A NADPH Oxidase–Dependent Redox Signaling Pathway Mediates the Selective Radiosensitization Effect of Parthenolide in Prostate Cancer Cells

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Abstract
Cancer cells are usually under higher oxidative stress compared with normal cells. We hypothesize that introducing additional reactive oxygen species (ROS) insults or suppressing antioxidant capacity may selectively enhance cancer cell killing by oxidative stress–generating agents through stress overload or stress sensitization, whereas normal cells may be able to maintain redox homeostasis under exogenous ROS by adaptive response. Here, we show that parthenolide, a sesquiterpene lactone, selectively exhibits a radiosensitization effect on prostate cancer PC3 cells but not on normal prostate epithelial PrEC cells. Parthenolide causes oxidative stress in PC3 cells but not in PrEC cells, as determined by the oxidation of the ROS-sensitive probe H2DCFDA and intracellular reduced thiol and disulfide levels. In PC3 but not PrEC cells, parthenolide activates NADPH oxidase, leading to a decrease in the level of reduced thioredoxin, activation of phosphoinositide 3-kinase/Akt, and consequent FOX-O3a phosphorylation, which results in the downregulation of FOXO3a targets antioxidant enzyme manganese superoxide dismutase and catalase. Importantly, when combined with radiation, parthenolide further increases ROS levels in PC3 cells whereas it decreases radiation-induced oxidative stress in PrEC cells, possibly by increasing reduced glutathione levels. Together, the results show that parthenolide selectively activates NADPH oxidase and mediates intense oxidative stress in prostate cancer cells by both increasing ROS generation and decreasing antioxidant defense capacity. The results support the concept of exploiting the intrinsic differences in the redox status of cancer cells and normal cells as targets for selective cancer killing. Cancer Res; 70(7): 2880–90. ©2010 AACR.

Introduction
Selectively killing cancer without harming normal tissue is a fundamental challenge in cancer therapy. Elevated oxidative stress and aberrant redox homeostasis are frequently observed in cancer cells compared with their normal cell counterparts. For example, prostate cancer cells often have increased reactive oxygen species (ROS) generation from mitochondria (1) or NADPH oxidase (2), and decreased antioxidant enzymes, such as manganese superoxide dismutase (MnSOD), CuZnSOD, and catalase (3, 4). A small shift toward an oxidizing condition in cells may lead to elevated proliferation and induction of adaptive response. However, a high oxidizing condition often results in cell injury and cell death. Persistent high ROS in cancer cells often leads to increased cell proliferation and adaptive responses that may contribute to tumorigenesis, metastasis, and treatment resistance. Further exposure to exogenous ROS is hypothesized as pushing tumor cells, which already have high constitutive oxidative stress levels, to cell death, whereas normal cells may still maintain redox homeostasis through adaptive responses. Therefore, regulating intracellular redox state may represent an ideal strategy to selectively sensitize cancer cells to oxidative stress–inducing therapy, such as radiotherapy.

Parthenolide is a sesquiterpene lactone derived from the traditional herbal medicine feverfew. The biological activity of parthenolide is thought to be mediated through its α-methylene-γ-lactone moiety, which can react with nucleophiles, especially with cysteine thiol groups, in a Michael addition reaction. Thiols (-SH) are important in integrating intracellular redox changes with cellular signaling transduction pathways. Several regulatory proteins, such as kinases, phosphatases, and transcription factors, have cysteines on their active sites. Oxidation and reduction of cysteine thiols affect protein functions or act as the molecular switch for their downstream signaling cascades (5). The chemical properties of parthenolide make it a good candidate for modifying cellular redox signaling and give it great potential in cancer therapy. Oxidative stress is a major mechanism for parthenolide–induced cell death (6). Our previous study showed that parthenolide sensitizes human prostate cancer cells to radiation treatment by inhibiting the NF-κB pathway (7). However, whether the radiosensitization effect of parthenolide is selective to prostate cancer cells, but not to normal prostate...
cells, and whether parthenolide differentially regulates intracellular redox state in cancer and normal cells are unknown.

NADPH oxidase is an important source of ROS, which accounts, at least partially, for increased levels of ROS in prostate cancer (2, 8). The first discovered NADPH oxidase is phagocyte NADPH oxidase. It is a multisubunit enzyme localized in cell membranes, consisting of membrane-bound components (gp91phox and p22phox) and cytosolic components (p47phox, p67phox, p40phox, and Rac) that translocate to the membrane upon activation. Homologues of gp91phox (Nox2), including Nox1-5, Duox1 (dual oxidase), and Duox2, have been identified and named Nox (NADPH oxidase) proteins in nonphagocytic cells. Their activation requires p47phox paralogue Nox1 (Nox organizer 1) and p67phox paralogue Nox1a (Nox activator 1), or calcium binding (9). Nox proteins catalyze the transfer of an electron to O2 to generate O2•−, which is then dismutated to H2O2. It has been shown that prostate tumor more is likely (86%) to have Nox1 staining compared with benign prostate tissue (62%; refs. 8, 10) and that Nox-induced ROS is an important contributor to X-ray radiation–induced cell death (11, 12). Whether parthenolide activates NADPH oxidase in prostate cancer cells is unknown.

In a previous study, we found that parthenolide activates the phosphatidylinositol 3-kinase (PI3K)/Akt pathway in prostate cancer cells (7). Although activated Akt is known to inhibit apoptosis and promote cell survival, recent studies suggest that the PI3K/Akt pathway may induce oxidative stress and trigger cell death under certain conditions (13). The mechanisms by which Akt induces ROS may involve the stimulation of mitochondrial oxidative metabolism and reduction of antioxidant defense through FOXO suppression.

The FOXO transcription factors are mammalian homologues of DAF-16, which regulates longevity in Caenorhabditis elegans. Among the four FOXO family members (FOXO1, FOXO3a, FOXO4, and FOXO6), FOXO1 and FOXO3a are the most highly expressed FOXO proteins in human prostate (14). The downstream targets of FOXOs may be involved in cell cycle and cell death regulation, differentiation and development, cellular stress response, and energy metabolism control.

**Materials and Methods**

**Cell culture and treatment.** Human prostate cancer cell lines PC3 and DU145 were obtained from the American Type Culture Collection and were cultured as previously described (7). Human normal prostate epithelial PrEC cells were purchased from Lonza and were maintained in PrEGM medium (Lonza). Parthenolide, NADPH oxidase inhibitor diphenylene iodonium (DPI, Sigma), and PI3K inhibitor wortmannin (Cell Signaling) were dissolved in DMSO.

**MTT assay.** Cells were treated with parthenolide for 24 h and exposed to radiation or were sham irradiated. Twenty-four hours after radiation, the parthenolide-containing medium was replaced with normal culturing medium for a total parthenolide treatment of 48 h. After four cell doubling times (~4 d for PC3 cells and 8 d for PrEC cells; ref. 26), MTT assay was performed as previously described (7).

**Cell growth curve.** Cells were treated as described above for the MTT assay. The mean number of cells per well was obtained every other day after radiation from the triplicate average. The results were plotted on a log-linear scale and fitted into an exponential growth curve.

**Dichlorofluorescein assay.** Dichlorofluorescein (DCF) assay was performed using carboxy-H2DCFDA (sensitive to oxidation; Invitrogen) and oxidized carboxy-DCFDA (insensitive to oxidation; Invitrogen) as optimized by Wan and colleagues (27). The fluorescence in cells preloaded with carboxy-H2DCFDA was normalized to that in cells preloaded with carboxy-DCFDA (ratio of H2DCFDA/DCFDA) to control for the cell number, dye uptake, and ester cleavage differences between different treatment groups.

**Detection of reduced thiols and disulfides.** Protein thiols were labeled by 3-N-maleimido-propionyl biocytin (MPB) and detected by avidin-biotin technology on the blots as previously described by Wan and colleagues (28). To detect protein disulfides, samples were first treated with N-ethylmaleimide (Sigma) to block the free thiol groups. Then, 2-mercaptoethanol (Sigma) was added to reduce the disulfide bond. The N-ethylmaleimide–blocked, 2-mercaptoethanol–reduced protein was then treated with MPB for disulfide labeling. Labeled proteins were subjected to SDS-PAGE, followed by detection with horseradish peroxidase–conjugated streptavidin.

**NADPH oxidase activity assay.** This assay was performed as previously described by Cui and Douglas (29). Photoemission generated by the reaction of superoxide radical and lucigenin in terms of relative luminescence units (RLU) was measured every minute for 15 min. Reaction velocity was calculated as the change of RLU per minute per microgram of protein.

**Glutathione assay.** Total glutathione (GSH) and oxidized glutathione (GSSG) were measured using the recycling assay of the Glutathione Assay kit (Cayman Chemical). The
amount of reduced GSH was calculated by subtracting the amount of GSSG from total GSH (GSHt − 2GSSG).

**Western blot analysis.** Western blot analysis was performed as previously described (7) using corresponding antibodies against Akt and phospho-Akt (Ser473), FOXO3a and phospho-FOXO3a (Ser253; Cell Signaling), actin (Sigma), Nox1 (Santa Cruz), catalase (Santa Cruz), and MnSOD (Upstate). Representative blots and quantification from three independent experiments are shown.

**Knocking down Nox1 using small interfering RNA.** Cells were transiently transfected with Nox1 small interfering RNA (siRNA) (Santa Cruz Biotechnology) and control siRNA by using Oligofectamine (Invitrogen).

**Electrophoretic mobility shift assay.** Double-stranded oligonucleotides corresponding to the MnSOD promoter region containing consensus FOXO3a binding element (DBE, Daf-16 family protein–binding element, 5′-TTCTGAGCTGTGAAACAAGCCAGCCCTT-3′; ref. 18) were labeled with [32P] ATP. The assay was performed as previously described (7).

**Chromatin immunoprecipitation assay.** Cells were collected and processed using ChIP-IT kit (Active Motif). The MnSOD promoter fragment containing DBE was amplified. The sequences of primer set were as follows: upper-strand primer, 5′-CACCCCACAACGATGACCCTAAGTCATTC-3′; lower-strand primer, 5′-CTAGGCTTCCGGTAAGTGGAATGGGAAAAC-3′.

**SOD mimetic treatment and colony survival assay.** Cells were treated with parthenolide or DMSO for 24 h before radiation exposure. Twenty-four hours after radiation, normal culture medium replaced the medium containing parthenolide. SOD mimetic (MnTe-2-PyP5+) was added concurrently with parthenolide and kept in the medium throughout. Colony survival assay was conducted as previously described (7).

**FOXO3a transient transfection and determination of cell survival by trypan blue exclusion assay.** Cells were transfected with HA-FOXO3a-WT (wild-type), HA-FOXO3a-TM (triple mutant; Addgene; ref. 15), or pECE vector control plasmid using Lipofectamine 2000 (Invitrogen). HA-FOXO3a-TM has three mutations at Akt phosphorylation sites (Thr32, Ser253, and Ser315), leading to constitutively active FOXO3a. After transfection, the medium was replaced with complete medium with or without parthenolide and incubated overnight. Cells were then treated with 6 Gy radiation or were sham irradiated. Forty-eight hours after radiation, cells were processed for trypan blue exclusion assay and collected for Western blot analysis.

**Statistical analysis.** Statistical analysis was performed using either Student’s t test (for two-group comparison) or one-way ANOVA (for multiple-group comparison). Data were reported as mean ± SEM.

**Results**

The radiosensitization effect of parthenolide is selective to prostate cancer PC3 cells but not to normal prostate epithelial PrEC cells. Previously, we showed that parthenolide synergistically enhances the sensitivity of prostate cancer cells to radiation treatment using colony survival assay (7). In the present study, we compared the effect of parthenolide in prostate cancer PC3 cells and normal prostate epithelial PrEC cells. Because PrEC cells did not form colonies in vitro, we performed MTI assay under the growth condition that provided a relative cell survival comparable with the clonogenic assays (7). PrEC cells are more resistant to parthenolide-induced cytotoxicity compared with PC3 cells (Fig. 1A). The subcytotoxic dose of parthenolide (1 μmol/L) was chosen to study the combination effect of parthenolide and radiation on cell survival. As shown in Fig. 1B, 6 Gy radiation decreases cell viability in PC3 cells (by 53%) and in PrEC cells (by 41%). Parthenolide (1 μmol/L) reduces cell viability and enhances radiation-induced cytotoxicity in PC3 cells but not in PrEC cells. Comparisons of growth curves for these two cell lines also reveal a selective radiosensitization effect of parthenolide (Fig. 1C) in PC3 cells. Radiation (6 Gy) decreases cell growth rate by ~50% in both cell lines (Fig. 1D). Parthenolide alone decreases cell number without obvious change in growth rate in both cell lines. A combination of parthenolide and radiation shows radiosensitization in PC3 cells by both decreased cell number and lower growth rate. However, the growth rate in the combination treatment group is the same as the untreated group for PrEC cells.

**Parthenolide induces oxidative stress in PC3 cells but not in PrEC cells.** We then explored potential determinants for the selectivity of the effect of parthenolide. Because oxidative stress is the major mechanism for both parthenolide-induced (6) and radiation-induced cell death, we compared the effect of parthenolide on cellular ROS level in PC3 and PrEC cells by DCF assay. As shown in Fig. 2A, radiation significantly increases normalized carboxy-H2DCFDA fluorescence, a general indicator of cellular ROS level, in both PC3 and PrEC cells. However, parthenolide alone increases the ROS level in prostate cancer PC3 cells but not in normal prostate PrEC cells. When combined with radiation, parthenolide further increases the cellular ROS level in PC3 cells, consistent with the radiosensitization effect. Interestingly, in PrEC cells, parthenolide decreases radiation-induced ROS level (significant at 5 μmol/L), suggesting an antioxidant response.

Parthenolide exerts its effect mainly by targeting thiol groups, which may lead to the depletion of intracellular glutathione and protein thiols and induction of ROS (6, 30). We therefore detected the presence of protein thiols and disulfides in PC3 and PrEC cells by MPB labeling. As shown in Fig. 2B, 5 μmol/L parthenolide significantly decreases reduced protein thiols and increases disulfide staining in PC3 cells. The decrease in the reduced protein thiols may result from the direct reaction of parthenolide with protein thiols or the oxidation of thiol groups due to parthenolide-induced oxidative stress. Interestingly, in PrEC cells, parthenolide does not significantly change the protein thiols and disulfides levels, which is consistent with the selective induction of oxidative stress in PC3 cells.

**Parthenolide activates NADPH oxidase in PC3 cells but not in PrEC cells.** Oxidative stress is the imbalance between prooxidants and antioxidants. Both increased production of ROS and decreased antioxidants can lead to oxidative stress. One major source of ROS generation in prostate cancer cells

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Figure 1. The radiosensitization effect of parthenolide is selective to prostate cancer PC3 cells but not to normal prostate epithelial PrEC cells. A, MTT assay. Cell viability was measured after 48 h of parthenolide (PN) treatment. B, the combination effect of parthenolide and radiation on cell viability was compared in PC3 and PrEC cells by the MTT assay. *, $P < 0.05$ compared with DMSO-treated control. C, cell growth curve. Cell numbers were plotted on a log-linear scale. Equations derived from the exponential growth curve fit $Y = Y_{\text{start}} \times \exp(K \times X)$ are shown. Cell number begins at $Y = Y_{\text{start}}$ and increases exponentially with rate constant $K$. IR, irradiation. D, comparison of the growth rate constant in PC3 and PrEC cells after parthenolide and radiation treatment. The growth rate constant ($K$) was obtained from the exponential growth curve fit in C and normalized to the DMSO control group for each cell line, respectively. *, $P < 0.05$ compared with DMSO treated control.
Figure 2. Parthenolide induces oxidative stress in PC3 cells but not in PrEC cells. A, DCF assay. Cells were treated with parthenolide or DMSO for 24 h; the ratio of carboxy-H$_2$DCFDA (oxidation sensitive) to carboxy-DCFDA (oxidation insensitive) was compared. *, $P < 0.05$ compared with no radiation control. #, $P < 0.05$ compared with DMSO-treated no radiation control. §, $P < 0.05$ compared with the indicated group. B, detection of protein thiols and disulfides. Cells were treated with DMSO or parthenolide for 24 h and harvested for thiol and disulfide detection by MPB labeling. C, NADPH oxidase activity assay. Cells were treated with DMSO or parthenolide for 24 h in the absence or presence of 0.5 μmol/L DPI. Reaction velocity (v) was calculated as the change in RLU per minute per microgram of protein. *, $P < 0.05$ compared with DMSO-treated no DPI control. #, $P < 0.05$ compared with no DPI control.
is NADPH oxidase (2, 8). We measured NADPH oxidase activity to probe whether it is involved in parthenolide-induced oxidative stress in prostate cancer PC3 cells. Our results (Fig. 2C) show that parthenolide enhances NADPH oxidase activity dose dependently in PC3 cells, which can be inhibited by DPI, a NADPH oxidase inhibitor. However, in PrEC cells, NADPH oxidase activity is not significantly affected by parthenolide.

**Parthenolide decreases reduced thioredoxin in PC3 cells as a downstream event of NADPH oxidase activation but increases glutathione in PrEC cells.** Because parthenolide targets thiols, we also detected two important thiol-containing small-molecule antioxidants, GSH and thioredoxin. As shown in Fig. 3A, total GSH level is slightly increased in PC3 cells and significantly increased in PrEC cells by parthenolide. The reduced GSH/GSSG ratio is not significantly changed by parthenolide in PC3 cells but is increased 2.4-fold in PrEC cells, which may lead to the protective effect against radiation-induced oxidative stress observed by DCF assay. Our result is contrary to a report that parthenolide can deplete intracellular GSH in cancer cells (6). Because the reactivities of thiol groups are inversely related to their pKa, parthenolide may more readily react with protein thiols that have low pKa than with GSH, which has a high pKa of 8.8. The induction of GSH may be due to the activation of the Nrf2/ARE (antioxidant/electrophile response element) pathway (31) because Keap1, the negative regulator of Nrf2, contains numerous low pKa cysteines (32).

Globally decreased protein thiols have been observed in PC3 cells but not in PrEC cells after parthenolide treatment (Fig. 2B). We examined reduced thioredoxin, the active form of thioredoxin, by using thioredoxin antibody to pull down the thiol-labeled protein sample. Parthenolide significantly decreases reduced thioredoxin in PC3 cells without altering the thioredoxin protein amount (Fig. 3B). However, in the presence of DPI, the decrease caused by parthenolide in the reduced thioredoxin is abolished, suggesting that this is a downstream event of NADPH oxidase activation. It is likely that thioredoxin was oxidized by Nox-derived ROS.

**Activation of NADPH oxidase by parthenolide is upstream of PI3K/Akt activation in PC3 cells.** The PI3K/Akt pathway has been known to be activated by growth factors and oxidative stress (33), so we tested whether activation of the PI3K/Akt pathway is a downstream event of NADPH oxidase activation in PC3 cells. The PI3K inhibitor wortmannin prevents activation of Akt by parthenolide, as indicated by the decrease of p-Akt/Akt ratio. However, activation of NADPH oxidase by parthenolide is not affected (Fig. 4A). We then identified Nox1 as the major Nox isoform in PC3 cells by real-time PCR (data not shown). Knockdown down Nox1 by siRNA inhibits both parthenolide-induced NADPH oxidase activation and Akt activation (Fig. 4B), suggesting that Nox1-dependent NADPH oxidase activation by parthenolide is upstream of PI3K/Akt activation. This was further confirmed by using the NADPH oxidase inhibitor DPI. In the presence of DPI, Akt activation by parthenolide and phosphorylation of FOXO3a, the downstream target of Akt kinase, are both prevented (Fig. 4C).

**Activation of Akt by parthenolide induces FOXO3a phosphorylation and suppresses its downstream targets, antioxidant enzymes MnSOD and catalase, in prostate cancer cells but not in PrEC cells.** FOXO3a is a main target of activated Akt. There are three conserved Akt phosphorylation sites: T32, S233, and S253. Parthenolide significantly decreases the phosphorylation of FOXO3a at T32, S233, and S253 in PC3 cells (Fig. 4B). However, the phosphorylation of FOXO3a at these sites is not altered in PrEC cells, suggesting that Akt activation by parthenolide is upstream of FOXO3a phosphorylation.

**Activation of Akt by parthenolide also induces phosphorylation of the downstream target of FOXO3a, atp-1, in PC3 cells but not in PrEC cells.** Akt activation by parthenolide induces phosphorylation of the downstream target of FOXO3a, atp-1, in PC3 cells but not in PrEC cells. At present, it is not known which kinase specifically phosphorylates atp-1. However, Akt is known to induce phosphorylation of other substrates, such as caspase-9, through atp-1. These findings suggest that Akt activation by parthenolide is upstream of atp-1 phosphorylation.

**Figure 3. Effect of parthenolide on thiol-containing antioxidants GSH and thioredoxin.** A, GSH assay. Cells were treated with DMSO or parthenolide for 24 h before radiation and then harvested at 24 h after radiation for GSH detection. *, P < 0.05; **, P < 0.001 compared with DMSO control. B, detection of reduced thioredoxin (TRxre) by immunoprecipitating (IP) MBP-labeled protein sample with thioredoxin antibody, followed by avidin detection. Total thioredoxin protein is detected by thioredoxin antibody.
sites on FOXO3a: Thr32, Ser253, and Ser315 (34). Consistent with our previous study (7), parthenolide increases Akt phosphorylation in prostate cancer PC3 and DU145 cells (Fig. 5A). Consequently, the phosphorylation on Ser253 in FOXO3a is increased by parthenolide in a dose-dependent manner. Radiation only slightly increases Akt and FOXO3a phosphorylation. This may be explained by the fact that radiation only induces transient activation of Akt, which peaks at 1 hour after radiation and then drops at 6 hours after radiation (7).

In DU145 cells, the total FOXO3a level is increased after radiation treatment, consistent with Yang’s observation in osteosarcoma cells (20). Parthenolide decreases total FOXO3a level in DU145 cells, which may be due to Akt activation-induced FOXO3a degradation by proteasome (35). Although FOXO3a phosphorylation increases in prostate cancer cells, in normal prostate epithelial PrEC cells, the combination of parthenolide with radiation does not enhance but slightly decreases FOXO3a phosphorylation.

Figure 4. Activation of NADPH oxidase by parthenolide is upstream of PI3K/Akt activation in PC3 cells. A, PI3K inhibitor wortmannin (Wort) does not suppress parthenolide-induced Nox activation. PC3 cells were treated with DMSO or parthenolide for 24 h. Wortmannin was added 1 h before and throughout the treatment period. *, P < 0.05 compared with the indicated groups. B, knocking down Nox1 suppresses parthenolide-induced Nox activation and Akt activation. Cells were treated with DMSO or parthenolide for 24 h after transfection. *, P < 0.05 compared with the indicated groups. C, NADPH oxidase inhibitor DPI suppresses parthenolide-induced Akt activation and FOXO3a phosphorylation. PC3 cells were treated with DMSO or parthenolide for 24 h in the absence or presence of DPI.
Akt-mediated phosphorylation has been shown to induce FOXO3a cytoplasmic sequestration and thereby suppress its DNA binding activity. This is confirmed by an electrophoretic mobility shift assay (EMSA; Fig. 5B). Radiation enhances FOXO3a DNA binding activity in PC3 cells. Parthenolide inhibits FOXO3a DNA binding dose dependently in PC3 cells but not in PrEC cells. Chromatin immunoprecipitation assay verified that FOXO3a indeed binds to the promoter of its target gene. As shown in Fig. 5C, radiation enhances FOXO3a binding to the MnSOD promoter region.

Figure 5. Activation of Akt by parthenolide induces FOXO3a inhibitory phosphorylation and suppresses its downstream targets, MnSOD and catalase, in prostate cancer cells but not in PrEC cells. Cells were treated with DMSO or parthenolide for 24 h before radiation. A, whole-cell lysates were prepared 6 h after radiation for Western blot analysis. B, nuclear extract was isolated 6 h after radiation for EMSA. C, chromatin immunoprecipitation assay was performed at 6 h after radiation. Input controls were prepared before adding the antibody. Anti–RNA polymerase II antibody was used, and the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) promoter region was amplified as negative control. D, whole-cell lysates were prepared 24 h after radiation for Western blot analysis.
Figure 6. Suppression of antioxidant enzymes by parthenolide is involved in its radiosensitization effect. A, colony survival assay was performed with SOD mimetic in PC3 cells. *, P < 0.05 compared with untreated control. **, P < 0.05 compared with parthenolide alone and radiation alone. #, P < 0.05 compared with parthenolide in combination with radiation. B, overexpression of FOXO3a protects PC3 cells against the effect of parthenolide as determined by trypan blue exclusion assay. *, P < 0.05 compared with vector-transfected cells under the same treatment condition. C, Western blots to confirm overexpression of FOXO3a in PC3 cells. D, schematic of the differential effects of parthenolide in prostate cancer and normal prostate epithelial cells.
which is suppressed by parthenolide, consistent with the EMSA result.

FOXO3a regulates a wide range of target genes. Because radiation kills cells, in part through generation of ROS, we detected FOXO3a targets—antioxidant enzymes catalase and MnSOD—in prostate cancer PC3 and DU145 cells (Fig. 5D). MnSOD protein level is increased after radiation in both cell lines, but is suppressed by parthenolide. Parthenolide also decreases catalase levels dose dependently in both cell lines. Consistent with FOXO3a DNA binding activity, MnSOD and catalase levels in PrEC cells are not changed by parthenolide.

**Suppression of antioxidant enzymes by parthenolide contributes to its radiosensitization effect.** To confirm the role of antioxidant enzymes in the selective radiosensitization effect of parthenolide, we treated PC3 cells with a SOD mimetic, MnTE-2-PyP5++. SOD mimetic partially abolishes the radiosensitization effect of parthenolide in PC3 cells as determined by colony survival assay (Fig. 6A).

We then overexpressed FOXO3a in PC3 cells to investigate whether overexpression of FOXO3a can rescue cells from parthenolide-induced radiosensitization effect by induction of antioxidant enzymes. Overexpression of FOXO3a-WT and FOXO3a-TM decreases the survival of untreated cells, possibly due to the induction of apoptotic targets of FOXO3a (19). We therefore normalized all untreated cell viability to 100% to eliminate basal survival differences among three different transfection groups. After normalization, we observed that FOXO3a overexpression does not significantly affect cell sensitivity to radiation treatment. However, overexpression of FOXO3a, especially constitutively active FOXO3a-TM, in PC3 significantly decreases catalase and MnSOD levels in prostate cancer PC3 cells (Fig. 6B). The expression of exogenous HA-FOXO3a was confirmed by Western blot. The basal levels of the antioxidant enzymes catalase and MnSOD are higher when active FOXO3a-TM is overexpressed (Fig. 6C). These data show that FOXO3a plays an important role in maintaining the cellular antioxidant enzymes catalase and MnSOD, which are involved in the radiosensitization effect of parthenolide.

**Discussion**

The selective cytotoxicity of parthenolide to cancer cells has been reported in human acute myelogenous leukemia stem and progenitor cells (36). Our current study confirms these findings in prostate cancer cells and extends to show that the radiosensitization effect of parthenolide is selective to prostate cancer cells but not to normal prostate epithelial PrEC cells (Fig. 1). Our results also indicate that parthenolide “rejuvenates” irradiated normal prostate cells because cell growth rate after radiation is restored to the untreated control level when combined with parthenolide treatment. Parthenolide decreases radiation-induced ROS in PrEC cells (Fig. 2A), which correlates with increased GSH levels (Fig. 3A). Thus, the apparent antioxidant property of parthenolide in normal prostate cells may be due, in part, to the increased GSH level and may account for the rejuvenation of irradiated PrEC cells.

The selective targeting of cancer cells by parthenolide is of great interest. Our study shows for the first time that differential modulation of intracellular redox state by parthenolide in prostate cancer and normal prostate cells is involved in its selective radiosensitization effect. Cancer cells and normal cells have different redox status, which may be targeted for selective cancer killing. Prostate cancer PC3 cells have higher Nox1 and lower MnSOD, catalase (Supplementary Fig. S1), and GSH levels (Fig. 3A) compared with normal prostate PrEC cells, which may be responsible for the high oxidative stress levels in prostate cancer cells. In response to the constitutively high oxidative stress in prostate cancer cells, elevated proliferation and induction of adaptive response are observed; for example, higher thioredoxin levels in prostate cancer cells (Supplementary Fig. S2) and constitutively active NF-κB (37), which may be involved in treatment resistance (38–40). Parthenolide further increases oxidative stress in prostate cancer cells by activating NADPH oxidase and suppressing the antioxidants thioredoxin, MnSOD, and catalase. The redox imbalance in prostate cancer cells caused by parthenolide enhances cellular sensitivity to oxidative stress–inducing radiotherapy (Fig. 6D). However, in normal prostate cells, due to high GSH (Fig. 3A) and glutathione S-transferase (GST; ref. 41) levels, parthenolide may more readily conjugate with GSH under the catalysis of GSTs and then be exported outside the cells, leading to cellular resistance to parthenolide.

How parthenolide activates Nox remains unknown. Because the amount of Nox1 is not increased (Fig. 4B) by parthenolide, parthenolide may activate Nox by facilitating the assembly of the multisubunit enzyme complex. The full activation of Nox1 requires p22h•h, Noxo1, Noxa1, and Rac1 (9). It has been shown that modulation of cysteine thiol can activate Ras (42, 43). Because Rac1 is a member of the Ras superfamily of GTPases, it is possible that parthenolide may modify the cysteine thiol on Rac1 and increase its activity. In addition to direct oxidative damage, Nox-derived ROS plays an important role in redox signaling due to its highly regulated activation. Activation of Nox can activate PI3K/Akt by the oxidation and inactivation of phosphatases that dephosphorylate PI3K or AKT kinase by Nox-derived ROS. The PI3K/Akt/FOXO3a cascade leads to downregulation of the antioxidant enzymes MnSOD and catalase, which is also involved in the oxidative stress induced by parthenolide.

In summary, our study shows that in prostate cancer cells, parthenolide induces dramatic oxidative stress through NADPH oxidase activation. On one hand, NADPH oxidase activation not only increases ROS generation but also decreases reduced thioredoxin or acts as a second messenger to activate the PI3K/Akt pathway, leading to a decrease in antioxidant defense capacity through FOXO3a suppression. On the other hand, parthenolide increases GSH levels but does not activate NADPH oxidase in normal prostate epithelial cells. Thus, the selective induction of oxidative stress by parthenolide in prostate cancer cells accounts for its selective radiosensitization effect. Our results also imply that modulating the intracellular redox state might be an ideal way for cancer therapy to achieve selective cancer killing.
Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Dr. Ines Batinic-Haberle (Department of Radiation Oncology, Duke University Medical Center, Durham, NC) for kindly providing the SOD mimetic used in this study.

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Grant Support

NIH grants CA49797 and CA115801.

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Received 12/18/2009; revised 01/27/2010; accepted 02/02/2010; published OnlineFirst 03/16/2010.
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Cancer Res 2010;70:2880-2890. Published OnlineFirst March 16, 2010.

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