

α_1 -Adrenergic Receptors Activate AKT via a Pyk2/PDK-1 Pathway That Is Tonically Inhibited by Novel Protein Kinase C Isoforms in Cardiomyocytes

Jianfen Guo, Abdelkarim Sabri, Hasnae Elouardighi, Vitalyi Rybin, Susan F. Steinberg

Abstract—AKT is a potent antiapoptotic kinase, but its role in the cardioprotective actions of α_1 -adrenergic receptors (ARs) remains uncertain, because α_1 -ARs typically induce little-to-no AKT activation in most cardiomyocyte models. This study identifies a prominent α_1 -AR-dependent AKT activation pathway that is under tonic inhibitory control by novel protein kinase Cs (nPKCs) in neonatal rat cardiomyocyte cultures. We also implicate Pyk2, Pyk2 complex formation with PDK-1 and paxillin, and increased PDK-1–Y373/376 phosphorylation as the mechanism that links α_1 -AR activation to increased AKT phosphorylation. nPKCs (which are prominent α_1 -AR effectors) interfere with this α_1 -AR-dependent AKT activation by blocking Pyk2/PDK-1/paxillin complex formation and PDK-1–Y373/376 phosphorylation. Additional studies used an adenoviral-mediated overexpression strategy to show that Pyk2 exerts dual controls on antiapoptotic PDK-1/AKT and proapoptotic c-Jun N-terminal kinase (JNK) pathways. Although the high nPKC activity of most cardiomyocyte models favors Pyk2 signaling to JNK (and cardiac apoptosis), the cardioprotective actions of Pyk2 through the PDK-1/AKT pathway are exposed when PKC or JNK activation is prevented. Collectively, these studies identify JNK and AKT as functionally distinct downstream components of the α_1 -AR/Pyk2 signaling pathway. We also implicate nPKCs as molecular switches that control the balance of signaling via proapoptotic JNK and antiapoptotic PDK-1/AKT pathways, exposing a novel mechanism for nPKC-dependent regulation of cardiac hypertrophy and failure. (*Circ Res.* 2006;99:1367-1375.)

Key Words: AKT ■ PDK-1 ■ Pyk2 ■ JNK ■ cardiomyocytes ■ apoptosis

AKT is a potent antiapoptotic serine/threonine kinase that has been implicated in growth regulatory pathways in the heart. AKT is expressed as 3 isoforms (AKT1, AKT2, and AKT3) that share a similar N-terminal pleckstrin homology and C-terminal catalytic domain structure. AKT is stimulated by phosphatidylinositol 3-kinase, the enzyme that converts phosphatidylinositol 4,5-bisphosphate to phosphatidylinositol 1,4,5-trisphosphate, a lipid that anchors AKT (through its pleckstrin homology domain) to the plasma membrane. Membrane-targeted AKT is then sequentially phosphorylated at highly conserved activation loop (T308) and C-terminal hydrophobic domain (S473) phosphorylation motifs. AKT-T308 phosphorylation has been attributed to 3-phosphoinositide-dependent protein kinase-1 (PDK-1), whereas the controls of AKT-S473 phosphorylation in any particular cell type remain controversial; many enzymes with S473 kinase activity (as well as a specific AKT-S473 phosphatase) have been identified, presumably evolving to allow for stimulus- and/or cell-specific regulatory controls.^{1,2} Once activated, AKT phosphorylates a broad range of cellular substrates that are implicated in the control of cell proliferation, survival, and differentiation.

AKT has attracted considerable interest as a therapeutic target for cardiac disorders because it provides meaningful cardioprotection in both in vitro models of cardiomyocyte apoptosis and in vivo experimental models of heart failure and cardiac ischemia.³ Whereas most studies have focused on the role of the phosphatidylinositol 3-kinase/AKT pathway in the growth-promoting and prosurvival actions of tyrosine kinase and cytokine receptors, there is convincing evidence that AKT also plays a role in the β -adrenergic receptor (β -AR) pathway, leading to cardiomyocyte hypertrophy, insulin resistance, and cardioprotection.^{4,5} The literature implicating AKT as a physiologically relevant effector of other cardiac G protein-coupled receptors has generally been less persuasive. In particular, α_1 -ARs are considered to be cardioprotective, but α_1 -ARs trigger little or no increase in AKT activity in most cardiomyocyte models.⁶ Based on our previous studies demonstrating that novel protein kinase C (nPKC) isoforms (which are prominent downstream effectors of agonist-occupied α_1 -ARs) act to curtail AKT activation in cardiomyocytes,⁷ we reasoned that an α_1 -AR pathway leading to AKT activation might be dynamically regulated through

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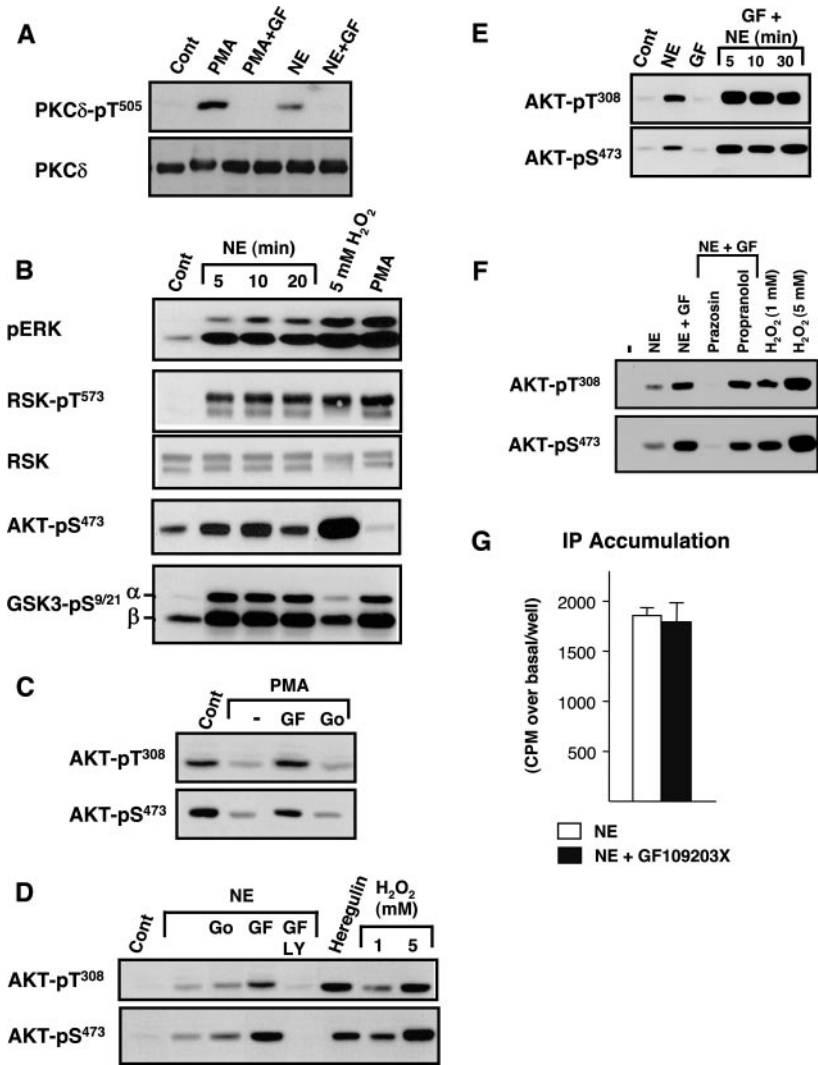


Figure 1. The effect of α_1 -ARs to activate AKT is blunted by nPKC isoforms. Cardiomyocytes were treated with vehicle, NE (10 μ mol/L, for 5 minutes unless indicated otherwise), H₂O₂ (1 or 5 μ mol/L for 15 minutes), PMA (300 nmol/L, 20 minutes), heregulin (1 nmol/L, 30 minutes), or isoproterenol (10 μ mol/L, for 5 minutes). Agonists were added following a 45-minute pretreatment with GF109203X (GF) (5 μ mol/L), Go6976 (Go) (10 μ mol/L), LY294002 (LY) (10 μ mol/L), prazosin (0.1 μ mol/L), or propranolol (1 μ mol/L) as indicated. Immunoblotting and measurements of inositol phosphate accumulation were according to Materials and Methods.

both stimulatory and inhibitory inputs. This study provides novel evidence that α_1 -ARs are hardwired to an AKT activation pathway involving the activation of Pyk2, the formation of a Pyk2/PDK-1/paxillin complex, and PDK-1-Y373/376 phosphorylation, but this pathway is exposed in cardiomyocytes only when nPKC isoforms are inhibited.

Materials and Methods

The preparation of neonatal rat ventricular cardiomyocyte cultures, measurements of inositol phosphate accumulation, and immunoblotting (on lysates or immunoprecipitated proteins) were performed according to methods described previously or according to the instructions of the manufacturer.^{7,8} All antibodies were from Cell Signaling Technology with the following exceptions: anti-PKC δ (Santa Cruz Biotechnology); anti-pY (Upstate Biotechnology); anti-Pyk2 (BD Transduction Laboratories); and anti-paxillin and anti-paxillin-pY31 (BioSource). In immunoblotting experiments, all panels in each figure are from a single gel (exposed for a uniform duration); detection was with enhanced chemiluminescence. All results were replicated in at least 4 experiments on separate culture preparations. Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining was performed for detection of apoptotic cells according to the instructions of the manufacturer (Boehringer).

Results

nPKCs Limit α_1 -AR Activation of AKT

Figure 1A shows that norepinephrine (NE) and 4-phorbol myristate 13-acetate (PMA) exert similar effects to activate PKC δ (tracked by the increase in PKC δ -T505 phosphorylation that accompanies PKC δ translocation to membranes and is required for optimal PKC δ activation⁸). NE and PMA also activate the extracellular signal-regulated kinase (ERK)/p90 ribosomal S6 kinase pathway and increase glycogen synthase kinase (GSK)-3 phosphorylation (Figure 1B). In contrast, AKT regulation by NE and PMA is discordant; AKT phosphorylation is increased by NE and suppressed by PMA. Figure 1C shows that the effect of PMA to inhibit AKT phosphorylation is abrogated when cultures are pretreated with GF109203X (a conventional PKC and nPKC isoform inhibitor), but not Go6976 (a selective conventional PKC inhibitor, which does not inhibit PDK-1 under our experimental conditions; Figure I in the online data supplement, available at <http://circres.ahajournals.org>). These results confirm our previous findings that AKT signaling is tonically inhibited by a nPKC isoform.⁷

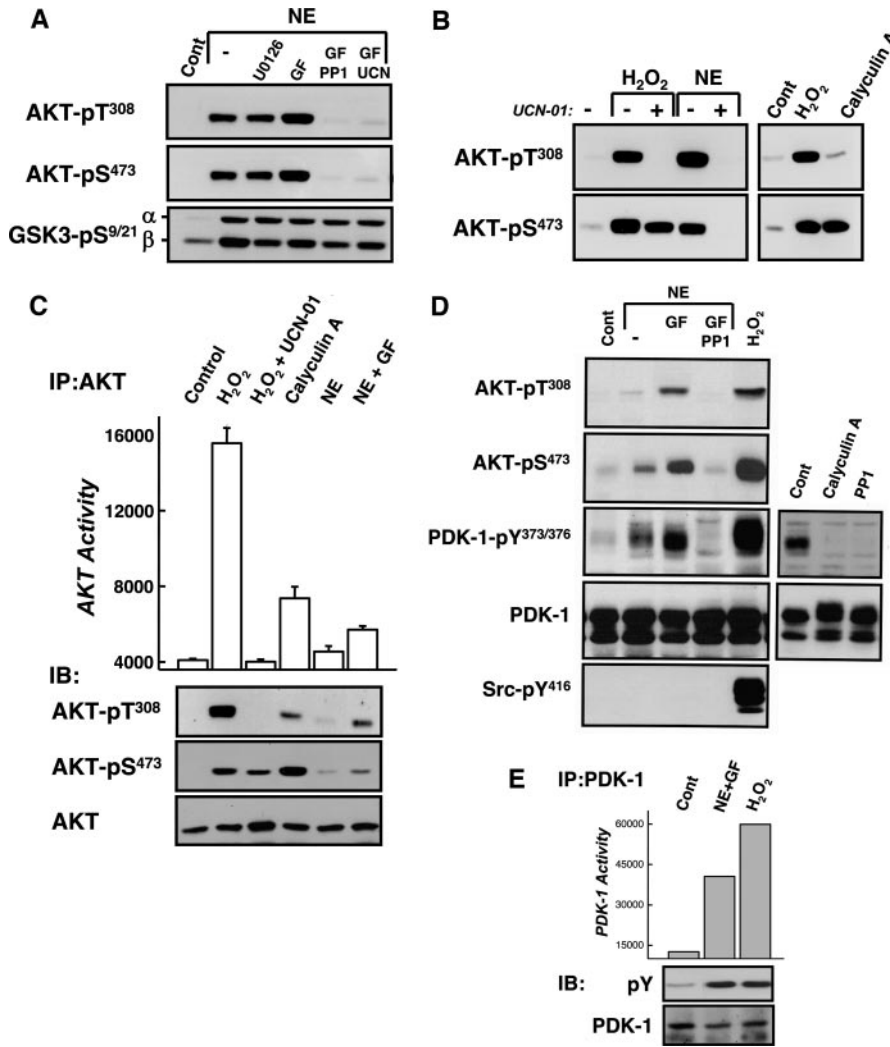


Figure 2. α_1 -ARs activate AKT via tyrosine phosphorylation of PDK-1. Cardiomyocytes were pretreated for 45 minutes with vehicle, PP1 (10 μmol/L) (A and D), UCN-01 (1 μmol/L) (A and B), U0126 (5 μmol/L) (A), or GF109203X (GF) (5 μmol/L) (A and D) and then stimulated with NE (10 μmol/L), H₂O₂ (5 mmol/L), or calyculin A (0.1 μmol/L), each for 15 minutes, as indicated. Cell extracts were subjected to immunoblotting (A, B, and D), immunoprecipitation with anti-AKT antibodies and immunocomplex kinase assays with AKT/SGK substrate peptide (Upstate Biotechnology) (C) or immunoprecipitation with anti-PDK-1 antibodies followed by immunoblotting for PDK-1 tyrosine phosphorylation and protein recovery and immunocomplex kinase assays using PDK-1 autophosphorylation as a measure of PDK-1 activity (E).

Reasoning that nPKCs are downstream components of the NE-activated α_1 -ARs, we tested the hypothesis that nPKCs might act to curtail NE-dependent AKT activation. Figure 1D and 1E shows that NE induces a relatively modest increase in AKT phosphorylation (relative to the considerably more robust response triggered by heregulin or H₂O₂) and that NE-dependent AKT activation is markedly augmented (approaching levels in heregulin- or H₂O₂-treated cultures) when cultures are pretreated with GF109203X, but not Go6976. Figure 2A shows that NE-dependent AKT activation is not influenced by U0126, effectively excluding a potential regulatory role for ribosomal S6 kinase (a GF109203X-sensitive enzyme and downstream target of the α_1 -AR/nPKC signaling pathway⁹). The effect of NE+GF109203X to increase AKT phosphorylation is sustained for at least 30 minutes; GF109203X alone does not significantly alter basal AKT phosphorylation (Figure 1E). Because NE is a mixed α_1 -/ β_1 -AR agonist, and AKT has been identified as a downstream effector of both α_1 - and β -ARs, it was important to identify the AR subtype that mediates NE-dependent AKT activation. Figure 1F and supplemental Figure II show that NE activates AKT in both GF109203X- and vehicle-treated cultures via an α_1 -AR pathway that is blocked by the α_1 -AR antagonist

prazosin, but not the β -AR blocker propranolol. NE-dependent AKT phosphorylation is via the canonical phosphatidylinositol 3-kinase pathway (which is blocked by the phosphatidylinositol 3-kinase inhibitor LY294002; Figure 1D).

As an initial attempt to expose the nPKC-sensitive target(s) in the α_1 -AR/AKT activation pathway, we examined the effect of GF109203X on α_1 -AR-dependent phospholipase C activation. Figure 1G shows that the effect of NE to promote inositol phosphate accumulation is identical in vehicle- and GF109203X-treated cultures, effectively excluding a trivial explanation of our findings, namely that GF109203X augments α_1 -AR-dependent AKT phosphorylation by blocking PKC-dependent α_1 -AR desensitization. Rather, these results localize the nPKC-sensitive target to a more distal component of the α_1 -AR/AKT activation pathway.

α_1 -ARs Increase AKT Phosphorylation in GF109203X-Treated Cardiomyocytes by Activating PDK-1

We used a pharmacological approach to define the molecular components of the pathway linking α_1 -ARs to AKT phosphorylation. Figure 2A shows that the effects of NE+GF109203X to increase AKT-T308 and AKT-S473

phosphorylation are blocked by PP1 (a general Src nonreceptor tyrosine kinase inhibitor) and UCN-01 (a 7-hydroxystaurosporine derivative that is a relatively selective inhibitor of PDK-1¹⁰). Of note, NE also increases GSK-3 phosphorylation, but this response is not augmented by GF109203X or blocked by UCN-01. These results indicate that NE increases GSK-3 phosphorylation via a mechanism that does not involve AKT phosphorylation. This could involve a PKC-dependent pathway, because PMA increases GSK-3 phosphorylation without activating AKT (Figure 1B).

Figure 2A shows that α_1 -ARs increase AKT-T308 and -S473 phosphorylation in a coordinated manner, consistent with the prevailing model of AKT phosphorylations as coordinated events that synergistically activate the enzyme. In contrast, the H₂O₂-dependent increases in AKT-T308 and -S473 phosphorylation are via distinct mechanisms; UCN-01 fully abrogates the H₂O₂-dependent increase in AKT-S308 phosphorylation, but UCN-01 induces only a relatively minor reduction in H₂O₂-dependent AKT-S473 phosphorylation (Figure 2B). Calyculin A (an equipotent PP1 and PP2A inhibitor) also preferentially increases AKT-S473 phosphorylation, in association with a considerably more modest increase in phospho-AKT-T308. These results identify AKT dephosphorylation at T308 and S473 as uncoupled events (which is consistent with the emerging model that implicates phosphatases, rather than kinases, in the divergent controls of AKT-T308 and -S473 phosphorylation²). These actions of calyculin A are quite distinct from the cellular actions of NE (which increases AKT phosphorylation at both T308 and S473), suggesting that the effect of α_1 -ARs to activate AKT cannot be attributed to the activation of a calyculin A-sensitive phosphatase. Importantly, Figure 2C shows that the T308/S473-phosphorylated enzyme recovered from H₂O₂-treated cardiomyocytes is highly active, whereas H₂O₂+UCN-01 selectively increases AKT-S473 phosphorylation without increasing AKT activity. Calyculin A also markedly increases AKT-S473 phosphorylation in association with a relatively modest increase in AKT-T308 phosphorylation and AKT activity (relative to the high level of activity recovered from H₂O₂-treated cultures). Similar results have been obtained previously in only overexpression models, where there is evidence that full AKT catalytic competence requires phosphorylation at both T308 and S473 (indicating that stimulus-specific differences in AKT-T308 and -S473 phosphorylation are functionally important¹¹). Finally, Figure 2C shows that the GF109203X-dependent increase in NE-dependent AKT phosphorylation results in enhanced AKT activity.

Studies with UCN-01 provided a rationale to examine whether α_1 -ARs activate AKT through a mechanism involving PDK-1. PDK-1 was initially described as a constitutively active enzyme that is anchored to membranes and not regulated by growth factor-activated signaling pathways. However, recent studies have exposed PDK-1 regulation through stimulus-induced changes in its conformation, phosphorylation, and/or subcellular localization within cells. In particular, there is recent evidence that the intrinsic catalytic activity of PDK-1 is regulated through tyrosine phosphorylation. PDK-1 tyrosine phosphorylation is an ordered process;

PDK-1-Y9 phosphorylation (by a PP1-insensitive protein kinase) generates a consensus sequence for the SH2-domain of Src, leading to the recruitment of Src and Src-dependent PDK-1-Y373/376 phosphorylation (which increases PDK-1 activity¹²). We used an immunologic approach with anti-PDK-1-pY373/376 phosphorylation site-specific antibodies to determine whether PDK-1-Y373/376 phosphorylation contributes to NE-dependent AKT activation. Figure 2D shows that cardiomyocytes express two molecular forms of PDK-1. PDK-1-pY373/376 immunoreactivity is detected at very low levels (as a single band that comigrates with the larger PDK-1 isoform) in unstimulated cultures. Similar molecular heterogeneity of PDK-1 isoform expression has been identified in mouse tissues, where alternative splicing is believed to generate a smaller PDK-1 splice variant that lacks the tyrosine corresponding to Y9 of human PDK-1 and is not regulated through tyrosine phosphorylation.¹³ Basal PDK-1-Y373/376 phosphorylation is increased by H₂O₂ and suppressed by PP1 and calyculin A; calyculin A also decreases PDK-1 mobility in SDS-PAGE, indicative of serine/threonine phosphorylation. NE alone induces a modest increase in PDK-1-pY373/376 immunoreactivity; NE markedly increases PDK-1-pY373/376 immunoreactivity (to a level comparable to the stimulatory effect of H₂O₂) when cells are pretreated with GF109203X to inhibit PKC isoforms. Figure 2E shows that the NE+GF109203X- and H₂O₂-dependent increases in PDK-1-pY373/376 immunoreactivity are associated with increased PDK-1 activity. Finally, Figure 2D shows that the NE+GF109203X-dependent increase in PDK-1-Y373/376 phosphorylation is abrogated by PP1, but NE (alone or with GF109203X) does not increase Src activation loop Y416 phosphorylation (which accompanies, and provides a surrogate measure of, Src activation). H₂O₂ markedly increases Src-pY416 immunoreactivity and serves as a control in these experiments.

α_1 -ARs Activate Pyk2 and Promote Pyk2/PDK-1/Paxillin Complex Formation in GF109203X-Treated Cardiomyocytes

Because PDK-1-Y373/376 phosphorylation could not be linked to Src activation, we considered a potential role for Pyk2, another nonreceptor protein tyrosine kinase with scaffolding function that has been recovered in PDK-1/Src complexes and has been implicated in a Src-dependent PDK-1-Y373/376 phosphorylation pathway.¹⁴ Pyk2 activation is through a tyrosine phosphorylation-dependent mechanism that is tracked by immunoprecipitating active Pyk2 using an anti-pY antibody followed by Western blotting with anti-Pyk2 antibodies. Figure 3 shows that NE increases Pyk2 tyrosine phosphorylation at 1 minute and that this response is sustained for at least another 20 minutes of agonist stimulation. The effect of NE to activate Pyk2 is relatively specific; Pyk2 activation by thrombin (an agonist for PAR-1, another Gq-coupled receptor) is at the limits of detection, and NE does not significantly increase the tyrosine phosphorylation of FAK (a structurally related nonreceptor tyrosine kinase with distinct signaling functions). NE-dependent Pyk2 phosphorylation is mediated by an α_1 -AR-dependent pathway (blocked by prazosin, and not propranolol) and a PP1-

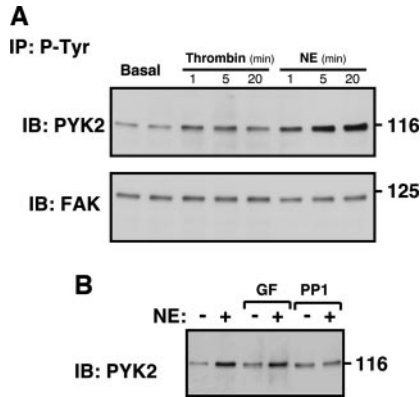


Figure 3. α_1 -ARs activate Pyk2 via a PKC-independent pathway. Cardiomyocytes were treated with thrombin (1 U/mL) or NE (10 μ mol/L) for the indicated intervals (A); stimulations followed a 45-minute pretreatment with vehicle, GF109203X (5 μ mol/L), or PP1 (10 μ mol/L) (B). Extracts were immunoprecipitated with anti-pY antibodies followed by immunoblotting for Pyk2 or FAK.

sensitive kinase (Figure 3B and data not shown). Although PKC isoforms have been implicated in some Pyk2 activation pathways (in response to other stimuli and in other cell types), the α_1 -AR-dependent Pyk2 activation pathway in cardiomyocytes is not PKC dependent (ie, is not inhibited by GF109203X).

Further studies using an immunoprecipitation approach identified Pyk2/PDK-1 complex formation as a GF109203X-sensitive component of the α_1 -AR signaling pathway. Figure 4A shows that (1) PDK-1 constitutively interacts with tyrosine phosphorylated proteins that migrate with apparent molecular weights of \approx 116 and 68 kDa; the 68-kDa protein is best detected with longer exposures of the gel; (2) these interactions are increased by NE+GF109203X, but not by NE alone; and (3) NE+GF109203X does not lead to the appearance of new PDK-1 binding partners. H₂O₂ treatment (which was used as a control and is depicted as a much shorter exposure of the gel) promotes even greater PDK-1 complex formation with the 116- and 68-kDa proteins (and the de novo appearance of a \approx 75-kDa tyrosine phosphorylated protein in PDK-1 pulldowns).

Tyrosine phosphorylated PDK-1-binding partners were identified using a candidate immunoblotting approach based on their mobilities in SDS-PAGE. Figure 4B shows that the 116- and 68-kDa tyrosine phosphorylated proteins recovered in PDK-1 pulldowns comigrate with Pyk2 and paxillin (a cytoskeletal linker protein with adapter function¹⁵). Pyk2 and paxillin interact with PDK-1 constitutively (at low levels) in resting cardiomyocytes. NE+GF109203X and H₂O₂ (but not NE alone) increase the recovery of PDK-1/PYK2 and PDK-1/paxillin complexes. Figure 4C shows that NE promotes a modest increase in paxillin phosphorylation at Y31 (a tyrosine residue that contribute to the adapter function of paxillin¹⁵). NE also decreases paxillin-pY31 mobility in SDS-PAGE. GF109203X blocks the NE-dependent Pyk2 band shift (without influencing the NE-dependent increase in paxillin-pY31 phosphorylation), consistent with previous evidence that the paxillin mobility shift is attributable to serine phosphorylation.

α_1 -ARs Activate c-Jun N-terminal Kinase via a PKC-Dependent Mechanism

Pyk2 is reported to activate c-Jun N-terminal Kinase (JNK) in neonatal rat cardiomyocytes and other cell types.^{16,17} Because JNK can act as a positive modulator of AKT (by phosphorylating AKT-T450, a modification that is believed to substitute for S473 phosphorylation and “prime” AKT for subsequent PDK-1-dependent T308 phosphorylation¹⁸), we examined whether JNK might be another GF109203X-sensitive target of the α_1 -AR. Figure 5A shows that NE increases the phosphorylation of 2 major JNK isoforms (p54/p46-JNK) and that this response is blocked by GF109203X and SP600125 (a JNK inhibitor), but not by U0126 (an inhibitor of ERK). Of note, SP600125 also blocks the NE-dependent increase in AKT phosphorylation (Figure 5B). This is not caused by a nonspecific effect of SP600125 on the ERK signaling cascades, because SP600125 does not block ERK activation (and AKT phosphorylation is not blocked when the ERK pathway is interrupted by U0126). SP600125 does not block H₂O₂-dependent AKT phosphorylation, arguing against a nonspecific effect on PDK-1 (or a toxic effect of the inhibitor). Collectively, these results

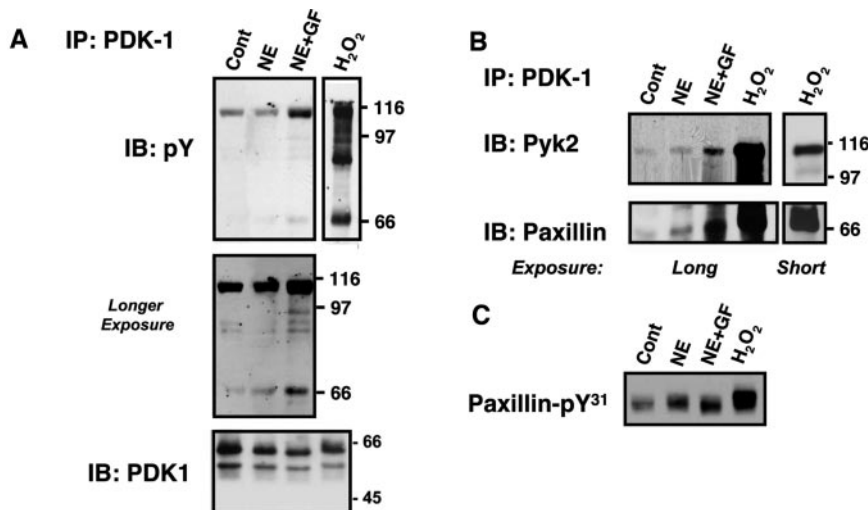


Figure 4. α_1 -ARs promote Pyk2/PDK-1/paxillin complex formation when PKC activity is blocked. Drug treatments were as in Figure 2. A and B, Cell extracts were subject to immunoprecipitation with anti-PDK-1 antibodies followed by immunoblotting with antibodies to phospho-tyrosine (with a short exposure presented for the H₂O₂-treated sample), Pyk2, paxillin, or PDK-1 (to verify equal protein recovery and loading). C, Anti-paxillin-Y31 immunoblotting on cell extracts.

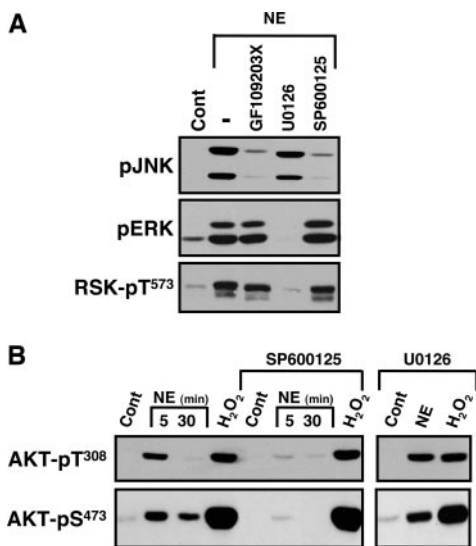


Figure 5. α_1 -AR activation of AKT and JNK in cardiomyocytes with intact PKC activity. Immunoblots of cell extracts from cardiomyocytes pretreated with GF109203X (5 μ mol/L), U0126 (5 μ mol/L), or SP600125 (10 μ mol/L) followed by stimulation with vehicle, NE (10 μ mol/L), or 5 mmol/L H_2O_2 for 10 minutes, unless otherwise indicated.

identify a PKC-dependent pathway that links α_1 -ARs to the activation of JNK and a modest increase in AKT activity in cells with intact PKC activity.

AKT and JNK Are Downstream Effectors of Pyk2

We previously used an adenoviral overexpression strategy to link Pyk2 activation to cardiomyocyte apoptosis.¹⁹ Although our previous studies identified JNK as a downstream component of the cardiac Pyk2-activated signaling pathway, a role for JNK as a proapoptotic effector of Pyk2 (or AKT as an additional Pyk2 effector) was not considered.

Figure 6 shows that Pyk2 overexpression results in a pronounced increase in phospho-JNK (which is not further increased by NE or H_2O_2). Pyk2 overexpression also increases phospho-AKT (particularly at T308) in association with an increase in PDK-1 tyrosine phosphorylation). The Pyk2-dependent increase in AKT phosphorylation represents a submaximal response; AKT phosphorylation is increased further when Pyk2-overexpressing cultures are treated with NE or H_2O_2 . These results suggest that the actions of Pyk2, on balance, favor signaling to the JNK pathway (ie, the Pyk2-dependent pathway leading to JNK activation dominates over the AKT activation pathway). Pyk2 overexpression does not lead to ERK activation (which serves as a control in these experiments).

The autophosphorylation site (Y402F-substituted) Pyk2 mutant provides a strategy to expose Pyk2 signaling events mediated through Src,¹⁶ because the Y402F substitution prevents the bimolecular Pyk2-Y402 transautophosphorylation that generates a docking site for the Src-SH2 domain, leading to Src-dependent Pyk2-Y579/580 phosphorylation/activation. Figure 6A shows that Pyk2-Y402F does not increase JNK or AKT phosphorylation.

We previously demonstrated that Pyk2 overexpression leads to a decrease in the abundance of paxillin (and other

focal adhesion-associated proteins), a loss of myofibrillar organization at peripheral contacts, and pronounced cellular apoptosis.¹⁹ Of note, these previous studies were performed on subconfluent cardiomyocyte cultures maintained in serum for a relatively short (18-hour) interval before adenoviral infections.¹⁹ This study was performed on confluent cultures maintained in serum for 4 days before initiating adenoviral infections and other experimental protocols. These more resilient cultures tolerate Pyk2 overexpression with only modest changes in cell morphology (and a more modest increase in TUNEL staining indicative of apoptosis; Figure 6D). Under these conditions, Pyk2 overexpression does not lead to a gross change in paxillin protein levels or basal paxillin-Y31 phosphorylation. Nevertheless, the NE-dependent increment in paxillin-Y31 phosphorylation is markedly exaggerated in Pyk2-overexpressing cultures (Figure 6A).

We used a pharmacological approach to further explore the role of JNK and AKT in Pyk2 responses. Figure 6C provide unanticipated evidence that chronic UCN-01 treatment increases JNK phosphorylation and induces a modest increase in TUNEL staining (indicative of cardiomyocyte apoptosis) in β -galactosidase (β -gal)-overexpressing cultures (Figure 6C and 6D). Whereas β -gal-overexpressing cultures remain grossly viable for 48 hours in the presence of UCN-01, the effect of UCN-01 to abrogate Pyk2-dependent AKT-T308 phosphorylation (which limits AKT activation) is associated with a marked increase in apoptosis (in association with a drop in paxillin protein recovery) in Pyk2-overexpressing cardiomyocyte cultures with high JNK activity. These cytotoxic actions of UCN-01 in Pyk2-overexpressing cardiomyocytes are reminiscent of the proapoptotic actions of UCN-01 in human leukemia cells, where UCN-01 induces apoptosis by synergistically inhibiting cytoprotective actions of AKT and stimulating proapoptotic actions of JNK.²⁰ In contrast, SP600125 prevents the Pyk2-dependent increase in JNK phosphorylation without blocking AKT; SP600125 prevents apoptosis in Pyk2-overexpressing cultures in association with an increase in paxillin protein abundance. These results identify Pyk2 as a kinase with dual roles in both cytoprotective and proapoptotic mechanisms (through AKT and JNK, respectively).

Discussion

AKT has garnered considerable interest over the past decade as a cardioprotective enzyme that inhibits apoptosis and preserves (and in some cases actually improves) cardiac function in clinically relevant cardiac disease models.³ Although there is ample evidence that various cardioprotective stimuli (such as growth factor and cytokine receptors) activate AKT, the role of AKT as a functionally important α_1 -AR effector has remained uncertain. Indeed, we recently identified increased AKT phosphorylation in transgenic mice with cardiac-restricted α_{1A} -AR overexpression,²¹ but most studies in cardiomyocyte cultures fail to identify α_1 -AR-dependent activation of AKT. This study provides novel evidence that α_1 -ARs are hardwired to an AKT activation pathway that is mediated by a novel mechanism involving the formation of a multiprotein Pyk2/paxillin/PDK-1 complex and PDK-1-

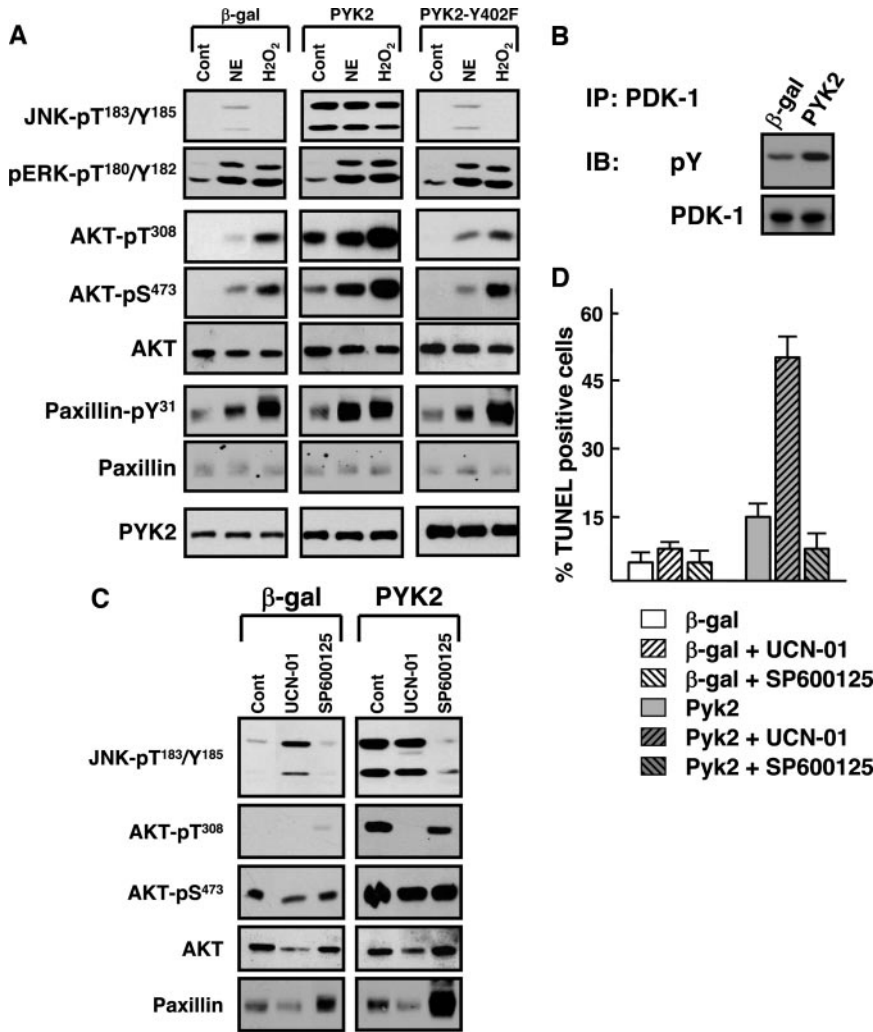


Figure 6. PYK2 overexpression activates JNK and AKT and increases paxillin-pY31 phosphorylation. Adenoviral-mediated gene transfer was used to overexpress wild-type Pyk2, Pyk2-Y402F, and β -gal as a control. A, Immunoblot analysis of total cell lysates treated for 10 minutes with vehicle, NE (10 mmol/L), or H_2O_2 (5 mmol/L) to compare ERK, JNK, and AKT activation and paxillin-pY31 phosphorylation. With regard to immunoblotting for JNK, it is worth noting that (1) the NE-dependent increase in JNK phosphorylation in Ad- β -gal cultures appears modest because all results (for Ad- β -gal, Ad-Pyk2, and Ad-Pyk2-Y402F) are depicted at identical exposure times: NE-dependent JNK activation is robust with longer exposures. (2) H_2O_2 (0.1 mmol/L) activates JNK, but 5 mmol/L H_2O_2 (which is used in these experiments) does not. B, Cardiomyocyte extracts were subjected to immunoprecipitation with anti-PDK-1 antibodies followed by immunoblotting with anti-pY and anti-PDK-1. C and D, Pyk2 overexpression was in the presence of UCN-01 (1 μ mol/L) or SP600125 (10 μ mol/L) followed by immunoblotting to compare JNK and AKT activity, as well as AKT and paxillin protein abundance and imaging by fluorescence microscopy (300 to 500 cardiomyocytes per condition) to score cells undergoing apoptosis as TUNEL positive. Pyk2 overexpression increases apoptosis; UCN-01 increases (and SP600125 decreases) apoptosis in Pyk2-overexpressing cultures. * $P < 0.05$ by paired t test with Bonferroni correction for multiple comparisons.

Y373/376 phosphorylation and that this pathway is blocked by nPKC isoforms (which are prominent components of the α_1 -AR signaling pathway); nPKCs prevent Pyk2/paxillin/PDK-1 complex formation and PDK-1-Y373/376 phosphorylation (Figure 7). This study also identifies a PKC-dependent α_1 -AR pathway that activates JNK; we show that JNK (or another SP600125-sensitive kinase) contributes to the low level of α_1 -AR-dependent AKT activation in cardiomyocytes with intact PKC activity. Finally, we identify Pyk2 as an α_1 -AR effector with dual functions to activate both

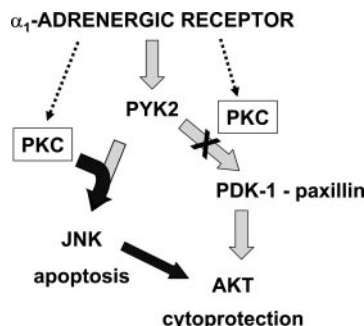


Figure 7. Schematic of the pathway linking α_1 -ARs to AKT. See text.

proapoptotic JNK and antiapoptotic AKT pathways. Although the robust PKC activity characteristic of most cardiomyocyte models favors Pyk2 signaling to JNK (rather than AKT, likely explaining the general designation of Pyk2 as a proapoptotic cardiac enzyme), this study identifies a more versatile and contextual role for Pyk2, with Pyk2 function dictated by conditions (or maneuvers) that alter PKC activity (and thereby the balance of signaling via JNK and AKT pathways).

These studies link α_1 -ARs to a PP1/UCN-01-sensitive mechanism involving Pyk2 and PDK-1 that leads to a coordinate increase in AKT-T308 and -S473 phosphorylation (in GF109203X-treated cultures); this suggests that PDK-1 or T308-phosphorylated AKT is the physiologic AKT-S473 kinase under these conditions. In contrast, H_2O_2 and calyculin A influence AKT-T308 and -S473 phosphorylation via divergent regulatory controls. The effect of calyculin A to increase AKT-S473 phosphorylation in association with only a minor increase in AKT-T308 phosphorylation was not predicted based on (1) early studies implicating PP2A as a physiologically relevant AKT-T308 and -S473 phosphatase²² and (2) more recent studies showing that PP2A dephosphorylates AKT at T308 and a different calyculin A-insensitive pleckstrin homology domain-containing leucine-rich repeat pro-

tein phosphatase dephosphorylates AKT at S473.² However, our studies exposed an additional effect of calyculin A to decrease PDK-1–Y373/376 phosphorylation; a fall in PDK-1–Y373/376 phosphorylation might be predicted to decrease basal PDK-1 activity and limit PDK-1-dependent AKT-T308 phosphorylation, providing a plausible explanation for the preferential increase in phospho-AKT–S473 in calyculin A–treated cells. Importantly, *in vitro* kinase assays demonstrate that AKT–S473 phosphorylation alone (in H₂O₂+UCN-01–treated cultures) is not sufficient to increase native AKT enzyme activity. These results are consistent with previous evidence that heterologously overexpressed AKT-T308A does not function as an active kinase *in vivo* in cells (and that phosphorylation at both T308 and S473 act synergistically to activate the enzyme¹¹). Collectively, these results identify important stimulus-specific differences in the regulatory controls of AKT-T308 and S473 phosphorylation that have functional consequences. It is important to note that current concepts regarding AKT activation mechanisms (and the role of AKT in cardioprotection) are based largely on studies that rely on changes in AKT-pS473 immunoreactivity as the sole criteria for AKT activation (because AKT-T308 and S473 phosphorylation have generally been viewed as tightly coupled events and highly specific/sensitive anti-AKT-T308 phosphorylation site–specific antibodies were not commercially available). Results reported herein emphasize the limitations of this approach and the importance of more comprehensive analyses of AKT activation.

Although PKC isoforms and the ERK/mitogen-activated protein kinase cascade have been the focus of most studies exploring α_1 -AR–dependent mechanisms that trigger cardiac remodeling, this study identifies an additional α_1 -AR–dependent pathway involving Pyk2 and JNK in neonatal rat cardiomyocyte cultures. It is interesting to note that an α_1 -AR–dependent pathway leading to JNK activation is not detected in adult cardiomyocytes with exceedingly low levels of Pyk2 expression.^{23,24} These results could be construed as evidence that age- or disease-dependent differences in Pyk2 expression (as occurs in pressure overload hypertrophy²⁴) might play a functionally important role to calibrate the α_1 -AR–dependent JNK activation pathway. With regard to PKC isoforms (which also are regulated in models of cardiac hypertrophy and failure), we show that PKC activity is required for α_1 -AR activation of JNK activation, whereas it is dispensable for α_1 -AR activation of Pyk2. This result was somewhat surprising based on previous evidence that PMA treatment or PKC α overexpression activates Pyk2 (and phorbol ester–sensitive PKCs link endothelin receptors to Pyk2 activation) in a similar cardiomyocyte culture model.^{25,26} However, the cardiac α_1 -AR/Pyk2 pathway has not previously been scrutinized, and there is ample precedent for PKC-independent Pyk2 activation pathways in other cell types.²⁷

We previously demonstrated that Pyk2 activates JNK, promotes cytoskeletal remodeling, and induces certain aspects of the pathologic hypertrophic gene reprogram.¹⁹ This study identifies an additional role for Pyk2 to activate AKT via a novel mechanism involving the formation of a multi-protein Pyk2/paxillin/PDK-1 complex and PDK-1–Y373/376

phosphorylation. Paxillin is presumed to act as a molecular scaffold to facilitate signaling through this α_1 -AR pathway, explaining the cardioprotective actions recently attributed to paxillin overexpression. These results suggest that decreased paxillin expression (as observed in cultures chronically treated with UCN-01) serves as both a marker and mediator of apoptosis. In keeping with the well-established role of phosphorylation to control the scaffolding function of paxillin, this study shows that α_1 -AR activation and Pyk2 overexpression increase paxillin–Y31 phosphorylation; the α_1 -AR/PKC pathway also slows the electrophoretic mobility of paxillin in SDS-PAGE and influences paxillin interactions with binding partners. It is worth noting that the LIM domains of paxillin contain numerous PKC consensus phosphorylation motifs (that are implicated in the control of focal contact assembly, cell adhesion to matrix, and cell spreading¹⁵) and that paxillin contains 2 highly conserved tyrosine/serine motifs (Y88/S89 and Y118/S119) that are particularly attractive candidates for regulation by PKC. It will be interesting to examine whether PKC-dependent phosphorylation at these tyrosine/serine motifs prevents protein docking to adjacent phosphotyrosine-based motifs and interferes with the scaffolding function of paxillin in future studies.

This study identifies an α_1 -AR/Pyk2 pathway leading to the activation of both JNK and AKT. The precise biological consequences of JNK activation in this context were not necessarily predictable based on previous literature that identifies dual roles for JNK in both stress-induced apoptosis and cytoprotective mechanisms.¹⁸ In fact, studies reported suggest that JNK links α_1 -ARs to a low level of AKT activation in cardiomyocytes with intact PKC activity. However, this JNK-dependent AKT activation pathway does not afford meaningful cytoprotection, because the proapoptotic actions of JNK via other mechanisms are more prominent in this setting. Similarly, AKT has been linked to both adaptive and maladaptive cardiac growth responses, depending on the identity of the AKT isoform, the duration of AKT activation, and/or AKT compartmentalization to the cytosol or nucleus.^{28–30} This study identifies a cytoprotective α_1 -AR/AKT activation pathway that does not promote GSK-3 phosphorylation. This mimics the actions of nuclear-targeted AKT (which also provides cytoprotection without inducing cardiac hypertrophy or phosphorylating GSK-3³⁰); studies to determine whether the NE-dependent AKT signaling pathway is limited to only a subset of the potential cellular AKT targets because of compartmentalization are ongoing. Importantly, nPKCs appear to function as molecular switches to control the balance of signaling via proapoptotic JNK and antiapoptotic AKT pathways. The effect of nPKC inhibitors to expose a dormant α_1 -AR–dependent AKT activation pathway identifies a rationale for the use of nPKC-targeted therapeutics to modulate the apoptosis program, prevent cell damage, and preserve cardiac function.

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Disclosures

None.

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