Guggulsterone-Induced Apoptosis in Human Prostate Cancer Cells Is Caused by Reactive Oxygen Intermediate–Dependent Activation of c-Jun NH_2-Terminal Kinase

Shivendra V. Singh,[1,2,3] Sunga Choi,[4] Yan Zeng,[3] Eun-Ryeong Hahm,[1,3] and Dong Xiao[1,3]

Departments of [1]Pharmacology and [2]Urology and [3]University of Pittsburgh Cancer Institute, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania and [4]Department of Life Sciences, Hallym University, Gangwon-do, Korea

Abstract

Guggulsterone, a constituent of Indian Ayurvedic medicinal plant *Commiphora mukul*, causes apoptosis in cancer cells but the sequence of events leading to cell death is poorly understood. We now show that guggulsterone-induced cell death in human prostate cancer cells is caused by reactive oxygen intermediate (ROI)–dependent activation of c-Jun NH_2-terminal kinase (JNK). Exposure of PC-3 and LNCaP cells to apoptosis inducing concentrations of guggulsterone resulted in activation of JNK and p38 mitogen-activated protein kinase (p38 MAPK) in both cell lines and activation of extracellular signal-regulated kinase 1/2 (ERK1/2) in LNCaP cells. The guggulsterone-induced apoptosis in PC-3/LNCaP cells was partially but statistically significantly attenuated by pharmacologic inhibition (SP600125) as well as genetic suppression of JNK activation. On the other hand, pharmacologic inhibition of p38 MAPK activation in PC-3 or LNCaP cells (SB202190) and ERK1/2 activation in LNCaP cells (PD98059) did not protect against guggulsterone-induced cell death. The guggulsterone treatment caused generation of ROI in prostate cancer cells but not in a normal prostate epithelial cell line (PrEC), which was also resistant to guggulsterone-mediated JNK activation. The guggulsterone-induced JNK activation as well as cell death in prostate cancer cells was significantly attenuated by overexpression of catalase and superoxide dismutase. In addition, guggulsterone treatment resulted in a decrease in protein level and promoter activity of androgen receptor in LNCaP cells. In conclusion, the present study reveals that the guggulsterone-induced cell death in human prostate cancer cells is regulated by ROI-dependent activation of JNK and guggulsterone inhibits promoter activity of androgen receptor. [Cancer Res 2007;67(15):7439–49]

Introduction

Prostate cancer is one of the most commonly diagnosed malignancies and a leading cause of cancer-related deaths among men in the United States (1). Prostate carcinogenesis is a multistep process involving progression from localized and low-grade lesions to high-grade and metastatic carcinomas. Molecular mechanism underlying onset and progression of prostate cancer is not fully understood, but age, race, diet, and androgen secretion and metabolism are the identifiable risk factors for this malignancy (2, 3). Therapeutic options exist for localized disease, including surgery, radiation therapy, and hormonal therapy. Androgen ablation is a frequently prescribed treatment option for prostate cancer (4). This treatment modality, however, is palliative and has limited scope especially for hormone-refractory prostate cancers (4). Moreover, chemo and radiation therapies are largely ineffective against advanced prostate cancer (5, 6). Prostate cancer is usually diagnosed in the sixth and seventh decades of life, which allows a large window of opportunity for intervention to prevent or slow progression of the disease. Therefore, clinical development of agents that are nontoxic to normal prostate epithelial cells but can delay onset and/or progression of human prostate cancer could have a significant effect on disease-related cost, morbidity, and mortality for a large segment of population. Guggulsterone [4,17(20)-pregnadien-3,16-dione], a plant sterol derived from the gum resin of the *Commiphora mukul*, meets these criteria and seems promising for prevention of prostate cancer.

Guggulsterone or gum guggul have been used extensively in Indian Ayurvedic medicine for the treatment of different disorders, including bone fracture, arthritis, inflammation, cardiovascular disease, and lipid disorders (7–11). Recent studies have shown that guggulsterone is an antagonist of farnesoid X receptor (12, 13). In addition, guggulsterone has been shown to increase transcription of bile salt export pump (14). Our interest in guggulsterone stemmed from a recent study documenting suppression of nuclear factor-κB (NF-κB) activation in tumor cells by this phytochemical (15). NF-κB is a transcription factor belonging to the Rel family of proteins that are involved in regulation of expression of various genes including inflammatory cytokines, chemokines, cell adhesion molecules, growth factors, and IFNs (16, 17). NF-κB activation is considered a prosurvival signal because this transcription factor regulates expression of several antiapoptotic genes, including *cIAP1, XIAP, Bfl-1/A1, Bcl-2, cFLIP*, and *survivin* (18–25).

Because NF-κB is constitutively activated in a variety of hematologic and solid tumor cells, including prostate cancer cells (26–31), we raised the question of whether guggulsterone can suppress proliferation of human prostate cancer cells. Indeed, we found that guggulsterone inhibits growth of PC-3 human prostate cancer cells by causing apoptotic cell death (32). Interestingly, growth of a normal prostate epithelial cell line (PrEC) is minimally affected by guggulsterone treatment even at concentrations that are highly cytotoxic to the PC-3 cells (32). We also found that guggulsterone-induced cell death in PC-3 cells is independent of Bcl-2 but correlates with induction of Bax and Bak proteins and activation of caspase-9, caspase-8, and caspase-3 (32). Despite these advances, not much is known about the upstream signal(s) that triggers guggulsterone-induced apoptosis. The present study provides compelling experimental evidence to indicate that

Requests for reprints: Shivendra V. Singh, 2.32A Hillman Cancer Centre Research Pavilion, 5117 Centre Avenue, Pittsburgh, PA 15213. Phone: 412-623-3263; Fax: 412-623-7828; E-mail: singhs@upmc.edu.

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guggulsterone-induced apoptosis in human prostate cancer cells is initiated by reactive oxygen intermediate (ROI)–mediated activation of c-Jun NH_2-terminal kinase (JNK).

Materials and Methods

Reagents. *Z*-guggulsterone was purchased from Steraloids. Tissue culture medium and fetal bovine serum (FBS) were from Invitrogen; *N*-acetylcysteine (NAC) was from Sigma; and hydroethidine and 6-carboxy-2′,7′-dichlorodihydrofluorescein (DCF) diacetate (H_2DCFDA) were from Molecular Probes. The mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) kinase (MEK) 1 inhibitor PD98059, p38 MAPK inhibitor SB202190, and JNK inhibitor SP600125 were purchased from Calbiochem. The ELISA kit for quantitation of cytoplasmic histone-associated DNA fragmentation was from Roche Diagnostics. Antibodies specific for detection of total and phospho-(Tyr^{204})-ERK1/2, total and phospho-(Thr^{183}/Tyr^{185})-JNK, and total and phospho-(Tyr^{182})-p38 MAPK and androgen receptor were from Santa Cruz Biotechnology; the antibodies against Cu,Zn-superoxide dismutase (Cu,Zn-SOD), manganese SOD (Mn-SOD), and catalase were from Calbiochem; the anti-Flag antibody was from Sigma; and anti-actin antibody was from Oncogene Research Products.

Cell lines and cell culture. The PC-3, LNCaP, and DU145 human prostate cancer cell lines were obtained from the American Type Culture Collection. Monolayer cultures of PC-3 and DU145 cells were maintained as described previously (32, 33). Monolayer cultures of LNCaP cells were maintained in RPMI 1640 supplemented with 10% (v/v) nonheat inactivated FBS, antibiotics, 10 mmol/L HEPES, 1 mmol/L sodium pyruvate, and 2.4 mg/mL glucose. Normal prostate epithelial cell line PrEC (Clonetics) was maintained as described previously (32). Each cell line was maintained in a humidified atmosphere of 95% air and 5% CO_2 at 37°C.

Immunoblotting. Stock solution of guggulsterone was prepared in DMSO and diluted with fresh complete medium. Cells were treated with DMSO (final concentration, 0.1%) or desired concentrations of guggulsterone for specified time intervals and lysed as described previously (32). Preparation of cell lysates and immunoblotting was done as described previously (32, 33). The immunoreactive bands were visualized by enhanced chemiluminescence method. Each membrane was stripped and reprobed with anti-actin antibody to normalize for differences in protein loading.

Determination of apoptosis. Apoptosis induction by guggulsterone was assessed by analysis of cytoplasmic histone-associated DNA fragmentation as described previously (32). In some experiments, cells were pretreated for 2 h with desired MAPK inhibitor before guggulsterone treatment and assessment of apoptosis.

Measurement of ROI generation. Intracellular ROI generation was measured by flow cytometry following staining with hydroethidine and H_2DCFDA as described by us previously (33). Briefly, 3×10^5 cells were plated in 60-mm culture dishes, allowed to attach overnight, and exposed to DMSO (control) or different concentrations of guggulsterone for specified time intervals. The cells were stained with 2 μmol/L hydroethidine and 5 μmol/L 2′,7′,-dichlorodihydrofluorescein diacetate (H_2DCFDA) for 30 min at 37°C. The cells were collected and the fluorescence was analyzed using a Coulter Epics XL Flow Cytometer. In some experiments, cells were pretreated with NAC for 2 h before guggulsterone exposure and analysis of ROI generation.

Adenoviral infection. Adenoviral constructs encoding enhanced green fluorescence protein (Ad.EGFP), catalase (Ad.Catalase), Flag-tagged JNK binding domain (JBD) of JNK interacting protein-1 (JIP-1; Ad.JBD), Cu,Zn-SOD (Ad.Cu,Zn-SOD), and Mn-SOD (Ad.Mn-SOD) were a generous gift from Dr. Yong Lee (University of Pittsburgh, Pittsburgh, PA). For infection, DU145 or LNCaP cells (2.5×10^5) were plated in 60-mm culture dishes, allowed to attach, and infected with Ad.EGFP, Ad.Catalase, Ad.JBD, Ad.Cu,Zn-SOD, or Ad.Mn-SOD [50 multiplicity of infection (MOI) for DU145 and 100 MOI for LNCaP]. After 36 h of incubation at 37°C, the cells were treated with DMSO or desired concentrations of guggulsterone for specified time intervals and processed for immunoblotting, analysis of ROI generation, or analysis of cytoplasmic histone-associated DNA fragmentation.

Luciferase assay. LNCaP cells were seeded at a density of 2×10^5 cells per well in 12-well plates. After overnight culture, cells were transiently cotransfected with 0.5 μg pARLUC plasmid (a generous gift from Dr. William H. Walker, Department of Cell Biology and Physiology, University of Pittsburgh) and 0.05 μg pRL-CMV plasmid using Fugene 6 transfection reagent. Twenty-four hours after transfection, the cells were exposed to desired concentration of guggulsterone for 24 h, washed with ice-cold PBS, and harvested in reporter lysis buffer. The samples were centrifuged and a 20 μL supernatant fraction was used for measurement of dual luciferase activity (Promega) using a luminometer. The luciferase activity was normalized and expressed as a ratio of firefly luciferase to *Renilla* luciferase units.

Results

Guggulsterone treatment activated JNK and p38 MAPK in PC-3 and LNCaP cells. Guggulsterone has been shown to suppress growth of cancer cells including acute myeloid leukemia and PC-3 human prostate cancer cell lines by causing apoptosis (32, 34) but the sequence of events leading to cell death is poorly defined. To gain insights into the mechanism of guggulsterone-induced apoptosis, we determined its effect on activating phosphorylations of JNK1/2 (Thr^{183}/Tyr^{185}), p38 MAPK (Tyr^{182}), and ERK1/2 (Tyr^{204}), which are implicated in regulation of cell survival and apoptosis by different stimuli including various cancer preventive agents (35–40). Figure 1*A* summarizes data on effect of guggulsterone treatment on activating phosphorylations of different MAPKs in PC-3 cells. Exposure of PC-3 cells to growth-suppressive and apoptosis-inducing concentrations of guggulsterone (20 and 40 μmol/L; refs. 32, 34) resulted in rapid and concentration-dependent increase in Thr^{183}/Tyr^{185} phosphorylation of JNK1/2, which was evident as early as 30 min after treatment (Fig. 1*A*). Guggulsterone-mediated hyperphosphorylation of JNK1/2 was not due to increase in the levels of total JNK1/2 proteins (data not shown). Guggulsterone treatment also increased phosphorylation of p38 MAPK at Tyr^{182} that was clearly evident at 2- to 4-h time points (Fig. 1*A*). Similar to JNK1/2, guggulsterone treatment did not cause any appreciable change in total p38 protein level (data not shown). On the other hand, exposure of PC-3 cells to guggulsterone resulted in suppression of ERK1/2 phosphorylation at Tyr^{204}. For instance, constitutive activation (phosphorylation) of ERK1/2 was decreased by ~80% to 90% following a 4-h treatment of PC-3 cells with 20 and 40 μmol/L guggulsterone compared with DMSO-treated control as judged by densitometric scanning of the immunoreactive bands after correction for actin loading control (Fig. 1*A*). Guggulsterone-mediated suppression of ERK1/2 phosphorylation was not due to a decrease in the levels of ERK1/2 proteins (data not shown).

To test whether guggulsterone-mediated changes in MAPK phosphorylation observed in PC-3 were restricted to this cell line, we determined the effect of guggulsterone treatment on phosphorylation of JNK1/2, p38 MAPK, and ERK1/2 using LNCaP cells and the results are shown in Fig. 1*B*. The LNCaP cell line is androgen responsive and expresses wild-type (WT) p53, whereas PC-3 cells are androgen independent and lack functional p53. Similar to PC-3 cells, guggulsterone treatment resulted in rapid and marked increase in phosphorylation of JNK1/2 and p38 MAPK in LNCaP cells (Fig. 1*B*). Unlike PC-3 cells, phosphorylation of ERK1/2 was increased by treatment with guggulsterone, at least at 1-h time point at both 20 and 40 μmol/L concentrations (Fig. 1*B*). Guggulsterone-mediated hyperphosphorylation of ERK1/2 in LNCaP cells correlated with induction of ERK1/2

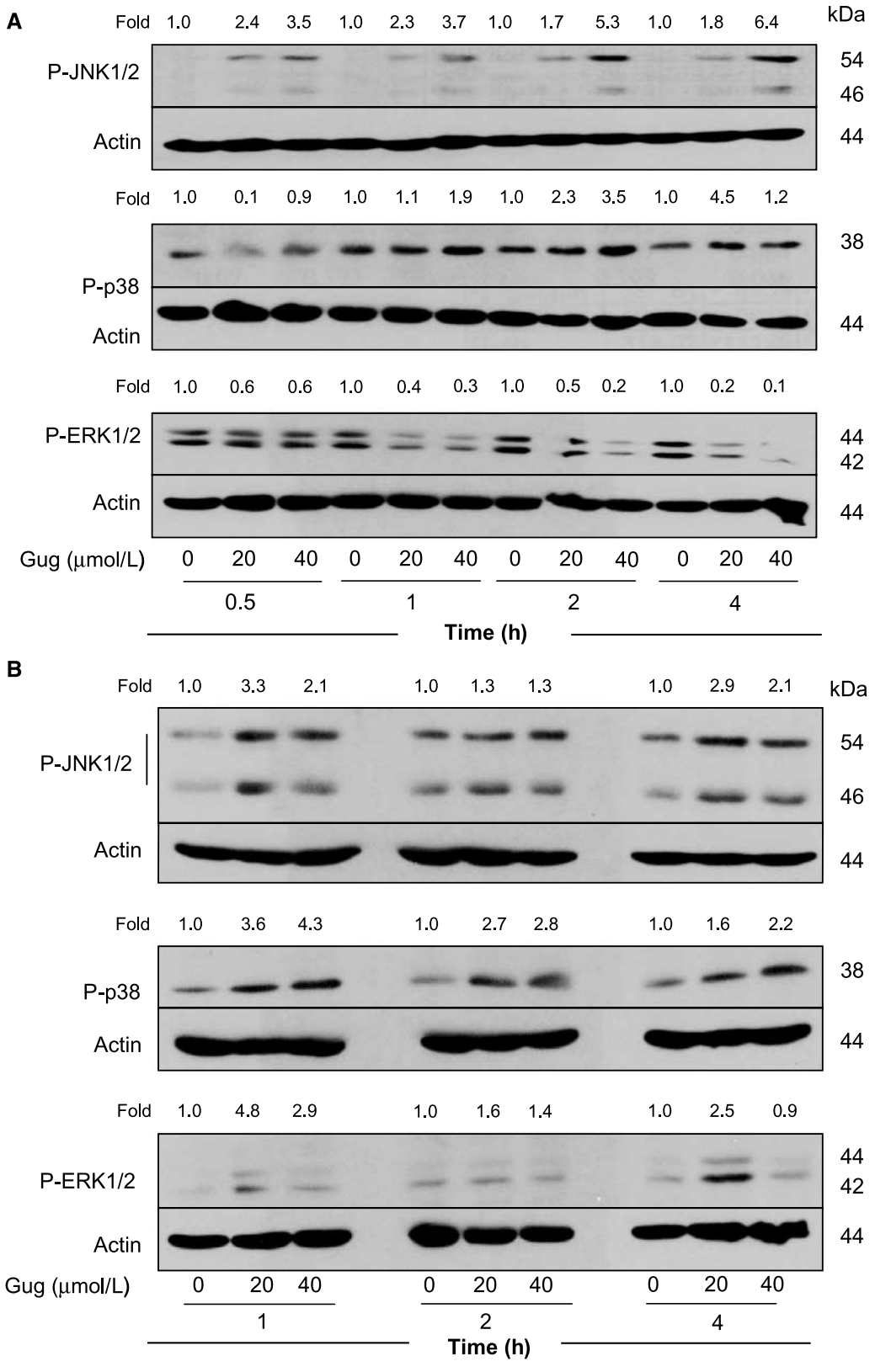

Figure 1. Immunoblotting for phospho-JNK1/2 (*P-JNK1/2*), phospho-p38 MAPK (*P-p38*), and phospho-ERK1/2 (*P-ERK1/2*) using lysates from PC-3 (*A*) and LNCaP (*B*) cells treated with DMSO (control) or the indicated concentrations of guggulsterone (*Gug*) for specified time intervals. The blots were stripped and reprobed with anti-actin antibody to correct for differences in protein level. Densitometric scanning data after correction for actin loading control are shown on top of bands. Immunoblotting for each protein was done at least twice using independently prepared lysates and the results were similar.

protein expression (data not shown). Collectively, these results indicated that guggulsterone treatment increased activating phosphorylations of both JNK1/2 and p38 MAPK in human prostate cancer cell lines irrespective of their androgen responsiveness or p53 status, albeit with different time course kinetics. On the other hand, guggulsterone treatment exhibited differential response on ERK1/2 phosphorylation in PC-3 (decrease) versus LNCaP cells (increase).

Guggulsterone-induced DNA fragmentation was attenuated by pharmacologic inhibition of JNK1/2 activation in PC-3 and LNCaP cells. Next, we proceeded to determine the functional significance of MAPK activation in guggulsterone-induced apoptosis using pharmacologic inhibitors of JNK1/2 (SP600125), p38 MAPK (SB202190), and MEK1 (PD98059; an upstream kinase implicated in activation of ERK1/2). The guggulsterone-mediated hyperphosphorylation of JNK1/2 in PC-3 cells was fully blocked in the presence of SP600125 (Fig. 2*A, left*, compare *lanes 2* and *4*). The cytoplasmic histone-associated DNA fragmentation (apoptosis) resulting from a 24-h exposure of PC-3 cells to both 20 and 40 μmol/L guggulsterone was partially but statistically significantly attenuated by pharmacologic inhibition of JNK1/2 activation (Fig. 2*A, right*). Guggulsterone-mediated hyperphosphorylation of p38 MAPK was abrogated in the presence of 20 μmol/L SB202190 (data not shown). However, pharmacologic inhibition of p38

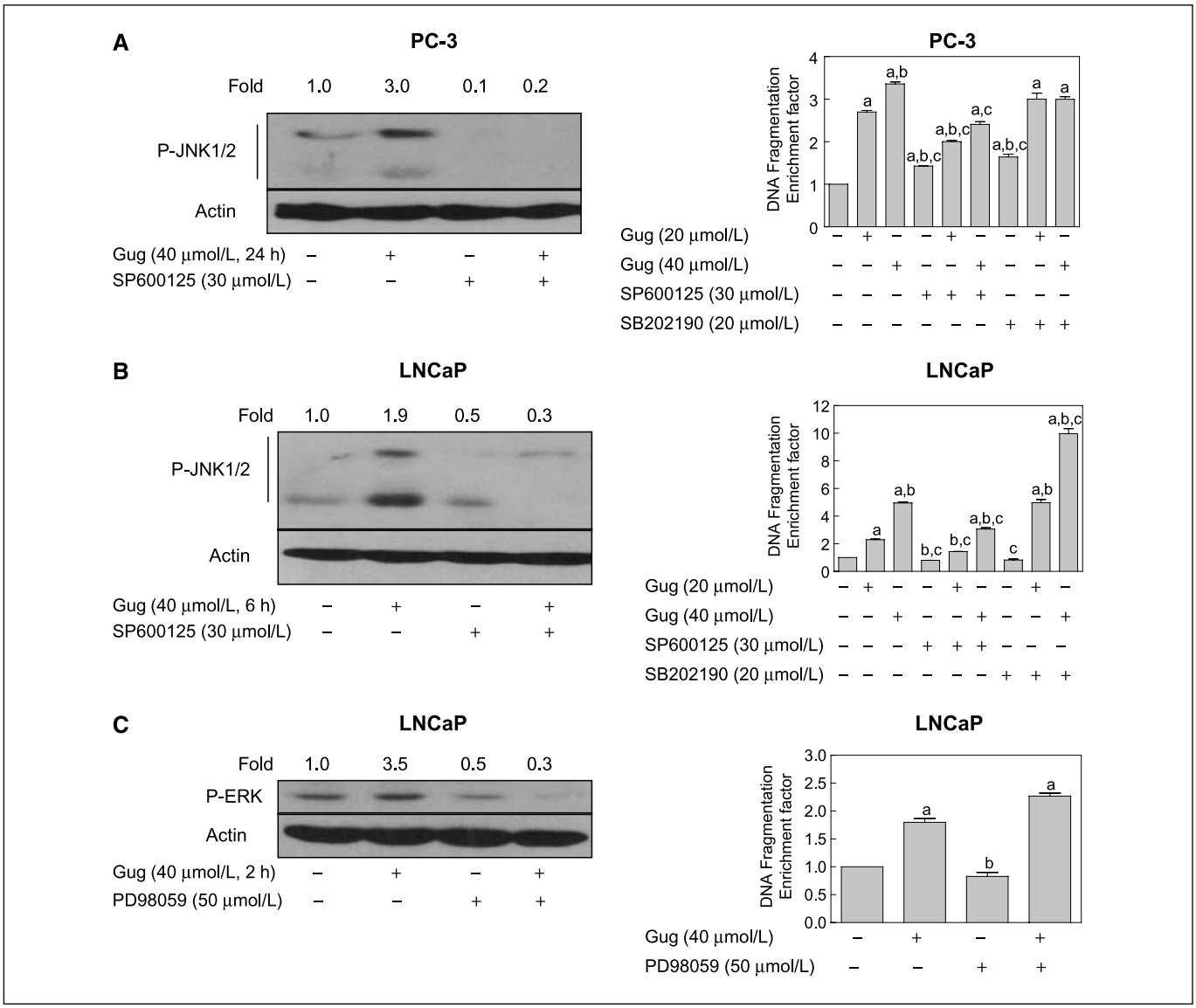

Figure 2. *A,* effect of SP600125 (JNK1/2 inhibitor) and SB202190 (p38 MAPK inhibitor) on guggulsterone-induced JNK1/2 hyperphosphorylation (*left*) and/or cytoplasmic histone-associated DNA fragmentation (*right*) in PC-3 cells. *B,* effect of SP600125 (JNK1/2 inhibitor) and SB202190 (p38 MAPK inhibitor) on guggulsterone-induced JNK1/2 hyperphosphorylation (*left*) and/or cytoplasmic histone-associated DNA fragmentation (*right*) in LNCaP cells. *C,* effect of PD98059 (MEK1-ERK1/2 inhibitor) on guggulsterone-induced ERK1/2 hyperphosphorylation (*left*) and cytoplasmic histone-associated DNA fragmentation (*right*) in LNCaP cells. Cells were treated with DMSO (control) or the indicated concentrations of SP600125, SB202190, or PD98059 for 2 h. The cells were then either left untreated (DMSO or inhibitor alone controls) or exposed to guggulsterone (20 or 40 μmol/L) for specified time interval in the presence of the inhibitor. The cells were collected and processed for immunoblotting or analysis of cytoplasmic histone-associated DNA fragmentation. *Columns,* mean ($n = 3$); *bars,* SE. a, b, and c, $P < 0.05$, significantly different compared with DMSO-treated control, 20 μmol/L guggulsterone treatment alone, and 40 μmol/L guggulsterone treatment alone, respectively, as judged by one-way ANOVA followed by Tukey's (*A* and *B*) or Bonferroni's (*C*) multiple comparison test. Similar results were observed in two independent experiments.

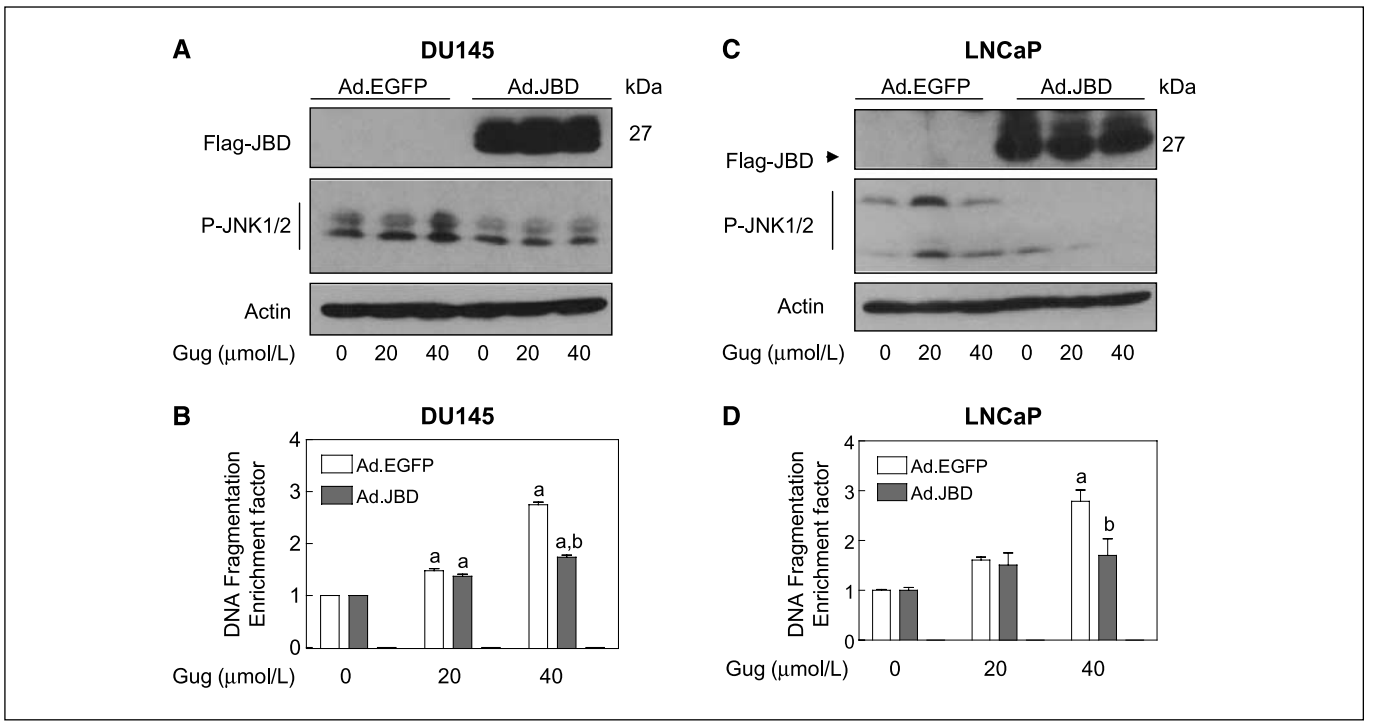

Figure 3. Immunoblotting for Flag-JBD (*top*) and phospho-JNK1/2 (*bottom*) in DU145 (*A*) and LNCaP (*C*) cells infected with adenovirus encoding EGFP (Ad.EGFP) and JBD of JIP-1 (Ad.JBD) following a 4-h treatment with DMSO (control) or the indicated concentrations of guggulsterone. The blots were stripped and reprobed with anti-actin antibody to correct for differences in protein loading. Cytoplasmic histone-associated DNA fragmentation in DU145 (*B*) and LNCaP (*D*) cells infected with Ad.EGFP and Ad.JBD following a 24-h treatment with DMSO (control) or the indicated concentrations of guggulsterone. *Columns,* mean ($n = 3$); *bars,* SE. a and b, $P < 0.05$, significantly different compared with corresponding DMSO-treated control and cells infected with Ad.EGFP, respectively, by one-way ANOVA followed by Tukey's multiple comparison test. Similar results were observed in two independent experiments.

MAPK activation by SB202190 did not have any appreciable effect on guggulsterone-induced cytoplasmic histone-associated DNA fragmentation in PC-3 cells (Fig. 2*A, right*).

To confirm that the JNK1/2 dependence of guggulsterone-induced apoptosis was not restricted to the PC-3 cell line, we determined the effect of SP600125 on guggulsterone-mediated apoptosis using LNCaP cells. As shown in Fig. 2*B* (*left*), SP600125 offered protection against guggulsterone-mediated hyperphosphorylation of JNK1/2 in LNCaP cells as well. Similar to PC-3 cells, the cytoplasmic histone-associated DNA fragmentation resulting from a 24-h exposure of LNCaP cells to 20 and 40 μmol/L guggulsterone was statistically significantly attenuated by SP600125 (Fig. 2*B, right*). Interestingly, unlike PC-3 cells, a 2-h pretreatment of LNCaP cells with p38 MAPK inhibitor SB202190, which resulted in full inhibition of p38 MAPK activation (data not shown), caused an increase in guggulsterone-mediated cytoplasmic histone-associated DNA fragmentation especially at 40 μmol/L concentration (Fig. 2*B, right*). Collectively, these results pointed toward an important role of JNK1/2 in regulation of guggulsterone-induced cell death in human prostate cancer cells.

Because guggulsterone treatment resulted in activation of ERK1/2 in LNCaP cells (Fig. 1*B*), we proceeded to determine possible contribution of this MAPK in regulation of cell death in LNCaP cells. As can be seen in Fig. 2*C* (*left*), guggulsterone-mediated increase in Tyr^{204} phosphorylation of ERK1/2 was completely abolished in the presence of 50 μmol/L PD98059. However, pharmacologic inhibition of ERK1/2 activation by PD98059 did not have any appreciable effect on guggulsterone-induced cytoplasmic histone-associated DNA fragmentation (Fig. 2*C, right*). These results argued against ERK1/2 involvement in guggulsterone-mediated apoptosis in LNCaP cells.

Overexpression of JBD of JIP-1 attenuated guggulsterone-induced JNK1/2 activation and apoptosis. The relationship between JNK1/2 activation and apoptosis induction by guggulsterone was further investigated by determining the effect of adenovirus-mediated overexpression of Flag-tagged JBD of JIP-1 on guggulsterone-induced apoptosis. JIP-1 is a cytoplasmic scaffold protein that selectively binds to and inhibits activity of JNK but not other members of the MAPK cascade (41, 42). Overexpression of JBD of JIP-1 has been shown to inhibit JNK activation in response to different stimuli (43, 44). Because PC-3 cells are not easily infected with adenoviruses, we used another androgen-independent human prostate cancer cell line (DU145) for these studies. Overexpression of JBD of JIP-1 in DU145 cells infected with Flag-tagged JBD (Ad.JBD) was confirmed by immunoblotting using anti-Flag antibody (Fig. 3*A, top*). Cells infected with an adenoviral construct encoding EGFP (Ad.EGFP) were used as a control. An average infection efficiency of ~60% to 80% was observed in different experiments as judged by fluorescence microscopic analysis of EGFP (data not shown). In DU145 cells infected with Ad.EGFP (control), exposure to 20 and 40 μmol/L guggulsterone resulted in hyperphosphorylation of JNK1/2 compared with DMSO-treated control (Fig. 3*A, bottom*). A similar guggulsterone treatment in DU145 cells infected with Ad.JBD failed to significantly increase phosphorylation of JNK1/2 over DMSO-treated control. A 24-h exposure to guggulsterone resulted in a concentration-dependent and statistically significant increase in cytoplasmic histone-associated DNA fragmentation in DU145 cells infected with Ad.EGFP, which was relatively less

pronounced in cells infected with Ad-JBD especially at 40 μmol/L concentration (Fig. 3*B*).

The effect of overexpression of JBD of JIP-1 on guggulsterone-induced JNK1/2 activation and cytoplasmic histone-associated DNA fragmentation was also determined in LNCaP cells and the results are summarized in Fig. 3*C* and *D*. Similar to DU145 cells, treatment of control LNCaP cells (infected with Ad.EGFP) with guggulsterone caused an increase in the levels of phospho-JNK1/2 compared with DMSO-treated control. On the other hand, the guggulsterone-mediated hyperphosphorylation of JNK1/2 was not observed in LNCaP cells infected with Ad.JBD (Fig. 3*C, bottom*). Consistent with the results of studies using JNK1/2 inhibitor SP600125 (Fig. 2*B*), the cytoplasmic histone-associated DNA fragmentation induced by a 24-h exposure to 40 μmol/L guggulsterone was statistically significantly inhibited in LNCaP cells overexpressing JBD of JIP-1 (Fig. 3*D*). These results provided additional evidence for involvement of JNK1/2 in regulation of guggulsterone-induced apoptosis.

Guggulsterone treatment caused ROI generation in PC-3 and LNCaP cells. Previous studies have shown that JNK-mediated apoptosis in some systems is associated with generation of hydrogen peroxide as a second messenger (45, 46). We raised the question of whether guggulsterone-induced JNK1/2 activation in human prostate cancer cells involved ROI generation. To address this question, initially, we determined the effect of guggulsterone treatment on ROI generation using PC-3 and LNCaP cells. Intracellular ROI generation in control (DMSO treated) and guggulsterone-treated PC-3/LNCaP cells was assessed by flow cytometry. The H_2DCFDA is cleaved by nonspecific cellular esterases and oxidized in the presence of H_2O_2 and other peroxides to yield fluorescent DCF that can be monitored by flow cytometry. The guggulsterone-treated PC-3 cells exhibited a dose-dependent increase in DCF fluorescence compared with control (Fig. 4*A, left*). For instance, the DCF fluorescence in PC-3 cells treated for 4 h with 20, 40, and 60 μmol/L guggulsterone was increased by ~1.6-, 1.8-, and 2.0-fold, respectively ($P < 0.05$ compared with DMSO-treated control at each guggulsterone concentration as judged by one-way ANOVA followed by Dunnett's test) compared with vehicle-treated control (Fig. 4*A, left*). In time course experiments using 40 μmol/L guggulsterone, statistically significant increase in DCF fluorescence over DMSO-treated control in LNCaP cells was evident as early as 4 h after treatment and increased gradually with increasing exposure time (Fig. 4*A, right*). To test whether guggulsterone-mediated activation of JNK1/2 in prostate cancer cells was linked to ROI generation, we determined the levels of phospho-JNK1/2 in PC-3/LNCaP cells treated for 4 h with 20 μmol/L guggulsterone in the absence or presence of 4 mmol/L NAC (2-h pretreatment), a known antioxidant. As can be seen in Fig. 4*B*, guggulsterone-mediated activation of JNK1/2 in both PC-3 (Fig. 4*B, left*) and LNCaP cells (Fig. 4*B, right*) was significantly attenuated in the presence of NAC. These results indicated that guggulsterone-mediated activation of JNK1/2 in prostate cancer cells was indeed caused by ROI generation.

Normal prostate epithelial cell line PrEC was resistant to ROI generation and JNK1/2 activation by guggulsterone. We have shown previously that PrEC normal prostate epithelial cell line is resistant to growth inhibition and apoptosis induction by guggulsterone (32). We raised the question of whether resistance of PrEC cells to guggulsterone-induced apoptosis was due to lack of ROI generation and consequently JNK1/2 activation. We addressed this question by determining the effect of guggulsterone treatment on ROI generation and JNK activation using PrEC cells. As can be seen in Fig. 4*C* (*left*), exposure of PrEC cells to 40 μmol/L guggulsterone failed to cause an increase in DCF fluorescence even after 6 h of treatment, indicating lack of ROI generation. Moreover, guggulsterone treatment resulted in marginal increase in JNK1/2 phosphorylation in PrEC cells even after 4 h of treatment with 40 μmol/L concentration (Fig. 4*C, right*). These results indicated that PrEC cells were resistant to ROI generation as well as JNK1/2 activation by guggulsterone, which may explain lack of apoptosis by this agent in PrEC cells (32).

Overexpression of catalase attenuated guggulsterone-mediated JNK1/2 activation and apoptosis in DU145 cells. To further examine the relationship between ROI generation, JNK1/2 activation, and apoptosis in our model, we determined the effect of adenovirus-mediated overexpression of catalase, an enzyme responsible for conversion of hydrogen peroxide to water molecules, on guggulsterone-mediated JNK1/2 activation and apoptosis using DU145 cells and LNCaP cells. The level of catalase protein was markedly higher in DU145 (Fig. 5*A*) and LNCaP cells (data not shown) infected with Ad.Catalase compared with control cells infected with Ad.EGFP as revealed by immunoblotting. Activation of JNK caused by treatment with 40 μmol/L guggulsterone in DU145 (Fig. 5*B*) and LNCaP cells (data not shown) was markedly suppressed by overexpression of catalase as indicated by immunoblotting for phospho-JNK1/2 levels. Catalase overexpression also conferred statistically significant protection against guggulsterone-induced apoptosis as judged by analysis of cytoplasmic histone-associated DNA fragmentation in DU145 cells. For instance, a 24-h exposure of Ad.EGFP cells to 40 μmol/L guggulsterone resulted in ~3-fold increase in cytoplasmic histone-associated DNA fragmentation relative to DMSO-treated control (Fig. 5*C*). The cytoplasmic histone-associated DNA fragmentation was increased by only ~40% on a similar guggulsterone treatment in Ad.Catalase cells (Fig. 5*C*). The guggulsterone-mediated generation of ROI was also suppressed significantly in catalase-overexpressing cells compared with cells infected with Ad.EGFP (Fig. 5*D*). These results confirmed that guggulsterone-mediated JNK1/2 activation and apoptosis in human prostate cancer cells were linked to ROI generation.

SOD overexpression conferred significant protection against guggulsterone-induced JNK1/2 activation and apoptosis. Superoxide is the primary oxygen-free radical produced by mitochondria and is rapidly removed by conversion to hydrogen peroxide in a reaction catalyzed by SOD (47). At least three SODs exist in mammalian cells, including a cytosolic Cu,Zn-SOD (SOD1), an intramitochondrial Mn-SOD (SOD2), and an extracellular Cu,Zn-SOD (SOD3). To test whether guggulsterone-mediated H_2O_2 production, which was revealed by an increase in DCF fluorescence (Fig. 4*A*) and consequently JNK1/2 activation in our model involved mitochondrial superoxide, we determined the effects of Cu,Zn-SOD and Mn-SOD overexpression on guggulsterone-induced JNK1/2 activation using DU145 cells. As can be seen in Fig. 6*A*, infection of DU145 cells with an adenovirus encoding Mn-SOD (Ad.Mn-SOD cells) resulted in overexpression of Mn-SOD, whose expression was very weak in cells infected with Ad.EGFP. Treatment of Ad.EGFP cells with 20 and 40 μmol/L guggulsterone caused between 8- and 16-fold increase in the levels of phospho-JNK1/2 compared with DMSO-treated control. The guggulsterone-mediated hyperphosphorylation of JNK1/2 was markedly suppressed in Mn-SOD–overexpressing DU145 cells

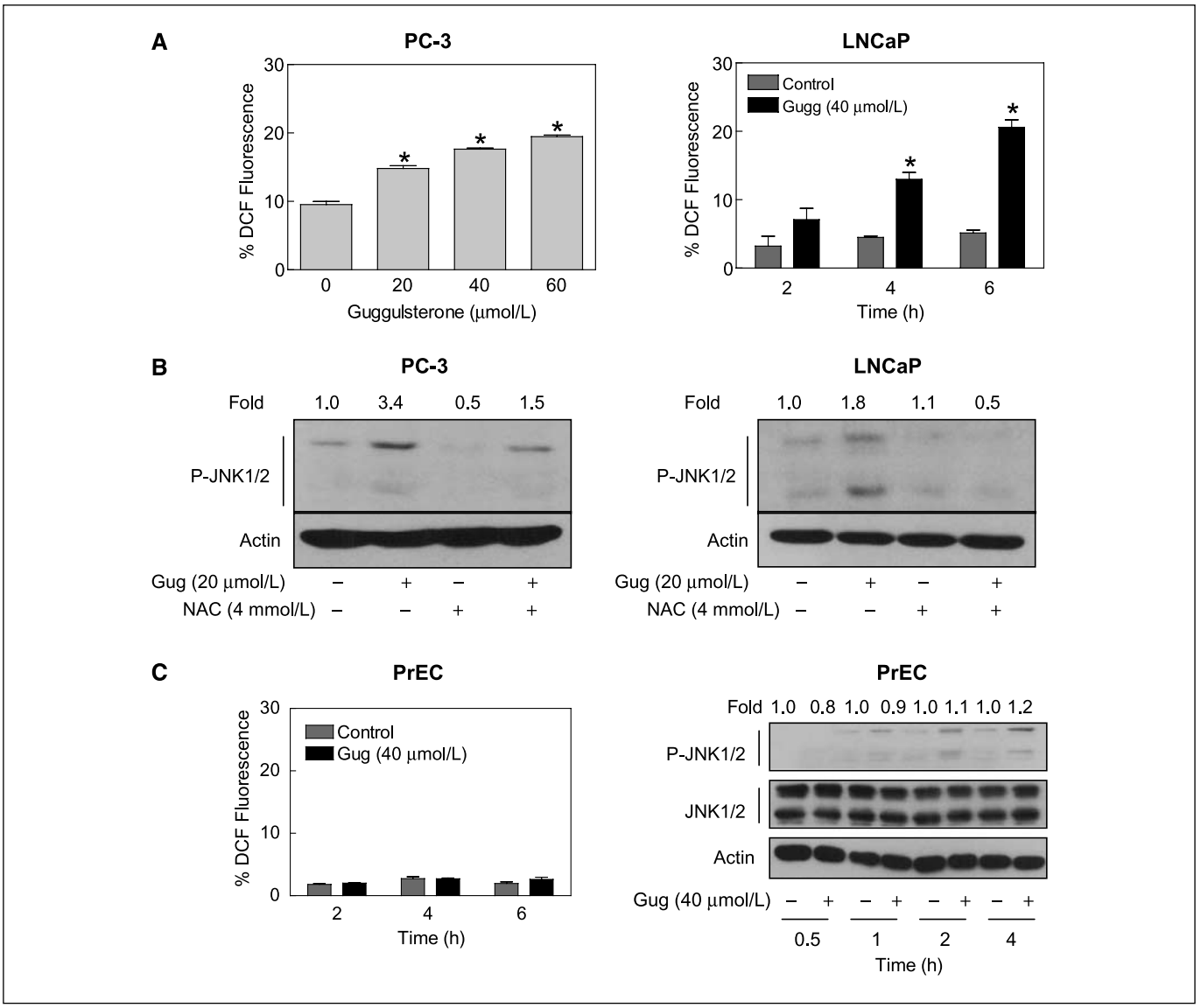

Figure 4. *A,* percentage of DCF fluorescence in PC-3 (*left*) and LNCaP (*right*) cells following treatment with DMSO or different concentrations of guggulsterone for 4 h (PC-3 cells) or treatment with 40 μmol/L guggulsterone for the indicated times (LNCaP cells). *Columns,* mean ($n = 3$); *bars,* SE. *, $P < 0.05$, significantly different compared with DMSO control by one-way ANOVA followed by Dunnett's test (PC-3 cells) or by paired *t* test (LNCaP cells). Similar results were observed in replicate experiments. *B,* effect of NAC on guggulsterone-induced JNK1/2 hyperphosphorylation in PC-3 cells (*left*) or LNCaP cells (*right*). The cells were pretreated with either DMSO (control) or 4 mmol/L NAC for 2 h. The cells were then either left untreated (DMSO or NAC alone controls) or exposed to 20 μmol/L guggulsterone for 4 h in the presence of NAC. The blots were stripped and reprobed with anti-actin antibody to correct for differences in protein loading. Similar results were observed in two independent experiments. Densitometric scanning data after correction for actin loading control are shown on top of bands. *C,* effect of guggulsterone treatment (40 μmol/L) on ROI generation (*left*) and JNK1/2 phosphorylation (*right*) in PrEC normal prostate epithelial cell line. *Columns,* mean ($n = 3$); *bars,* SE.

(Fig. 6*A*). Similarly, guggulsterone-induced activation of JNK1/2 was markedly attenuated in DU145 cells infected with Ad.Cu,Zn-SOD compared with Ad.EGFP cells (Fig. 6*A*). Overexpression of both Mn-SOD and Cu,Zn-SOD conferred statistically significant protection against guggulsterone-induced cytoplasmic histone-associated DNA fragmentation especially at 40 μmol/L concentration compared with Ad.EGFP cells (Fig. 6*B*). These results pointed toward mitochondrial superoxide generation in guggulsterone-mediated JNK activation and apoptosis.

Guggulsterone treatment inhibited androgen receptor promoter activity in LNCaP cells. We raised the question of whether guggulsterone treatment affects expression of androgen receptor. We addressed this question by determining the effect of guggulsterone treatment on protein level of androgen receptor by immunoblotting using LNCaP cells. The PC-3 cell lysate was included as a negative control. As can be seen in Fig. 6*C*, androgen receptor protein expression was detectable in LNCaP cells but not in the androgen-independent cell line PC-3. The guggulsterone treatment (40 μmol/L) caused a modest decrease (~30–40% decrease relative to control) in protein level of androgen receptor especially at the 16- and 24-h time points (Fig. 6*C*). Next, we determined the effect of guggulsterone treatment on promoter activity of androgen receptor by luciferase reporter assay using pARLUC plasmid (48), and the results are summarized in Fig. 6*D*. Exposure of LNCaP cells to 40 μmol/L guggulsterone for 24 h resulted in ~40% decrease in androgen receptor promoter

activity ($P < 0.05$ by one-way ANOVA followed by Dunnett's test). A decrease in androgen receptor promoter activity was also evident at 20 μmol/L guggulsterone concentration, although the difference did not reach statistical significance. These results indicated that the guggulsterone-mediated decline in androgen receptor protein level was probably due to repression of the androgen receptor promoter activity.

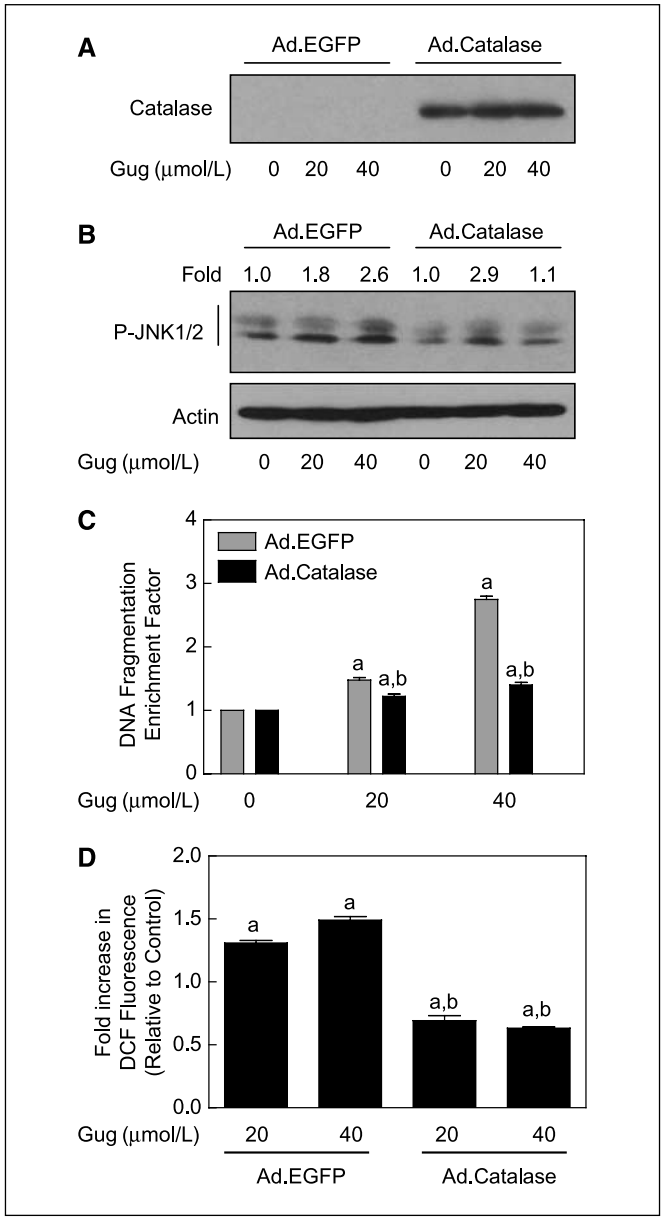

Figure 5. Immunoblotting for catalase (*A*) and phospho-JNK1/2 (*B*) in DU145 cells infected with adenovirus encoding EGFP (Ad.EGFP) or catalase (Ad.Catalase) following a 4-h treatment with either DMSO (control) or the indicated concentrations of guggulsterone. The blots were stripped and reprobed with anti-actin antibody to correct for differences in protein level. Densitometric scanning data after correction for actin loading control are shown on top of bands. *C,* analysis of cytoplasmic histone-associated DNA fragmentation in Ad.EGFP or Ad.Catalase cells following a 24-h treatment with either DMSO (control) or the indicated concentrations of guggulsterone. *Columns,* mean ($n = 3$); *bars,* SE. a and b, $P < 0.05$, significantly different compared with corresponding DMSO-treated control and Ad.EGFP cells, respectively, by one-way ANOVA followed by Tukey's multiple comparison test. *D,* fold increase in DCF fluorescence relative to DMSO-treated control in LNCaP cells infected with Ad.EGFP or Ad.Catalase and treated for 2 h with the indicated concentrations of guggulsterone. *Columns,* mean ($n = 3$); *bars,* SE. a and b, $P < 0.05$, significantly different compared with corresponding DMSO-treated control and Ad.EGFP cells, respectively, by one-way ANOVA followed by Tukey's multiple comparison test.

Discussion

An understanding of the mechanism by which guggulsterone causes apoptotic cell death in human prostate cancer cells but not in normal prostate epithelial cell line is necessary for its further clinical development because this knowledge could lead to identification of mechanism-based biomarkers potentially useful in future clinical trials. The present study indicates that the cell death caused by guggulsterone in human prostate cancer cells is tightly linked to activation of JNK1/2. The correlation between guggulsterone-mediated apoptosis induction and JNK1/2 activation is not a cell line–specific effect and not affected by p53 status either. This conclusion is based on the following observations: (*a*) guggulsterone treatment causes activation of JNK1/2 in both androgen-responsive (LNCaP) and androgen-independent (PC-3 and DU145) human prostate cells with WT (LNCaP) as well as mutant p53 expression (DU145); (*b*) the guggulsterone-induced apoptosis in human prostate cancer cells is partially but statistically significantly attenuated by pharmacologic inhibition of JNK1/2 activation; (*c*) genetic suppression of JNK1/2 activation by ectopic expression of JBD of JIP-1 confers partial yet significant protection against guggulsterone-induced cytoplasmic histone-associated DNA fragmentation especially at higher concentration; and (*d*) guggulsterone treatment causes very weak activation of JNK1/2 in PrEC normal prostate epithelial cells, which are resistant to growth arrest and apoptosis induction by guggulsterone (32). Because guggulsterone-induced apoptosis in prostate cancer cells is only partially attenuated by pharmacologic inhibition as well as genetic suppression of JNK1/2 activation, it is reasonable to conclude that JNK1/2 activation alone may not fully account for the cell death in our model.

Unlike JNK1/2, effect of guggulsterone on p38 MAPK and ERK1/2 seems cell line specific. For instance, despite activation of p38 MAPK on treatment with guggulsterone in both PC-3 and LNCaP cells, pharmacologic inhibition of p38 MAPK has different effect on guggulsterone-induced cytoplasmic histone-associated DNA fragmentation in these cells. In PC-3 cells, apoptosis induction by guggulsterone is not influenced by inhibition of p38 MAPK activation. On the other hand, pharmacologic inhibition of p38 MAPK activation in LNCaP cells leads to potentiation of guggulsterone-induced cell death. Similarly, guggulsterone treatment suppresses constitutive ERK1/2 activation in PC-3 cells but increases its phosphorylation at Tyr^{204} in LNCaP cells; although pharmacologic inhibition of ERK1/2 activation in LNCaP cells fails to confer significant protection against guggulsterone-induced cytoplasmic histone-associated DNA fragmentation. Although the molecular basis for cell line–specific effects of guggulsterone on p38 MAPK and ERK1/2 activation remains elusive, the possibility of p53 involvement in differential response cannot be ignored. For example, ERK1/2- and p38 MAPK–dependent phosphorylation of p53 at Ser^{15} has been implicated in UV-induced apoptosis (49, 50). Further studies are needed to explore the possible role of p53 in differential response and cellular outcome of guggulsterone-mediated p38 MAPK/ERK1/2 activation in PC-3 versus LNCaP cells. Nonetheless, it is clear that JNK1/2 activation positively correlates with guggulsterone-induced apoptosis in human prostate cancer cells and seems

independent of androgen responsiveness or p53 status. We are tempted to speculate that JNK1/2 activation may be a viable biomarker of guggulsterone response at least for prostate cancer.

The guggulsterone-mediated JNK1/2 activation in human prostate cancer cells correlates with generation of ROI. Guggulsterone-mediated JNK1/2 activation and/or apoptosis are significantly attenuated by small-molecule antioxidant NAC and/or ectopic expression of enzymes responsible for inactivation of hydrogen peroxide (catalase) or dismutation of superoxide anion (Cu,Zn-SOD and Mn-SOD). Although activation of JNK1/2 was not studied, guggulsterone-mediated generation of ROI has been observed in acute myeloid leukemia cell line U937 (34). Collectively, these results indicate that ROI generation is probably an important event in guggulsterone-induced apoptosis. However, the fundamental questions requiring further investigation are how guggulsterone induces oxidative stress and how ROI generation triggers JNK1/2 signal transduction pathways. Mitochondria are an important source of cellular ROI, which are generated during incomplete reduction of oxygen primarily by redox cycling of ubiquinone from the electrons that escape during normal oxidative phosphorylation (47). Possibly, guggulsterone targets mitochondria to trigger ROI generation, which is highly likely because JNK1/2 activation and apoptosis induction by this agent are significantly attenuated by overexpression of

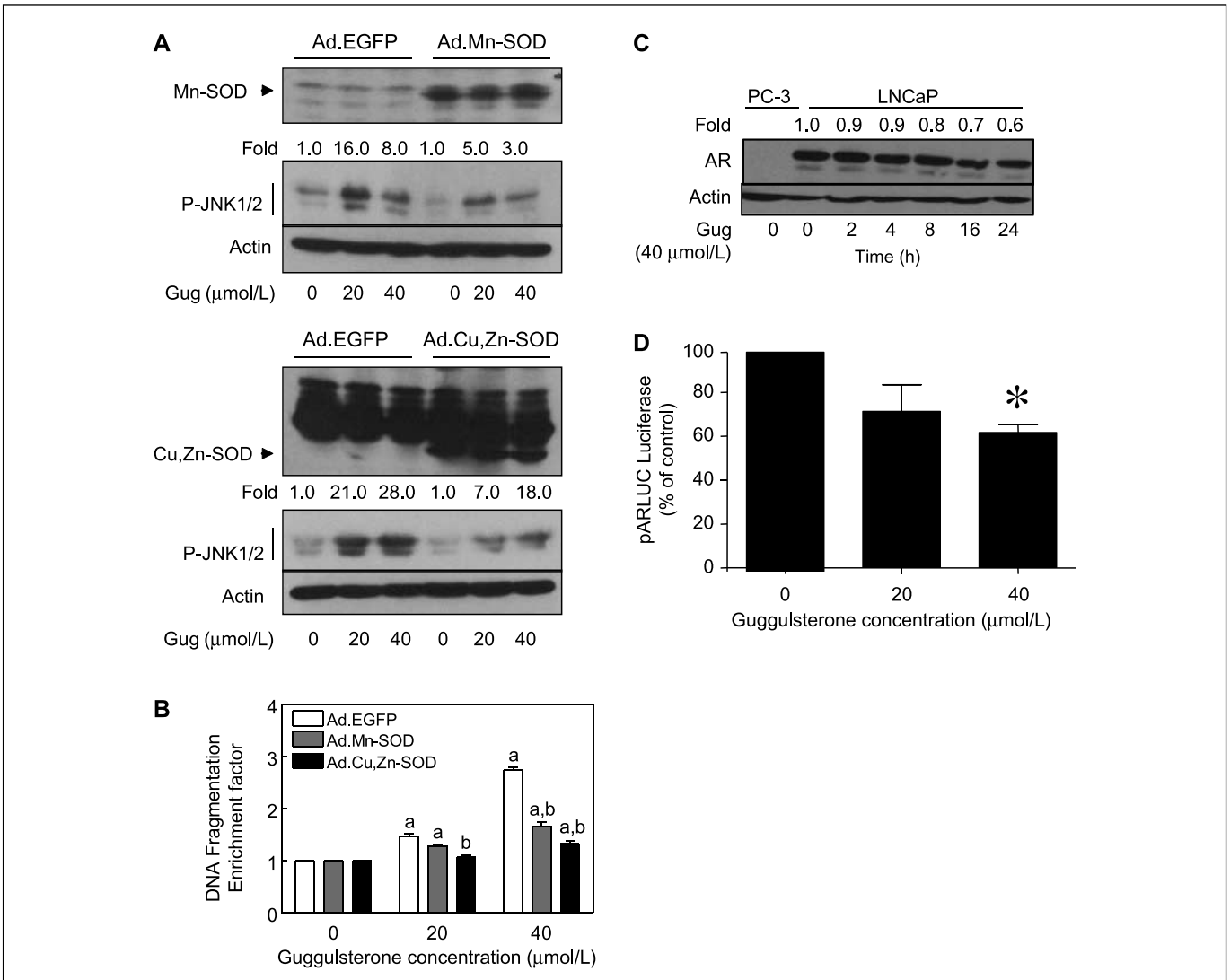

Figure 6. *A,* immunoblotting for Mn-SOD, Cu,Zn-SOD, and phospho-JNK1/2 using lysates from DU145 cells infected with adenovirus encoding EGFP (Ad.EGFP) or Mn-SOD (Ad.Mn-SOD) or Cu,-Zn-SOD (Ad.Cu,Zn-SOD) and treated for 4 h with either DMSO (control) or the indicated concentrations of guggulsterone. The blots were stripped and reprobed with anti-actin antibody to correct for differences in protein level. Densitometric scanning data after correction for actin loading control are shown on top of bands. *B,* cytoplasmic histone-associated DNA fragmentation in DU145 cells infected with Ad.EGFP, Ad.Mn-SOD, or Ad.Cu,Zn-SOD and treated for 24 h with either DMSO (control) or the indicated concentrations of guggulsterone. *Columns,* mean (n = 3); *bars,* SE. a and b, $P < 0.05$, significantly different compared with DMSO-treated control and cells infected with Ad.EGFP, respectively, by one-way ANOVA followed by Tukey's multiple comparison test. The experiment was repeated twice with comparable results. *C,* immunoblotting for androgen receptor protein expression using PC-3 cell lysate (negative control; *lane 1*) or cellular lysates from LNCaP cells treated with 40 μmol/L guggulsterone for the indicated times. The blot was stripped and reprobed with anti-actin antibody to correct for differences in protein level. Densitometric scanning data after correction for actin loading control are shown on top of the bands. Similar results were observed in replicate experiments. *D,* effect of guggulsterone treatment (24 h) on androgen receptor promoter activity as determined by luciferase reporter assay. *Columns,* mean (n = 3); *bars,* SE. *, $P < 0.05$, significantly different compared with DMSO-treated control by one-way ANOVA followed by Dunnett's test.

intramitochondrial Mn-SOD. At the same time, the possibility that guggulsterone depletes intracellular reduced glutathione to trigger ROI generation cannot be ruled out. With regards to the connection between ROI generation and JNK activation, the increase in steady-state levels of intracellular ROI can be detected through redox-sensing molecules, including thioredoxin and glutaredoxin. These molecules bind to apoptosis signal-regulating kinase 1 (ASK1) and suppress its activation (43). Thioredoxin and glutaredoxin dissociate from ASK1 during oxidative stress and their dissociation from ASK1 activates the ASK1/SEK1/JNK1 signal transduction pathway (43). We postulate that guggulsterone-mediated ROI generation is detected by redox-sensing molecules leading to activation of ASK1/JNK1/2 signal transduction pathway.

We used LNCaP and PC-3/DU145 cell lines as well-characterized representatives of androgen-responsive and androgen-independent human prostate cancer cells, respectively, to gauge into possible effect of androgen responsiveness on apoptosis induction by guggulsterone. Although guggulsterone-induced apoptosis was observed in all three cell lines, we also observed a modest decrease in protein level of androgen receptor in LNCaP cells. The guggulsterone-mediated decline in androgen receptor protein level correlated with inhibition of androgen receptor promoter activity as revealed by the luciferase reporter assay. These results suggest that the guggulsterone-mediated decline in protein level of androgen receptor is probably the result of repression of the androgen receptor promoter. Previous studies have shown that androgen receptor promoter activity is stimulated by overexpression of NF-κB subunits in Sertoli cells (48). Because guggulsterone treatment inhibits NF-κB activation (15), it seems reasonable to postulate that guggulsterone-mediated inhibition of androgen receptor promoter in LNCaP cells may be caused by inhibition of NF-κB. However, further studies are needed to systematically explore this possibility.

In conclusion, the present study offers novel insights into the mechanism of guggulsterone-induced apoptosis in human prostate cancer cells and shows a tight link between ROI generation, JNK1/2 activation, and the cell death process. Based on these observations, we propose that JNK1/2 activation may be a useful biomarker for guggulsterone response.

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References

1. Jemal A, Siegel R, Ward E, et al. Cancer statistics, 2006. CA Cancer J Clin 2006;56:106–30.
2. Ross RK, Henderson BE. Do diet and androgens alter prostate cancer risk via a common etiologic pathway? J Natl Cancer Inst 1994;86:252–4.
3. Whittemore AS, Kolonel LN, Wu AH, et al. Prostate cancer in relation to diet, physical activity, and body size in blacks, whites, and Asians in the United States and Canada. J Natl Cancer Inst 1995;87:652–61.
4. Laufer M, Denmeade SR, Sinibaldi VJ, Carducci MA, Eisenberger MA. Complete androgen blockade for prostate cancer: what went wrong? J Urol 2000;164:3–9.
5. Ramakrishna NR, DeWeese TL. In: Prostate cancer: biology, genetics, and the new therapeutics. Chung LWK, Isaacs WB, Simons JW, editors. New Jersey: Humana Press; 2001. p. 387–413.
6. Gilligan T, Kantoff PW. Chemotherapy for prostate cancer. Urology 2002;60:94–100.
7. Gujral ML, Sareen K, Tangri KK, Amma MK, Roy AK. Antiarthritic and anti-inflammatory activity of gum guggul (*Balsamodendron mukul* Hook). Indian J Physiol Pharmacol 1960;4:267–73.
8. Sharma JN, Sharma JN. Comparison of the anti-inflammatory activity of *Commiphora mukul* (an indigenous drug) with those of phenylbutazone and ibuprofen in experimental arthritis induced by mycobacterial adjuvant. Arzneimittelforschung 1977;27:1455–7.
9. Sinal CJ, Gonzalez FJ. Guggulsterone: an old approach to a new problem. Trends Endocrinol Metab 2002;13:275–6.
10. Urizar NL, Moore DD. GUGULIPID: a natural cholesterol-lowering agent. Annu Rev Nutr 2003;23:303–13.
11. Tripathi YB, Tripathi P, Malhotra OP, Tripathi SN. Thyroid stimulatory action of (Z)-guggulsterone: mechanism of action. Planta Med 1988;4:271–7.
12. Urizar NL, Liverman AB, Dodds DT, et al. A natural product that lowers cholesterol as an antagonist ligand for FXR. Science 2002;296:1703–6.
13. Wu J, Xia C, Meier J, Li S, Hu X, Lala DS. The hypolipidemic natural product guggulsterone acts as an antagonist of the bile acid receptor. Mol Endocrinol 2002;16:1590–7.
14. Cui J, Huang L, Zhao A, et al. Guggulsterone is a farnesoid X receptor antagonist in coactivator association assays but acts to enhance transcription of bile salt export pump. J Biol Chem 2003;278:10214–20.
15. Shishodia S, Aggarwal BB. Guggulsterone inhibits NF-κB and IκBα kinase activation, suppresses expression of anti-apoptotic gene products, and enhances apoptosis. J Biol Chem 2004;279:47148–58.
16. Baeuerle PA, Henkel T. Function and activation of NF-κB in the immune system. Annu Rev Immunol 1994;12:141–79.
17. Miyamoto S, Verma IM. Rel/NF-κB/IκB story. Adv Cancer Res 1995;66:255–92.
18. Chu ZL, McKinsey TA, Liu L, Gentry JJ, Malim MH, Ballard DW. Suppression of tumor necrosis factor-induced cell death by inhibitor of apoptosis c-IAP2 is under NF-κB control. Proc Natl Acad Sci U S A 1997;94:10057–62.
19. You M, Ku PT, Hrdlickova R, Bose HR. ch-IAP1, a member of the inhibitor-of-apoptosis protein family, is a mediator of the anti-apoptotic activity of the v-Rel oncoprotein. Mol Cell Biol 1997;17:7328–41.
20. Stehlik C, de Martin R, Kumabashiri I, Schmid JA, Binder BR, Lipp J. Nuclear factor (NF)-κB-regulated X-chromosome-linked iap gene expression protects endothelial cells from tumor necrosis factor α-induced apoptosis. J Exp Med 1998;188:211–6.
21. Schwenzer R, Siemienski K, Liptay S, et al. The human tumor necrosis factor (TNF) receptor-associated factor 1 gene (TRAF1) is up-regulated by cytokines of the TNF ligand family and modulates TNF-induced activation of NF-κB and c-Jun N-terminal kinase. J Biol Chem 1999;274:19368–74.
22. Grumont RJ, Rourke IJ, Gerondakis S. Rel-dependent induction of A1 transcription is required to protect B cells from antigen receptor ligation-induced apoptosis. Genes Dev 1999;13:400–11.
23. Zong WX, Edelstein LC, Chen C, Bash J, Gelinas C. The prosurvival Bcl-2 homolog Bfl-1/A1 is a direct transcriptional target of NF-κB that blocks TNFα-induced apoptosis. Genes Dev 1999;13:382–7.
24. Zhu L, Fukuda S, Cordis G, Das DK, Maulik N. Anti-apoptotic protein survivin plays a significant role in tubular morphogenesis of human coronary arteriolar endothelial cells by hypoxic preconditioning. FEBS Lett 2001;508:369–74.
25. Kreuz S, Siegmund D, Scheurich P, Wajant H. NF-κB inducers upregulate cFLIP, a cycloheximide-sensitive inhibitor of death receptor signaling. Mol Cell Biol 2001;21:3964–73.
26. Bargou RC, Emmerich F, Krappmann D, et al. Constitutive nuclear factor-κB-RelA activation is required for proliferation and survival of Hodgkin's disease tumor cells. J Clin Invest 1997;100:2961–9.
27. Shattuck-Brandt RL, Richmond A. Enhanced degradation of I-κBα contributes to endogenous activation of NF-κB in Hs294T melanoma cells. Cancer Res 1997;57:3032–9.
28. Giri DK, Aggarwal BB. Constitutive activation of NF-κB causes resistance to apoptosis in human cutaneous T cell lymphoma HuT-78 cells. Autocrine role of tumor necrosis factor and reactive oxygen intermediates. J Biol Chem 1998;273:14008–14.
29. Reuther JY, Reuther GW, Cortez D, Pendergast AM, Baldwin AS. A requirement for NF-κB activation in Bcr-Abl-mediated transformation. Genes Dev 1998;12:968–81.
30. Dong G, Chen Z, Kato T, Van Waes C. The host environment promotes the constitutive activation of nuclear factor-κB and proinflammatory cytokine expression during metastatic tumor progression of murine squamous cell carcinoma. Cancer Res 1999;59:3495–504.
31. Palayoor ST, Youmell MY, Calderwood SK, Coleman CN, Price BD. Constitutive activation of IκB kinase α and NF-κB in prostate cancer cells is inhibited by ibuprofen. Oncogene 1999;18:7389–94.
32. Singh SV, Zeng Y, Xiao D, et al. Caspase-dependent apoptosis induction by guggulsterone, a constituent of Ayurvedic medicinal plant *Commiphora mukul*, in PC-3 human prostate cancer cells is mediated by Bax and Bak. Mol Cancer Ther 2005;4:1747–54.
33. Xiao D, Herman-Antosiewicz A, Antosiewicz J, et al. Diallyl trisulfide-induced G_2-M phase cell cycle arrest in

human prostate cancer cells is caused by reactive oxygen species-dependent destruction and hyperphosphorylation of Cdc25C. Oncogene 2005;24:6256–68.
34. Samudio I, Konopleva M, Safe S, McQueen T, Andreeff M. Guggulsterones induce apoptosis and differentiation in acute myeloid leukemia: identification of isomer-specific antileukemic activities of the pregnadienedione structure. Mol Cancer Ther 2005;4:1982–92.
35. Widmann C, Gibson S, Jarpe MB, Johnson GL. Mitogen-activated protein kinase: conservation of a three-kinase module from yeast to human. Physiol Rev 1999;79:143–80.
36. Cobb MH. MAP kinase pathways. Prog Biophys Mol Biol 1999;71:479–500.
37. Davis RJ. Signal transduction by the JNK group of MAP kinases. Cell 2000;103:239–52.
38. Ding M, Lu Y, Bowman L, et al. Inhibition of AP-1 and neoplastic transformation by fresh apple peel extract. J Biol Chem 2004;279:10670–6.
39. Bode AM, Dong Z. Signal transduction pathways in cancer development and as targets for cancer prevention. Prog Nucleic Acid Res Mol Biol 2005;79:237–97.
40. Hundley TR, Rigas B. Nitric oxide-donating aspirin inhibits colon cancer cell growth via mitogen-activated protein kinase activation. J Pharmacol Exp Ther 2006;316:25–34.
41. Dickens M, Rogers JS, Cavanagh J, et al. A cytoplasmic inhibitor of the JNK signal transduction pathway. Science 1997;277:693–6.
42. Yasuda J, Whitmarsh AJ, Cavanagh J, Sharma M, Davis RJ. The JIP group of mitogen-activated protein kinase scaffold proteins. Mol Cell Biol 1999;19:7245–54.
43. Saitoh M, Nishitoh H, Fujii M, et al. Mammalian thioredoxin is a direct inhibitor of apoptosis signal-regulating kinase (ASK) 1. EMBO J 1998;17:2596–606.
44. Harding TC, Xue L, Bienemann A, et al. Inhibition of JNK by overexpression of the JNL binding domain of JIP-1 prevents apoptosis in sympathetic neurons. J Biol Chem 2001;276:4531–4.
45. Wang X, Martindale JL, Liu Y, Holbrook NJ. The cellular response to oxidative stress: influences of mitogen-activated protein kinase signalling pathways on cell survival. Biochem J 1998;333:291–300.
46. Xiao D, Choi S, Johnson DE, et al. Diallyl trisulfide-induced apoptosis in human prostate cancer cells involves c-Jun N-terminal kinase and extracellular-signal regulated kinase-mediated phosphorylation of Bcl-2. Oncogene 2004;23:5594–606.
47. Raha S, Robinson BH. Mitochondria, oxygen free radicals, and apoptosis. Am J Med Genet (Semin Med Genet) 2001;106:62–70.
48. Delfino FJ, Boustead JN, Fix C, Walker WH. NF-κB and TNF-α stimulate androgen receptor expression in Sertoli cells. Mol Cell Endocrinol 2003;201:1–12.
49. She QB, Chen N, Dong Z. ERKs and p38 kinase phosphorylate p53 protein at serine 15 in response to UV radiation. J Biol Chem 2000;275:20444–9.
50. Bulavin DV, Saito S, Hollander MC, et al. Phosphorylation of human p53 by p38 kinase coordinates N-terminal phosphorylation and apoptosis in response to UV radiation. EMBO J 1999;18:6845–54.

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Shivendra V. Singh, Sunga Choi, Yan Zeng, et al.

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