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p66^{shc} Is Indispensable for Phenethyl Isothiocyanate-induced Apoptosis in Human Prostate Cancer Cells

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Abstract

Naturally-occurring phenethyl isothiocyanate (PEITC) selectively inhibits growth of cancer cells by causing apoptosis but the mechanism of cell death induction is not fully understood. We now demonstrate, for the first time, that growth factor adapter protein p66^{shc} is indispensable for PEITC-induced apoptosis. Mouse embryonic fibroblasts derived from p66^{shc} knockout mice were significantly more resistant to PEITC-mediated growth inhibition, cytoplasmic histone-associated apoptotic DNA fragmentation, and caspase-3 activation compared with wild-type fibroblasts. The PEITC treatment resulted in induction as well as increased Ser36 phosphorylation of p66^{shc} in PC-3 and LNCaP human prostate cancer cells. Knockdown of p66^{shc} protein conferred significant protection against PEITC-mediated cytoplasmic histone-associated DNA fragmentation as well as production of reactive oxygen species in both PC-3 and LNCaP cells. The PEITC-treated PC-3 and LNCaP cells exhibited increased binding of p66^{shc} with prolyl isomerase Pin1, a protein implicated in translocation of p66^{shc} to mitochondria. Consistent with these results, treatment of PC-3 cells with PEITC resulted in translocation of p66^{shc} to the mitochondria as judged by immunoblotting using cytosolic and mitochondrial fractions and immunofluorescence microscopy. Growth suppression and apoptosis induction in tumor xenografts *in vivo* by oral administration of PEITC to the PC-3 tumor bearing male athymic mice was accompanied by statistically significant increase in the level of Ser36 phosphorylated p66^{shc}. Collectively, these results provide novel insight into the critical role of p66^{shc} in regulation of PEITC-induced apoptotic cell death in human prostate cancer cells.

Keywords

Phenethyl isothiocyanate; p66^{shc}; Apoptosis

Introduction

Despite continuous evolution of targeted therapies and significantly improved screening efforts (*e.g.*, digital rectal examination and screening for prostate specific antigen), prostate cancer remains a leading cause of cancer-related deaths among men in the United States (1). Because many of the risk factors associated with prostate cancer development (*e.g.*, genetic predisposition and age) are not modifiable, novel agents to prevent and treat this devastating disease are highly attractive to reduce disease-related cost, morbidity, and mortality for a large segment of population. Natural products have received increasing attention in recent years for discovery novel cancer chemopreventive/therapeutic agents (2). Epidemiological studies have suggested that dietary intake of cruciferous vegetables may lower the risk of

different malignancies including cancer of the prostate (3–5). For example, a multicenter case-control study of African-American, white, Japanese, and Chinese men (n= 1619) with histologically confirmed prostate cancer and matched controls (n= 1618; matched by ethnicity, age, region of residence) showed an inverse association between intake of cruciferous vegetables and the risk of prostate cancer (4).

Anticarcinogenic effect of cruciferous vegetables is credited to naturally-occurring chemicals with an isothiocyanate moiety ($-N=C=S$), which are produced by myrosinase-mediated hydrolysis of corresponding glucosinolates (6,7). Phenethyl-ITC (PEITC) is one such naturally-occurring ITC compound that has garnered tremendous research interest for a variety of reasons, including anticancer effect and favorable safety profile (8–13). For example, PEITC is a potent inhibitor of pulmonary tumorigenesis in rats induced by tobacco-specific carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (8,9). The PEITC administration was shown to offer significant protection against diethylnitrosamine-induced hepatocellular adenoma development in mice (10). The PEITC-mediated prevention of *N*-nitrosobenzylmethylamine-induced esophageal cancer in rats was associated with modulation of carcinogen-induced gene expression (11,12).

More recent studies, including those from our laboratory, have revealed that PEITC has the ability to suppress growth of cancer cells in culture and *in vivo* (14–19). Mechanism behind anticancer effect of PEITC is not fully understood but known cellular responses to this promising agent include c-Jun N-terminal kinase- and extracellular signal-regulated kinase-mediated apoptosis induction (14,15), G2/M phase cell cycle arrest (16), suppression of nuclear factor- κ B-regulated gene expression (17), inhibition of angiogenesis *in vitro* and *ex vivo* (20), and Atg5-mediated autophagy (21). We have also shown previously that oral administration of PEITC significantly retards growth of PC-3 human prostate cancer cells subcutaneously implanted in male athymic mice without causing weight loss or any other side effects (19,21).

Previous studies have indicated that the PEITC-mediated proapoptotic signal transduction is intimately linked to production of reactive oxygen species (ROS) (19,22). However, the molecular circuitry of PEITC-mediated ROS production and apoptotic cell death is still not fully understood. We now demonstrate, for the first time, that growth factor adapter protein p66^{Shc} is essential for PEITC-induced ROS production as well as apoptosis in human prostate cancer cells.

Materials and Methods

Reagents

PEITC (purity >99%) was purchased from Sigma-Aldrich. Reagents for cell culture were purchased from GIBCO. Hydroethidine (HE) and 6-carboxy-2',7'-dichlorodihydrofluorescein diacetate (H₂DCFDA) were purchased from Molecular Probes. The p66^{Shc}-targeted small interfering RNA (siRNA) was procured from Santa Cruz Biotechnology and a non-specific control siRNA was from Qiagen. A kit for quantitation of cytoplasmic histone-associated DNA fragmentation was from Roche Diagnostics. Antibodies against p66^{Shc}, phospho-(Ser36)-p66^{Shc}, and heat shock protein 70 (HSP70) were from Santa Cruz Biotechnology; an antibody against prolyl isomerase (Pin1) was from Cell Signaling Technology; anti-cytochrome *c* oxidase IV (COXIV) antibody was from Molecular Probes; and an antibody against actin was from Oncogene Research Products. Caspase-3 activation was measured by flow cytometry using a kit from Cell Signaling Technology. Protein kinase C β (PKC β)-selective inhibitor [3-(1-(3-imidazol-1-ylpropyl)-1H-indol-3-yl)-4-anilino-1H-pyrrole-2,5-dione; hereafter abbreviated as PKC β -I] was purchased from EMD Biosciences.

Cell lines and cell culture

The PC-3 and LNCaP cell lines were obtained from the American Type Culture Collection, and validated by analysis of known markers (*e.g.*, expression of p53 and androgen-responsiveness). Monolayer cultures of PC-3 (an androgen-independent cell line lacking functional p53) and LNCaP cells (an androgen-responsive cell line with wild-type p53) were maintained as described by us previously (15,23). Immortalized MEFs derived from wild-type and p66^{Shc}^{-/-} mice were generously provided by Dr. T. Finkel and Dr. S. Nemoto (National Institutes of Health, Bethesda, MD), and cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum, 0.1 mmol/L non-essential amino acids, 0.1 μmol/L 2-mercaptoethanol, and antibiotics.

Western blotting

Stock solution of PEITC was prepared in dimethyl sulfoxide (DMSO) and diluted with complete medium immediately before use. An equal volume of DMSO (final concentration <0.1%) was added to the controls. Cell lysates were prepared as previously described by us (15,24). Cytosolic and mitochondrial fractions from DMSO-treated control and PEITC-treated cells were prepared using a kit from BioVision according to the manufacturer's recommendations. Immunoblotting was performed essentially as described by us previously (24).

Cell viability assay and measurement of apoptosis

Effect of PEITC treatment on cell viability was determined by trypan blue dye exclusion assay as described by us previously (25). Apoptosis induction by PEITC was assessed by analysis of cytoplasmic histone-associated DNA fragmentation using a kit from Roche Diagnostics. Activation of caspase-3 was determined by flow cytometry using a kit from Cell Signaling Technology as recommended by the supplier.

RNA interference of p66^{Shc}

The PC-3 or LNCaP cells (1×10^5) were seeded in six-well plates and allowed to attach by overnight incubation. The cells were transfected with 200 nmol/L of a control non-specific siRNA or p66^{Shc}-targeted siRNA using Oligofectamine (Invitrogen). Twenty-four hours after transfection, the cells were treated with DMSO (control) or specified concentration of PEITC for desired time period. The cells were collected, washed with phosphate-buffered saline (PBS), and processed for immunoblotting or analysis of cytoplasmic histone-associated DNA fragmentation and ROS production as described previously (25,26).

Analysis of interaction between p66^{Shc} and Pin1 or HSP70

Aliquots containing 300 μg of total lysate protein or mitochondria-enriched fractions from PC-3 or LNCaP cells treated with DMSO (control) or 5 μmol/L PEITC (8 h treatment) were incubated overnight at 4°C with 6 μg of anti-p66^{Shc} antibody. Protein G-agarose beads (50 μL; Santa Cruz Biotechnology) were then added to each sample and the incubation was continued for an additional 2 h at 4°C. The immunoprecipitates were washed five times with lysis buffer and subjected to sodium-dodecyl sulfate polyacrylamide gel electrophoresis followed by immunoblotting using anti-Pin1 antibody (total lysate) or anti-HSP70 antibody (mitochondria-enriched fraction).

Immunocytochemical analysis for mitochondrial localization of p66^{Shc}

The PC-3 cells (1×10^5) were plated on coverslips and treated with 5 μmol/L PEITC or DMSO (control) for 8 h. The cells were then treated with 200 nmol/L MitoTracker Red at 37°C for 30 min to stain mitochondria. After washing with PBS, the cells were fixed in 2% paraformaldehyde overnight at 4°C and permeabilized using 0.1% Triton X-100 in PBS for

10 min. The cells were washed with PBS, blocked with 0.5% bovine serum albumin in PBS for 1 h, and incubated with anti-p66^{Shc} antibody overnight at 4°C. The cells were then washed with PBS, incubated with Alexa Fluor 488-conjugated secondary antibody (Molecular probes) for 1 h at room temperature. Subsequently, the cells were washed with PBS and treated with 10 ng/mL 4',6-diamidino-2-phenylindole (DAPI) for 5 min at room temperature to stain nucleus. The cells were washed twice with PBS and examined under a Leica fluorescence microscope at 100× magnification.

Immunohistochemical analyses for total and phosphorylated p66^{Shc} and Pin1 in PC-3 tumor xenografts

We used tumor tissues from a previously published xenograft study (21) to determine *in vivo* effect of PEITC administration on expression of total and Ser36-phosphorylated p66^{Shc} and Pin1. Tumor tissues from the control and PEITC-treated mice were fixed in 10% neutral-buffered formalin, dehydrated, embedded in paraffin, and sectioned at 4–5 μm thickness. Immunohistochemistry was performed as described by us previously (27). At least three non-overlapping images from each tissue section were captured and analyzed using Image ProPlus software (Media Cybernetics).

Statistical analysis

Each experiment was repeated at least twice to ensure reproducibility of the results. Paired *t*-test or one-way ANOVA followed by Bonferroni's multiple comparison test was used to determine significance of difference in measured variables between groups. Difference was considered significant at $P < 0.05$.

Results

p66^{Shc} deficiency conferred resistance toward PEITC-induced apoptosis

We have shown previously that the PEITC-induced apoptosis in PC-3 cell line is intimately linked to the production of ROS (19). Because electron transfer between cytochrome *c* and p66^{Shc}, which is a splice variant of cytoplasmic adapter proteins p52^{Shc}/p46^{Shc} that are involved in signal transduction from activated tyrosine kinases to Ras (28), has been shown to cause ROS-dependent mitochondrial apoptosis (29), we raised the question of whether PEITC-induced apoptosis was mediated by the p66^{Shc} protein. Initially, we used MEFs derived from wild-type (p66-WT) and p66^{Shc} knockout (p66-KO) mice to test possible involvement of this protein in PEITC-induced apoptosis. Immunoblotting confirmed absence of the p66^{Shc} isoform (but not p52^{Shc} or p46^{Shc}) in p66-KO MEFs (Fig. 1A). Exposure of MEFs derived from p66-WT mice to PEITC resulted in a concentration-dependent and statistically significant decrease in cell viability (Fig. 1B). The MEFs derived from the p66-KO mice were statistically significantly more resistant to PEITC-mediated growth retardation compared with p66-WT cells (Fig. 1B). The PEITC-mediated suppression of p66-WT MEF viability correlated with apoptosis induction as evidenced by cytoplasmic histone-associated DNA fragmentation (Fig. 1C) and caspase-3 activation (Fig. 1D). In agreement with cell viability data (Fig. 1B), the p66-KO MEFs were protected against PEITC-mediated cytoplasmic histone-associated apoptotic DNA fragmentation (Fig. 1C) as well as caspase-3 activation (Fig. 1D) in comparison with p66-WT MEFs.

We raised a question of whether p66^{Shc} dependence of PEITC-induced apoptosis was unique to this compound. We addressed this question using benzyl isothiocyanate (BITC), a structural analogue of PEITC. The MEFs derived from p66-KO mice were significantly more resistant to growth inhibition (supplemental Fig. S1, panel A) as well as cytoplasmic histone-associated DNA fragmentation (supplemental Fig. S1, panel B) compared with p66-

WT MEFs. These results indicated that involvement of p66^{Shc} in apoptosis regulation is not unique to the PEITC.

PEITC-induced apoptosis and ROS production was inhibited by RNA interference of p66^{Shc}

Next, we determined the effect of PEITC treatment (5 $\mu\text{mol/L}$, 24 h) on levels of total and Ser36 phosphorylated p66^{Shc} because oxidative stress-induced Ser36 phosphorylation of p66^{Shc} has been shown to be critical for its translocation to mitochondria after recognition by Pin1 (30). The PEITC-treated PC-3 cells exhibited a modest yet marked increase in protein level of total p66^{Shc}, which was accompanied by ~2.5-fold increase in level of Ser36 phosphorylated p66^{Shc} over DMSO-treated control (Fig. 2A). Next, we utilized siRNA technology to directly test contribution of p66^{Shc} in regulation of PEITC-induced apoptosis. As can be seen in Fig. 2B, the level of p66^{Shc} protein was decreased by about 60% by transient transfection of PC-3 cells with p66^{Shc}-targeted siRNA compared with cells transfected with a control non-specific siRNA. Similar to un-transfected cells (Fig. 2A), the PEITC treatment (5 $\mu\text{mol/L}$, 24 h) caused an increase in levels of total and Ser36 phosphorylated p66^{Shc} in comparison with DMSO-treated control in PC-3 cells transfected with the non-specific siRNA. The PEITC-mediated induction as well as Ser36 hyperphosphorylation of p66^{Shc} was markedly suppressed in PC-3 cells transfected with the p66^{Shc}-targeted siRNA (Fig. 2B). Twenty-four hour exposure of non-specific control siRNA transfected PC-3 cells to 5 $\mu\text{mol/L}$ PEITC resulted in about 2.8-fold increase in cytoplasmic histone-associated DNA fragmentation compared with DMSO-treated control (Fig. 2C). The p66^{Shc} depleted cells were nearly completely resistant to PEITC-induced apoptotic DNA fragmentation compared with cells transfected with control siRNA (Fig. 2C). Because PEITC-mediated apoptosis correlates with ROS production (19,22), we also determined ROS generation by PEITC treatment (5 $\mu\text{mol/L}$; 4 h treatment) in cells transfected with control siRNA and p66^{Shc}-targeted siRNA. As shown in Fig. 2D, the PEITC-mediated oxidation of H₂DCFDA (a measure of peroxide production) over DMSO-treated control was observed in non-specific siRNA transfected PC-3 cells but not in the p66^{Shc} depleted cells.

We raised the question of whether involvement of p66^{Shc} protein in regulation of PEITC-induced apoptosis was unique to the PC-3 cell line or influenced by androgen responsiveness and p53 status. We addressed these questions using LNCaP cell line. The p53 connection was particularly intriguing because p66^{Shc} protein has been shown to be a downstream target of the tumour suppressor p53 (31). Similar to PC-3 cells, the PEITC treatment (5 $\mu\text{mol/L}$; 24 h) resulted in induction as well as increased Ser36 phosphorylation of p66^{Shc} in LNCaP cells (Fig. 3A). Moreover, a 50% depletion of p66^{Shc} protein level by transient transfection of LNCaP cells with the p66^{Shc}-targeted siRNA (Fig. 3B) caused statistically significant protection against PEITC-induced DNA fragmentation (Fig. 3C) as well as peroxide production (Fig. 3D) compared with cells transfected with non-specific siRNA. Collectively, these results indicated that the p66^{Shc} protein was essential for apoptosis resulting from PEITC treatment in prostate cancer cells irrespective of their androgen-responsiveness or the p53 status. Based on these results, we also conclude that p66^{Shc} protein functions upstream of ROS production in the molecular circuitry of PEITC-induced apoptosis.

PEITC-induced apoptosis was inhibited by pharmacologic inhibition of PKC β

We used a cell permeable pharmacologic inhibitor of PKC β to determine its role in PEITC-mediated increase in Ser36 phosphorylation of p66^{Shc}. As shown in supplemental Fig. S2 (panel A), increased Ser36 phosphorylation of p66^{Shc} resulting from PEITC exposure (5 $\mu\text{mol/L}$; 24 h) was significantly inhibited in the presence of PKC β -I in PC-3 cells. Moreover, co-treatment of PC-3 cells with PKC β -I conferred statistically significant

protection against PEITC-induced cytoplasmic histone-associated apoptotic DNA fragmentation (supplemental Fig. S2, panel B). These results indicated that the PEITC-mediated Ser36 hyperphosphorylation of p66^{Shc} was mediated by PKC β .

PEITC treatment promoted mitochondrial translocation of p66^{Shc}

Prolyl isomerase Pin1 has been shown to play an important role in mitochondrial translocation of p66^{Shc} (30). Initially, we determined the effect of PEITC treatment on protein level of Pin1 by immunoblotting (Fig. 4A). The protein level of Pin1 was decreased by about 30–80% by a 24 h treatment of PC-3 and LNCaP cells with 2.5 and 5 $\mu\text{mol/L}$ PEITC (Fig. 4A). Despite reduction in Pin1 protein level, the PEITC treatment (5 $\mu\text{mol/L}$, 8 h) resulted in increased binding of p66^{Shc} protein with Pin1 in both cell lines as revealed by immunoprecipitation using anti-p66^{Shc} antibody followed by immunoblotting with anti-Pin1 antibody (Fig. 4B). The blot was also probed with anti-p66^{Shc} antibody to ensure equal immunoprecipitation (Fig. 4B).

Next, we proceeded to test whether PEITC treatment caused mitochondrial translocation of p66^{Shc} by immunoblotting using isolated cytosolic and mitochondrial fractions from PC-3 cells. As can be seen in Fig. 4C, the p66^{Shc} protein was detectable in both cytosolic and mitochondrial fractions of DMSO-treated control PC-3 cells. However, the mitochondrial level of p66^{Shc} protein was increased by about 2.8-fold upon 8 h treatment of PC-3 cells with 5 $\mu\text{mol/L}$ PEITC compared with DMSO-treated control (Fig. 4C, *upper panel*). Probing of the blot with anti-COXIV antibody confirmed purity of the mitochondrial fraction. The PEITC-mediated translocation of p66^{Shc} to the mitochondria was confirmed by immunofluorescence microscopy (Fig. 4C, *lower panel*). Intensity of the p66^{Shc}-associated green fluorescence was very weak in DMSO-treated control PC-3 cells. On the other hand, 8 h treatment of PC-3 cells with 2.5 $\mu\text{mol/L}$ PEITC resulted in marked increase in green fluorescence due to induction of p66^{Shc} protein (Fig. 4C), which was consistent with the immunoblotting data shown in Fig. 2A. Moreover, the PEITC-treated PC-3 cells exhibited increased mitochondrial localization of p66^{Shc} as evidenced by appearance of yellow-orange color due to the merging of MitoTracker-associated red fluorescence and p66^{Shc}-associated green fluorescence (Fig. 4C, *lower panel*). Collectively, these results indicated that PEITC treatment caused increased interaction of p66^{Shc} with Pin1 and resulted in mitochondrial translocation of p66^{Shc}.

PEITC treatment inhibited interaction between p66^{Shc} and mitochondrial HSP70

Previous studies have shown that mitochondrial p66^{Shc} complexes with HSP70, and disruption of this interaction is necessary for regulation of trans-membrane potential (32). We therefore tested the possibility whether PEITC treatment affected interaction between p66^{Shc} and HSP70. As can be seen in Fig. 4D, the p66^{Shc}-HSP70 complex was detectable in DMSO-treated control PC-3 cells as evidenced by immunoprecipitation using anti-p66^{Shc} antibody followed by immunoblotting with the anti-HSP70 antibody (Fig. 4D). The level of p66^{Shc}-HSP70 complex was decreased by about 80% upon 8 h treatment of PC-3 cells with 5 $\mu\text{mol/L}$ PEITC (Fig. 4D). These results showed that PEITC treatment disrupted interaction between p66^{Shc} and HSP70.

Oral administration of PEITC increased Ser36 phosphorylation of p66^{Shc} in PC-3 xenografts

We have shown previously that oral administration of PEITC inhibits growth of PC-3 cells implanted in male athymic mice in association with apoptosis induction (21). The average tumor volume on the day of sacrifice (38 days after tumor cell implantation) in mice gavaged with 9 μmol PEITC was lower by about 48% compared with the vehicle-treated control mice (21). Fraction of apoptotic bodies was about 4-fold higher in tumor sections

from PEITC-treated mice compared with control tumors (21). In the present study, we used tumor sections from these mice to determine *in vivo* significance of our cellular findings. As shown in Fig. 5A, the tumor sections from PEITC-treated mice exhibited statistically significant increase in the level of Ser36 phosphorylated p66^{Shc} compared with control tumor sections, which was consistent with data in cultured PC-3 cells (Fig. 2A). Even though PEITC treatment resulted in a modest increase in the level of total p66^{Shc} (Fig. 5B), the difference did not reach statistical significance. In agreement with cellular data (Fig. 4A), the level of Pin1 protein was ~50% lower ($P < 0.05$ compared with control by paired *t*-test) in the tumor sections from PEITC-treated mice compared with control tumor sections (Fig. 5C). These results showed that oral administration of PEITC resulted in increased Ser36 phosphorylation of p66^{Shc} as well as down-regulation of Pin1 protein in PC-3 tumor xenograft *in vivo*.

Discussion

Previous studies have shown that cells lacking p66^{Shc} protein resist oxidation of ROS-sensitive chemical probes and display reduced accumulation of endogenous oxidative stress marker 8-oxo-guanosine (31,33). Furthermore, the p66^{Shc} knockout mice exhibit diminished levels of systemic (isoprostane) as well as intracellular (8-oxo-guanosine) markers of oxidative stress (31,33,34). Genetic deletion of p66^{Shc} in cells also confers protection against apoptosis induced by ultraviolet radiation, staurosporin, and growth factor deprivation (32,35,36). Moreover, the p66^{Shc} knockout mice are resistant to apoptosis induction by paraquat and ischemia (34,35,37). Because proapoptotic effect of PEITC in cancer cells is linked to ROS production (19,22), we raised the question of whether molecular circuitry of PEITC-induced apoptosis was dependent on p66^{Shc}. Results presented herein indicate that the p66^{Shc} protein is indeed essential for PEITC-induced apoptosis. This conclusion is substantiated by the observed resistance of immortalized MEFs derived from p66^{Shc}^{-/-} mice to growth suppression and apoptosis induction by PEITC compared with WT fibroblasts. Moreover, knockdown of p66^{Shc} protein by RNA interference significantly inhibits PEITC-induced apoptosis in PC-3 and LNCaP cells. Another important conclusion from the present study is that p66^{Shc} protein functions upstream of ROS production in execution of PEITC-induced apoptosis. Previous studies have shown that electron transfer between cytochrome *c* and p66^{Shc} leads to production of hydrogen peroxide (29). It is probable that PEITC treatment promotes p66^{Shc}-mediated oxidation of reduced cytochrome *c* to trigger ROS production in our model. Further work is needed to directly test this possibility.

The PC-3 and LNCaP cells were used in the present study not only to authenticate generality of the p66^{Shc}-dependence of PEITC-induced apoptosis but also to determine possible influence of p53 in this response. The p53 status is known to influence stability of the p66^{Shc} protein in response to treatment with UV and hydrogen peroxide (31). The PEITC-mediated induction of p66^{Shc} protein expression was relatively more pronounced in the LNCaP cell line with intact p53 compared with PC-3 cells lacking functional p53. Because PEITC treatment results in modest induction of p66^{Shc} protein in the PC-3 cell line, we can conclude that the induction of p66^{Shc} protein in our model is not solely dependent on the p53 status. The LNCaP cell line is relatively more sensitive to PEITC-mediated apoptotic DNA fragmentation compared with PC-3 cells. On the other hand, the PC-3 cells express nearly 4-fold higher level of p66^{Shc} protein compared with LNCaP C-33 cells (38). A role for p66^{Shc} and ROS in androgen-induced prostate cancer cell proliferation has also been documented by the same group of investigators (39). Because the p66^{Shc} protein promotes PEITC-induced apoptosis in our model, we conclude that differential sensitivity of PC-3 and LNCaP cells to proapoptotic effect of PEITC *per se* is not related to difference in p66^{Shc} protein level.

Protein kinase C β -mediated Ser36 phosphorylation of p66^{Shc} has been documented to promote its mitochondrial accumulation in a prooxidant environment even though this protein lacks conventional mitochondria-targeting sequence (30). Instead, the oxidative stress-induced (e.g., H₂O₂) mitochondrial enrichment of p66^{Shc} is facilitated by interaction with peptidyl-prolyl isomerase Pin1 (30). The precise mechanism to explain Pin1-dependent mitochondrial import of p66^{Shc} is still not clear. The present study reveals that PEITC treatment not only intensifies Ser36 phosphorylation of p66^{Shc} in prostate cancer cells but also increases its binding with Pin1 as evidenced by immunoprecipitation-immunoblotting experiments. Consistent with these results mitochondrial enrichment of p66^{Shc} is evident upon treatment with PEITC. We also found that PEITC treatment reduces protein level of Pin1 in both PC-3 and LNCaP cell lines. Interestingly, Pin1 has been shown to facilitate cytokine-induced survival of eosinophils by suppressing Bax activation (40). It is plausible that down-regulation of Pin1 contributes to Bax activation in PEITC-induced apoptosis. This possibility is likely because cell death resulting from PEITC exposure is inhibited in cells lacking Bax (18).

In vivo validation of the cellular mechanistic observations is critical for identification of biomarkers(s) of response potentially useful in future clinical trials. Biomarker discovery is especially critical because cancer incidence is too rigorous of an end point for chemoprevention trials. We found that oral administration of PEITC to the PC-3 xenograft-bearing male athymic mice not only retards tumor growth in association with apoptosis induction (21) but also increases Ser36 phosphorylation of p66^{Shc} and down-regulates Pin1 protein level (present study). These observations suggest that Ser36 phosphorylated p66^{Shc} and Pin1 protein level may be viable biomarkers of PEITC response.

In conclusion, as summarized in Fig. 6 the results presented herein indicate that PEITC treatment enhances PKC β -mediated Ser36 phosphorylation of p66^{Shc}, which increases its binding with Pin1. The binding of p66^{Shc} with Pin1 promotes translocation of p66^{Shc} to the mitochondria. The p66^{Shc} protein localized to the mitochondria is released from HSP70 and causes ROS production leading to caspase-3 activation and apoptotic cell death.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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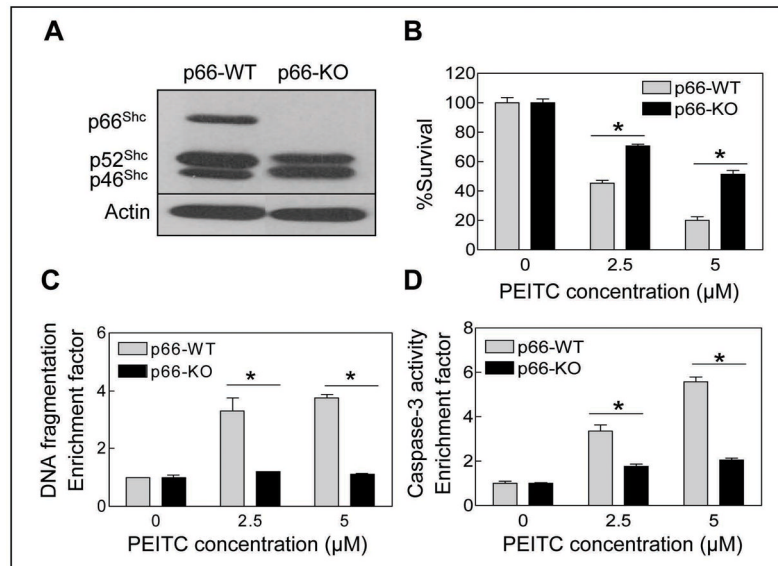
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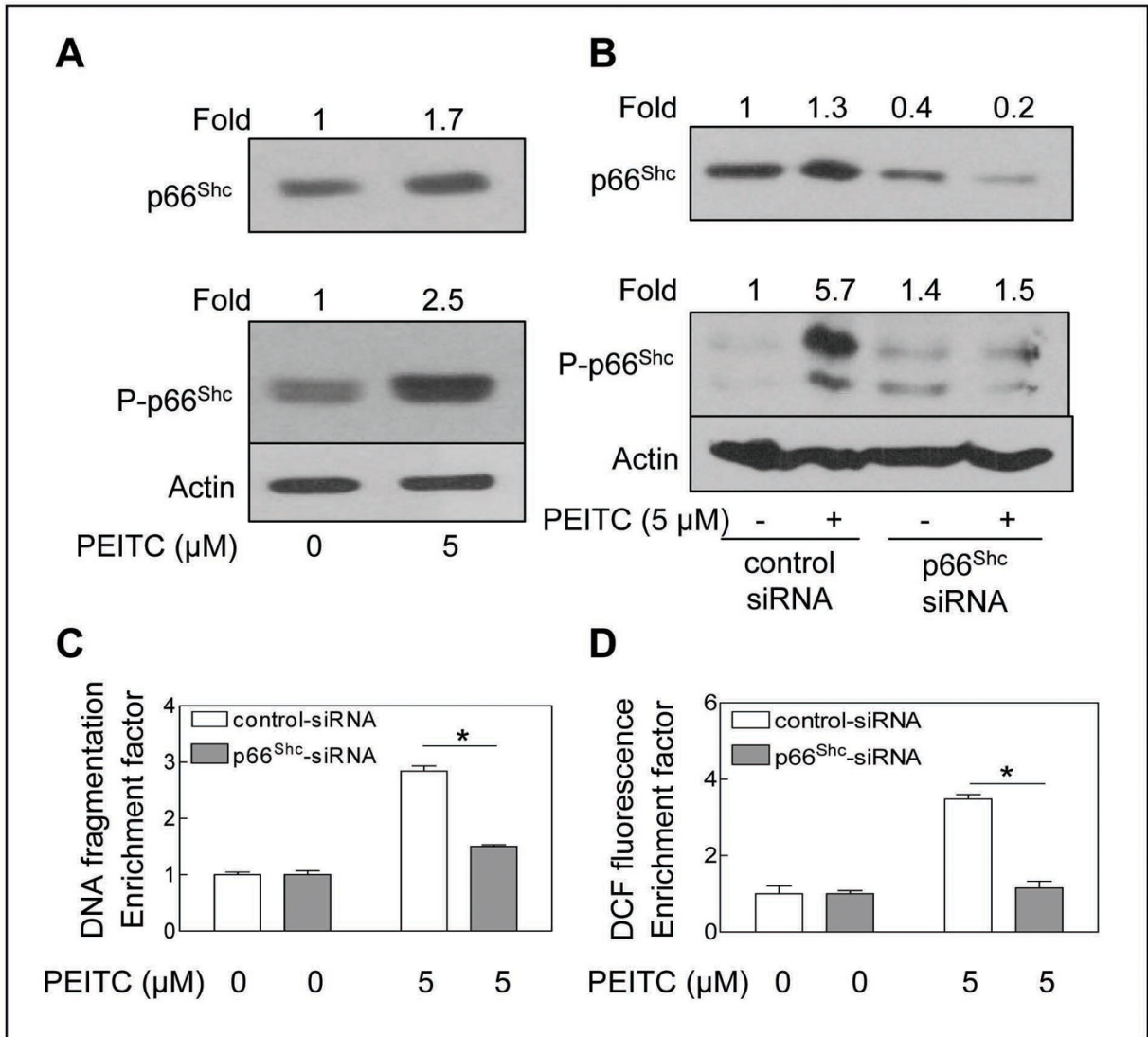
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**Fig. 1.**

A, immunoblotting for p66^{Shc} protein using lysates from untreated MEFs derived from wild-type (p66-WT) and p66^{Shc} knockout mice (p66-KO). The blot was stripped and re-probed with anti-actin antibody to ensure equal protein loading. Cell viability (B), cytoplasmic histone-associated DNA fragmentation (C), and caspase-3 activation (D) in p66-WT and p66-KO MEFs following 4 h treatment with DMSO (control) or the indicated concentrations of PEITC. Results are expressed as enrichment factor relative to DMSO-treated control. Each experiment was done at least twice in triplicate and representative data from a single experiment are shown. Columns, mean (n=3); bars, SE. *Significantly different ($P < 0.05$) between the indicated groups by paired *t*-test.

**Fig. 2.**

A, immunoblotting for total and Ser36 phosphorylated p66^{Shc} using lysates from PC-3 cells treated with DMSO (control) or 5 μmol/L PEITC for 24 h. B, immunoblotting for total and Ser36 phosphorylated p66^{Shc} using lysates from PC-3 cells transiently transfected with a control non-specific siRNA or p66^{Shc}-targeted siRNA and treated for 24 h with DMSO or 5 μmol/L PEITC. The blots were stripped and re-probed with anti-actin antibody to ensure equal protein loading. The numbers on top of the immunoreactive bands represent changes in protein levels relative to corresponding DMSO-treated control (panel A) or relative to DMSO-treated non-specific siRNA transfected PC-3 cells (panel B). C, cytoplasmic histone-associated apoptotic DNA fragmentation, and D, DCF fluorescence in PC-3 cells transiently transfected with a control non-specific siRNA or p66^{Shc}-targeted siRNA and treated for 24 h (panel C) or 4 h (panel D) with DMSO or 5 μmol/L PEITC. The results are expressed as enrichment factor relative to DMSO-treated control for PC-3 cells transiently transfected with the control non-specific siRNA. Each experiment was done twice and representative data from a single experiment are shown. Columns, mean (n=3); bars, SE. *Significantly different ($P<0.05$) between the indicated groups by paired *t*-test.

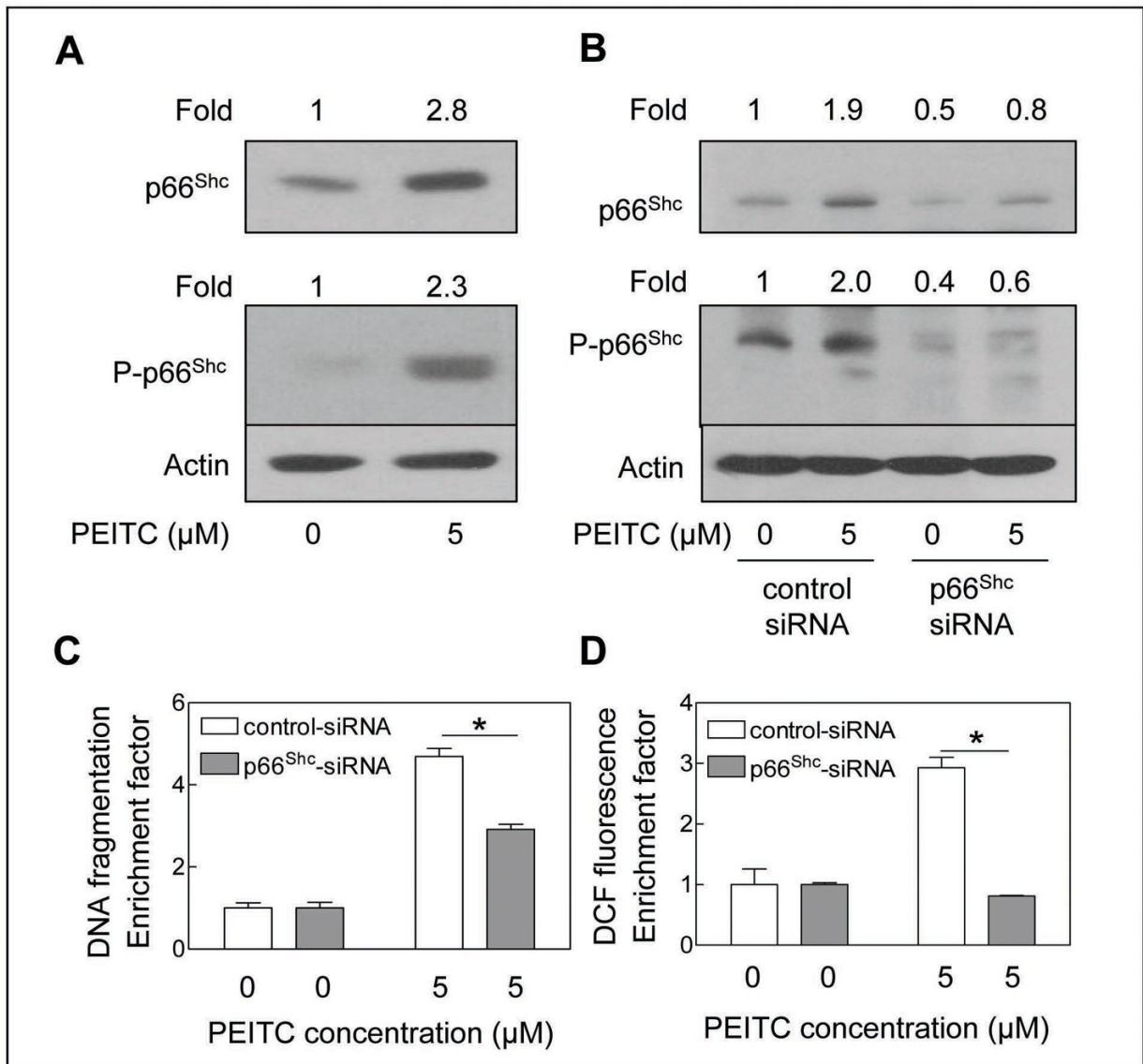
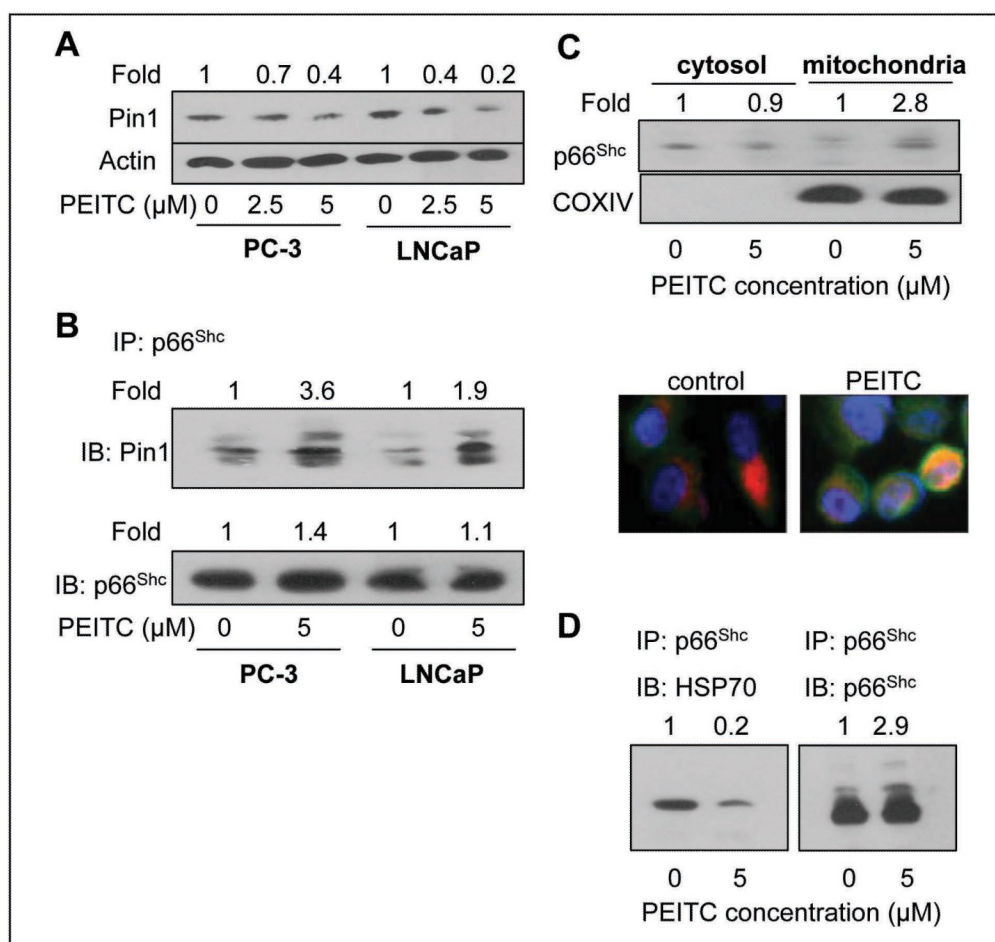


Fig. 3. *A*, immunoblotting for total and Ser36 phosphorylated p66^{Shc} using lysates from LNCaP cells treated with DMSO (control) or 5 μmol/L PEITC for 24 h. *B*, immunoblotting for total and Ser36 phosphorylated p66^{Shc} using lysates from LNCaP cells transiently transfected with a control non-specific siRNA or p66^{Shc}-targeted siRNA and treated for 24 h with DMSO or 5 μmol/L PEITC. The blots were stripped and re-probed with anti-actin antibody to ensure equal protein loading. The numbers on top of the immunoreactive bands represent changes in protein levels relative to DMSO-treated control (panel *A*) or relative to DMSO-treated non-specific control siRNA transfected cells (panel *B*). *C*, cytoplasmic histone-associated apoptotic DNA fragmentation, and *D*, DCF fluorescence in LNCaP cells transiently transfected with a control non-specific siRNA or p66^{Shc}-targeted siRNA and treated for 24 h (panel *C*) and 4 h (panel *D*) with DMSO or 5 μmol/L PEITC. The results are expressed as enrichment factor relative to DMSO-treated control for LNCaP cells transiently transfected with the non-specific siRNA. Each experiment was done twice and

representative data from a single experiment are shown. *Columns*, mean (n=3); *bars*, SE.
*Significantly different ($P<0.05$) between the indicated groups by paired *t*-test.

**Fig. 4.**

A, immunoblotting for Pin1 using lysates from PC-3 and LNCaP cells following 24 h treatment with DMSO or the indicated concentrations of PEITC. The blot was stripped and reprobbed with anti-actin antibody to ensure equal protein loading. The numbers on top of the immunoreactive bands represent change in protein levels relative to corresponding DMSO-treated control. B, immunoblotting for Pin1 using p66^{Shc} immunoprecipitates from PC-3 and LNCaP cells treated for 8 h with DMSO (control) or 5 μmol/L PEITC. The numbers on top of the immunoreactive bands represent change in levels relative to DMSO-treated control for each cell line. C, immunoblotting for p66^{Shc} using isolated cytosolic and mitochondrial fractions from PC-3 cells following 8 h treatment with DMSO or 5 μmol/L PEITC. The blot was reprobbed with anti-COXIV antibody to ensure purity of the mitochondrial fraction. The numbers on top of the immunoreactive bands represent change in levels relative to DMSO-treated control. Immunofluorescence microscopic analysis for p66^{Shc} localization in PC-3 cells following 8 h treatment with DMSO or 2.5 μmol/L PEITC (magnification 100×). The staining for mitochondria (MitoTracker red), p66^{Shc}, and nuclei are indicated by red, green and blue fluorescence, respectively. D, immunoblotting for HSP70 using p66^{Shc} immunoprecipitates (using anti-p66^{Shc} antibody) from control (DMSO, 8 h) or PEITC-treated (5 μmol/L, 8 h) PC-3 cells. The numbers on top of the immunoreactive bands represent change in levels relative to DMSO-treated control.

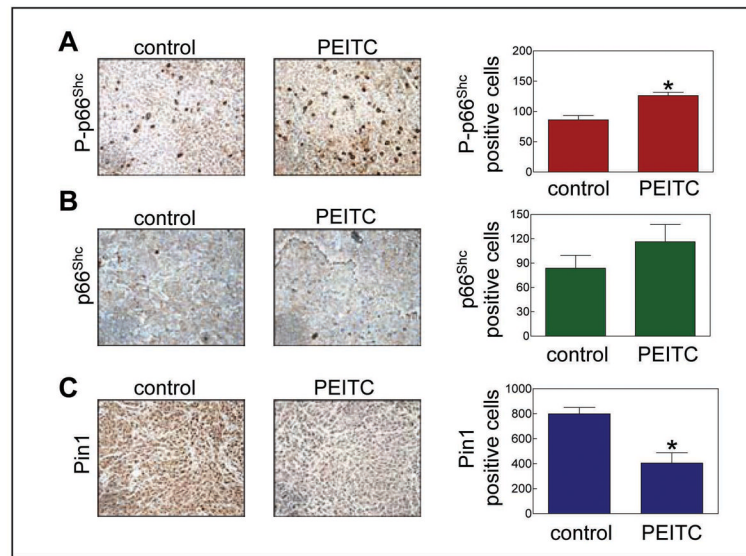


Fig. 5. Immunohistochemical analysis for expression of (A) Ser36 phosphorylated p66^{Shc}, (B) total p66^{Shc}, and (C) Pin1 (200× magnification) in representative tumor section of a vehicle-treated control mouse and a PEITC-treated mouse. *Right panel*, quantitation of immunohistochemical data. *Columns*, mean (n= 3); *bars*, SE. *Significantly different ($P < 0.05$) compared with control by paired *t*-test.

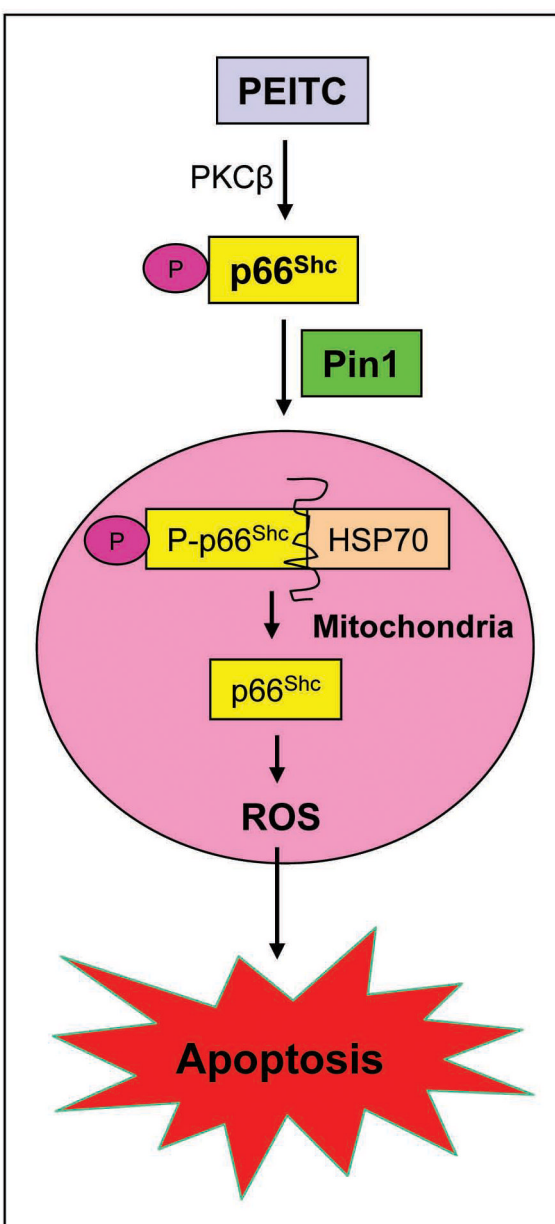


Fig. 6.

A mechanistic model explaining role of p66^{Shc} in PEITC-induced apoptosis. Based on the results of the present study, we conclude that PEITC treatment increases Ser36 phosphorylation of p66^{Shc}, which increases its binding with Pin1. The binding of p66^{Shc} with Pin1 promotes translocation of p66^{Shc} to the mitochondria. The p66^{Shc} protein localized to the mitochondria is released from HSP70 and causes ROS production leading to caspase-3 activation and eventual apoptotic cell death.