells already expressing Nox1, NOXO1, NOXA1, and AT₁R were further transfected for 24 h with the corresponding pCEFL plasmid with the use of Lipofectamine (Invitrogen). For each expression vector, control experiments were performed with cells transfected with the corresponding empty plasmid.

Small Interference RNA (siRNA) Transfection—siRNA of human PLD1 was kind gift of Sung ho Ryu (Pohang University of Science and Technology) (47). The siRNA of 21-nucleotide sequences corresponding to a human PLD1 sequence (nucleotides 1454–1474, AAGGUGGGACGACAAUAGAGCA) was purchased from Dharmacon Research Inc. (Lafayette, Colo.). siRNA of human PLD2 was purchased from Dharmacon Research Inc. (Lafayette, Colo.). The siRNA to a human PLD2 was siGENOME SMARTpool duplex (combination of 4 duplexes; Duplex1 sense, GGACAACCAAGAAGAAAUAUU; Duplex1 antisense, PUUUUCUUCUUGGUUGGUCCUU; Duplex2 sense, GGACCGGCCUUUCGAAGAUUU; Duplex2 antisense, PAUCUUCGAAAGGCCGGUCCUU; Duplex3 sense, GACCUGCACUACCGACUGAUU; Duplex3 antisense, PUCAGUCGGUAGUGCAGGUCCUU; Duplex4 sense, CAG-CAUGGCGGGACUAUAUUU; Duplex4 antisense, PAUAGUCCCCGCAUGCUGUU). Then 100 nM siRNA duplex was transiently transfected into HEK293 cells expressing AT₁R,

Nox1, NOXO1, and NOXA1 or AT₁R, Nox2, p47^{phox}, and p67^{phox} by using Lipofectamine 2000 (Invitrogen), and 48 h later the cells were harvested for measurement of superoxide production, reverse transcription (RT)-PCR, or immunoblot analysis.

RNA Extraction and RT-PCR—Total RNA was isolated from PLD1 or PLD2 siRNA-transfected HEK293 cells using Trizol Reagent (Invitrogen) and reverse-transcribed and amplified with SuperScriptTM III One-Step RT-PCR System with Platinum[®] TaqDNA Polymerase (Invitrogen). Specific primers and conditions used for the amplification of PLD1 or PLD2 were described previously (55). RT-PCR primer and control were purchased for set-specific primers for actin (Invitrogen).

Radioligand Binding—The extent of AT₁R expression on the cell surface was determined with ¹²⁵I-labeled Ang II as described (48). Total protein, determined with the BCA reagent (Bio-Rad), was used to normalize the receptor expression level.

Treatment with Inhibitors and Measurement of Superoxide Production—Reconstituted HEK293 or CHO cells were deprived of serum for 4 h, washed once in Hanks' balanced salt solution, and incubated for 10 or 30 min at 37 °C in the absence or presence of inhibitors. The cells were washed once with Hanks' balanced salt solution and then incubated with 1 μ M Ang II (in the continued absence or presence of inhibitor) and the luminol enhanced chemiluminescence substrate Diogenes (National Diagnostics). Chemiluminescence was measured over 40 min at 37 °C in 96-well microtiter chemiluminescence plates (5×10^5 cells per well in Hanks' balanced salt solution) with a Luminoskan luminometer (Labsystems) and was expressed in arbitrary units. All superoxide values (chemiluminescence units (CL)) presented correspond to the sum of chemiluminescence produced over 40 min period after the stimulation of 1 μ M Ang II. The luminescence was fully attributable to superoxide, given that it was completely abolished in the presence of superoxide dismutase.

RESULTS

Reconstitution of Ang II-induced Superoxide Generation in HEK293 Cells—Previous studies showed that transfection of several host cell lines with expression vectors for Nox1, NOXO1, and NOXA1 reconstituted superoxide generation, whereas those transfected with individual vectors did not (20–22). These studies differed, however, in whether the production of superoxide was stimulated by PMA; PMA further increased superoxide generation in cells expressing all three components (Nox1, NOXO1, NOXA1) of human origin (20, 21) but not in cells expressing the murine components (22). We repeated these reconstitution experiments in HEK293 cells expressing human components and measured the time course of superoxide generation. HEK293 cells that had been transfected with Nox1, NOXO1, and NOXA1 vectors produced superoxide in the absence of cell stimulation (Fig. 1A). PMA further increased superoxide generation, with this effect peaking at 10 min and decaying slowly thereafter. Transfection of HEK293 cells with an AT₁R vector in addition to those for Nox1, NOXO1, and NOXA1 rendered superoxide generation sensitive to Ang II (Fig. 1A). The Ang II-induced production of superoxide also

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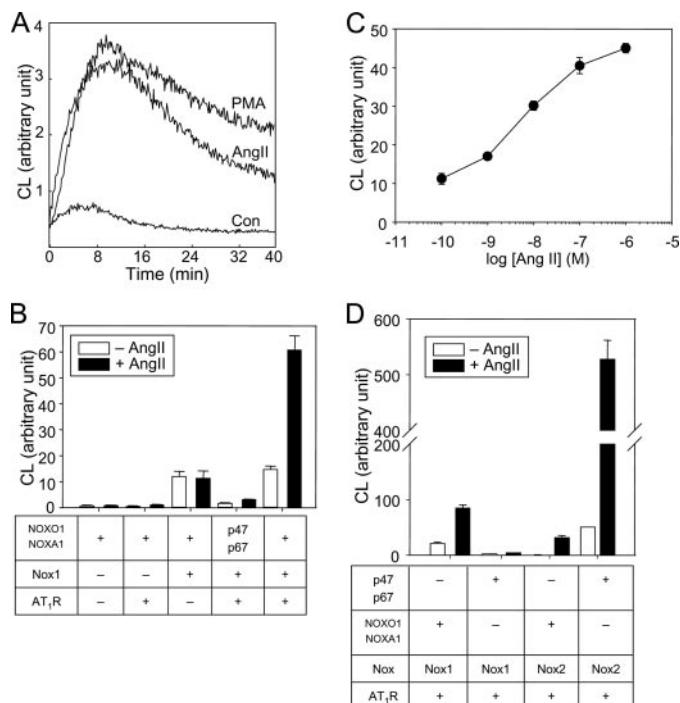


FIGURE 1. Reconstitution of Ang II-induced superoxide production in HEK293 cells. *A*, cells expressing exogenous AT₁R, Nox1, NOXO1, and NOXA1 were incubated in the absence (Con) or presence of Ang II (1 μ M) or PMA (0.3 μ M), and superoxide generation was continuously monitored by measurement of superoxide dismutase-inhibitable CL. *B* and *C*, HEK293 cells transfected with expression vectors for the indicated combinations of proteins were incubated in the absence or presence of Ang II (1 μ M) for 40 min and monitored by measurement of chemiluminescence. *D*, HEK293 cells transfected as in *B* were stimulated for 40 min with the indicated concentrations of Ang II and monitored for chemiluminescence. Data in *B–D* are the means \pm S.D. of values from three separate transfection experiments.

peaked at \sim 10 min but decayed faster than did the effect of PMA (Fig. 1A). The total amount of superoxide generated over 40 min in the presence of Ang II was about four times that produced constitutively in cells expressing Nox1, NOXO1, NOXA1, and AT₁R (Fig. 1A). All superoxide values presented hereafter correspond to the total amount produced during incubation of cells for 40 min.

The observation that superoxide production in cells expressing Nox1, NOXO1, and NOXA1, but not AT₁R, was not responsive to Ang II (Fig. 1B) suggests that any contribution of endogenous AT₁R to the effect of Ang II in cells expressing all four components was negligible. Replacement of NOXO1 and NOXA1 with their phagocyte counterparts, p47^{phox} and p67^{phox}, resulted in a marked reduction in both constitutive and Ang II-dependent superoxide production (Fig. 1B). The effect of Ang II on superoxide production in cells expressing exogenous AT₁R was concentration-dependent (Fig. 1C).

We also investigated Nox2 activation in HEK293 cells that had been systematically transfected with an AT₁R vector in addition to those for Nox2 and the pair of NOXO1 and NOXA1 or the pair of p47^{phox} and p67^{phox} (Fig. 1D). Ang II-dependent Nox2 activation was observed in the presence of both pairs with the activity supported by the pair of p47^{phox} and p67^{phox} being 17 times that supported by the pair of NOXO1 and NOXA1. The Ang II-stimulated superoxide production in HEK293 cells

expressing the Nox2 components (Nox2, p47^{phox}, and p67^{phox}) was 10 times that produced by the identical cells in the absence of Ang II stimulation. And Ang II-induced superoxide production in the cells expressing the Nox2 components was seven times higher than that in the cells expressing the Nox1 components (Nox1, NOXO1, and NOXA1).

Effect of AT₁R Mutations on Superoxide Generation—Certain downstream signaling pathways of AT₁R are triggered independently of G proteins via β -arrestin, which forms a complex with the activated receptor and acts as a scaffold to recruit various signaling proteins, including mitogen-activated protein kinases, apoptosis signal-regulating kinase 1, Src, Raf, and murine double minute 2 (Mdm2) (56, 57). β -Arrestin also functions as an adapter in clathrin-mediated endocytosis of AT₁R (57, 58). In addition to its interactions with G proteins and β -arrestin, activated AT₁R associates via its COOH-terminal Tyr-Ile-Pro-Pro motif with Jak2, PLC- γ 1, and SH2 domain-containing phosphatase 2 (SHP2) (57, 59, 60). To determine which pathways link ligation of AT₁R to Nox1 activation, we studied three AT₁R mutants (M17, del 221/222, Y319stop). The M17 mutant, which contains an Asp⁷⁴ \rightarrow Asn substitution as well as lacks residues 221–226 in the third cytoplasmic loop, is not able to couple to G proteins or to stimulate the generation of inositol phosphates but is only moderately impaired with regard to internalization compared with the wild-type receptor (48). The deletion of Ala²²¹ and Leu²²² in the del 221/222 mutant results in a complete loss of the ability to mediate Ang II-induced generation of inositol phosphates and to undergo internalization (50). The Y319stop mutant lacks the 41 residues downstream of Tyr³¹⁹ and, thus, does not contain the Tyr-Ile-Pro-Pro motif required for the binding of Jak2, PLC- γ 1, and SHP2 and is also unable to bind β -arrestin (57). This COOH-terminal deletion does not impair AT₁R coupling to G proteins but inhibits receptor internalization (61).

Transfection of HEK293 cells with vectors for each of these three AT₁R mutants yielded expression levels in the range of 0.4–0.5 pmol/mg of protein as determined by a radioligand binding assay (data not shown). Whereas Ang II did not stimulate superoxide generation in cells expressing the M17 or del 221/222 mutants (in addition to Nox1, NOXO1, and NOXA1), its effect was increased by 60% in cells expressing the Y319stop mutant (Fig. 2A). Similar results were obtained when the Nox2 components, instead of the Nox1 components, were expressed together with each of the three AT₁R mutants (Fig. 2B).

These data suggest that coupling to G proteins, but not to signaling proteins such as β -arrestin, Jak2, PLC- γ 1, and SHP2, is necessary for Ang II-induced superoxide production. They further indicate that internalization of AT₁R results in down-regulation of this activity.

Effects of Constitutively Active G α Subunits on Superoxide Generation—AT₁R is coupled to G α_i or G α_o and to G α_{12} or G α_{13} in addition to its principal transducer G $\alpha_{q/11}$ (1, 62). We assessed the role of G α subunits in Ang II-induced superoxide production with the use of HEK293 cells expressing AT₁R and constitutively active mutants (G α_q QL, G α_i QL, G α_{12} QL, G α_s QL) representing each subtype in addition to the Nox1 components (Nox1, NOXO1, and NOXA1) (Fig. 3A) or the Nox2 components (Nox2, p47^{phox}, and p67^{phox}) (Fig. 3C).

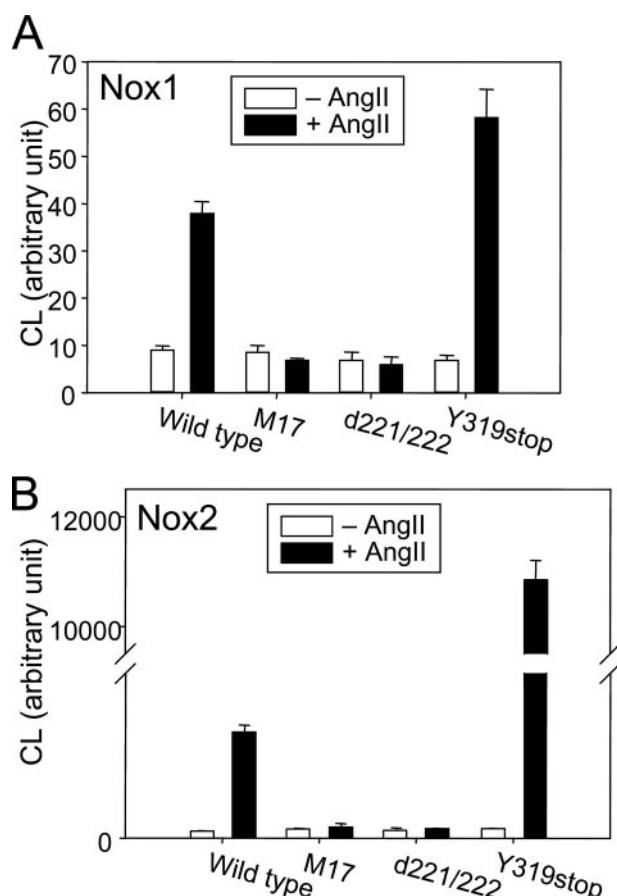


FIGURE 2. Effect of Ang II on superoxide production in HEK293 cells expressing various AT₁R mutants. Cells expressing either wild-type or mutant (M17, del 221/222, Y319stop) forms of AT₁R, in addition to the Nox1 components (Nox1, NOXO1, and NOXA1) (A) or the Nox2 components (Nox2, p47^{phox}, and p67^{phox}) (B), were incubated in the absence or presence of Ang II, and superoxide production was measured. Data are the means \pm S.D. of values from three separate transfection experiments.

Expression of the G α subunits was confirmed by immunoblot analysis with specific antibodies (Fig. 3, B and D). Expression of G α_q QL resulted in a ~20- and ~30-fold increase in superoxide production by Nox1 and Nox2 enzymes, respectively (Figs. 3, A and C). This magnitude of activation amounts to three to four times those by Ang II in cells not expressing G α mutants. Ang II did not induce an additional increase in superoxide generation in the cells expressing G α_q QL. The production of superoxide was also increased, although to a much lesser extent than that observed with G α_q QL, by expression of G α_s QL (~3-fold for both Nox1 and Nox2) or G α_{12} QL (~2-fold for both Nox1 and Nox2). In contrast to the cells expressing G α_q QL, those expressing G α_s QL or G α_{12} QL remained sensitive to the stimulatory effect of Ang II on superoxide generation. Expression of G α_i QL resulted in a slight decrease in the extents of both constitutive and Ang II-induced superoxide production by both Nox1 and Nox2.

Effect of PKC Inhibition on Superoxide Production—The major target of activated G $\alpha_{q/11}$ is PLC- β . PLC catalyzes the hydrolysis of PI 4,5-bisphosphate to generate diacylglycerol (DAG) and inositol 1,4,5-trisphosphate, which mediate the activation of PKC and intracellular Ca²⁺ mobilization, respectively. We examined the effect on Ang II-induced superoxide production of GF109203X, which inhibits various isoforms of PKC with a median inhibitory concentration (IC₅₀) of 10–20 nM by competing with ATP (63). GF109203X inhibited the stimulatory effect of Ang II on superoxide generation in a concentration-dependent manner, with ~60 and ~90% inhibition apparent at a concentration of 2 μ M in HEK293 expressing the Nox1 and Nox2 components, respectively (Fig. 4A). GF109203X (2 μ M) inhibited by ~40% the effect of G α_q QL on superoxide production in HEK293 cells expressing the Nox1 components (Fig. 4B) and by ~50% that in the cells expressing the Nox2 components (Fig. 4C). These results suggest that the stimulatory effects of both Ang II and G α_q QL on superoxide generation are mediated in part by PKC but are not fully dependent on kinase activation. The proportion of superoxide production dependent on PKC was difficult to evaluate quantitatively because the concentration-response relation for GF109203X was biphasic, and although it is more selective than are other PKC inhibitors, this agent inhibits other protein kinases (such as cAMP-dependent protein kinase with an IC₅₀ of 2 μ M).

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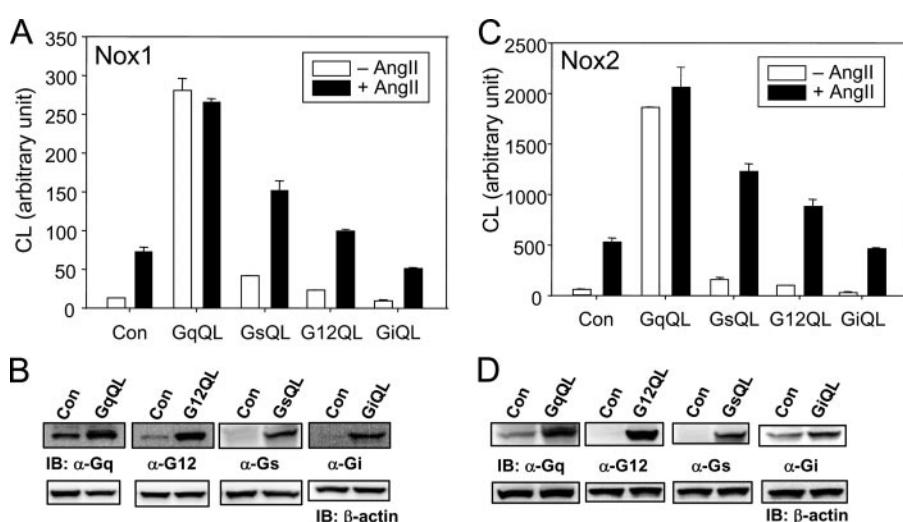


FIGURE 3. Effects of constitutively active G α proteins on superoxide production in HEK293 cells. A and C, cells expressing AT₁R, Nox1, NOXO1, and NOXA1 with or without (Con) the indicated constitutively active G α proteins (G α_q QL, G α_s QL, G α_{12} QL, G α_i QL) (A) and cells expressing AT₁R, Nox2, p47^{phox}, and p67^{phox} with or without (Con) the indicated constitutively active G α proteins (C) were incubated with or without Ang II and assayed for the production of superoxide. Data are the means \pm S.D. of values from three separate transfection experiments. B and D, lysates of the cells transfected as in A (B) and C (D) were subjected to immunoblot (IB) analysis with antibodies to each G α protein or to β -actin (internal control). Data are representative of two separate experiments.

Ang II-induced Superoxide Generation

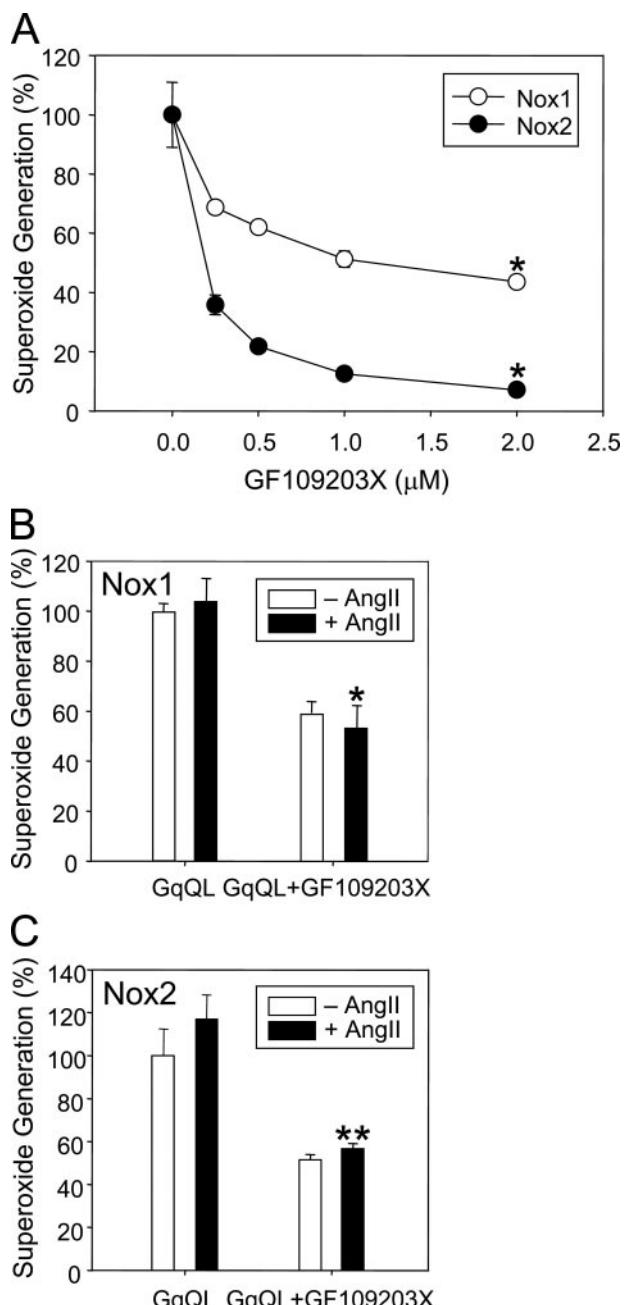


FIGURE 4. Effect of PKC inhibition on superoxide generation in HEK293 cells. *A*, cells expressing AT₁R, Nox1, NOXO1, and NOXA1 (open circle) or cells expressing AT₁R, Nox2, p47^{phox}, and p67^{phox} (closed circle) were treated with the indicated concentrations of GF109203X for 10 min and then assayed for the effect of Ang II on superoxide production. *B* and *C*, cells expressing AT₁R, Nox1, NOXO1, NOXA1, and Gα_qQL (*B*) or cells expressing AT₁R, Nox2, p47^{phox}, p67^{phox}, and Gα_qQL (*C*) were treated with 10 μM GF109203X for 30 min and then assayed for the effect of Ang II on superoxide generation. Data are expressed as a percentage of the value for cells incubated without inhibitor and are the means ± S.D. of values from four separate transfection experiments. *p* < 0.001 (*) and *p* < 0.05 (**) compared with no PKC inhibitor treatment.

acid to DAG provide a greater supply of the latter for the sustained activation of PKC (64). Inhibition of PLD by the broad specific inhibitor sphinganine results in a partial inhibition of Ang II-induced superoxide production in human VSMCs (65). Mammalian cells contain two PLD isoforms, PLD1 and PLD2 (66). To assess PLD function in superoxide generation, we

transfected the reconstituted HEK293 cells with either a PLD1- or PLD2-specific siRNA to deplete PLD1 and PLD2 separately (Fig. 5). The silencing was highly effective both at the mRNA and protein levels as evidenced by RT-PCR and immunoblot analyses (Figs. 5, *B* and *D*). PLD2 depletion led to ~20% reduction of Nox1 activity (Fig. 5*A*) and ~30% reduction of Nox2 activity (Fig. 5*C*), both measured after the stimulation of the reconstituted HEK293 cells with Ang II. In contrast, neither Nox1 nor Nox2 activity was inhibited by PLD1 depletion in the Ang II-stimulated reconstituted cells (Fig. 5, *A* and *C*).

Effect of Inhibition of the Protein-tyrosine Kinase Activities of Src or EGFR on Superoxide Production—Stimulation with Ang II induces activation of EGFR via a pathway involving Src family kinases in some cell types including VSMCs (67, 68). The Src-mediated activation of EGFR results in the production of PI 3,4,5-trisphosphate (PIP₃) by PI 3-kinase and the activation of Rac via a PIP₃-dependent guanine nucleotide exchange factor for Rac (RacGEF) in rat VSMCs (69). Ang II-induced production of ROS in rat VSMCs was almost completely abolished by inhibition of the function of Src, EGFR, PI 3-kinase, or Rac (69); thus, Ang II-induced production of ROS in these cells appears to occur mainly through an Src-EGFR-PI 3-kinase-RacGEF-Rac pathway.

We evaluated the possible contribution of EGFR activation to superoxide generation in reconstituted HEK293 cells with the use of two highly selective inhibitors; PP1, which inhibits the protein-tyrosine kinase activity of members of the Src family with IC₅₀ values of 5 nM for p56^{ck}, 6 nM for p59^{ck}, and 170 nM for p60^{src}, and AG1478, which inhibits that of the EGFR with an IC₅₀ of 3 nM (70). Prior incubation with 20 μM PP1 or 1 μM AG1478 had no effect on constitutive or Ang II-induced production of superoxide in reconstituted HEK293 cells expressing the Nox1 (Fig. 6*A*) or Nox2 components (Fig. 6*B*). To determine whether the apparent lack of EGFR participation in superoxide production was due to a low abundance of this receptor, we transfected HEK293 cells with an expression vector for EGFR in addition to those for AT₁R and the Nox1 components. Basal or Ang II-stimulated superoxide production was still unaffected by AG1478 in the resulting cells (Fig. 7*A*). It was shown previously that Ang II-induced EGFR transactivation is cell type-specific (53): Activation of the AT₁R by Ang II in AT₁R-transfected HEK293 cells did not lead to transactivation of the EGFR even though EGFR is expressed in these cells and can be activated by EGF, whereas other cell types including CHO cells showed transactivation of their expressed EGF receptors by Ang II. We, therefore, examined the EGFR participation in CHO cells by transfecting the cells with vectors for the Nox1 components in addition to those for AT₁R and EGFR. The constitutive production of superoxide in the reconstituted CHO cells was four to five times that apparent in HEK293 cells expressing the same components. Ang II induced an ~7-fold increase in superoxide generation in the reconstituted CHO cells, and neither this effect nor the basal level of superoxide production was sensitive to AG1478 (Fig. 7*C*). Expression of EGFR in the transfected HEK293 and CHO cells was confirmed by immunoblot analysis (*left panels* of Fig. 7, *B* and *D*), and functional competence of the expressed receptor was manifest by its EGF-dependent autophosphorylation (*right panels* of Fig.

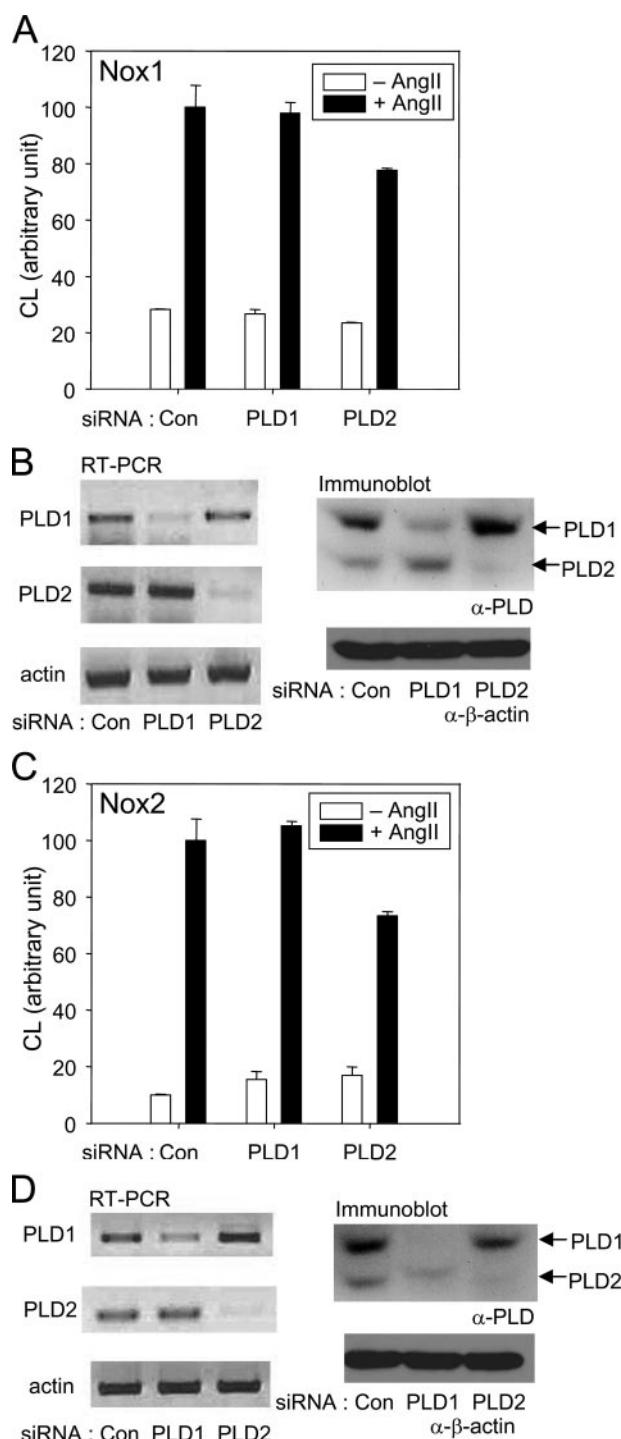


FIGURE 5. Effect of PLD depletion on Ang II-induced superoxide generation in HEK293 cells. *A* and *C*, cells expressing AT₁R, Nox1, NOXO1, and NOXA1 (*A*) and cells expressing AT₁R, Nox2, p47^{phox}, and p67^{phox} (*C*) were transfected with a control RNA (Con), PLD1-specific siRNA (PLD1), or PLD2-specific siRNA (PLD2). Forty-eight hours after transfection the cells were assayed for the effect of Ang II on superoxide production. Data are expressed as a percentage of the value for control cells stimulated with Ang II and are means \pm S.D. of values from three separate transfection experiments. *B* and *D*, the extent of PLD depletion was evaluated using RT-PCR (*left panels*) and immunoblot (*right panels*) analyses. RT-PCRs were carried out as described under “Experimental Procedures” using specific primers for PLD1, PLD2, or β -actin. Antibodies to PLD1, to PLD2, or to β -actin were used for immunoblot analyses.

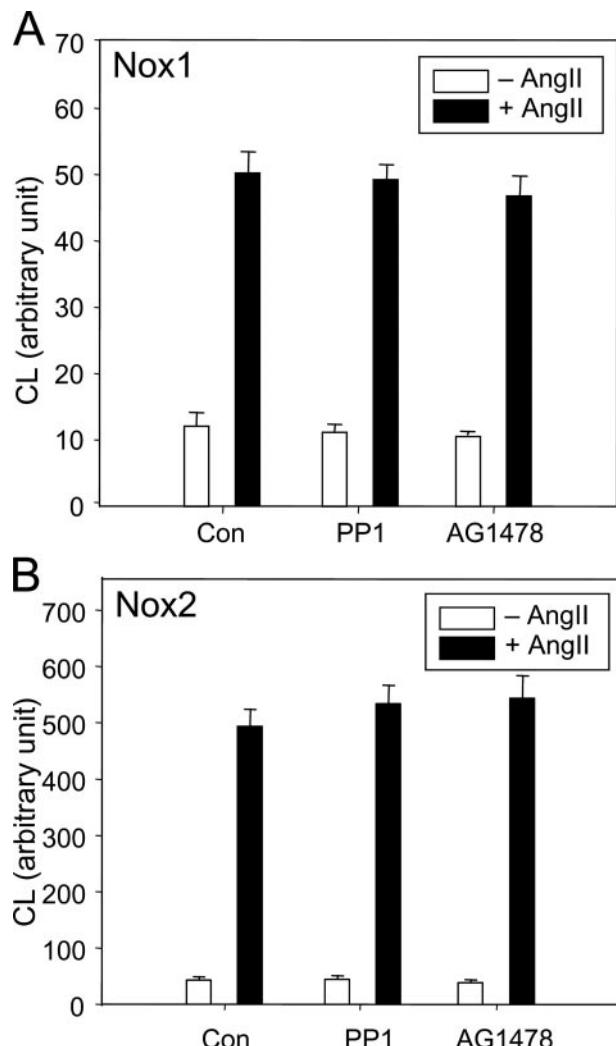


FIGURE 6. Effect of inhibition of Src or EGFR on Ang II-induced superoxide generation in HEK293 cells. Cells expressing AT₁R, Nox1, NOXO1, and NOXA1 (*A*) and cells expressing AT₁R, Nox2, p47^{phox}, and p67^{phox} (*B*) were incubated for 10 min in the absence (Con) or presence of 20 μ M PP1 or 1 μ M AG1478 and then assayed for the effect of Ang II on superoxide production. Data are the means \pm S.D. of values from three separate transfection experiments.

7, B and D). Stimulation of the EGFR-expressing HEK293 or CHO cells with EGF also elicited an \sim 3-fold increase in superoxide production, and this effect of EGF was completely inhibited by prior incubation of the cells with 1 μ M AG1478 (*right panels* of Fig. 7, *A* and *C*). Thus, it appears that both EGF- and Ang II-stimulated Nox1 activation pathways are competent in these transfected lines, although the Ang II receptor-stimulated pathway is not capable of cross-talking through the EGFR pathway, as observed in rat VSMCs (69).

Effect of Inhibition of PI 3-Kinase or Rac1 on Superoxide Generation—We next examined the effects of two PI 3-kinase inhibitors, LY294002 (IC_{50} of 1.4 μ M) and wortmannin (IC_{50} of 5 nM) (71, 72). LY294002 at saturating concentrations reduced the Ang II-induced superoxide production by \sim 20 and \sim 70%, respectively, in HEK293 cells reconstituted with the Nox1 and Nox2 components in addition to AT₁R (Fig. 8*A*). Wortmannin at saturating concentrations also inhibited the Ang II-induced

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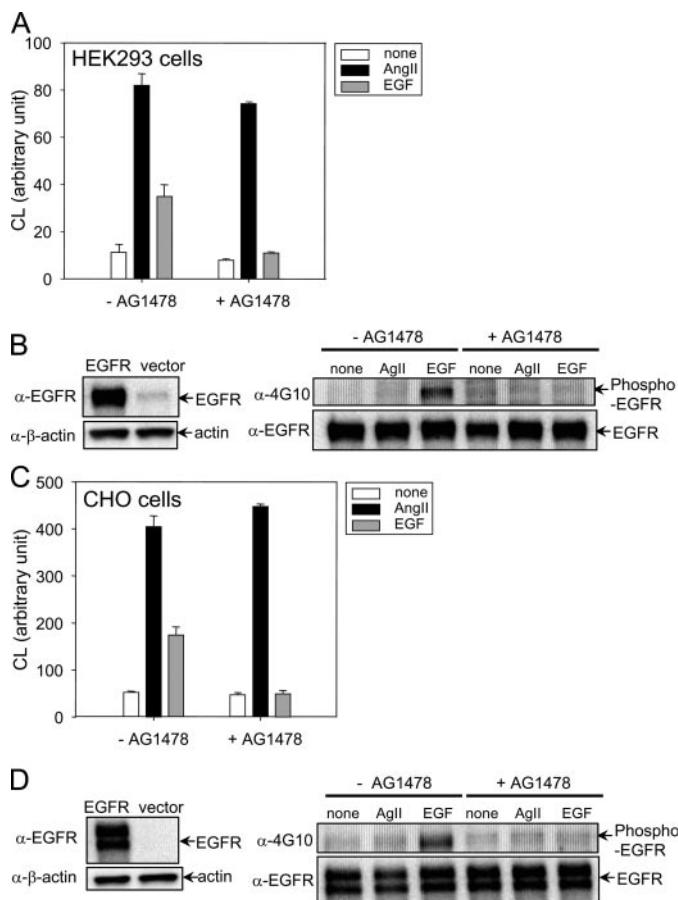


FIGURE 7. Effect of inhibition of EGFR on Ang II- or EGF-induced superoxide generation. A and C, HEK293 (A) or CHO (C) cells transfected with pcDNA3-EGFR, a vector for EGFR, in addition to vectors for AT₁R, Nox1, NOXO1, and NOXA1 were incubated in the absence or presence of 1 μ M AG1478 for 30 min and then assayed for the effect of Ang II or EGF on superoxide generation. B and D, lysates of cells transfected with pcDNA3-EGFR (EGFR) or the empty plasmid (vector) in addition to vectors for AT₁R, Nox1, NOXO1, and NOXA1 were subjected to immunoblot analysis with antibodies to EGFR or to β -actin (left panels). Lysates of the cells transfected and treated as in A and C were subjected to immunoblot analysis with antibodies to phosphotyrosine (4G10) or to β -actin (right panels). Superoxide assay results in all panels are means \pm S.D. of values from three separate transfection experiments.

superoxide production by Nox2 ($\sim 75\%$) more significantly than that by Nox1 ($\sim 20\%$) (Fig. 8B).

The possible role of Rac1 in superoxide generation was investigated by expression of dominant negative (RacN17) or constitutively active (RacV12) mutants of Rac1 in reconstituted HEK293 or CHO cells (Fig. 9). Expression of AU5 epitope-tagged Rac1 proteins was detected by immunoblot analysis with antibodies to AU5 or to Rac1 (Figs. 9, B, D, and F). Although the expression level of AU5-RacN17 was two to three times that of endogenous Rac1 in both cell types, RacN17 did not affect either basal or Ang II-induced superoxide production (Fig. 9, A, and C). Furthermore, expression of Myc epitope-tagged RacN17 also had no effect in this regard (data not shown). Expression of AU5-RacN17, however, caused $\sim 45\%$ inhibition of Ang II-induced superoxide generation in HEK293 cells reconstituted with Nox2 components (Fig. 9E). In contrast, expression of AU5-RacV12 at a level similar to that of endogenous Rac1 increased the basal production of superoxide about

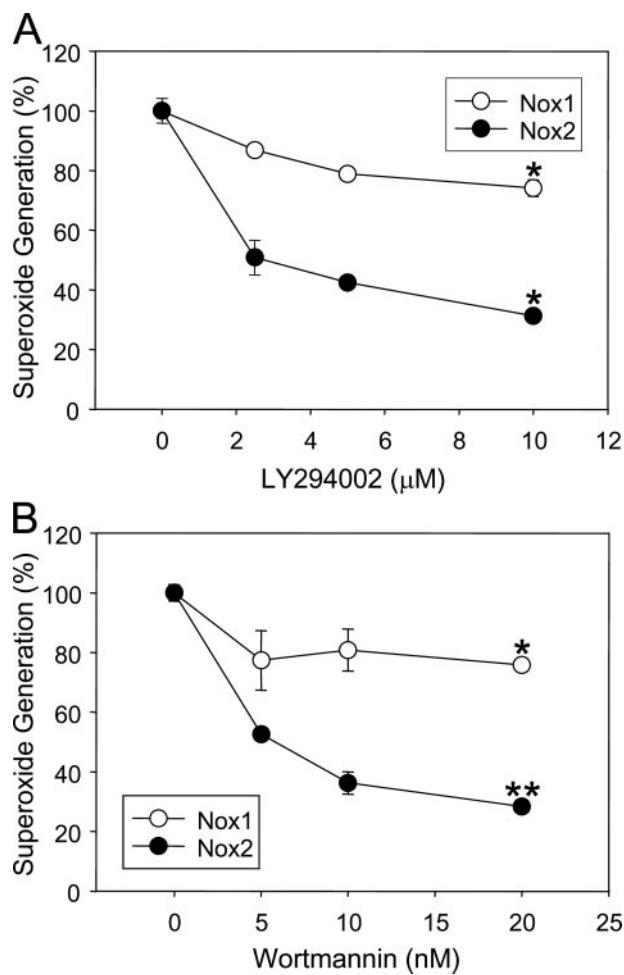


FIGURE 8. Effect of PI 3-kinase inhibition on superoxide generation in HEK293 cells. Cells expressing AT₁R, Nox1, NOXO1, and NOXA1 (open circle) or cells expressing AT₁R, Nox2, p47^{phox}, and p67^{phox} (closed circle) were incubated for 10 min in the presence of the indicated concentrations of LY294002 (A) or wortmannin (B) and were then assayed for the effect of Ang II on superoxide production. Data are expressed as a percentage of the value for cells stimulated with Ang II in the absence of inhibitor and are means \pm S.D. of values from four separate transfection experiments. $p < 0.05$ (*) and $p < 0.001$ (**) compared with no PI 3-kinase inhibitor treatment.

2-fold in both the HEK293 and CHO cells reconstituted with the Nox1 components (Fig. 9, A and C), indicating that the Nox1-NOXO1-NOXA1 system has the capacity to respond to activation by Rac1. The stimulatory effect of Ang II was not affected by expression of AU5-RacV12; the slight increase in superoxide production induced by RacV12 expression in Ang II-treated HEK293 cells was likely attributable to the increase in basal production, and a similar effect was not apparent in CHO cells likely because the production machinery was near saturation in the presence of Ang II. As reported previously (17), superoxide generation in the cells reconstituted with the Nox2 components was profoundly affected upon expression of the dominant negative or constitutively active Rac derivative (Fig. 9E).

DISCUSSION

Because the first demonstration of Ang II-induced generation of ROS in VSMCs (5), the mechanism of this effect has been of interest because it underlies many of the biological and

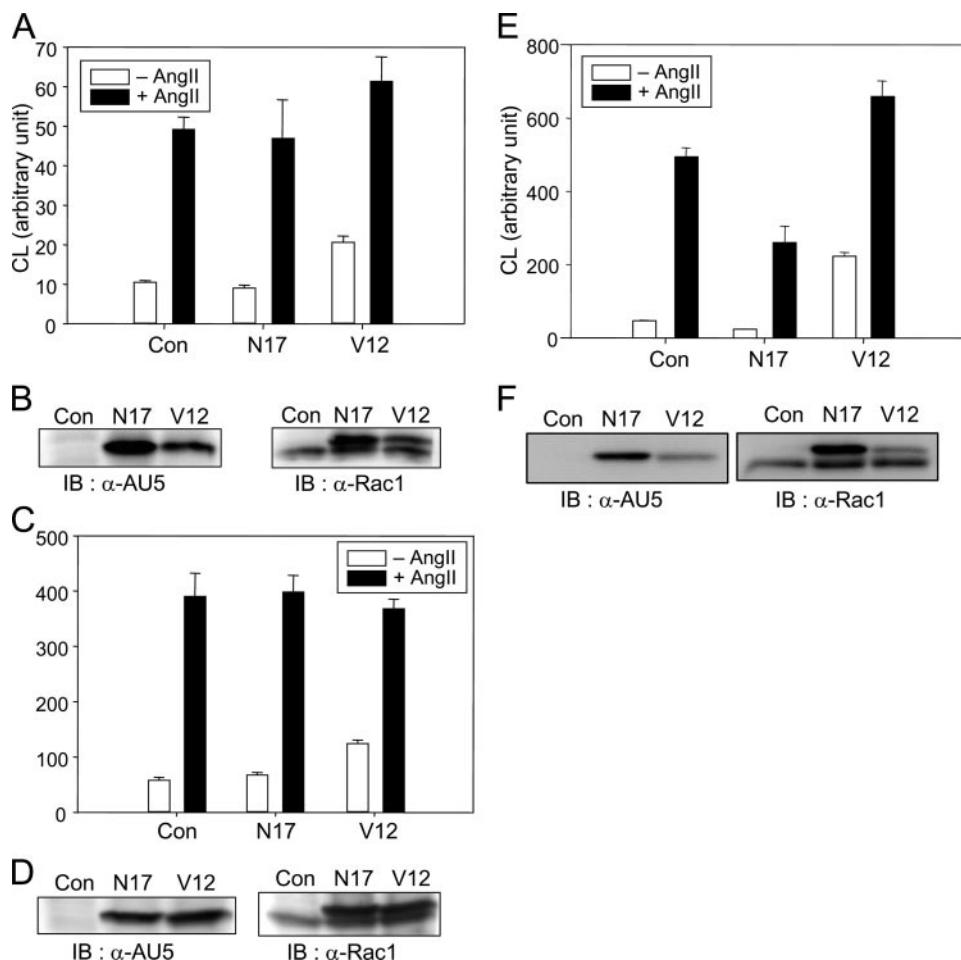


FIGURE 9. Role of Rac1 in superoxide generation. HEK293 (A and B) or CHO (C and D) cells expressing AT₁R, Nox1, NOXO1, and NOXA1 were transfected with expression plasmids for RacN17 (N17) or RacV12 (V12) or with the corresponding empty vector (Con). HEK293 cells (E and F) expressing AT₁R, Nox2, p47^{phox}, and p67^{phox} were also transfected with expression plasmids for RacN17 (N17) or RacV12 (V12) or with the corresponding empty vector (Con). The cells were then incubated in the absence or presence of Ang II and assayed for superoxide production (A, C, and E); data are the means \pm S.D. of values from three separate transfection experiments. Cell lysates were also subjected to immunoblot analysis (IB) with antibodies to AU5 or to Rac1 (B, D, and F); the results are representative of three separate experiments.

pathophysiological actions of Ang II. Various enzymes, including Nox1 (31, 33, 34), Nox2 (40, 42–44, 73), and Nox 4 (74), have been implicated in Ang II-induced superoxide production on the basis of indirect evidence. We have now shown that heterologous expression of the Nox1 components (Nox1, NOXO1, and NOXA1) or the Nox2 components (Nox2, p47^{phox}, and p67^{phox}) together with AT₁R in HEK293 or CHO cells renders them competent to produce superoxide in response to stimulation with Ang II, thereby demonstrating that both Nox1 and Nox2 are capable of mediating this effect of Ang II. The first functional reconstitution of Nox system was achieved when the Nox2 components were expressed in K562 and COS-7 cells to demonstrate PMA-dependent superoxide production (16, 75). Subsequently, expression of human Nox1 components in HEK293 cells was shown to confer both constitutive and enhanced generation of superoxide in response to PMA (20, 21), whereas the murine system exhibited only basal activity that was not further increased by cell stimulation (22). Our results confirm that PMA is indeed able to increase constitutive activity of the human Nox1 system severalfold in

HEK293 cells (Fig. 1A). The combination of NOXO1 and NOXA1 supported the Ang II-induced activation of Nox1 to a much greater extent than did the combination of p47^{phox} and p67^{phox}, whereas the opposite is true for Ang II-dependent Nox2 activation. The 4–5-fold increase in superoxide generation in response to Ang II observed in the presence of NOXO1 and NOXA1 represents the first direct demonstration of Nox1 activation in cells stimulated via any type of cell surface receptor.

β -Arrestin (56, 57) and several proteins (Jak2, PLC- γ 1, SHP2) that specifically bind to the COOH terminus of activated AT₁R (57, 59, 60) serve as transducers of Ang II signaling, which may activate signaling pathways independent of G protein activation. In addition, activation of certain signaling events mediated by G protein-coupled receptors requires receptor internalization (76). We evaluated the abilities of various AT₁R mutants defective in the interaction with transducers or in internalization to mediate Ang II-induced superoxide production in HEK293 cells expressing the Nox components. Our results indicated that coupling of AT₁R to G proteins is essential for this effect of Ang II, whereas interaction with β -arrestin, Jak2, PLC- γ 1, or SHP2 is not (Figs. 2 and 3). Furthermore, an AT₁R

mutant defective in internalization and desensitization manifested an enhanced ability to mediate Ang II-induced superoxide generation (Fig. 2). This is consistent with the role of G protein activation in ROS generation, since the same mutant also showed similarly increased Ang II-induced inositol phosphate response in previous studies (48, 77).

Our data showing that, among the various constitutively active G α subunits tested, G α _qQL conferred the highest level of superoxide production for both Nox1 and Nox2 systems (Fig. 3) are consistent with the previous observation that G α _{q/11} is the major transducer of AT₁R signaling. Expression of G α _sQL also markedly increased superoxide generation (Fig. 3). The only known effector of G α _s is adenylyl cyclase. Treatment of HEK293 cells expressing the Nox1 components with 8-bromo-cAMP, a cell-permeable analog of cAMP, had no effect on superoxide generation (data not shown), however, suggesting that the observed effect of G α _qQL was not mediated by adenylyl cyclase. Expression of G α ₁₂QL induced a smaller increase in superoxide production (Fig. 3). Effector molecules that directly interact with G α _{12/13} include several RhoGEFs, Bruton's tyro-

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sine kinase, Ras GTPase-activating proteins, and cadherin (78). It is not known whether any of these effectors mediate Nox1 activation. Given that AT₁R is able to activate Gα_{12/13} (1, 62, 79), however, it is possible that the latter mediates Ang II-induced superoxide production.

The marked increases in superoxide production induced by PMA and Gα_qQL in the reconstituted cells suggest that PKC contributes to the corresponding effect of Ang II. Inhibition of PKC with GF109203X at a maximally effective concentration reduced the effect of Ang II on Nox1-mediated superoxide production by ~60% and that on Nox2-mediated production by ~90% (Fig. 4A). The PKC inhibition reduced the effect of Gα_qQL on Nox1 activation and that on Nox2 activation by 40 to 50% (Fig. 4, B and C). These results suggest that the AT₁R-Gα_{q/11}-PLC-β-PKC pathway mediates a substantial extent of the Ang II-induced activation of both Nox1 and Nox2. Activation of Nox2 in phagocytic cells requires phosphorylation of p47^{phox} on several serine residues located in its AID and its subsequent translocation to the cell membrane (10, 11, 13, 20). Given that NOXO1 does not contain a domain equivalent to the AID of p47^{phox} (20–22), the mechanism by which PKC activates the Nox1-NOXO1-NOXA1 system remains unclear. The results with GF109203X suggest that PKC inhibition does not completely block Ang II-induced Nox1 activation. The PKC-independent Nox 1 activation pathway might be mediated by Gα_{12/13} or by Ca²⁺ mobilized as a result of AT₁R-Gα_{q/11}-PLC-β signaling. Activation of phospholipase A₂ by intracellular Ca²⁺ and consequent production of arachidonic acid contribute to Ang II-induced production of ROS in VSMCs (80). The results with GF109203X also suggest that PKC inhibition blocked less than 50% of the Gα_qQL-induced Nox1 and Nox2 activation. This result might be in connection with recent observations that Gα_{q/11} activates RhoA-mediated signaling through direct interaction with leukemia-associated RhoGEF (51, 81, 82) and the COOH-terminal Src kinase independently of PLC-β (83). Furthermore, DAG (or PMA) activates signaling through molecules other than PKC including DAG kinases, chimaerins, and Ras guanyl nucleotide-releasing proteins (84).

PLD has been implicated in Ang II-induced superoxide production in VSMCs (65). Our current findings suggest that depletion of PLD2 but not of PLD1 results in an ~20–30% reduction in the superoxide productions driven by Nox1 and Nox2 (Fig. 5). Although PLD2 is primarily localized in the plasma membrane, PLD1 is found mainly in the Golgi apparatus and perinuclear vesicle regions (66). Thus, DAG molecules produced in the plasma membrane, but not those produced in the Golgi and endoplasmic reticulum membranes, appears to be important for the production of superoxide released extracellularly. It is also possible that PLD2-derived phosphatidic acid interacts with the PX domain of the Nox organizer proteins, NOXO1 and p47^{phox} (85). It is also interesting to note that PLD2 is capable of enhancing PKCζ activity through direct interaction between the PLD2-Phox domain and the PKC kinase domain (47).

The important difference between the reconstituted cells of the present study and VSMCs concerns the role of EGFR activation. The Src-mediated activation of EGFR is essential for activation of Rac and of ROS production by Ang II in VSMCs

(69), whereas no effect of inhibition of either Src or EGFR activity on Ang II-induced ROS generation was observed in the present study (Figs. 6 and 7). Several observations might explain this difference. (i) Although Nox1 and Nox2 are expressed in VSMCs, the major Nox isozyme coupled to AT₁R in these cells remains unclear. (ii) The product of Nox activity measured in our study was superoxide released extracellularly, whereas intracellular production of ROS was monitored in VSMCs. The mechanisms of Nox activation may depend on the subcellular sites of oxidase assembly. Indeed, detailed studies on PMA-stimulated activation of the Nox2 complex in neutrophils have shown that extracellular and intracellular production of ROS is achieved by distinctly different pathways; whereas the extracellular production was insensitive to wortmannin, the intracellular production was inhibited by the PI 3-kinase inhibitor (86). (iii) The amount of superoxide produced by the reconstituted cells in the present study was much greater than that of ROS produced by endogenous Nox in VSMCs. The main component of ROS measured inside cells is H₂O₂, which is detected by its reaction with a dichlorofluorescein derivative. This reagent competes for H₂O₂ with many endogenous H₂O₂-removing enzymes and thiols, as indicated by the observation that the introduction of catalase, peroxidases, or thiols reduces fluorescence intensity. The fluorescence generated by the dye, thus, reflects only a fraction of H₂O₂ produced inside cells.

The products of PI 3-kinase modulate the function of several proteins that underlie activation of Nox2. Thus, PIP₃ is necessary for efficient activation of PKCσ, which phosphorylates p47^{phox} (87, 88); PIP₃ and PI 3,4-bisphosphate facilitate targeting of p47^{phox} to the membrane by binding to its PX domain (89); PIP₃ promotes GDP-GTP exchange on Rac by binding to the pleckstrin homology domain of certain RacGEF proteins (90). Given that the membrane localization of NOXO1 does not require 3'-phosphorylated phosphoinositides (24) and that Rac does not appear to be essential for Ang II-induced Nox1 activation, the PX domain of NOXO1 and RacGEF are less likely targets of the products of PI 3-kinase in the reconstituted cells, explaining the lower sensitivity to PI 3-kinase inhibitors of superoxide production in the Nox1-expressing cells compared with that in the Nox2-expressing cells (Fig. 8).

Assembly and activation of the Nox2-p47^{phox}-p67^{phox} complex requires at least two independent but coordinated events; that is, phosphorylation of p47^{phox} in its AID and the conversion of GDP-bound Rac2 to Rac2-GTP. Stimulus-dependent activation of Nox2 is, thus, inhibited by expression of the dominant negative Rac1 mutant RacN17 (17). Evidence suggests that Rac2-GTP interacts directly with both Nox1 and p67^{phox} in the assembled complex of Nox1, p22^{phox}, p47^{phox}, and p67^{phox} and that it facilitates electron flow from NADPH to molecular oxygen (10, 11, 13, 14, 29). The tetratricopeptide repeat in the NH₂-terminal region of p67^{phox} has been identified as the site of Rac2 binding (91). Similarly, NOXA1 associates via its NH₂-terminal tetratricopeptide repeat with Rac1-GTP (20), and Rac1 was shown to bind to an exogenously expressed COOH-terminal fragment of Nox1 in cells stimulated with platelet-derived growth factor (92).

An essential role of Rac in activation of Nox2 has been demonstrated using various cells reconstituted with the

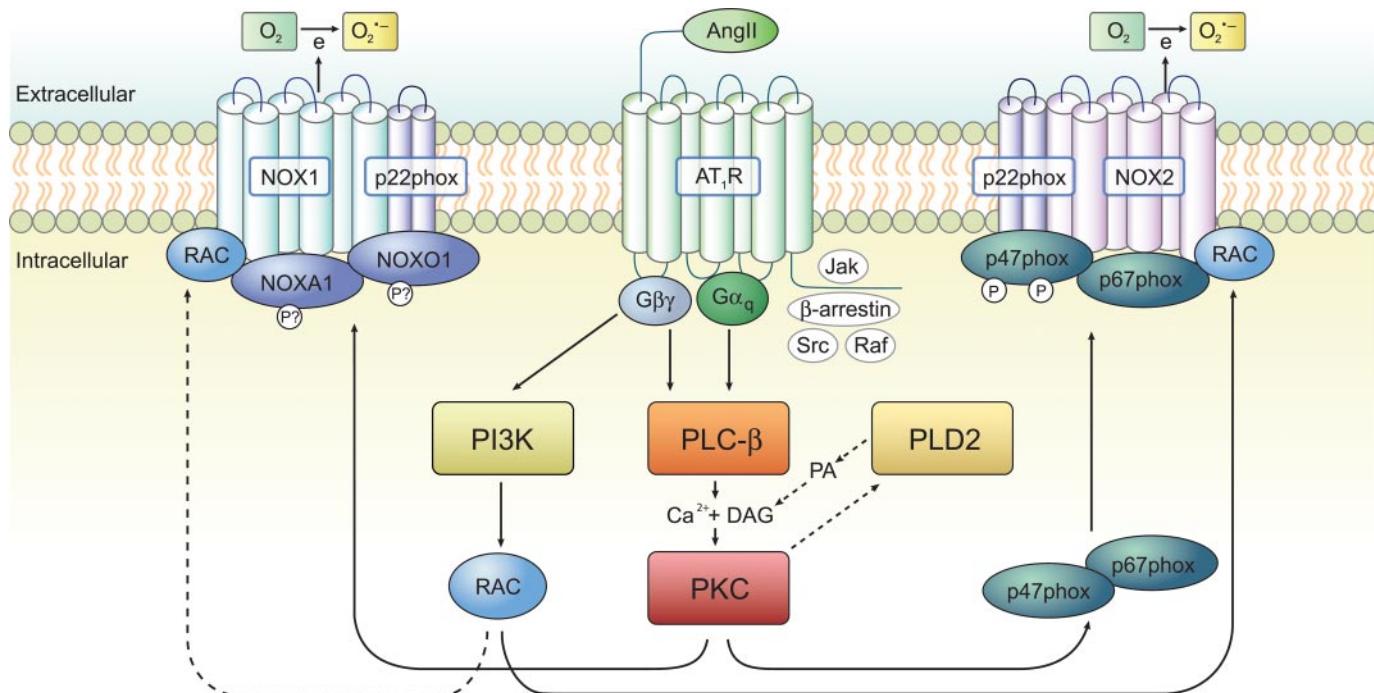


FIGURE 10. Pathways leading to AT₁R-mediated activation of Nox1 and Nox2. Although activated AT₁R elicits a number of signaling cascades by interacting with various signaling molecules such as G proteins, β -arrestin, Src, Raf, and Jak2, only the signaling pathways involving G proteins appear to be important for Ang II-dependent activation of both Nox1 and Nox2. Engagement of AT₁R enables G α_q and G $\beta\gamma$ activation of PLC- β . Production of DAG and increased intracellular calcium after the PLC- β activation triggers PKC activation. Activated PKC phosphorylates p47^{phox}, which results in the translocation of the cytosolic p47^{phox}-p67^{phox} complex to form the membrane-associated Nox2 complex. Assembly of the functional Nox2 complex requires participation of Rac, which occurs mainly as the result of G $\beta\gamma$ -dependent activation of PI 3-kinase. In the case of Nox1 complex, assembly of the membrane-associated complex does not depend on PKC, but its catalytic function is greatly enhanced as the result of PKC activation via unknown mechanism. Participation of Rac in the assembled Nox1 complex can further enhance its activity but not a critical factor as seen in Nox2 activation. PLD2 but not PLD1 contributes in some degree to the AT₁R-dependent activation of both Nox1 and Nox2. This is likely due to the fact that PLD2 activation after the PLC- β -PKC activation produces phosphatidic acid, which supplements DAG pool needed for long-term activation of PKC. Solid lines denote major pathways, whereas dotted lines indicate pathways that are not essential or contribute less significantly. IB, immunoblot; Con, empty vector.

Nox2 components (17, 91, 93). PMA-dependent activation of Nox2 is inhibited by expression of the dominant negative Rac1 mutant RacN17 (93), the constitutively active Rac mutants Rac61L and RacV12 enhance Nox2 activity in the absence of other stimulation signals, and a mutant of p67^{phox} that cannot interact with Rac as the result of a substitution of a critical residue in the tetratricopeptide repeat region does not support superoxide production by Nox2. As expected, both Ang II-dependent and -independent superoxide production in HEK293 cells reconstituted with AT₁R along with the Nox2 components were inhibited by RacN17 and activated by RacV12 (Fig. 9E).

Recent studies suggest that superoxide production by the Nox1-NOXO1-NOXA1 system is facilitated also through the Rac-NOXA1 interaction (20, 93, 94). Superoxide production in HeLa and HEK293 cells reconstituted with the Nox1 components was, however, reduced by only 50% upon a near complete depletion of Rac by RNA-mediated interference and was not affected by overexpression of the dominant negative RacN17 (93, 94), suggesting that Rac is not essential for superoxide production by the Nox1 components. In agreement with the suggestion, we found that neither Ang II-stimulated nor unstimulated superoxide production in HEK293 and CHO cells, both expressing AT₁R and the Nox1 components, was inhibited by overexpression of RacN17 (Fig. 9, A and C).

In summary, we find that the Nox1-NOXO1-NOXA1 complex as well as the Nox2-p47^{phox}-p67^{phox} complex are targets of

activation by Ang II signaling. Pathways leading to their activation are shown in Fig. 10. The activation of the Nox1 complex is largely dependent on a pathway comprising G $\alpha_{q/11}$, phospholipase C- β , and protein kinase, whereas the activation of the Nox2 complex requires additional pathways involving PI 3-kinase and Rac.

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