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Activation Loop Phosphorylation Controls Protein Kinase D-Dependent Activation of Nuclear Factor κ-B

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Running title: Resveratrol blocks PKD-mediated NF-κB activation.

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Non-standard abbreviations: ATCC, American Type Culture Collection; DMEM, Dulbecco's modified Eagle's medium; IKK, IκB kinase; LPS, lipopolysaccharide; MBP, myelin basic protein; NF-κB, nuclear factor κ-B; PH, pleckstrin homology; PKD, protein kinase D; PKC, protein kinase C; PMA, 12-phorbol 13-myristate acetate; TBS, Tris-buffered saline; TNF-α, tumor necrosis factor-α.

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ABSTRACT

Activation of the inducible transcription factor NF-κB (Nuclear Factor κ-B) occurs in cells exposed to oxidative stress, and the serine/threonine kinase PKD (protein kinase D) is critical for signal relay to NF-κB. We have recently delineated two coordinated events which control PKD activation in response to oxidative stress, phosphorylation at Tyr463 by the tyrosine kinase Abl, and phosphorylation at the activation loop Ser738/Ser742 by the PKC isoform PKCδ. The result is fully active PKD which controls NF-κB activation through the IKK complex. Here, we investigate the mechanism by which PKD controls IKK/NF-κB activation. Resveratrol, a potent anti-oxidant, blocks both PKD activation and NF-κB induction. Specifically, resveratrol blocked PKD activation loop phosphorylation and activity, and this was due to a specific inhibition of the Ser738/Ser742 kinase, PKCδ. Conversely, resveratrol did not affect Abl kinase activity and had no effect on Tyr463 phosphorylation. Moreover, we show that the mechanism by which resveratrol inhibits NF-κB is by blocking the translocation of PKD to the IKK complex, specifically by inhibiting Ser738/Ser742 phosphorylation. We therefore propose that rather than acting as an anti-oxidant, resveratrol specifically blocks oxidative stress-dependent NF-κB activation by interfering with PKD phosphorylation and association with the IKK complex.

INTRODUCTION

Exposure of cells and tissues to DNA and protein-damaging reactive oxygen species (ROS) and oxidative stress typically results in cell death, and depending on the cell type and dose of ROS, death can occur either by necrosis or apoptosis (Martindale and Holbrook, 2002). Consequently, cells have developed protective mechanisms in the form of pro-survival signaling pathways which are activated in response to such insults, and these result in the induction of *de novo* gene transcription and the up-regulation of survival and repair genes (Kops et al., 2002; Mercurio and Manning, 1999). The inducible NF-κB (Nuclear Factor κ-B) transcription factor is a good example of such a mechanism, because activation of NF-κB is often observed in cells exposed to oxidative stress. NF-κB activation has been linked to increased cellular survival in cells exposed to a variety of insults, including oxidative stress (Li and Karin, 1999). In addition to the well characterized cytokine-induced NF-κB activation pathways, we recently described a signaling pathway which results in the activation of NF-κB in cells specifically exposed to ROS such as H_2O_2 (Storz and Toker, 2003). In this pathway, a key player is the serine/threonine kinase PKD (Protein Kinase D), which relays a signal from ROS to the activation of the canonical IKK/NF-κB signalosome. The net consequence is an increase in the survival of cells exposed to increasing concentrations of oxidative stress, such that in cells in which the PKD/NF-κB pathway is blocked, cells are less protected from ROS-induced death.

PKD, formerly known as PKCμ, is a member of a novel sub-family of protein kinases which include two additional isoforms, PKD2 and PKD3/PKCν (Van Lint et al., 2002). PKDs have been shown to be important for G-protein-mediated signaling, Golgi sorting, immune cell signaling, cell growth and cell survival (Baron and Malhotra, 2002; Matthews et al., 2000; Rey et al., 2001; Storz and Toker, 2003). In addition to interaction with a number of regulatory proteins

(Hausser et al., 1999; Johannes et al., 1999; Storz et al., 2000), PKD activity is under the strict control of phosphorylation at two key sites in the catalytic kinase core, the activation loop serines Ser738 and Ser742. Phosphorylation of these two sites is absolutely required for the kinase to achieve catalytic competence, and studies from several laboratories have shown that these residues are directly phosphorylated by several isoforms of the PKC (Protein Kinase C) family (Brandlin et al., 2002; Storz et al., 2004; Waldron and Rozengurt, 2003). Our recent studies have focused on the mechanism by which oxidative stress mediates PKD activation, and we have shown that two synergistic signaling pathways are required to promote efficient activation of PKD in response to ROS. First, the tyrosine kinase Src activates Abl, which then directly phosphorylates PKD at Tyr463 in the amino-terminal PH (Pleckstrin Homology) domain (Storz and Toker, 2003). This initial step facilitates the second, rate-limiting step, phosphorylation of Ser738/Ser742 in the activation loop, mediated by PKCδ, which itself is also activated by Src (Storz et al., 2004). The net result is a fully active, catalytically competent PKD which relays the signal to IKK/NF-κB by an as yet unknown mechanism .

Numerous chemical inhibitors have been used to investigate the various pathways which converge on the IKK/NF-κB complex. Of these, resveratrol (trans-3,4',5-trihydroxystilbene), a known anti-oxidant, has been shown to block NF-κB in response to oxidative stress (Manna et al., 2000). Resveratrol has been shown to function in the prevention of various human pathological processes including inflammation, atherosclerosis and carcinogenesis (Cal et al., 2003; Tinhofer et al., 2001). Resveratrol can also promote apoptosis (Dorrie et al., 2001), and though the precise mechanism by which resveratrol exerts its function is not known, suppression of NF-κB has been proposed as one such mechanism (Estrov et al., 2003). For example, resveratrol blocks the phosphorylation and translocation of the p65 subunit of NF-κB in TNF-α

(Tumor Necrosis Factor-α)-stimulated cells, resulting in reduced transcriptional activity (Manna et al., 2000). However, resveratrol-mediated inhibition of NF-κB is not restricted to TNF signaling, since it has also been shown to blunt H_2O_2-, PMA- and LPS-mediated NF-κB activation (Manna et al., 2000). More recent studies have suggested that resveratrol is an inhibitor of PKD (Haworth and Avkiran, 2001; Stewart et al., 2000), but again, the mechanism remains undefined.

Here, we have used resveratrol in combination with molecular genetic approaches to investigate the mechanism by which PKD promotes NF-κB activation in cells exposed to oxidative stress. Our results do not support the notion that resveratrol acts as a general anti-oxidant to block NF-κB activation, rather that it specifically blunts PKD activation loop phosphorylation and activation. We further characterize this mechanism and show that resveratrol blocks the association of PKD with the IKK complex. The net effect is an effective inhibition of the PKD/NF-κB pathway which we propose explains the previous findings of inhibition of NF-κB by resveratrol in cells exposed to ROS.

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MATERIALS AND METHODS

Cell Culture, Antibodies, Reagents and Purified Proteins—The HeLa cell line was purchased from ATCC and maintained in high glucose DMEM supplemented with 10% fetal bovine serum. The α-PKD (C-20), α-PKCδ (C-20), α-IKKβ (H-4), α-IKKγ(FL-419), and α-Abl (24-11) antibodies were from Santa Cruz (Santa Cruz, CA), α-phospho-Ser738/742-PKD (α-pSer744/748 in mouse PKD/ α-pSer738/742 in human PKD), α-phospho-Ser916-PKD (α-pSer916 in mouse PKD/ α-pSer910 in human PKD) from Cell Signaling Technologies (Beverly, MA), α-Src from Upstate Biotechnology (Waltham, MA). α-HA was purified in-house from the 12CA5 hybridoma. The polyclonal α-pY463 antibody has been described (Storz and Toker, 2003). TNF was a kind gift from H. Wajant (Julius-Maximilians Universität Würzburg, Germany). Pervanadate was prepared as previously described (Storz et al., 2003). H_2O_2 (30%) was from Fisher Scientific. Resveratrol was from Biomol (Plymouth Meeting, PA). The PKD-specific substrate peptide used was AALVRQMSVAFFFK. Bovine brain phosphatidylserine and sn-1,2-dioleoylglycerol were from Avanti Polar Lipids (Alabaster, AL). Recombinant PKD or PKD* (kinase inactive, PKD.K612W) was expressed in insect cells after infection with baculovirus harboring HA-tagged PKD or PKD* in pFAST-Bac (Invitrogen Life Technologies) and purified on a Ni-NTA affinity column.

Expression Plasmids and Transfections—PKD expression plasmids (amino-terminal HA-tagged PKD in pcDNA3 and the constitutive active HA-PKD.Y463E and HA-PKD.SS738/742EE or inactive HA-PKD.K612W and HA-PKD.SS738/742AA mutants) have been described (Storz et al., 2004; Storz and Toker, 2003). PKD.S910A and PKD.S910E expression plasmids are based

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on an amino-terminal HA-PKD in pcDNA3. Mutagenesis was carried out by PCR using QuikChange (Stratagene, La Jolla, CA) with the following primer pairs for PKD.S910A: 5'CTCGGTGAGCGTGTCGCCATCCTCTGACTCGAG3' and 5'CTCGAGTCAGAGGATGGCGACACGCTCACCGAG3'; for PKD.S910E: 5'CTCGGTGAGCGTGTCGAAATCCTCTGACTCGAG3' and 5'CTCGAGTCAGAGGATTTCGACACGCTCACCGAG3'. Other expression plasmids were obtained from A. Israel (IKKβ), B. Schaffhausen (v-Abl p120, Src.Y527F), S. Ohno (PKCδRR144/145AA), T. Maniatis (GST-IκBα a.a.5-55) and R. van Etten (GST-CRK a.a.120-212). Superfect (Qiagen, Valencia, CA) was used for transient transfections in luciferase assays, and TransIT HeLa Monster reagent (Mirus, Madison, WI) was used for all other transfections according to the manufacturer's instructions. Cells were stimulated or harvested 24 hr after transfection.

Immunoblotting and Immunoprecipitation —Cells were stimulated or harvested 24 hr after transfection and lysed in lysis buffer (50 mM Tris/HCl pH 7.4, 1% Triton X-100, 150 mM NaCl, 5 mM EDTA pH 7.4) plus protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO). The lysates were used either for immunoblot analysis or proteins were immunoprecipitated by a 1 hr incubation with the respective antibody (2 μg), followed by a 30 min incubation with protein G-agarose (Amersham Biosciences, Piscataway, NJ). Immune complexes were washed three times with TBS (50 mM Tris/HCl pH 7.4, 150 mM NaCl), and resolved by SDS-PAGE or subjected to kinase assays.

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PKD Kinase Assays—For *in vitro* kinase assays with immunoprecipitated PKD, following immunoprecipitation (α-HA for transfected PKD) and washing, 20 µl kinase buffer (50 mM Tris/HCl pH 7.4, 10 mM $MgCl_2$, 2 mM dithiothreitol) was added to the precipitates, and the kinase reaction was carried out for 20 min after addition of 10 µl of kinase substrate mix (150 µM PKD-specific substrate peptide, 50 µM ATP, 10 µCi [γ-^{32}P]-ATP in kinase buffer). To terminate, the samples were centrifuged and the supernatants spotted onto P81 phosphocellulose paper (Whatman, Clifton, NJ). The papers were washed three times with 0.75% phosphoric acid, once with acetone, dried and activity was determined by liquid scintillation counting. Protein expression was detected by immunoblotting. For *in vitro* kinase assays using purified PKD or PKD*, 1 µg of PKD was incubated with resveratrol, phosphatidylserine/diacylglycerol micelles and ATP (50 µM ATP, 10 µCi [γ-^{32}P]-ATP in kinase buffer) for 15 min at room temperature. Mixed phosphatidylserine/diacylglycerol lipid vesicles were prepared as previously described (Storz et al., 2003). The reactions were stopped with sample buffer and subjected to SDS-PAGE. Dried gels were analyzed on a Molecular Imager (Bio-Rad, Hercules, CA).

IKK, Abl and PKCδ Kinase Assays—After immunoprecipitation (α-PKCδ, α-Abl, α-IKKγ) and extensive washing, 20 µl kinase buffer (50 mM Tris/HCl pH 7.4, 10 mM $MgCl_2$, 2 mM dithiothreitol) was added to the precipitates, and the kinase reaction was carried out for 30 min by addition of 10 µl kinase substrate mix (IKK assays: 2 µg GST-IκBα a.a.5-55, 50 µM ATP, 10 µCi [γ-^{32}P]-ATP in kinase buffer; Abl assays: 2 µg GST-CRK a.a.120-212, 50 µM ATP, 10 µCi [γ-^{32}P]-ATP in kinase buffer; PKCδ assays: 1 µg MBP, 50 µM ATP, 10 µCi [γ-^{32}P]-ATP in kinase buffer). To terminate the kinase reaction, 30 µl of 2x SDS sample buffer was added and

the samples were resolved by SDS PAGE. The gels were dried and analyzed on a Molecular Imager (Bio-Rad, Hercules, CA). Protein expression was detected by immunoblotting.

Reporter Gene Assays —Cells were transiently co-transfected with a NF-κB-reporter construct (NF-κB-luc, 5 μg), 1 μg pCS2-(n)β-gal and the protein(s) of interest (1 μg) using Superfect (Qiagen). Cells stimulated 6 hr after transfection. 24 hr after transfection, assays for luciferase and β-galactosidase activity were performed on total cell lysates using standard assays and measured on a luminometer. Luciferase activity was normalized to the β-galactosidase activity. Protein expression was controlled by immunoblot analysis.

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RESULTS

Resveratrol inhibits oxidative stress-mediated NF-κB activation —Resveratrol is a naturally occurring phytoalexin produced by certain grapevines, possesses potent anti-oxidant properties and has also been shown to block both NF-κB and PKD activity in cells (Haworth and Avkiran, 2001; Stewart et al., 2000; Uhle et al., 2003). To investigate the mechanism by which PKD mediates NF-κB activation in response to oxidative stress signaling, we first evaluated the NF-κB response in HeLa cells exposed to H_2O_2 either in the presence of absence of resveratrol. Consistent with previous reports, resveratrol efficiently blocked oxidative stress-induced NF-κB luciferase reporter activity stimulated by either H_2O_2 or pervanadate, a global inhibitor of tyrosine phosphatases (Fig. 1A, B). As a control, TNF-induced NF-κB activation was also blocked by resveratrol. When IKK kinase assays were used as a read-out for NF-κB activation, resveratrol also completely blocked H_2O_2-induced IKK activation (Fig. 1D), suggesting that the resveratrol block lies upstream of the IKK signalosome. In contrast, resveratrol did not completely abolish TNF-α-induced IKK activation, though it was significantly reduced (Fig. 1E). This indicates a difference in the mechanisms by which resveratrol blocks oxidative stress- versus TNF-α-induced NF-κB activation. Next, we analyzed whether the inhibitory effects of resveratrol are exclusively due to its anti-oxidant function, or if it can specifically inhibit protein kinases which regulate IKK and NF-κB.

Resveratrol inhibits NF-κB activation mediated by active alleles of Src, Abl, PKCδ and PKD — In HeLa cells, the oxidative stress-induced NF-κB activation pathway is mediated by a signaling cascade which involves Src, Abl, PKCδ and PKD (Storz et al., 2003; Storz et al., 2004; Storz and

Toker, 2003). We therefore used active alleles of Src (Src.Y527F), Abl (v-Abl p120), PKCδ (PKCδRR144/145AA) or PKD (PKD.Y463E) to specifically activate NF-κB in the absence of any exogenous oxidative stress. Interestingly, resveratrol was still able to potently block NF-κB induction mediated by both active Src, active Abl, active PKCδ or active PKD (Fig. 2 A-D). This suggests that resveratrol can directly block one or more steps in the PKD/NF-κB pathway even in the absence of exogenous ROS. From these experiments, we conclude that resveratrol can potentially block signaling downstream of either Abl (both Abl and PKD) or downstream of PKCδ (both PKCδ and PKD), leading to inhibition of NF-κB induction in all cases. However, because these are activated alleles, this approach does not directly address whether resveratrol blocks the endogenous kinases in response to oxidative stress.

Resveratrol inhibits oxidative stress-mediated activation of PKCδ, but not Abl—To determine where in the PKD/NF-κB pathway resveratrol inhibits, we evaluated H_2O_2-induced activation of both Abl and PKCδ, the PKD upstream kinases. Interestingly, resveratrol had no effect on oxidative stress-induced endogenous Abl activation (Fig. 3A), but significantly (70%) blunted PKCδ activity as judged by MBP immune-complex kinase assays (Fig. 3B) . Thus, again, resveratrol is not acting as a general anti-oxidant, but functions to block PKCδ, which is upstream of PKD, and in turn upstream of NF-κB. Moreover, it is important to note that resveratrol also likely does not block Src activity, because we previously showed that in this pathway Abl is downstream of Src (Storz and Toker, 2003), and resveratrol does not inhibit Abl (Fig. 3A).

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Resveratrol does not inhibit PKD directly —One explanation for the above data is that resveratrol can directly block PKD, which is required for oxidative stress-mediated NF-κB activation. To test this question, we first analyzed PKD activity in HeLa cells exposed to H_2O_2. Resveratrol effectively blocked oxidative stress-stimulated PKD activation as measured in immune-complex substrate kinase assays as well as auto-phosphorylation (Fig. 4A). Next, we tested whether resveratrol can directly inhibit PKD using recombinant, purified enzyme in *in vitro* kinase assays. Interestingly, when PKD was incubated in the presence of mixed phosphatidylserine/diacylglycerol vesicles to activate its intrinsic kinase activity, resveratrol did not significantly block this activity at a dose which completely eliminates activity in stimulated cells (100 μM, Fig. 4B). Finally, a kinase-inactive PKD (PKD*, PKD.K612W) control (Fig. 4B, left panel) clearly demonstrated that the phosphorylation of PKD seen in this assay is exclusively due to auto-phosphorylation and not caused by contaminating kinases in the purified PKD preparation. We therefore conclude that resveratrol does not directly inhibit PKD kinase activity, rather that the inhibition of PKD observed in cells must be due to a block in its activation mechanism.

Dose Response of PKCδ, PKD and NF-κB Inhibition by Resveratrol – If resveratrol blocks NF-κB activation by blocking the upstream kinases PKCδ and PKD, then the same concentrations of resveratrol which block NF-κB should also block these kinases. To test this, HeLa cells were exposed to increasing doses of resveratrol (0 – 200μM) followed by stimulation with H_2O_2. As predicted, the dose-response of inhibition of activation of both PKCδ (Fig. 5A), PKD (Fig. 5B and NF-κB (Fig. 5C) closely correlated, such that 50 to 100μM resveratrol potently blocked all three activities.

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Phosphorylation of PKD at Ser910 is not required for NF-κB activation —We next investigated the mechanism by which resveratrol blocks PKD activation in cells. Ser910 has been described as an *in vitro* and *in vivo* auto-phosphorylation site in PKD (Matthews et al., 1999). It has also been shown that PMA stimulates Ser910 phosphorylation, and that this is blocked by resveratrol (Haworth and Avkiran, 2001). However, we found that exposure of HeLa cells to oxidative stress, a potent stimulus for PKD, did not result in Ser910 phosphorylation (Fig. 6A). Furthermore, we used a mutational strategy to investigate the functional role of Ser910 in NF-κB activation. We found that mutation of Ser910 to either non-phosphorylatable Ala, or phospho-mimetic Glu, had no effect on NF-κB induction by H_2O_2 compared to wild-type PKD (Fig. 6B). Finally, because we previously showed that oxidative stress promotes association of PKD with the IKK complex, we investigated the relevance of Ser910 in this complex formation. Consistent with the lack of an effect on NF-κB luciferase activity, neither Ser910 mutant was compromised in association with the IKK complex in co-immunoprecipitation studies, again compared with wild-type PKD (Fig. 6C).

Resveratrol inhibits PKD activation loop phosphorylation—Having eliminated Ser910 as the mechanism by which resveratrol blocks PKD activation, we next evaluated Tyr463 and Ser738/Ser742 phosphorylation, both of which are induced in oxidative stress-dependent PKD regulation (Storz et al., 2004; Storz and Toker, 2003). In HeLa cells, resveratrol did not block oxidative stress-stimulated PKD tyrosine phosphorylation, measured with anti-phosphotyrosine (Fig. 7A), or with anti-pY463 (Fig. 7B). Conversely, resveratrol significantly blocked activation loop phosphorylation of PKD at Ser738/Ser742 (Fig. 7C). These data are in complete agreement with the ability of resveratrol to block PKCδ (the Ser738/Ser742 kinase, Fig. 3B) and the lack of

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inhibition towards Abl (the Tyr463 kinase, Fig. 3A). Consistent with this model, resveratrol also blocked the association of PKD with PKCδ which is observed in cells stimulated with H_2O_2 (Fig. 7D).

Resveratrol blocks translocation of PKD to the IKK complex —To further evaluate the mechanism by which PKD controls IKK/NF-κB activation, we determined whether inhibition of complex formation could account for the inhibitory effects of resveratrol on NF-κB activity. We have previously shown that PKD can be co-immunoprecipitated with the IKK complex following exposure of cells to H_2O_2 (Storz and Toker, 2003). We now show that resveratrol completely blocks this association, when PKD is immunoprecipitated and IKK is revealed (Fig. 8A), and when IKK is immunoprecipitated and PKD is immunoblotted (data not shown). Secondly, a PKD.Y463E mutant, which is constrictively phosphorylated at the activation loop Ser738/Ser742 by endogenous PKCδ (Storz et al., 2004), is also constitutively associated with IKK, but again this is blocked by resveratrol (Fig. 8B), consistent with the ability of resveratrol to block activation of PKCδ (Fig. 3B). Next, we investigated the importance of PKD kinase activity and activation loop phosphorylation in translocation to the IKK complex. Translocation of PKD was not dependent on PKD activity, since the both wild-type and kinase-inactive PKD (PKD.K612W) also efficiently co-immunoprecipitated with both IKK and PKCδ in cells stimulated with H_2O_2 (Fig. 8C). Conversely, the activation loop PKD mutant (S738A/S742A) was not associated with IKK, and also showed impaired (approximately 50%) association with PKCδ, though this was not complete. Finally, the constitutively active PKD activation loop mutant (S738E/S742E) was constitutively associated with IKK in the absence of stimulation, and a significant proportion was still associated with IKK in cells treated with resveratrol (Fig. 8D).

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Since PKD activation loop phosphorylation is blocked by resveratrol (Fig. 7C), we conclude from these data that inhibition of translocation of PKD to the IKK complex is the mechanism by which resveratrol blocks the PKCδ/PKD/NF-κB pathway in response to oxidative stress.

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DISCUSSION

Resveratrol is a known potent anti-oxidant and has shown significant efficacy as an anti-inflammatory and anti-tumorigenic agent *in vitro* and *in vivo* (Fremont, 2000). However, not all of the effects of resveratrol can be fully explained by its anti-oxidant and anti-cyclooxygenase activities, because it can also block cell cycle progression and cell growth (Joe et al., 2002). Recent studies have shown that in cells, resveratrol also potently blocks NF-κB, and separate studies have shown that it can also function as an inhibitor of PKD (Ashikawa et al., 2002; Haworth and Avkiran, 2001). Because we previously linked activation of PKD to NF-κB induction in cells exposed to oxidative stress (Storz and Toker, 2003), we sought to investigate the mechanism by which resveratrol blocks NF-κB so as to further understand how PKD promotes NF-κB activation. Specifically, our goal was to determine if resveratrol simply functions as a non-specific anti-oxidant to block ROS-mediated NF-κB activation, or whether it can more specifically block one or more steps in the PKD/IKK/NF-κB signaling module. Contrary to the notion that resveratrol functions solely as an anti-oxidant, our data point to this compound as a specific antagonist of two key steps in this pathway, phosphorylation of the PKD activation loop, and interaction of PKD with the IKK complex.

Previous studies have addressed whether resveratrol can directly inhibit PKD activity *in vitro*. In one study, Stewart *et al.* demonstrated a reduced autophosphorylation of purified PKD with increasing doses of resveratrol (Stewart et al., 1999). An IC_{50} of 50 μM was reported for inhibition of PKD. A separate group also demonstrated that purified PKD can be inhibited by resveratrol *in vitro*, however, here an IC_{50} of 200 μM was reported (Haworth and Avkiran, 2001). Using similar *in vitro* kinase assays, we find that highly purified, recombinant PKD is

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only modestly inhibited (approx. 20%) by resveratrol at a dose of 100 μM (Fig. 4). Clearly, this cannot account for the complete inhibition of PKD activity observed in cells treated with the same dose of resveratrol (Fig. 4A and 5B). Therefore, a mechanism other than direct inhibition of intrinsic kinase activity must occur in cells to explain inhibition of PKD, as well as NF-κB, by this compound. We therefore turned our attention to phosphorylation of PKD at three distinct sites: Ser910 at the very carboxyl-terminus, Tyr463 in the PH domain, and Ser738/Ser742 in the activation loop.

Contrary to what has been reported for PMA-stimulated PKD regulation, we were unable to detect an increase in Ser910 phosphorylation induced by oxidative stress (Fig. 6). Moreover, a mutational approach to investigate the potential role of Ser910 in NF-κB induction revealed that this site, if it is indeed phosphorylated, is actually dispensable for NF-κB activation (Fig. 6). Similar to Ser910, resveratrol also did not block the oxidative stress-stimulated phosphorylation of PKD at Tyr463 (Fig. 7A and 7B). We recently showed that Tyr463 is phosphorylated by a Src-Abl signaling pathway in cells exposed to ROS (Storz and Toker, 2003), and consistent with the finding that resveratrol does not inhibit Tyr463 phosphorylation, it also had no effect on both Src and Abl activity stimulated by H_2O_2. Therefore, resveratrol does not function as a non-specific inhibitor to blunt all pathways leading to the activation of PKD.

In contrast to Ser910 and Tyr463, treatment of cells with resveratrol did significantly inhibit phosphorylation of PKD at the activation loop residues Ser738/Ser742 (Fig. 7C). Because in oxidative stress signaling these sites are directly phosphorylated by the novel PKC isoform PKCδ(Storz et al., 2004), we tested whether resveratrol could block PKCδ activity. As expected, the reduced PKD activation loop phosphorylation seen in cells treated with resveratrol was indeed due to a block in PKCδ activity, when oxidative stress was the stimulus (Fig. 3B).

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Previous studies further support this model, as PKC activity in cells can be blocked with resveratrol concentrations as low as 2 μM (Slater et al., 2003). It has been suggested that one potential mechanism for PKC inhibition is competition with phorbol ester binding by interaction of resveratrol with C1 domains (Slater et al., 2003). Moreover, Yu *et al*. recently demonstrated inhibition of PKC isolated from HeLa cells (Yu et al., 2001). The cumulative evidence therefore supports a role for PKCδ, rather than PKD, as an intracellular target for resveratrol. Moreover, competition of resveratrol for diacylglycerol would also be an explanation for the slight inhibition of purified PKD (which also has two copies of the C1 domain) by resveratrol (Fig. 4B), although it is well established that the primary mechanism controlling PKD activation is activation loop phosphorylation (Iglesias et al., 1998). Taken together, we propose that resveratrol functions to block the PKCδ-mediated phosphorylation of PKD at Ser738/Ser742. We also propose that this is a specific inhibitory mechanism towards PKD, because resveratrol also does not block the Abl tyrosine kinase, such that PKD Tyr463 phosphorylation in resveratrol-treated cells is unaffected.

We took advantage of the mechanism by which resveratrol blocks PKD to investigate how PKD signals to NF-κB, since resveratrol has been shown to be a potent inhibitor of this transcription factor. Given that resveratrol not only blocks NF-κB in response to oxidative stress, but also in cells stimulated with the 'classical' NF-κB agonists LPS, PMA and TNF-α, it is likely that it functionally blocks at the level of the IKK complex. This is because all of these ligands initiate signaling pathways which ultimately converge at the level of the IKK signalosome, which then relays the signal to IκBα degradation and ultimately to NF-κB activation. Our data are consistent with this notion, because in response to oxidative stress, PKD translocates to the IKK complex, activates IKKβ, and promotes IκBα degradation (Storz and

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Toker, 2003). In the present study, we have evaluated the mechanism. Firstly, dose-response experiments showed that resveratrol concentrations which blocked NF-κB activity also blocked both PKCδ and PKD activation by oxidative stress (Fig. 5). Secondly, resveratrol blocked the oxidative stress-induced translocation of PKD to the IKK complex, measured by co-immunoprecipitation (Fig. 8A). We also show that PKD kinase activity is dispensable for PKD association with both IKK and PKCδ (Fig. 8C). Conversely, Ser738/Ser742 phosphorylation is required for PKD translocation to IKK, because a PKD mutant in which these residues cannot be phosphorylated does not associate with IKK following H_2O_2 stimulation, whereas a constitutively active PKD mutant with glutamate residues at the activation loop is constitutively associated with IKK (Fig. 8D). We therefore propose that resveratrol blocks NF-κB activation in response to oxidative stress by specifically blocking the activation loop phosphorylation of PKD, mediated by PKCδ, which in turn blocks association of PKD with IKK.

While it is not yet known whether the same mechanism functions in TNF-α signaling, it is tempting to speculate that this is indeed the case, because Johannes *et al.* have demonstrated activation of PKD by TNF-α (Johannes et al., 1998). More importantly, PKCδ has also been linked to TNF-α-mediated IκBα degradation and NF-κB activation (Vancurova et al., 2001). It is also worth noting that other studies have also shown that various other PKC isoforms, including PKCζ, PKCβII, PKCδ, PKCε and PKCθ have been linked to the activation of NF-κB. For example, PMA-stimulated NF-κB activation has been shown to be mediated by PKCε (Hirano et al., 1995), whereas PKCδ and PKCζ appear to participate in TNF-α-induced NF-κB induction (Leitges et al., 2001; Vancurova et al., 2001). Conventional PKCβ and novel PKCθ have been shown to control NF-κB induction in T- and B-cells (Bauer et al., 2001; Sun et al., 2000). Since resveratrol can potentially block some or all of these PKCs, possibly by competing

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for the natural ligand diacylglycerol, it is likely that PKC is the common target linking resveratrol inhibition of IKK and NF-κB in response to all NF-κB agonists. This hypothesis is supported by the finding that some of these PKCs have been shown to be inhibited by resveratrol *in vivo* (Slater et al., 2003), although clearly more work is needed to substantiate this hypothesis.

Although our studies support a specific function for resveratrol in blocking PKCδ-mediated PKD activation and its subsequent translocation to the IKK complex, we cannot exclude that in certain NF-κB activation pathways, resveratrol may also act as a global anti-oxidant. For example, TNF-α has been shown to promote the release of ROS (Shrivastava and Aggarwal, 1999) and thus an additional explanation for the block of NF-κB by resveratrol in response to TNF-α as well as oxidative stress could indeed be its anti-oxidant activity. However, we do not favor this model because oxidative stress indirectly activates kinases such as Src or PKCδ by inhibiting tyrosine and serine/threonine phosphatases. Thus, if resveratrol were merely acting as an anti-oxidant, one would expect that activation of NF-κB by activated alleles of Src, Abl or PKCδ would be unaffected by resveratrol, yet it clearly blocks NF-κB induction by these upstream kinases (Fig. 2). Moreover, we also evaluated specificity and demonstrated that resveratrol directly inhibits the intrinsic kinase activity of PKCδ, but not that of Abl (Figs. 3 and 7). This excludes a non-specific inhibition of general kinase activity by resveratrol, for example, by depleting cellular ATP levels, as has been demonstrated for the compound rottlerin, previously thought to be a specific PKCδ inhibitor (Soltoff, 2001).

Taken together, our data demonstrate that in oxidative stress signaling, resveratrol can block the activation of NF-κB at the level of PKD. This allowed us to investigate the mechanism by which PKD controls NF-κB induction at the level of the IKK complex. We found that inhibition of PKD activation loop phosphorylation and loss of translocation to IKK results in a

block in NF-κB activation. We therefore propose that the concerted action of these two events is responsible for the resveratrol-mediated inhibition of NF-κB in oxidative stress signaling. What remains to be determined is how activation loop phosphorylation controls access of PKD to IKK, leading to IKKβ activation. This is currently under investigation in our laboratory.

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FIGURE LEGENDS

FIG. 1. **Resveratrol inhibits oxidative stress-stimulated NF-κB activation.** (A-C) HeLa cells were transfected with NF-κB and β-gal reporter genes. 6 hr after transfection cells were treated with 100 μM resveratrol and 8 hr after transfection stimulated with H_2O_2 (500 nM), pervanadate (PV, 75 μM) or TNF-α (50 ng/ml) for 16 hr. Luciferase and β-gal reporter gene assays were performed. (D, E) HeLa cells were treated with resveratrol (1 hr, 100 μM) and stimulated with H_2O_2 (500 nM, 10 min) or TNF-α (50 ng/ml, 5 min). The IKK complex was immunoprecipitated (α-IKKγ) and an IKK substrate phosphorylation kinase assay using GST-IκBα as a substrate was performed. IKK expression in cells was determined by immunoblot analysis (α-IKKα/β). All results are typical of three independent experiments.

FIG. 2. **Resveratrol inhibits NF-κB activation mediated by active alleles of Src, Abl, PKCδ and PKD.** (A-D) HeLa cells were transfected with NF-κB and β-gal reporter genes and active Src (Src.Y527F), active Abl (v-Abl p120), active PKCδ (PKCδ.RR144/145AA) or active PKD (PKD.Y463E). 6 hr after transfection cells were treated with 100 μM resveratrol for 16 hr. Luciferase and β-gal reporter gene assays were performed. Protein expression in cells was controlled by immunoblot analysis. All results are typical of three independent experiments.

FIG. 3. **Resveratrol inhibits PKCδ, but not Abl in response to oxidative stress.** HeLa cells were treated with resveratrol (100 μM, 1 hr) and stimulated with H_2O_2 (10 μM, 10 min). Endogenous Abl (A) or PKCδ (B) were immunoprecipitated and substrate phosphorylation

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kinase assays were performed. Abl and PKCδ expression in cells was controlled by immunoblot analysis. All results are typical of three independent experiments.

FIG. 4. **(A) Resveratrol blocks oxidative stress-stimulated PKD activation.** PKD was over-expressed in HeLa cells. Cells were treated with resveratrol (100 μM, 1 hr) and stimulated with H_2O_2 (10 μM, 10 min). PKD was immunoprecipitated and substrate-phosphorylation or auto-phosphorylation assays were performed. PKD expression was controlled by immunoblot analysis (α-PKD). **(B) Resveratrol does not directly inhibit PKD.** Baculovirus-infected, insect cell-expressed, purified recombinant PKD or PKD* (kinase inactive mutant, PKD.K612W) were incubated with the indicated doses of resveratrol in the presence or absence of PS/DG vesicles. An *in vitro* auto-phosphorylation kinase assay was performed. All results are typical of two independent experiments.

FIG. 5. **Kinetics of PKCδ, PKD and NF-κB inhibition.** (A, B) HeLa cells were treated with resveratrol as indicated (1 hr) and stimulated with H_2O_2 (10 μM, 10 min). Endogenous PKCδ (A) or over-expressed PKD (B) were immunoprecipitated and substrate phosphorylation kinase assays were performed. PKCδ and PKD expression in cells was controlled by immunoblot analysis. (C) HeLa cells were transfected with NF-κB and β-gal reporter genes. 6 hr after transfection cells were treated with resveratrol as indicated and 8 hr after transfection stimulated with H_2O_2 (500 nM) for 16 hr. Luciferase and β-gal reporter gene assays were performed. All results are typical of two independent experiments.

FIG. 6. **Phosphorylation of PKD at Ser910 is not required for NF-κB activation** (A) HeLa cells were treated with resveratrol (100 μM, 1 hr) and stimulated with H_2O_2 (10 μM, 10 min).

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Endogenous PKD was immunoprecipitated and probed for Ser910 phosphorylation (α-pS910). Blots were stripped and re-probed for total PKD. (B) HeLa cells were transfected with NF-κB and β-gal reporter genes and PKD wild-type (wt) or mutants. 6 hr after transfection cells were treated with 500 nM H_2O_2 for 16 hr. Luciferase and β-gal reporter gene assays were performed. Protein expression in cells was controlled by immunoblot analysis. (C) PKD or PKD mutants and IKKβ were co-expressed in HeLa cells. Cells were stimulated with H_2O_2 (10 μM, 10 min). PKD was immunoprecipitated (α-HA) and probed for co-immunoprecipitation of IKKβ. Blots were then stripped and re-probed for total PKD. IKKβ expression was controlled by immunoblot analysis (α-IKKα/β). The results are typical of three independent experiments.

FIG. 7. **Resveratrol inhibits the phosphorylation of PKD at the activation loop but not at Tyr463.** (A - C) PKD was overexpressed in HeLa cells. HeLa cells were treated with resveratrol (100 μM, 1 hr) and stimulated with H_2O_2 (10 μM, 10 min). PKD was immunoprecipitated and probed for phosphorylation with α-pY (A) or α-pY463 (B) or α-pS738/742 (C) antibodies. Blots were stripped and re-probed for total PKD (α-PKD). (C) HeLa cells were treated with resveratrol (100 μM, 1 hr) and stimulated with H_2O_2 (10 μM, 10 min). Endogenous PKD was immunoprecipitated (α-PKD) and co-immunoprecipitated PKCδ was detected by immunoblotting with α-PKCδ. Blots were then stripped and re-probed for total PKD (α-PKD). All results are typical of two independent experiments.

FIG. 8. **Resveratrol blocks oxidative stress-mediated translocation of PKD to the IKK complex.** (A – D) PKD (wild-type, PKD.K612W (kinase inactive), PKD.Y463E or PKD.SS738/742EE mutants) and IKKβ were co-expressed in HeLa cells. HeLa cells were

treated with resveratrol (100 μM, 1 hr) and/or stimulated with H_2O_2 (10 μM, 10 min) as indicated. PKD (α-HA) was immunoprecipitated and probed for co-immunoprecipitation of IKKβ and PKCδ. Blots were then stripped and re-probed for total PKD. IKK expression was controlled by immunoblot analysis. All results are typical of three independent experiments.

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Figure 1

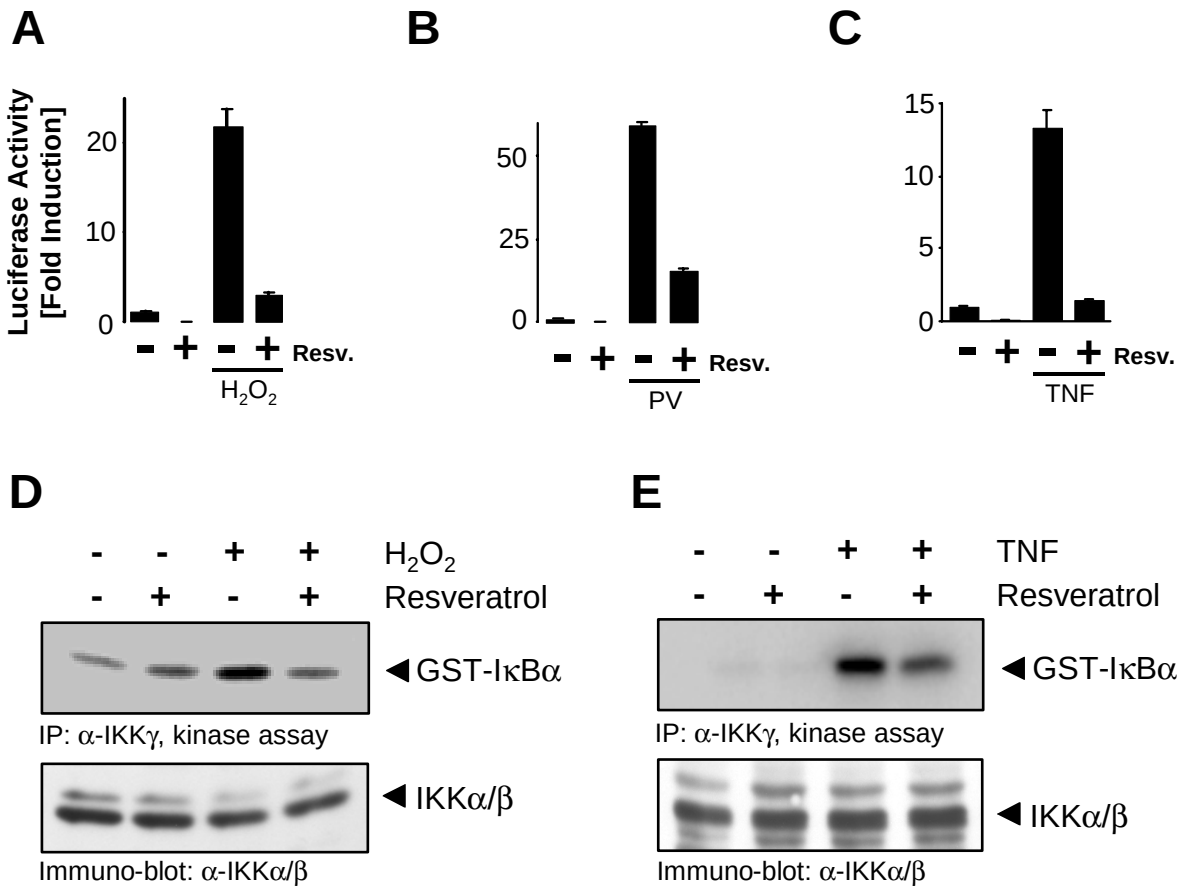

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Figure 2

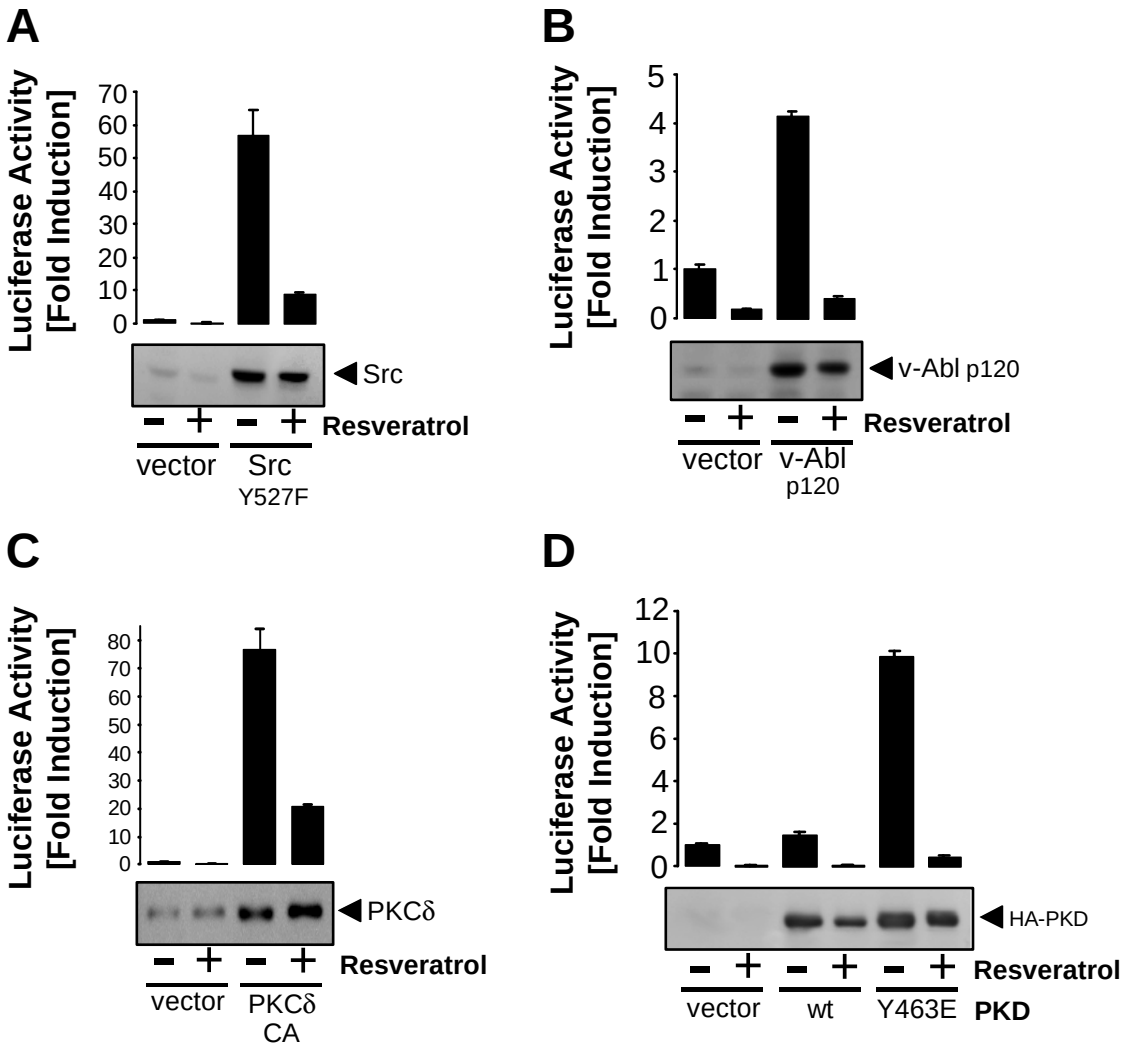

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Figure 3

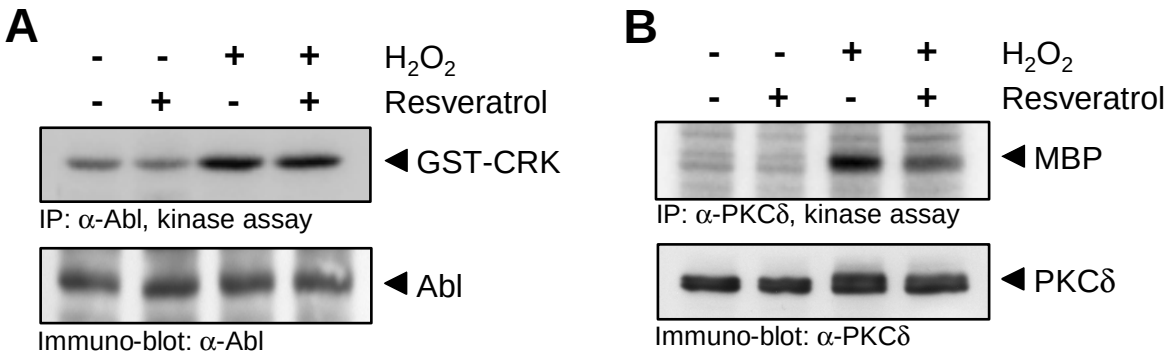

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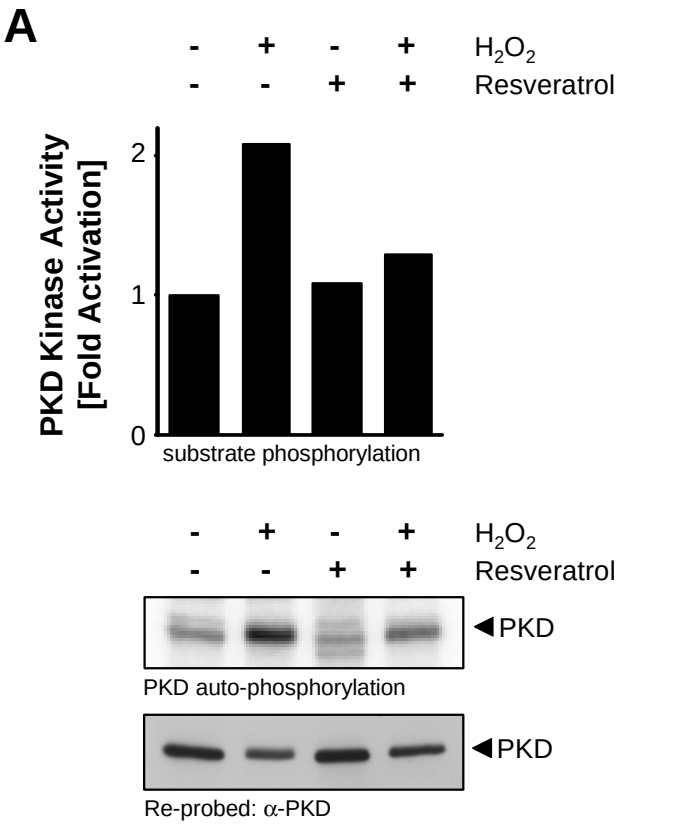
Figure 4



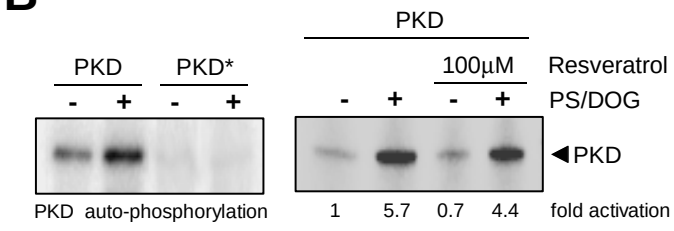


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Figure 5

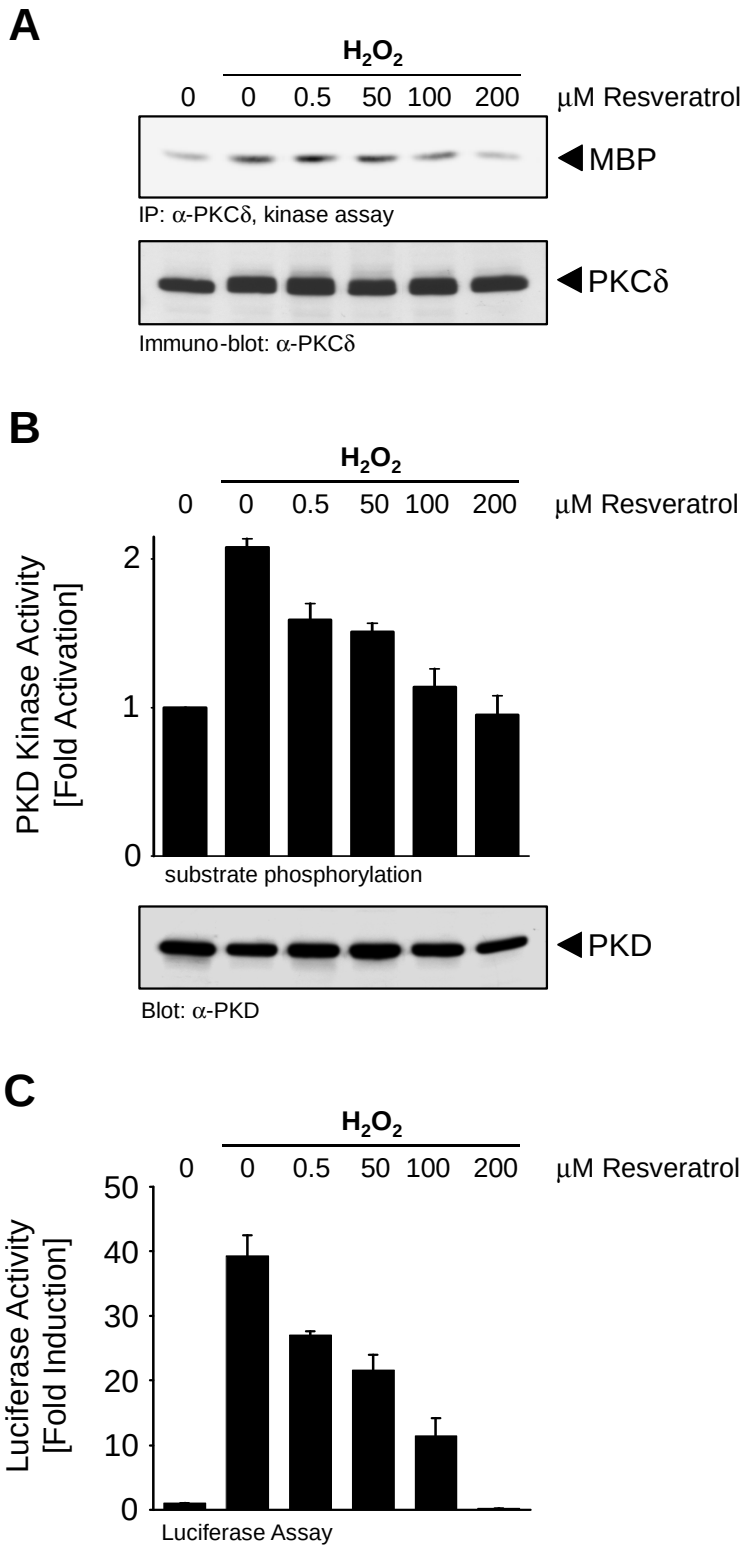

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Figure 6

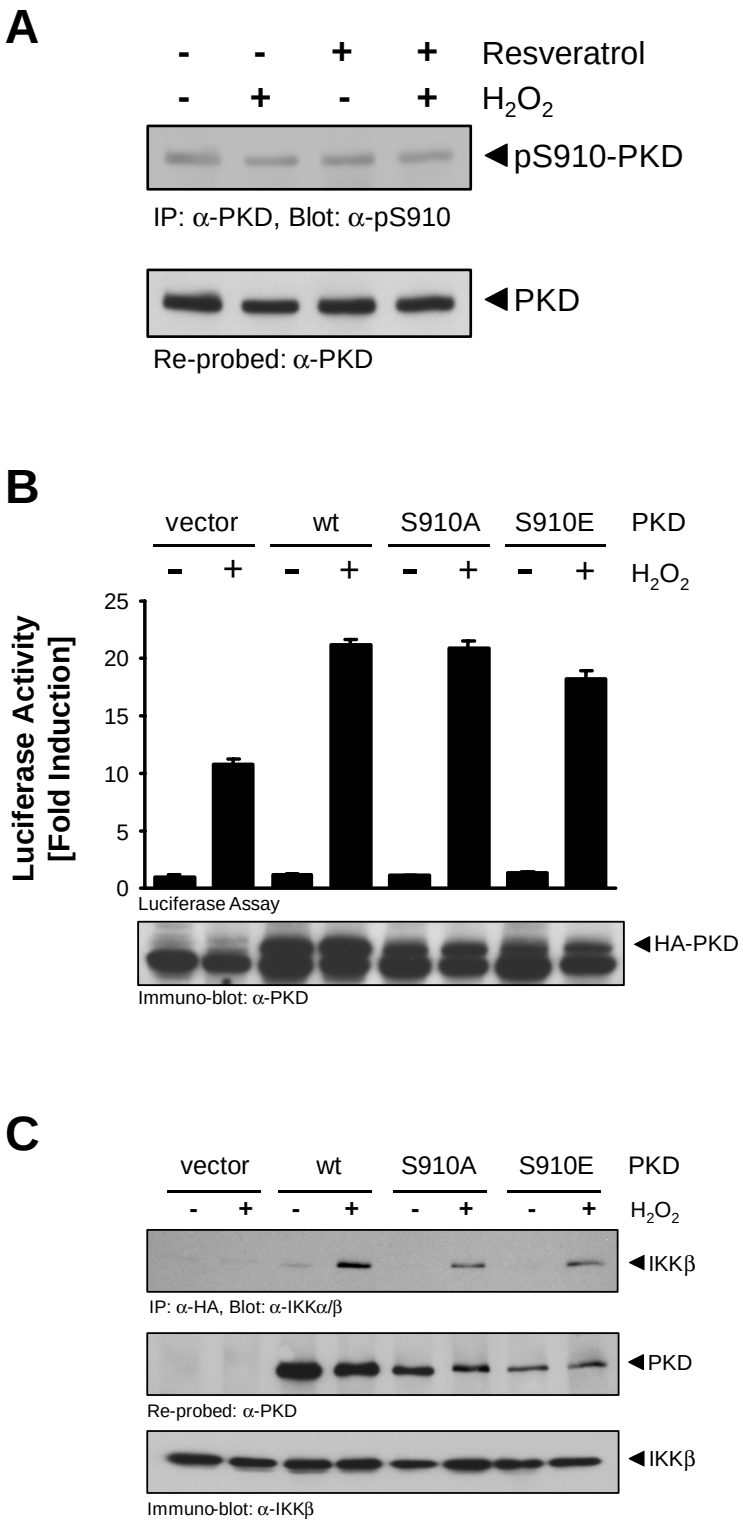

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Figure 7

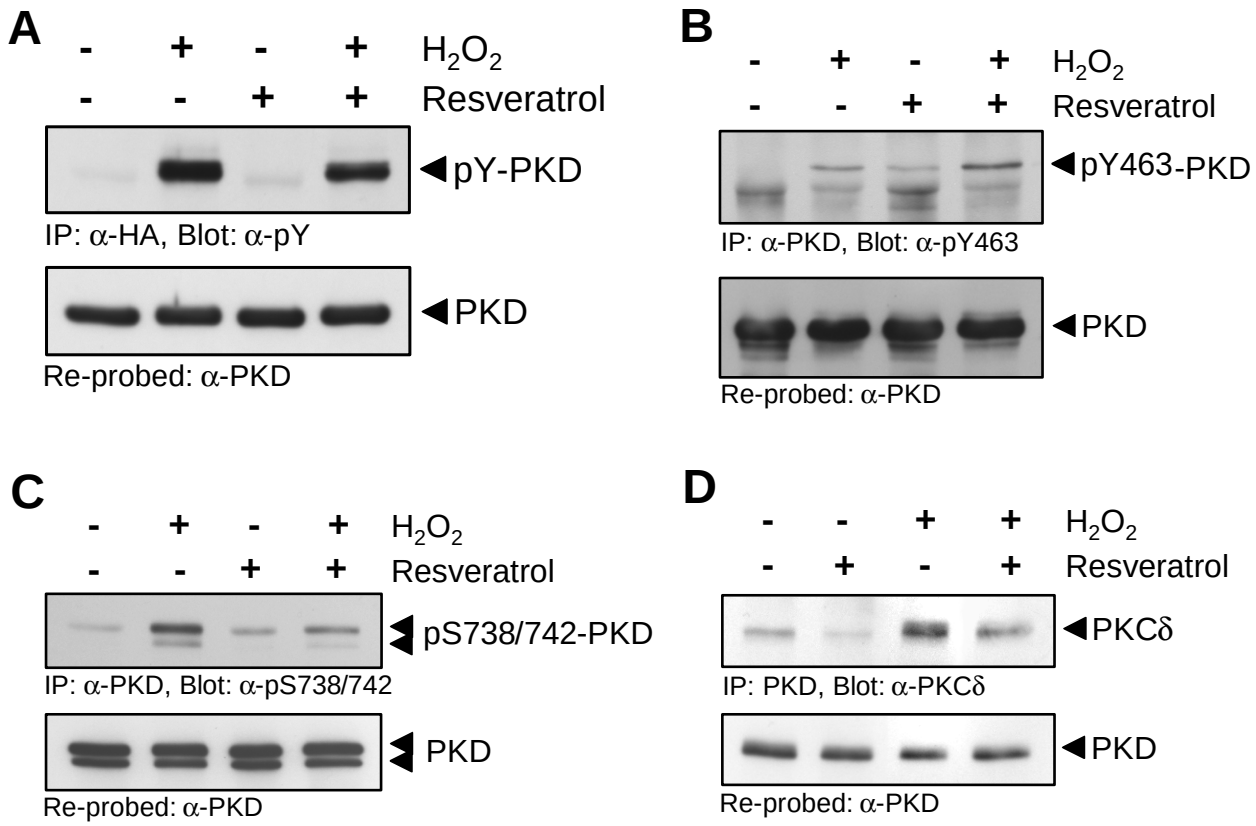

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Figure 8