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Human Chorionic Gonadotropin Modulates Prostate Cancer Cell Survival after Irradiation or HMG CoA Reductase Inhibitor Treatment

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ABSTRACT

The impact of human chorionic gonadotropin (hCG) on prostate carcinoma viability was investigated. Treatment of LNCaP and PC-3 cells with hCG modestly reduced cell viability within 96 h. Treatment of cells with hCG followed by exposure to ionizing radiation enhanced radiosensitivity. Exposure of LNCaP cells to hCG promoted activation of epidermal growth factor receptor (ERBB1) via a $G\alpha_i$-, mitogen-activated protein kinase kinase (MEK)1/2-, and metalloprotease-dependent paracrine mechanism, effects that were further enhanced after radiation exposure, and that were causal in prolonged intense activation of poly(ADP-ribose) polymerase (PARP). Inhibition of ERBB1, MEK1, or PARP1 function suppressed the radiosensitizing properties of hCG. Radiosensitization was also, in part, dependent upon c-Jun NH_2-terminal kinase 1/2 signaling. PARP1-dependent radiosensitization was suppressed by a pan-caspase inhibitor and by knockdown of apoptosis-inducing factor expression. Inhibition of phosphatidylinositol 3-kinase, expression of dominant-negative AKT, or treatment with the HMG CoA reductase inhibitor lovastatin suppressed AKT phosphorylation and enhanced the cytotoxic effects of hCG. The enhancing effect of lovastatin was reproduced by incubation with a geranylgeranyl transferase inhibitor and blocked by coexposure to geranylgeranyl pyrophosphate. Treatment with hCG and lovastatin decreased expression of $BCL\text{-}_{XL}$ and XIAP, and increased expression of IκB. The cytotoxic effects of hCG were enhanced by expression of dominant-negative IκB, and they were abolished by coexpression of activated AKT. Expression of activated AKT maintained $BCL\text{-}_{XL}$ levels in cells expressing dominant-negative IκB. The promotion of hCG lethality by lovastatin was abolished by overexpression of $BCL\text{-}_{XL}$, and was dependent upon activation of caspase-9. Thus, hCG, in combination with radiation and lovastatin, may represent a novel approach to kill prostate cancer cells.

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Human chorionic gonadotropin (hCG) is a heterodimeric glycoprotein hormone consisting of two subunits (α and β) that bind to the luteinizing hormone (LH) receptor, and whose major physiological functions are involved in the normal growth and differentiation of multiple male and female cell types in organs involved with sexual reproduction (Hong et al., 1999; Filicori et al., 2005). The α subunit is a common subunit shared with other hormones (e.g., luteinizing hor-

ABBREVIATIONS: hCG, human chorionic gonadotropin; LH, luteinizing hormone; PI3K, phosphatidylinositol 3-kinase; ERK, extracellular signal-regulated kinase; EGF, epidermal growth factor; TGF, transforming growth factor; NF-κB, nuclear factor-κB; HRP, horseradish peroxidase; ECL, enhanced chemiluminescence; PJ-34, *N*-(6-oxo-5,6-dihydrophenanthridin-2-yl)-2-(*N*,*N*-dimethylamino) acetamide, hydrochloride; JNK, c-Jun NH_2-terminal kinase; JNK-IP, c-Jun NH_2-terminal kinase-inhibitory peptide; MAPK, mitogen-activated protein kinase; GGPP, geranylgeranyl pyrophosphate; GGTI-286, [(2*R*)-1-[[4-[[(1*S*)-1-methoxycarbonyl-3-methyl-butyl]carbamoyl]-3-phenyl-phenyl]amino]-3-sulfanyl-propan-2-yl]azanium; AG-1478, 4-(3′-chloroanilino)-6,7-dimethoxy-quinazoline; PD184352, 2-[(2-chloro-4-iodo-phenyl)amino]-*N*-(cyclopropylmethoxy)-3,4-difluoro-benzamide; LY294002, 2-(4-morpholinyl)-8-phenyl-1(4*H*)-benzopyran-4-one hydrochloride; GM6001, (2*R*)-*N*′-hydroxy-*N*-[(1*S*)-2-(5*H*-indol-3-yl)-1-(methylcarbamoyl)ethyl]-2-(2-methylpropyl)butanediamide; VEH, vehicle; DMSO, dimethyl sulfoxide; PBS, phosphate-buffered saline; si, small interfering RNA; PAGE, polyacrylamide gel electrophoresis; AIF, apoptosis-inducing factor; PARP, poly(ADP-ribose) polymerase; MEK, mitogen-activated protein kinase kinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium; GPCR, G protein-coupled receptor; CMV, cytomegalovirus; dn, dominant-negative; L, lovastatin; z, *N*-benzyloxycarbonyl.

mone), whereas the β subunit is unique to hCG. In clinical use, hCG is often indicated for therapeutic application in children with low levels of hCG during puberty and exhibiting a lack of appropriate physical development, and administration of this hormone acts to promote sexual maturation (Styne, 1991). However, prolonged overexpression of hCG in adults, particularly in women, has been linked to the development of tumors (e.g., gestational trophoblastic diseases, choriocarcinoma/germ cell tumors, osteosarcoma, bladder cancer, and prostate cancer) (Sheaff et al., 1996; Huhtaniemi et al., 2005). In contrast, at least one study has argued that elevated hCG levels may be cancer-preventative for breast cancer (Salhab et al., 2005; Cole et al., 2006).

Prostate cancer is a disease of older men (>50 years). Upon initial presentation, the majority of patients present with tumors that are located within the prostate and tumor cells that are dependent upon androgen for growth and survival (Collette et al., 2006; Messing et al., 2006). Primary forms of therapy for these patients include surgery and radiotherapy. However, if tumor cell clonogens survive in the prostate after therapy or were already present at local regional sites (e.g., lymph nodes), tumors will eventually reoccur. At present, the primary mode of treating individuals with recurrent tumors is to ablate androgen signaling by use of a variety of agents (e.g., finasteride) (Flores et al., 2003; Ryan and Small, 2004). Androgen ablation therapy has the short-term effect of inducing tumor cell growth arrest and cell killing, with a parallel reduction in the plasma levels of prostate-specific antigen, followed by a longer term repopulation of androgen-independent prostate cancer cells that have adapted their intracellular signaling pathways to survive, including increased expression of growth factor receptors [e.g., epidermal growth factor receptor (also called ERBB1 and HER1) and insulin-like growth factor 1 receptor] and inactivation of tumor suppressor genes (e.g., phosphatase with sequence homology to tensin) with parallel activation of PDK-1-AKT survival signaling (Barton et al., 2001; Roberts, 2004; Sansal and Sellers, 2004). Specific inhibition of ERBB1 and/or phosphatidylinositol 3-kinase (PI3K)-AKT signaling has been proposed by others as a therapeutic approach to suppress androgen-independent prostate cancer cell growth and enhance tumor cell radiosensitivity (Formento et al., 2004). Because the development of androgen-independent prostate cancer is invariably eventually fatal, the development of novel therapies against prostate cancer has clinical value.

Many groups have shown that ERBB1 is activated in response to irradiation of various carcinoma cell types (for reviews, see Dent et al., 2003a,b). Radiation exposure in the range 1 to 2 Gy, via activation of the ERBB1, can activate the ERK1/2 pathway to a level similar to that observed by physiological, growth stimulatory, EGF concentrations (~0.1 nM). Radiation-induced reactive oxygen species seem to play an important role in the activation of ERBB family receptors. The actions of ERBB receptor autocrine ligands have also been shown to play important roles in the activation of receptors after radiation exposure. For example, TGFα mediates secondary activation of the ERBB1 and the downstream ERK1/2 pathway after irradiation of several carcinoma cell lines, including androgen-independent prostate cancer cells (Hagan et al., 2004). In this instance, radiation-induced activation of ERK1/2 promotes cleavage of pro-TGFα in the plasma membrane, leading to growth factor release where it feeds back onto the irradiated cell: a stimulus response-signaling loop (Shvartsman et al., 2002). Growth factors (e.g., insulin-like growth factor 1) have also been shown to promote ERBB1 and ERK1/2 activation in tumor cells by this circuitous route through the actions of other paracrine ligands (e.g., heparin-binding-EGF) (El-Shewy et al., 2004). In isolated rat luteal membranes, ERBB1 signaling has been shown to uncouple hCG from cAMP production, and in intact rat granulose cells hCG actions were potentiated by EGF, suggesting that ERBB1 signaling and hCG signaling may interact in hCG-responsive cell types (Hattori et al., 1995; Lamm et al., 1999).

In breast cancer cells cultured in vitro, hCG has previously been phenomenon logically shown to be tumoricidal as a single agent and to act as a radiosensitizer (Pond-Tor et al., 2002). The goals of the present studies were to examine whether hCG treatment can alter the overall viability of prostate cancer cells, to determine the molecular mechanisms by which hCG radiosensitize human prostate cancer cell lines in vitro, to determine whether inhibition of ERBB1, RAS, PI3K, and ERK1/2 signaling function was capable of changing the survival of prostate carcinoma cells treated with hCG. We discovered that hCG radiosensitized prostate cancer cells by causing hyperpoly(ADP-ribose) polymerase (PARP) 1 activation and that inhibition of PI3K and NF-κB function enhanced the toxic properties of hCG in androgen-dependent prostate cancer cells.

Materials and Methods

Materials

Cell lines were purchased from the American Type Culture Collection (Manassas, VA). Phospho-/total- (ERK1/2, JNK1/2, and p38 MAPK) antibodies, phospho-/total-AKT (S473), phospho-/total-ERBB1 (Tyr1068, Tyr1173, Tyr845, and Tyr992), and anti-PARP1 antibodies were purchased from Cell Signaling Technology Inc. (Danvers, MA). The anti-PARP1 ADP-ribosylation specific 10H monoclonal antibody was purchased from Axxora Life Sciences, Inc. (San Diego, CA). All the secondary antibodies (anti-rabbit-HRP, anti-mouse-HRP, and anti-goat-HRP) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Enhanced chemiluminescence (ECL) and terminal deoxynucleotidyl transferase dUTP nick-end labeling kits were purchased from PerkinElmer Life and Analytical Sciences (Boston, MA) and Roche (Mannheim, Germany), respectively. PJ-34 (CAS no. 344458-15-7) was purchased from Sigma-Aldrich (St. Louis, MO). Trypsin-EDTA, Dulbecco's modified Eagle's medium, RPMI 1640 medium, and penicillin-streptomycin were purchased from Invitrogen (Carlsbad, CA). Pertussis toxin, caspase inhibitors (zVAD, LEHD, and IETD), the JNK inhibitory peptide (JNK-IP) with membrane translocation sequence GGTI-286, farnesyl pyrophosphate, geranylgeranyl pyrophosphate (GGPP), AG-1478, LY294002, and lovastatin were purchased from Calbiochem (San Diego, CA). Human chorionic gonadotropin was purchased from ProSpec-Tany TechnoGene Ltd. (Rehovot, Israel). PD184352 was chemically synthesized in-house based on the published structure of the drug and stored as a powder in a nitrogen atmosphere under light-protected conditions at −80°C. Other reagents were as described previously (Qiao et al., 2002; Hagan et al., 2004; Caron et al., 2005a,b; Yacoub et al., 2006).

Methods

Culture and in Vitro Exposure of Cells to Drugs. All cell lines were cultured at 37°C [5% (v/v CO_2)] in vitro using RPMI 1640 medium supplemented with 10% (v/v) fetal calf serum. In vitro, AG-1478/PD184352/wortmannin/lovastatin treatment was from a

100 mM stock solution of each drug, and the maximal concentration of vehicle (VEH; DMSO) in media was 0.02% (v/v).

In Vitro Cell Treatments, SDS-Polyacrylamide Gel Electrophoresis, and Western Blot Analysis. For in vitro analyses of short-term apoptosis effects, cells were treated with vehicle (DMSO)/AG-1478/PD184352/LY294002/lovastatin or their combination for the indicated times. For apoptosis assays, cells were pretreated with zVAD, IETD, or LEHD (each 50 μM) as described previously (Hagan et al., 2004; Caron et al., 2005a,b; Yacoub et al., 2006). Cells were isolated and either subjected to trypan blue cell viability assay by counting in a light microscope or as indicated fixed to slides, and they were stained using a commercially available terminal deoxynucleotidyl transferase dUTP nick-end labeling assay kit according to manufacturer's instructions (PerkinElmer Life and Analytical Sciences).

Cells for in vitro colony formation assays were plated at 250 to 4000 cells per well in sextuplicate, and for in vitro assays 14 h after plating, cells were treated with either vehicle (DMSO), AG-1478/PD184352/LY294002/lovastatin, or the drug combinations as indicated for 48 h followed by drug removal. Fourteen to 28 days after exposure or tumor isolation, plates were washed in PBS, fixed with methanol, and stained with a filtered solution of crystal violet (5%, w/v). After washing with tap water, the colonies were counted both manually (by eye) and digitally using a ColCount plate reader (Oxford Optronics, Oxford, UK). Data presented are the arithmetic mean ± S.E.M. from both counting methods from multiple studies. Colony formation was defined as a colony of 50 cells or greater.

For SDS-PAGE and immunoblotting, cells were plated at 5×10^5 cells/cm^2 and treated with drugs at the indicated concentrations. After the indicated time of treatment, cells were lysed in whole-cell lysis buffer (0.5 M Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 1% β-mercaptoethanol, and 0.02% bromphenol blue), and the samples were boiled for 30 min. The boiled samples were loaded onto 10 to 14% SDS-PAG, and electrophoresis was run overnight. Proteins were electrophoretically transferred onto 0.22-μm nitrocellulose and immunoblotted with various primary antibodies against different proteins. All immunoblots were visualized by ECL or using an Odyssey LI-COR system (LI-COR Biosciences, Lincoln, NE). For presentation, ECL immunoblots were digitally scanned at 600 dpi by using PhotoShop 7.0 (Adobe Systems, Mountain View, CA), and their color was removed. Figures were generated in PowerPoint (Microsoft Corp., Redmond, WA).

Transfection of Cells with Small Interfering RNA Molecules to Suppress Apoptosis-Inducing Factor and Poly(ADP-Ribose) Polymerase Expression. Cells were plated as described above, and 24 h after plating, they were transfected. RNA interference or gene silencing for down-regulating the expression of PARP and apoptosis-inducing factor (AIF) was performed using validated target sequences designed by Ambion (Austin, TX). For transfection, 10 nM concentrations of the annealed siRNA targeting PARP or AIF, the positive sense control doubled-stranded siRNA targeting glyceraldehyde-3-phosphate dehydrogenase, or the negative control (a "scrambled" sequence with no significant homology to any known gene sequences from mouse, rat or human cell lines) were used. The small RNA sequences were transfected by electroporation at 600 V during 60 μs. Forty-eight or 72 h after transfection, the cells were treated with drugs, as noted above.

Recombinant Adenoviral Vectors, Generation, and Infection in Vitro. We generated and/or purchased previously noted recombinant adenoviruses to express constitutively activated and dominant-negative AKT and MEK1 proteins, dominant-negative ERBB1 (COOH-terminal 533 amino acid deletion; CD533), dominant-negative JNK1, dominant-negative caspase-9, dominant-negative IκB (S32A), and BCL-$_{XL}$ (Vector Biolabs, Philadelphia, PA). Prostate cancer cells were infected with these adenoviruses at an approximate m.o.i. of 25. Cells were further incubated for 24 h to ensure adequate expression of transduced gene products before any drug exposures/assays.

Data Analysis. Comparison of the effects of various treatments was performed using the Student's t test. Differences with a p value of <0.05 were considered statistically significant. Experiments shown are the means of multiple individual points ± S.E.M.

Results

Initial studies examined the killing of human prostate cancer cells exposed for 96 h to hCG as a single agent and in combination with radiation exposure, using trypan blue exclusion as a measure of cell viability. Treatment of LNCaP and PC-3 cells with low concentrations of hCG (2 mU/ml) caused a modest amount of cell killing as a single agent within 96 h, which was enhanced in a greater than additive manner after exposure to ionizing radiation (Fig. 1, A and B). An assessment was made to determine the duration of pretreatment time with hCG that was required to cause radiosensitization. Pretreatment of LNCaP and PC-3 cells with hCG for 12 or 6 h did not significantly radiosensitize prostate carcinoma cells, whereas pretreatment with hCG for 30 to 90 min enhanced the lethality of radiation (Fig. 1C; data not shown).

Previous studies by this laboratory have shown that ionizing radiation promotes cell death in paracrine regulated A431, DU145, and MDA-MB-231 carcinoma cells by mechanisms that require activation of both pro-caspase-8 and pro-caspase-9. Thus, we next determined the mechanism(s) by which hCG and ionizing radiation interact to kill LNCaP cells using MTT growth and trypan blue viability assays. In LNCaP cells, the pan-caspase inhibitor zVAD and to a lesser extent the caspase-9 inhibitor LEHD partially protected cells from the growth-inhibitory effect of ionizing radiation in MTT assays (Fig. 1D). Radiation exposure and hCG interacted to cause a greater than additive increase in cell death that was blunted by zVAD and to a lesser extent by LEHD (Fig. 1E). Overexpression of dominant-negative caspase-9, however, did not recapitulate the effects of LEHD in trypan blue assays, arguing that the intrinsic caspase pathway was playing a secondary role in the observed killing effect (data not shown). In agreement with our short-term cell-killing analyses, exposure to hCG-radiosensitized LNCaP and PC-3 cells in colony formation assays (Fig. 1F; data not shown).

A variety of cell types have been shown to respond to hCG via signaling from several G protein-coupled receptors (GPCRs), including the LH receptor, that are known to be coupled in a cell type-dependent manner to both Gα_s (typically linked to elevation of cAMP) and Gα_i subunits (typically linked to suppression of cAMP generation). Treatment of LNCaP cells with hCG promoted activation of ERK1/2 within 6 h, but it did not significantly alter the high basal level of AKT activity in these cells (Fig. 2A). Activation of p38 MAPK was not observed (data not shown). Pretreatment of LNCaP cells with a specific inhibitor of Gα_i subunit function, pertussis toxin, suppressed hCG-induced activation of ERK1/2 arguing that hCG activated this pathway in LNCaP cells in a Gα_i-dependent manner (Fig. 2B). Furthermore, treatment of LNCaP cells with the ERBB1 kinase inhibitor AG-1478 or expression of dominant-negative ERBB1-CD533 (CD533) also blocked hCG-induced ERK1/2 activation, which argues that ERBB1 is a key player in hCG-mediated Gα_i-dependent activation of ERK1/2 in LNCaP cells (Fig. 2B).

Based on our data in Fig. 2 and our findings in primary

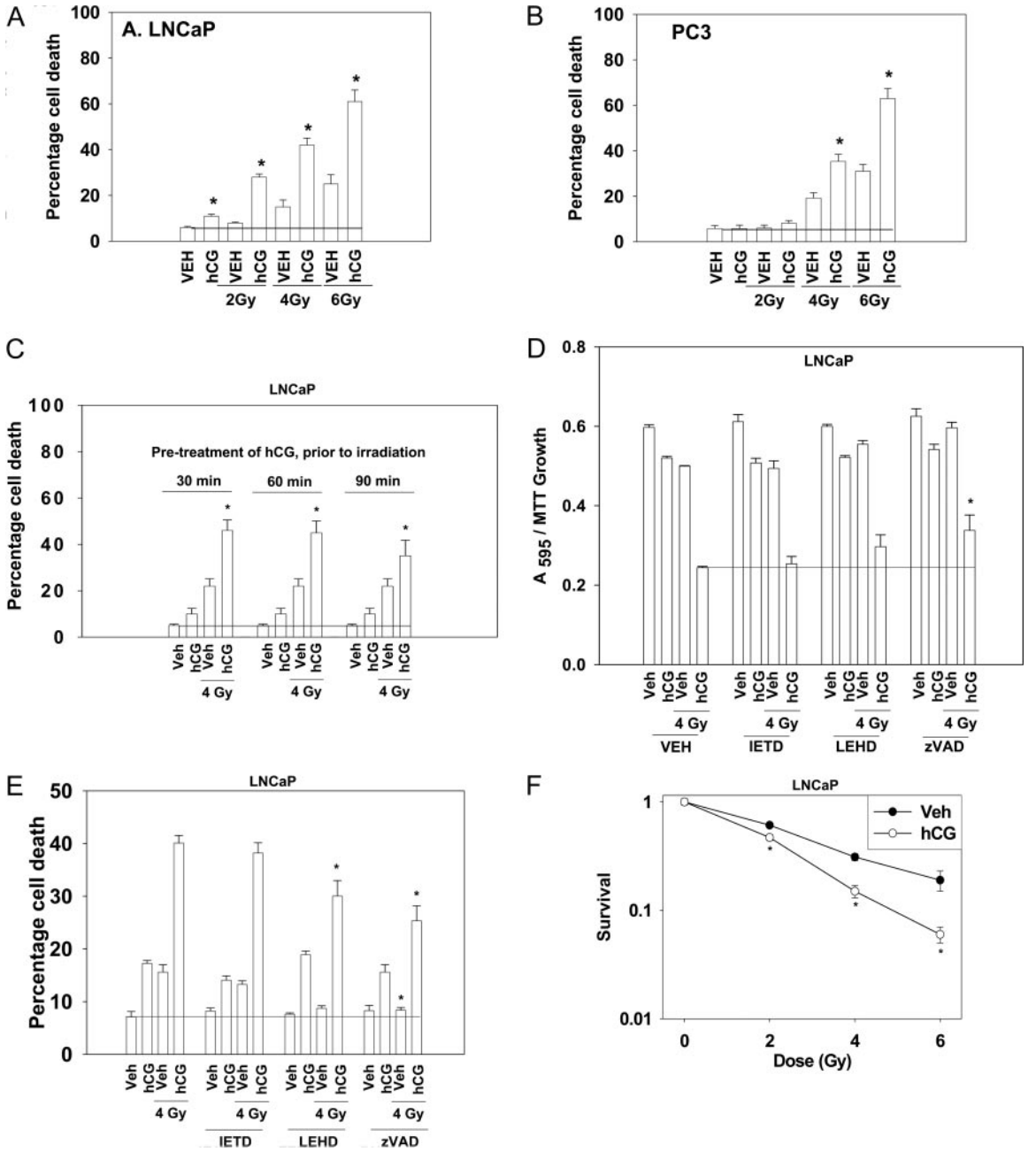

Fig. 1. Treatment of LNCaP and PC-3 cells with hCG enhances their radiosensitivity. LNCaP (A) and PC-3 (B) cells were cultured as described under *Materials and Methods*. Twenty-four hours after plating, cells were treated with 2 mU/ml hCG 30 min before radiation exposure (0–6 Gy). Cells were isolated 96 h after irradiation by trypsinization, the attached and floating cells were combined, and cell viability was determined by trypan blue exclusion assays using a light microscope (*, $p < 0.05$ value greater than corresponding vehicle/irradiated + vehicle value; $n = 3$/data point ± S.E.M. from three studies). C, LNCaP cells were cultured as described under *Materials and Methods*. Twenty-four hours after plating, cells were treated with 2 mU/ml hCG 30 to 90 min before radiation exposure (4 Gy). Cells were isolated 96 h after irradiation by trypsinization, the attached and floating cells were combined, and cell viability was determined by trypan blue exclusion assays using a light microscope (*, $p < 0.05$ value greater than corresponding vehicle/irradiated + vehicle value; $n = 3$/data point ± S.E.M. from three studies). D, LNCaP cells were cultured as described under *Materials and Methods*. Twenty-four hours after plating, cells were pretreated (30 min) with either the caspase-8 inhibitor IETD (50 μM), the caspase-9 inhibitor LEHD (50 μM), or the pan-caspase inhibitor zVAD (50 μM) followed by treatment with 2 mU/ml hCG 30 min before radiation exposure (4 Gy). Caspase inhibitors were resupplemented every 24 h. After 96 h of culture, cell viability numbers were determined MTT assays using a plate reader (*, $p < 0.05$ value greater than corresponding vehicle/irradiated + vehicle value; $n = 4$/data point ± S.E.M. from three studies). E, LNCaP cells were cultured as under *Materials and Methods*. Twenty-four hours after plating, cells were pretreated (30 min) with either the caspase-8 inhibitor IETD (50 μM), the caspase-9 inhibitor LEHD (50 μM), or the pan-caspase inhibitor zVAD (50 μM) followed by treatment with 2 mU/ml hCG 30 min before radiation exposure (4 Gy). Caspase inhibitors were resupplemented every 24 h. Cells were isolated 96 h after irradiation by trypsinization, the attached and floating cells were combined, and cell viability was determined by trypan blue exclusion assays using a light microscope (*, $p < 0.05$ value less than corresponding vehicle/irradiated + vehicle value; $n = 3$/data point ± S.E.M. from three studies). F, LNCaP cells were cultured as described under *Materials and Methods*. Twenty-four hours after plating (250–2500 cells/well of a six-well plate), cells were treated with 2 mU/ml hCG 30 min before radiation exposure (0–6 Gy). Ninety-six hours after irradiation, media containing hCG were removed, and fresh media lacking hCG were added. Colonies were permitted to form over the subsequent 28 days, after which the media were removed, and the cells were fixed and stained with crystal violet. Groups of >50 cells were considered to be colonies (*, $p < 0.05$ value less than corresponding vehicle/irradiated + vehicle value; $n = 6$/data point ± S.E.M. from two studies).

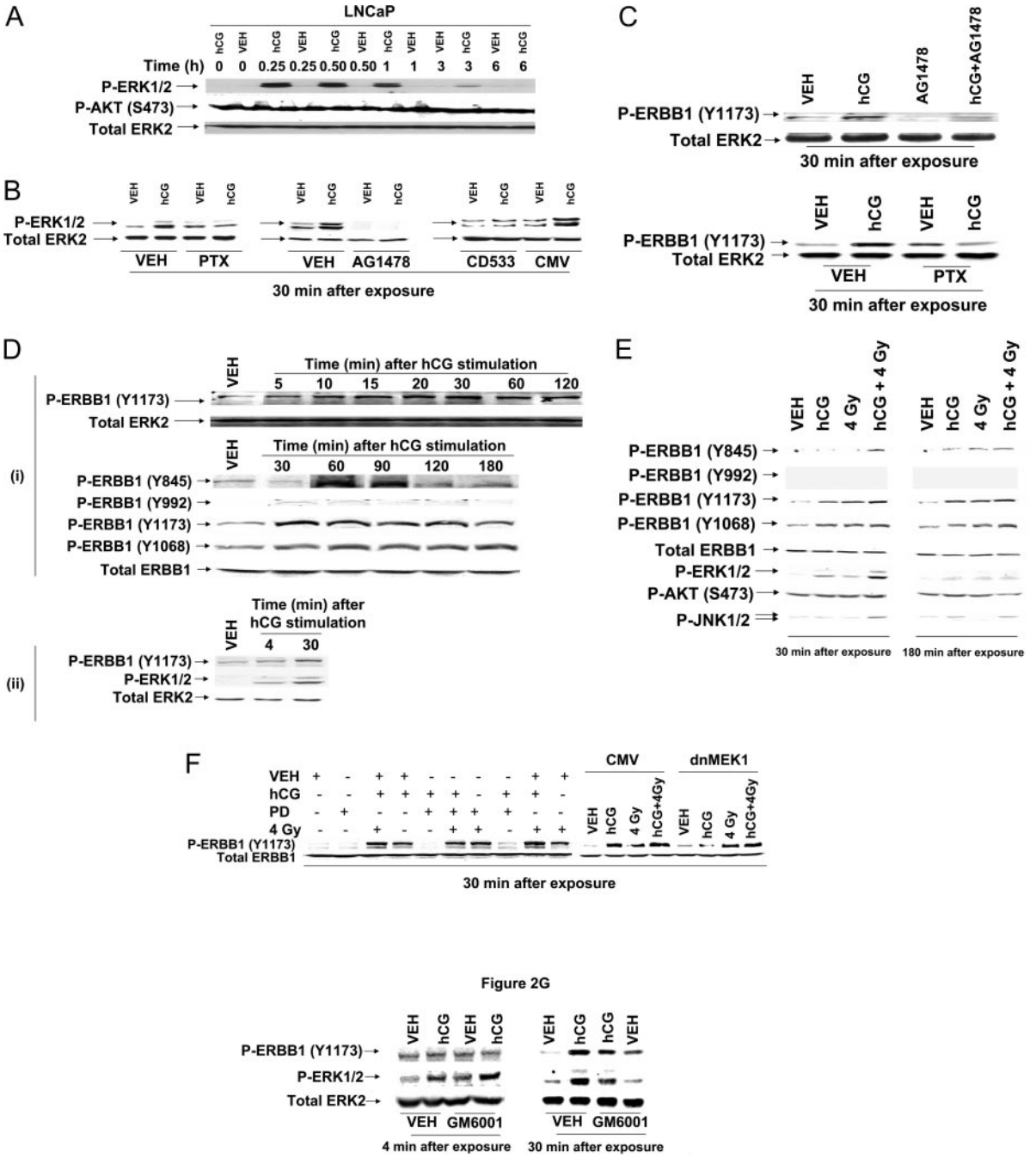

Figure 2G

Fig. 2. Paracrine activation of ERBB1 in LNCaP cells after treatment with hCG. A, LNCaP cells were cultured as described under *Materials and Methods*. Twenty-four hours after plating, cells were treated with 2 mU/ml hCG or VEH (phosphate-buffered saline). At the indicated times after treatment, cells were isolated, lysed in SDS-PAGE sample buffer, and equal protein concentrations were subjected to SDS-PAGE followed by immunoblotting to determine the phosphorylation status (activity status) of ERK1/2, JNK1/2, and AKT (S473) and the expression level of ERK2 protein. Data are from a representative study ($n = 3$). B, LNCaP cells were cultured as described under *Materials and Methods*. Twelve hours after plating, as indicated, cells were treated with vehicle (PBS) or 300 ng/ml pertussis toxin. Twenty-three hours after plating, as indicated, cells were treated with VEH (DMSO) or the ERBB1 inhibitor AG-1478 at 1 μM. Twenty-four hours after plating, cells were treated with 2 mU/ml hCG or VEH (phosphate-buffered saline). Cells were isolated 30 min after hCG treatment, lysed in SDS-PAGE sample buffer, and equal protein concentrations were subjected to SDS-PAGE followed by immunoblotting to determine the phosphorylation status (activity status) of ERK1/2 and the expression level of ERK2 protein. Data are from representative studies ($n = 3$). In parallel plates of LNCaP cells, 12 h after plating, as indicated, cells were infected with either empty vector virus (CMV) or with a virus to express dominant negative ERBB1 (CD533) at an m.o.i. of 25. Twenty-four hours after plating or 24 h after infection, as indicated, cells were treated with 2 mU/ml hCG or VEH (phosphate-buffered saline), and 30 min later they were irradiated (4 Gy). Treatment with AG-1478 or VEH (DMSO) was made as indicated in the figure. Thirty minutes after irradiation, cells were isolated, lysed in SDS-PAGE sample buffer and equal protein concentrations were subjected to SDS-PAGE followed by immunoblotting to determine the phosphorylation status (activity status) of ERK1/2 and the expression level of total ERK2 protein levels. Data are from a representative study ($n = 3$). C, LNCaP cells were cultured as described under *Materials and Methods*. Twelve hours after plating, as indicated, cells were treated with vehicle (PBS) or 300 ng/ml pertussis toxin. Twenty-three hours after plating, as indicated, cells were treated with VEH (DMSO) or the ERBB1 inhibitor AG-1478 at 1 μM. Twenty-four hours after plating, cells were treated with 2 mU/ml hCG or VEH (phosphate-buffered saline). Cells were isolated 30 min after hCG treatment, lysed in SDS-PAGE sample buffer, and equal protein concentrations were subjected to SDS-PAGE followed by immunoblotting to determine the phosphorylation status (activity status) of ERBB1 Tyr1173 and the expression level of ERK2 protein. Data are from a representative

hepatocytes treated with bile acids in which a conjugated bile acid activated a GPCR that in a $G\alpha_i$-dependent manner then caused activation of ERBB1 and ERK1/2, we next examined whether hCG also promoted activation of ERBB1 in LNCaP cells (Dent et al., 2005). Treatment of LNCaP cells with hCG increased phosphorylation of ERBB1 (Tyr1173), an effect that was blocked by the ERBB1-specific inhibitor AG-1478 and by treatment with pertussis toxin (Fig. 2C). Treatment of LNCaP cells with hCG promoted prolonged phosphorylation of ERBB1 at Tyr1173 and Tyr1068, transient late phosphorylation at Tyr845, and little phosphorylation at Tyr992 (Fig. 2D, i). It is noteworthy that treatment with hCG promoted ERK1/2 activation before ERBB1 phosphorylation (4 min; cf. 30 min after exposure) (Fig. 2D, ii).

The sites of ERBB1 tyrosine phosphorylation were then examined 30 and 180 min after combined hCG and radiation exposure. Treatment with hCG or irradiation increased tyrosine phosphorylation of ERBB1 at Tyr1068 and Tyr1173, 30 min after exposure (Fig. 2E). Combined treatment with hCG and radiation caused a modest further increase in Tyr1068 and Tyr1173 ERBB1 tyrosine phosphorylation, 30 min after exposure, and promoted Tyr845 phosphorylation. Combined exposure to hCG and radiation promoted further activation of ERK1/2, and of JNK1/2, 30 min after exposure, which had subsided within 180 min. ERBB1 Tyr1173 phosphorylation induced by hCG, 30 min after treatment, was abolished by prior incubation of cells with the specific MEK1/2 inhibitor PD184352 or by expression of dominant-negative MEK1, suggestive that hCG activated ERBB1 via an indirect mechanism (Fig. 2F).

Several studies have argued that GPCRs can promote a circuitous activation of ERBB1, via ERK1/2 and p38 MAPK pathway signaling, in which the MAPK pathways stimulate membrane metalloprotease activities, causing the release/activation of paracrine signaling factors that feed back onto the cell, thereby activating ERBB1 (Dent et al., 1999; Hagan et al., 2000, 2004; Shvartsman et al., 2002). Because inhibition of MEK1/2 blocked hCG-induced phosphorylation of ERBB1 Tyr1173, we tested whether hCG was promoting ERBB1 activation via a metalloprotease-dependent mechanism in our system. Cells were incubated with the metalloprotease inhibitor GM6001 and exposed to hCG; GM6001 abolished both ERBB1 Tyr1173 phosphorylation and ERK1/2 activation 30 min after hCG treatment, but not 4 min after treatment (Fig. 2G). We next attempted to determine the putative paracrine factor mediating hCG-induced activation of ERBB1. LNCaP cells do not express significant amounts of heregulin, TGFα, or heparin-binding-EGF, and functional inhibition of these growth factors by use of neutralizing antibodies did not significantly alter ERBB1 Tyr1173 phosphorylation in LNCaP cells after hCG treatment (data not shown). Thus, hCG activates ERBB1 by an indirect mechanism involving an initial modest ERBB1-independent activation of the MEK1/2-ERK1/2 pathway followed by secondary paracrine MEK1/2- and ligand-dependent intense activation of ERBB1 and the ERK1/2 pathway.

In Fig. 2, we noted that hCG activated ERBB1 and ERK1/2, and we next determined whether activation or inhibition of MEK1, AKT, and ERBB1 modulated the survival response of cells treated with hCG and exposed to radiation (Fig. 3). In short-term 96-h trypan blue cell-killing assays, expression of dominant-negative ERBB1 (CD533) suppressed the radiosensitizing effect of hCG (Fig. 3A). Expression of activated MEK1 did not alter hCG-induced cell killing, but it suppressed the lethality of hCG and radiation exposure, whereas expression of activated AKT suppressed the lethality of hCG, of radiation, and of hCG and radiation exposure (Fig. 3B). Expression of dominant-negative AKT enhanced hCG-induced cell killing and further enhanced the lethality of hCG and radiation exposure (Fig. 3C). Expression of dominant-negative MEK1 did not alter the lethality of hCG or of radiation, but surprisingly, based on our data with activated MEK1, it suppressed the lethality of hCG and radiation exposure. Although hCG and radiation combined to activate JNK1/2, the observed activation was relatively modest; nevertheless, inhibition of JNK1/2 signaling suppressed the lethality of hCG, radiation, and combined exposure to both agents (Fig. 3D). Together, the findings in Figs. 2 and 3 argue that activation of ERBB1, and potentially ERK1/2, is causal in the lethal interaction between hCG and radiation exposure, that a portion of the cell death signal is also mediated by the JNK1/2 pathway, and that PI3K-AKT signaling plays a major role in protecting LNCaP cells from hCG toxicity.

Because caspase inhibition only reduced the lethality of

(cont'd from previous page) study ($n = 3$). D, LNCaP cells were cultured as described under *Materials and Methods*. Twenty-four hours after plating, cells were treated with 2 mU/ml hCG or its vehicle (phosphate-buffered saline). After hCG treatment (0–180 min, as indicated), cells were isolated, lysed in SDS-PAGE sample buffer, and equal protein concentrations were subjected to SDS-PAGE followed by immunoblotting to determine the phosphorylation status (activity status) of ERBB1 Tyr1068/Tyr1173/Tyr992/Tyr845 and of ERK1/2 and the expression level of ERK2 and ERBB1 protein. Data are from a representative study ($n = 3$). E, LNCaP cells were cultured as described under *Materials and Methods*. Twenty-four hours after plating, cells were treated with 2 mU/ml hCG or its vehicle (phosphate-buffered saline). Thirty minutes after hCG treatment, cells were irradiated (4 Gy) and 30 min after irradiation cells were isolated, lysed in SDS-PAGE sample buffer, and equal protein concentrations were subjected to SDS-PAGE followed by immunoblotting to determine the phosphorylation status (activity status) of ERBB1 Tyr1068/Tyr1173/Tyr992/Tyr845, of ERK1/2 and JNK1/2, of AKT (S473), and the expression level of ERBB1 protein. Data are from a representative study ($n = 3$). F, LNCaP cells were cultured as described under *Materials and Methods*. Twenty-four hours after plating cells were pretreated, as indicated, with VEH (DMSO) or the MEK1/2 inhibitor PD184352 at 1 μM, and then treated 30 min afterward with 2 mU/ml hCG or its vehicle (phosphate-buffered saline). Thirty minutes after hCG treatment, cells were irradiated (4 Gy) and 30 min after irradiation cells were isolated, lysed in SDS-PAGE sample buffer, and equal protein concentrations were subjected to SDS-PAGE followed by immunoblotting to determine the phosphorylation status (activity status) of ERBB1 Tyr1068 and the expression level of ERBB1 protein. Data are from a representative study ($n = 3$). In parallel plates of LNCaP cells, 12 h after plating, as indicated, cells were infected with either empty vector virus (CMV) or with a virus to express dominant-negative MEK1 at an m.o.i. of 25. Twenty-four hours after infection, as indicated, cells were treated with 2 mU/ml hCG or VEH (phosphate-buffered saline), and 30 min later they were irradiated (4 Gy). Thirty minutes after irradiation, cells were isolated, lysed in SDS-PAGE sample buffer, and equal protein concentrations were subjected to SDS-PAGE followed by immunoblotting to determine the phosphorylation status (activity status) of ERBB1 and the expression level of total ERBB1 protein levels. Data are from a representative study ($n = 3$). G, LNCaP cells were cultured as described under *Materials and Methods*. Twenty-four hours after plating cells were pretreated, as indicated with VEH (DMSO) or the metalloprotease inhibitor GM6001 at 1 μM, and then with either treated 30 min afterward with 2 mU/ml hCG or its VEH (phosphate-buffered saline). At the indicated times after treatment, cells were isolated, lysed in SDS-PAGE sample buffer, and equal protein concentrations were subjected to SDS-PAGE followed by immunoblotting to determine the phosphorylation status (activity status) of ERK1/2 and of ERBB1 Tyr1068 and the expression level of ERK2 protein. Data are from a representative study ($n = 3$).

combined radiation and hCG treatment by ~50%, we investigated whether caspase-independent mechanisms play a role in hCG and radiation-induced cell death. Radiation and hCG acted in a greater than additive manner to activate PARP1 as judged by PARP1 ADP-ribosylation (Fig. 4A) (Schreiber et al., 2006). Hyperactivation of PARP1 has been linked to necrotic cell death caused by the depletion of cellular NAD^+ and ATP levels (Kolthur-Seetharam et al., 2006). Inhibition of ERBB1 using AG-1478 or expression of dominant-negative ERBB1 (CD533) abolished the activation of PARP1 caused by combined hCG and radiation treatment (Fig. 4B). Because inhibition of MEK1/2 also blocked hCG-induced activation of ERBB1, we next determined whether the profound activation of PARP1 caused by hCG and radiation treatment was dependent on ERK1/2 signaling. Expression of dominant-negative MEK1 blocked ERK1/2 activation and suppressed PARP1 activation after hCG and radiation treatment (Fig. 4C).

Inhibition of PARP1 function using low concentrations of a highly specific inhibitor of PARP family enzymes, PJ-34, or using siRNA to knock down PARP1 expression, abolished the potentiation of hCG lethality by radiation (Fig. 4, D and E).

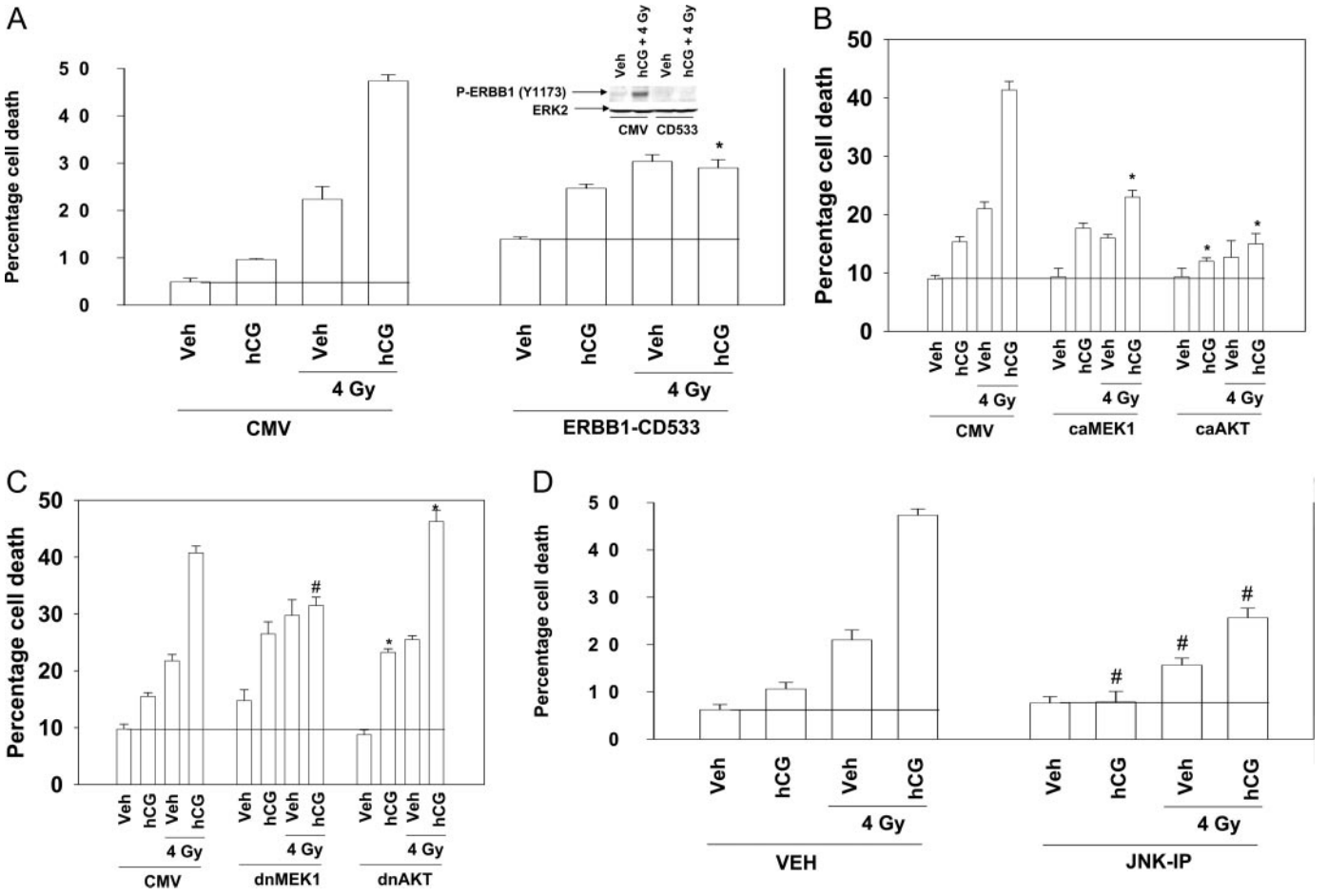

Fig. 3. A, inhibition of ERBB1 and activation of AKT and MEK1 suppress the radiosensitizing effects of hCG in LNCaP cells. B, LNCaP cells were cultured as described under *Materials and Methods*. Twelve hours after plating, cells were infected with either empty vector virus (CMV) or with a virus to express dominant-negative ERBB1 (CD533) at an m.o.i. of 25. Twenty-four hours after infection, cells were treated with 2 mU/ml hCG or VEH (phosphate-buffered saline), and 30 min later they irradiated (4 Gy). Cells were isolated 96 h after irradiation by trypsinization, the attached and floating cells were combined, and cell viability was determined by trypan blue exclusion assays using a light microscope (∗, $p < 0.05$ value less than corresponding vehicle/irradiated + vehicle value; n = 3/data point ± S.E.M. from three studies). Inset, expression of ERBB1-CD533 blocked hCG and radiation exposure-induced phosphorylation of ERBB1 Tyr1173. C, LNCaP cells were cultured as described under *Materials and Methods*. Twelve hours after plating, cells were infected with either empty vector virus (CMV) or with viruses to express constitutively active (ca) MEK1 or to express constitutively active (CA) AKT at an m.o.i. of 25. Twenty-four hours after infection, cells were treated with 2 mU/ml hCG or VEH (phosphate-buffered saline), and 30 min later they were irradiated (4 Gy). Cells were isolated 96 h after irradiation by trypsinization, the attached and floating cells were combined, and cell viability was determined by trypan blue exclusion assays using a light microscope (∗, $p < 0.05$ value less than corresponding vehicle/irradiated + vehicle value; n = 3/data point ± S.E.M. from three studies). D, LNCaP cells were cultured as described under *Materials and Methods*. Twelve hours after plating, cells were infected with either empty vector virus (CMV) or with viruses to express dominant-negative (dn) MEK1 or to express dn AKT at an m.o.i. of 25. Twenty-four hours after infection, cells were treated with 2 mU/ml hCG or VEH (phosphate-buffered saline), and 30 min later they were irradiated (4 Gy). Cells were isolated 96 h after irradiation by trypsinization, the attached and floating cells were combined, and cell viability was determined by trypan blue exclusion assays using a light microscope (∗, $p < 0.05$ value greater than corresponding vehicle/irradiated + vehicle value; n = 3/data point ± S.E.M. from three studies; #, $p < 0.05$ value less than corresponding vehicle/irradiated + vehicle value; n = 3/data point ± S.E.M. from three studies). E, LNCaP cells were cultured as described under *Materials and Methods*. Twenty-four hours after plating, cells were pretreated, as indicated, with VEH (DMSO) or the JNK1/2 JNK-IP at 10 μM, and then treated 30 min afterward with 2 mU/ml hCG or its vehicle (phosphate-buffered saline). Thirty minutes after hCG treatment, cells were irradiated (4 Gy). Cells were isolated 96 h after irradiation by trypsinization, the attached and floating cells were combined, and cell viability was determined by trypan blue exclusion assays using a light microscope (#, $p < 0.05$ value less than corresponding vehicle/irradiated + vehicle value; n = 3/data point ± S.E.M. from three studies).

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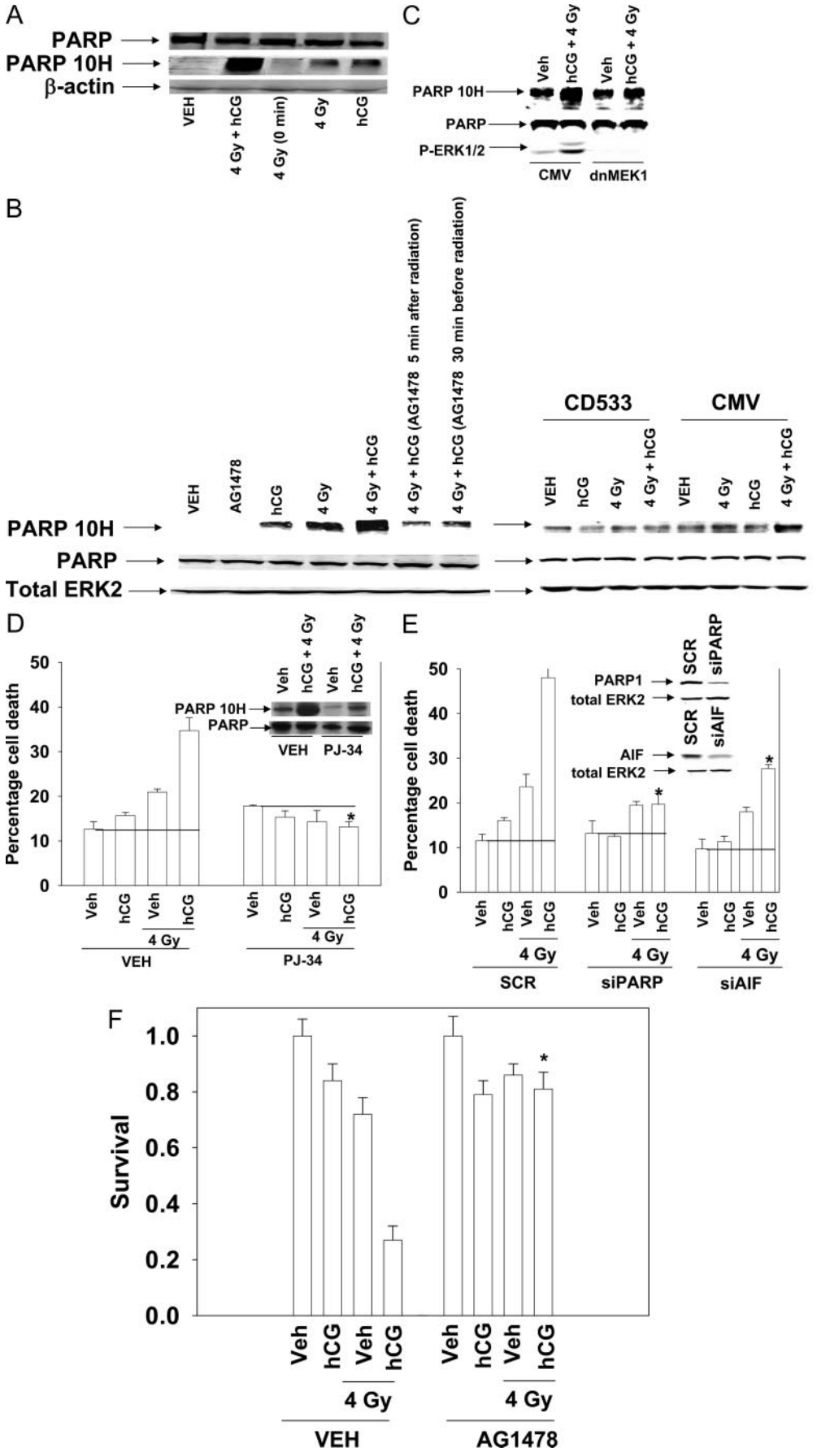


PARP1-induced cell death has also been linked by some groups to the cytotoxic actions of AIF (Kolthur-Seetharam et al., 2006). Suppression of AIF expression reduced the lethality of hCG and radiation exposure by ~50%, suggestive that AIF played a role in the caspase-independent death effect (Fig. 4E). In colony formation assays, transient inhibition of ERBB1 function also prevented the greater than additive induction of cell killing caused by hCG and radiation exposure (Fig. 4F). Together, these findings argue that hCG and radiation exposure interact in an ERBB1- and MEK1-dependent manner to promote the high levels of PARP1 activation that were responsible for LNCaP cell radiosensitization by hCG.

As a single agent, low concentrations of hCG caused modest levels of LNCaP cell death, which correlated with activation of ERBB1, ERK1/2, and JNK1/2. To further investigate the role of signal transduction pathways in the survival response of hCG-treated prostate cancer cells, we initially examined the abilities of ERBB1 and PI3K inhibitors to modify cell viability in hCG-treated LNCaP cells. In agreement with our viability data in Fig. 3A expressing ERBB1 CD533, treatment of cells with the ERBB1 inhibitor AG-1478 did not significantly reduce cell viability after hCG exposure, arguing that hCG-induced activation of ERBB1, per se, is not a toxic signal (Fig. 5). In agreement with our data in Fig. 3, B and C, expressing constitutively active and dominant-negative AKT proteins, and inhibition of PI3K signaling, using a small molecule kinase domain inhibitor LY294002, enhanced hCG lethality in LNCaP cells in a greater than additive manner.

Upstream of both the PI3K-AKT and ERK1/2 pathways are Ras/Rac/Rho small GTP binding proteins whose transducing functions are dependent upon their lipid modification (prenylation: farnesylation and geranylgeranylation) and subsequent membrane localization (van de Donk et al., 2003). A variety of specific inhibitors of protein farnesylation and/or geranylgeranylation have been generated that have been useful reagents in the laboratory and in animals, but they have proven relatively ineffective in the clinic, possibly due in part to the plasticity of tumor cell signaling in using different Ras/Rac/Rho proteins in the presence of such inhibitors to maintain cell signaling and viability (Fritz and Kaina, 2006). In an attempt to avoid these issues, we performed studies using the HMG CoA reductase inhibitor lovastatin. HMG CoA reductase is the rate-limiting step in mevalonate biosynthesis, and inhibition of this enzyme reduces the levels of both farnesyl pyrophosphate and geranylgeranyl pyrophosphate in cells, thereby impeding the lipid modification of many small GTP binding signal transducing proteins, which may thus inhibit signaling by multiple downstream kinase pathways.

Treatment of LNCaP cells with low clinically achievable concentrations of lovastatin (0.1–0.6 μM) enhanced the lethality of hCG to an extent that was at least the equivalent of our observations using a PI3K inhibitor (Fig. 6A, compare with data in Fig. 5). Incubation of cells with GGPP, but not farnesyl pyrophosphate, abolished the lethal interaction between hCG and lovastatin, arguing that the functional inhibition of a geranylgeranylated protein by lovastatin enhanced hCG lethality (Fig. 6B) (Thibault et al., 1996; van de Donk et al., 2003; Wu et al., 2004; Fritz and Kaina, 2006; Holstein et al., 2006). The impact of lovastatin on protein prenylation can be grossly determined by changes in the mobility of prenylated proteins on SDS-PAGE (Wu et al., 2004). Both the protein levels and SDS-PAGE mobility of Rho A, a membrane-associated geranylgeranylated protein, were

Fig. 4. ERBB1-dependent PARP activation is causal in the radiosensitizing effects of hCG in LNCaP cells. A, LNCaP cells were cultured as described under *Materials and Methods*. Twenty-four hours after plating, cells were treated with 2 mU/ml hCG or VEH (phosphate-buffered saline) followed 30 min later by irradiation (4 Gy). Thirty minutes after irradiation, cells were isolated, lysed in SDS-PAGE sample buffer, and equal protein concentrations were subjected to SDS-PAGE followed by immunoblotting to determine the ADP-ribosylation status (activity status) of PARP1 and the expression level of total PARP1 and β-actin protein levels. Data are from a representative study ($n = 3$). B, LNCaP cells were cultured as described under *Materials and Methods*. Twelve hours after plating, as indicated, cells were infected with either empty vector virus (CMV) or with a virus to express dominant negative ERBB1 (CD533) at an m.o.i. of 25. Twenty-four hours after plating or 24 h after infection, as indicated, cells were treated with 2 mU/ml hCG or VEH (phosphate-buffered saline), and 30 min later they were irradiated (4 Gy). Treatment with AG-1478 or VEH (DMSO) was made as indicated in the figure. Thirty minutes after irradiation, cells were isolated, lysed in SDS-PAGE sample buffer, and equal protein concentrations were subjected to SDS-PAGE followed by immunoblotting to determine the ADP-ribosylation status (activity status) of PARP1 and the expression level of total PARP1 and β-actin protein levels. Data are from a representative study ($n = 3$). C, LNCaP cells were cultured as described under *Materials and Methods*. Twelve hours after plating, as indicated, cells were infected with either empty vector virus (CMV) or with a virus to express dominant-negative MEK1 (dnMEK1) at an m.o.i. of 25. Twenty-four hours after plating or 24 h after infection, as indicated, cells were treated with 2 mU/ml hCG or VEH (phosphate-buffered saline), and 30 min later they were irradiated (4 Gy). Thirty minutes after irradiation, cells were isolated, lysed in SDS-PAGE sample buffer, and equal protein concentrations were subjected to SDS-PAGE followed by immunoblotting to determine the ADP-ribosylation status (activity status) of PARP1, the phosphorylation status (activity status) of ERK1/2, and the expression level of total PARP1 protein level. Data are from a representative study ($n = 3$). D, LNCaP cells were cultured as described under *Materials and Methods*. Twenty-four hours after plating, cells were pretreated with vehicle (DMSO) or with the PARP inhibitor PJ-34 at 5 μM followed 30 min later by treatment with vehicle (PBS) or with 2 mU/ml hCG. Thirty minutes after hCG treatment, cells were irradiated (4 Gy). Cells were isolated 96 h after irradiation by trypsinization, the attached and floating cells were combined, and cell viability was determined by trypan blue exclusion assays using a light microscope ($*$, $p < 0.05$ value less than corresponding vehicle/irradiated + vehicle value; $n = 3$/data point ± S.E.M. from two studies). Inset, cells were treated as indicated in the panel and isolated 30 min after irradiation, followed by SDS-PAGE to determine PARP1 ADP ribosylation in the presence or absence of PJ-34. E, LNCaP cells were cultured as described under *Materials and Methods*. Twelve hours after plating, as indicated, cells were transfected using Lipofectamine with either a scrambled siRNA (SCR) or with a siRNA molecules to suppress PARP1 or AIF expression (siPARP and siAIF; 10 nM each). Twenty-four hours after transfection, as indicated, cells were treated with 2 mU/ml hCG or VEH (phosphate-buffered saline), and 30 min later they irradiated (4 Gy). Cells were isolated 96 h after irradiation by trypsinization, the attached and floating cells were combined, and cell viability was determined by trypan blue exclusion assays using a light microscope ($*$, $p < 0.05$ value less than corresponding vehicle/irradiated + vehicle value; $n = 3$/data point ± S.E.M. from four studies). Inset, cells were treated as indicated in the panel and isolated 30 min after irradiation, followed by SDS-PAGE to determine PARP1 and AIF expression in the presence or absence of siPARP and siAIF, siRNA molecules, respectively. F, LNCaP cells were cultured as described under *Materials and Methods*. Twenty-four hours after plating (250–2500 cells/well of a six-well plate), cells were pretreated with vehicle (DMSO) or AG-1478 at 1 μM, and 30 min later they were treated with 2 mU/ml hCG. Thirty minutes after hCG exposure, cells were irradiated (4 Gy). Ninety-six hours after irradiation, media containing hCG and AG-1478 were removed, and fresh media lacking hCG/AG1478 were added. Colonies were permitted to form over the ensuing 28 days, after which the media were removed, and the cells were fixed and stained with crystal violet. Groups of >50 cells were considered to be colonies ($*$, $p < 0.05$ value greater than corresponding vehicle/irradiated + vehicle value; $n = 6$/data point ± S.E.M. from two studies).

not apparently reduced by individual exposure to hCG or to 0.6 μM lovastatin, but they were inhibited 12 to 48 h after combined lovastatin and hCG treatment (Fig. 6B, inset). Identical data LNCaP cell-killing data for hCG and lovastatin exposure were obtained when a geranylgeranyl transferase inhibitor (5 μM GGTI-286) was used in place of lovastatin (data not shown). Our findings 96 h after exposure were recapitulated in colony formation assays assessing LNCaP survival 14 to 28 days after lovastatin and hCG treatment (Fig. 6C). Similar cell-killing data to those in LNCaP cells were obtained combining hCG and lovastatin in 22RW1 and PC-3 prostate cancer cells as well as in SKOV3 ovarian carcinoma cells (Fig. 7, A–C). Together, these findings demonstrate that low clinically relevant concentrations of lovastatin enhance the lethality of hCG in multiple androgen-dependent and -independent carcinoma cell lines in vitro, which occurs by suppression of protein geranylgeranylation.

We then examined the impact of lovastatin and hCG on the activation status of signal transduction pathways in LNCaP cells. Lovastatin treatment, within 48 h, had suppressed basal and hCG-induced AKT activity (Fig. 8A). Combined exposure to hCG and lovastatin and promoted JNK1/2 activation (Fig. 8A). In agreement with our data using ionizing radiation, incubation of cells with a cell-permeable fragment of the JNK inhibitory protein JIP1 suppressed hCG lethality as a single agent and when combined with lovastatin (Fig. 8B). Expression of constitutively active AKT suppressed cell killing by hCG and by hCG and lovastatin treatment. Expression of dominant-negative AKT or dominant-negative IκB enhanced the lethality of lovastatin or hCG as single agents and abolished their cytotoxic interaction. In agreement with a role of IκB in modulating the lethal effects of lovastatin and hCG treatment, simultaneous exposure of LNCaP cells to both agents increased the expression of IκB and decreased expression of proteins whose expression can be regulated by NF-κB, the caspase inhibitor XIAP, and the mitochondrial protective protein BCL-$_{XL}$ (Fig. 8C).

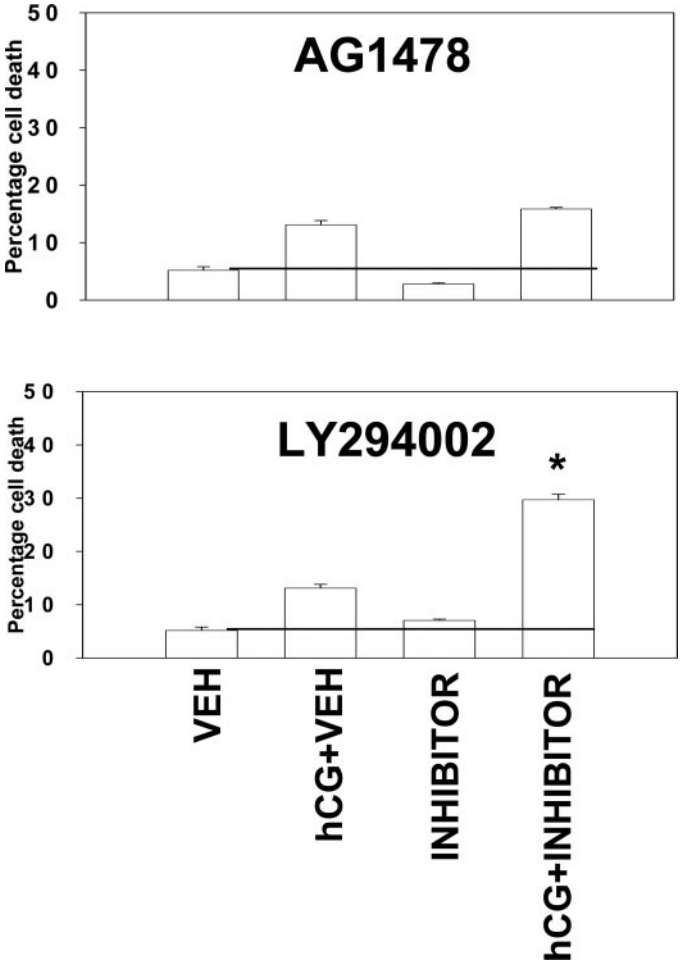


Fig. 5. Inhibition of PI3K enhances the toxicity of hCH in LNCaP cells. LNCaP cells were cultured as described under *Materials and Methods*. Twenty-four hours after plating, cells were pretreated with vehicle (DMSO) or with either the PI3K inhibitor LY294002 at 10 μM or the ERBB1 inhibitor AG-1478 at 1 μM, followed 30 min later by treatment with vehicle (PBS) or with 2 mU/ml hCG. Cells were isolated 96 h after irradiation by trypsinization, the attached and floating cells were combined, and cell viability was determined by trypan blue exclusion assays using a light microscope (*, $p < 0.05$ value greater than corresponding vehicle/irradiated + vehicle value; n = 3/data point ± S.E.M. from four studies).

Based on the findings in Fig. 8, A to C, we further examined the relationship between AKT signaling and the impact of dominant-negative IκB on hCG lethality in LNCaP cells. In a variety of cells, AKT has been argued to be an upstream activator of NF-κB (Mayo et al., 2003; Li et al., 2005). Expression of constitutively active AKT protected LNCaP cells from hCG toxicity in the absence, and in the presence, of coexpressed dominant-negative IκB, suggesting that AKT and NF-κB are independent/overlapping survival pathways against hCG toxicity in LNCaP cells and that activation of AKT can compensate for the loss of NF-κB function (Fig. 8D). Additional analysis of NF-κB activity in LNCaP cells using promoter reporter assays revealed that this cell line had very low basal levels of NF-κB activity compared with other tumor cell lines used in our laboratory, e.g., renal carcinoma cells, and treatment with either hCG or lovastatin did not significantly alter low basal NF-κB activity in LNCaP cells using an NF-κB reporter construct (data not shown).

The mechanism by which lovastatin potentiates hCG lethality was investigated in detail. Inhibition of caspase-8 did not alter the survival of cells treated with hCG, lovastatin, or the drug combination (data not shown). Treatment of cells with the pan-caspase inhibitor zVAD or the caspase-9 inhibitor LEHD reduced the lethality of the lovastatin and hCG combination (Fig. 9A). Expression of constitutively active AKT suppressed hCG and lovastatin-stimulated JNK1/2 activation, which correlated with enhanced survival (Fig. 9A, inset). In agreement with data using LEHD and with the concept that promotion of mitochondrial dysfunction is causal in hCG/lovastatin-induced death, overexpression of dominant-negative caspase-9 reduced the potentiation of hCG lethality by lovastatin (Fig. 9B). As noted in Fig. 8C, combined exposure of LNCaP cells to hCG and lovastatin reduced BCL-$_{XL}$ expression, and overexpression of BCL-$_{XL}$ maintained LNCaP survival when cells were treated with hCG and lovastatin (Fig. 9B). Expression of dominant-negative IκB suppressed BCL-$_{XL}$ levels that were rescued by expression of constitutively active AKT, which correlated with enhanced cell survival (Fig. 9B, inset) (Mayo et al., 2003; Li et al., 2005). These findings suggest that hCG and lovastatin lethality is mediated by inhibition of AKT signaling, leading to reduced BCL-$_{XL}$ expression and increased mitochondrial dysfunction.

Because radiation and lovastatin both enhanced the lethality of hCG in prostate cancer cells, we determined whether combined hCG and lovastatin treatment acted as a radiosensitizer. Trypan blue exclusion studies were performed 48 h after irradiation/hCG/lovastatin exposure, which was a time point chosen so as to minimize the observed lethal effects of either hCG and lovastatin treatment or hCG and radiation exposure compared with the majority of the data previously presented in the present article that was determined 96 h after exposure. After 48 h, treatment with hCG and lovastatin significantly enhanced LNCaP cell death 19.6 ± 0.9% above basal levels. After 48 h, exposure to radiation en-

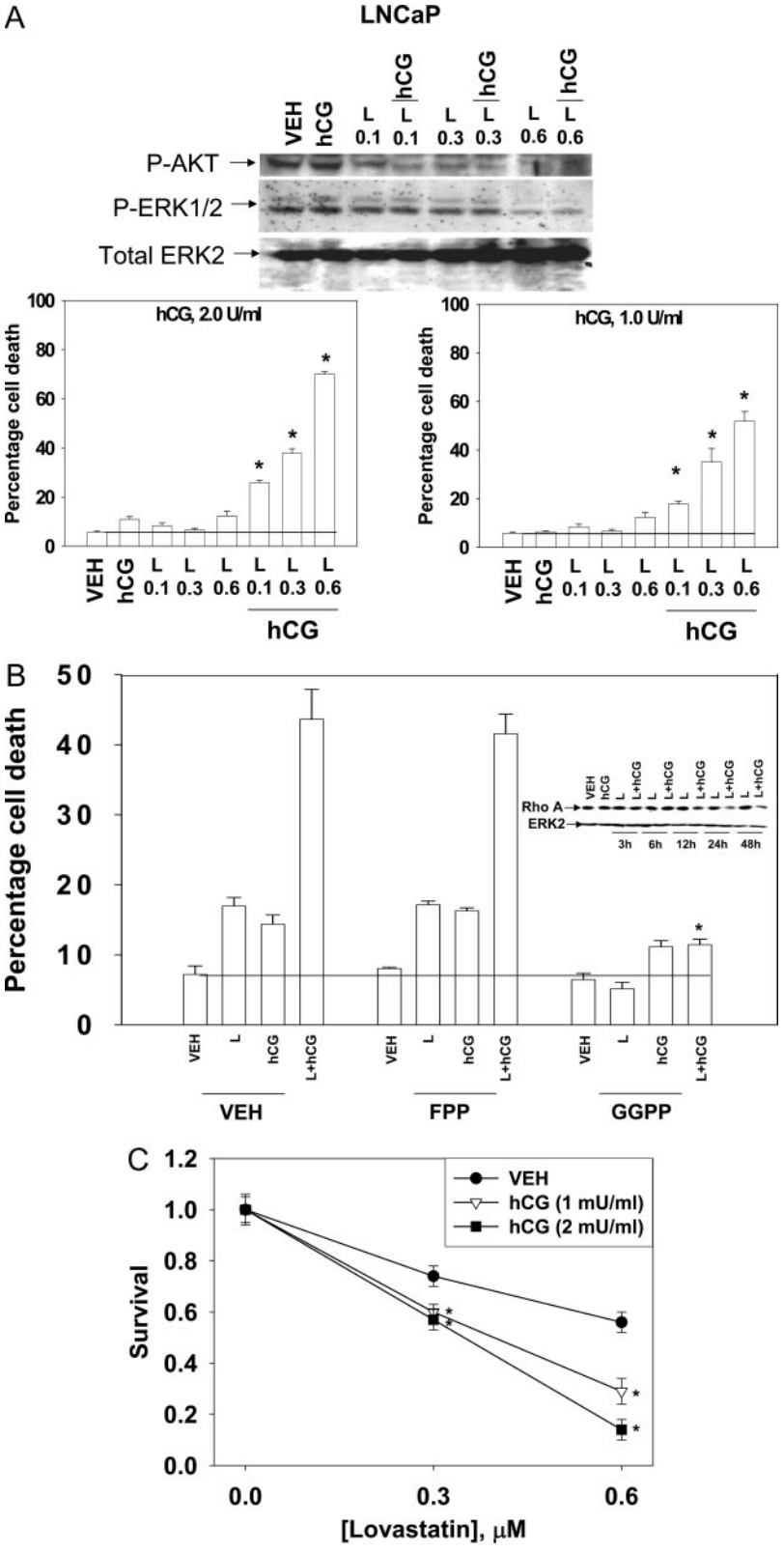
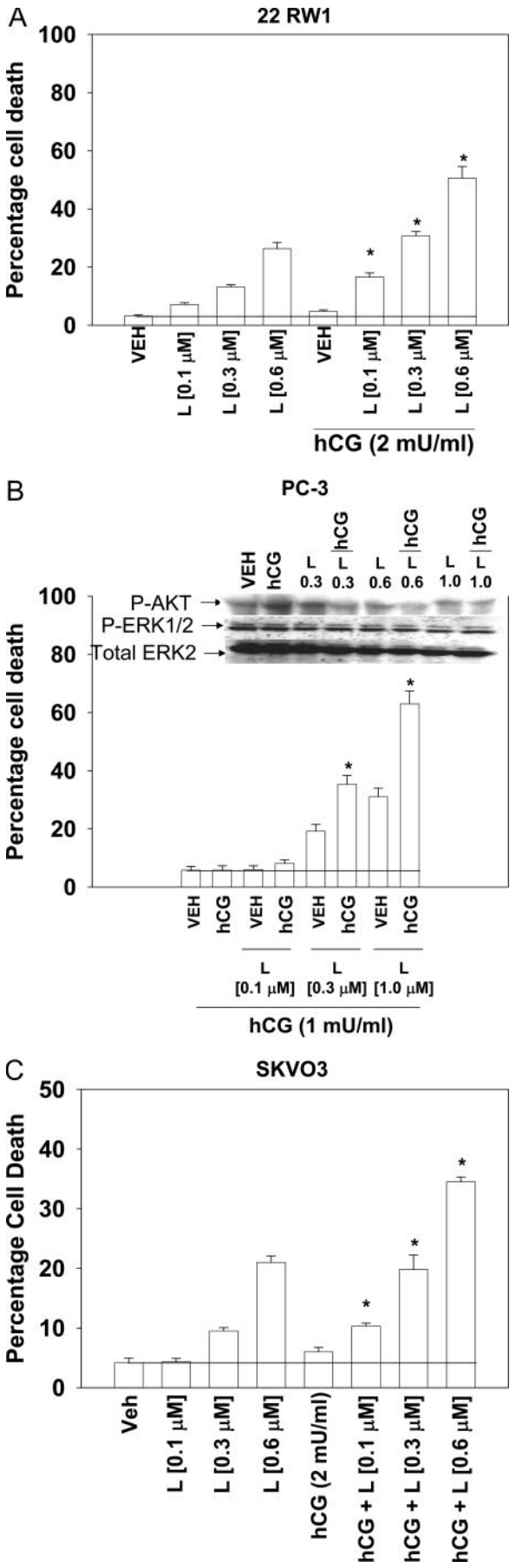


Fig. 6. The HMG CoA reductase inhibitor lovastatin enhances the toxicity of hCG in a greater than additive manner in LNCaP cells. A, LNCaP cells were cultured as described under *Materials and Methods*. Twenty-four hours after plating, cells were pretreated with vehicle (DMSO) or with the HMG CoA reductase inhibitor lovastatin (L; 0, 0.1, 0.3, and 0.6 μM, as indicated), followed 30 min later by treatment with vehicle (PBS) or with 1 or 2 mU/ml hCG, as indicated. Cells were isolated 96 h after irradiation by trypsinization, the attached and floating cells were combined, and cell viability was determined by trypan blue exclusion assays using a light microscope (*, $p < 0.05$ value greater than corresponding vehicle/irradiated + vehicle value; n = 3/data point ± S.E.M. from four studies). Inset, 48-h after hCG and lovastatin (L; 0–0.6 μM) exposure cells were lysed in SDS-PAGE sample buffer, and equal protein concentrations were subjected to SDS-PAGE followed by immunoblotting to determine the phosphorylation status (activity status) of ERK1/2 and AKT (S473) and the expression level of ERK2 protein. Data are from a representative study (n = 3). B, LNCaP cells were cultured as described under *Materials and Methods*. Twenty-three hours after plating, cells were treated with either vehicle (DMSO), 1 μM farnesyl pyrophosphate (FPP), or 1 μM GGPP. Twenty-four hours after plating, cells were pretreated with vehicle (DMSO) or with the HMG CoA reductase inhibitor lovastatin (L; 0.6 μM), followed 30 min later by treatment with vehicle (PBS) or with 2 mU/ml hCG. Cells were isolated 96 h after hCG treatment by trypsinization, the attached and floating cells were combined, and cell viability was determined by trypan blue exclusion assays using a light microscope (*, $p < 0.05$ value less than corresponding vehicle/irradiated + vehicle value; n = 3/data point ± S.E.M. from two studies). Inset, 48 h after hCG and lovastatin (L; 0.6 μM) exposure cells were lysed in SDS-PAGE sample buffer, and equal protein concentrations were subjected to SDS-PAGE followed by immunoblotting to determine the lipid modification status (activity status) of Rho A and the expression level of ERK2 protein. Data are from a representative study (n = 2). C, LNCaP cells were cultured as described under *Materials and Methods*. Twenty-four hours after plating (250–2500 cells/well of a six-well plate), cells were treated with lovastatin (L; 0–0.6 μM) 30 min before 2 mU/ml hCG. Ninety-six hours after hCG and lovastatin treatment, media containing hCG and lovastatin were removed, and fresh media lacking hCG/L were added. Colonies were permitted to form over the ensuing 28 days, after which the media were removed, and the cells were fixed and stained with crystal violet. Groups of >50 cells were considered to be colonies (*, $p < 0.05$ value less than corresponding vehicle/irradiated + vehicle value; n = 6/data point ± S.E.M. from two studies).



hanced LNCaP cell death 14.1 ± 0.8% above basal levels; however, hCG was not noted to enhance radiation-induced cell killing at this time point. Combined exposure to hCG, lovastatin, and radiation enhanced cell death 47.8 ± 1.8% above basal levels, which was significantly greater than the additive combination of hCG and lovastatin treatment and hCG and radiation exposure ($p < 0.05$ ± S.E.M.; $n = 6$). These findings suggest that treatment of LNCaP cells with hCG, lovastatin, and radiation promotes a greater amount of tumor cell killing than the combination of hCG with either radiation or lovastatin alone.

Discussion

hCG is a clinically used growth factor in the treatment of delayed puberty in adolescents (Delemarre-van de Waal, 2004; Soliman et al., 2005). Previous in vitro studies using hCG have demonstrated that this growth factor can radiosensitize mammary carcinoma cells, although no mechanistic analyses as to the mode of cell killing were performed (Pond-Tor et al., 2002). The present study was initiated to determine whether hCG radiosensitized human prostate carcinoma cells in vitro, and if so, the molecular mechanisms by which hCG caused radiosensitization. Additional experiments then determined whether modulation of known cytoprotective signal transduction pathway function(s) could subvert hCG signaling to promote prostate cancer cell death.

Treatment of prostate cancer cells with hCG promoted activation of ERBB1 as judged by receptor phosphorylation at multiple sites. Radiation exposure and hCG treatment interacted to promote further activation of ERBB1. The activation of ERBB1 by hCG was indirect, with hCG promoting ERBB1 activation via a paracrine loop involving signaling by

Fig. 7. The HMG CoA reductase inhibitor lovastatin enhances the toxicity of hCG in a greater than additive manner in multiple carcinoma cell types. A, 22RW1 prostate cancer cells were cultured as described under *Materials and Methods*. Twenty-four hours after plating, cells were pretreated with vehicle (DMSO) or with the HMG CoA reductase inhibitor lovastatin (L; 0, 0.1, 0.3, and 0.6 μM, as indicated), followed 30 min later by treatment with vehicle (PBS) or with 2 mU/ml hCG. Cells were isolated 96 h after hCG treatment by trypsinization, the attached and floating cells were combined, and cell viability was determined by trypan blue exclusion assays using a light microscope (*, $p < 0.05$ value greater than corresponding vehicle/irradiated + vehicle value; $n = 3$/data point ± S.E.M. from four studies). B, PC-3 prostate carcinoma cells were cultured as described under *Materials and Methods*. Twenty-four hours after plating, cells were pretreated with vehicle (DMSO) or with the HMG CoA reductase inhibitor lovastatin (L; 0, 0.1–1.0 μM, as indicated), followed 30 min later by treatment with vehicle (PBS) or with 1 mU/ml hCG. Cells were isolated 96 h after hCG treatment by trypsinization, the attached and floating cells were combined, and cell viability was determined by trypan blue exclusion assays using a light microscope (*, $p < 0.05$ value greater than corresponding vehicle/irradiated + vehicle value; $n = 3$/data point ± S.E.M. from four studies). Inset, 48 h after hCG and lovastatin (L; 0–1.0 μM) exposure, cells were lysed in SDS-PAGE sample buffer, and equal protein concentrations were subjected to SDS-PAGE followed by immunoblotting to determine the phosphorylation status (activity status) of ERK1/2 and AKT (S473) and the expression level of ERK2 protein. Data are from a representative study ($n = 3$). C, SKOV3 ovarian carcinoma cells were cultured as described under *Materials and Methods*. Twenty-four hours after plating, cells were pretreated with vehicle (DMSO) or with the HMG CoA reductase inhibitor lovastatin (L; 0, 0.1, 0.3, and 0.6 μM, as indicated), followed 30 min later by treatment with vehicle (PBS) or with 2 mU/ml hCG. Cells were isolated 96 h after hCG treatment by trypsinization, the attached and floating cells were combined, and cell viability was determined by trypan blue exclusion assays using a light microscope (*, $p < 0.05$ value greater than corresponding vehicle/irradiated + vehicle value; $n = 3$/data point ± S.E.M. from four studies).

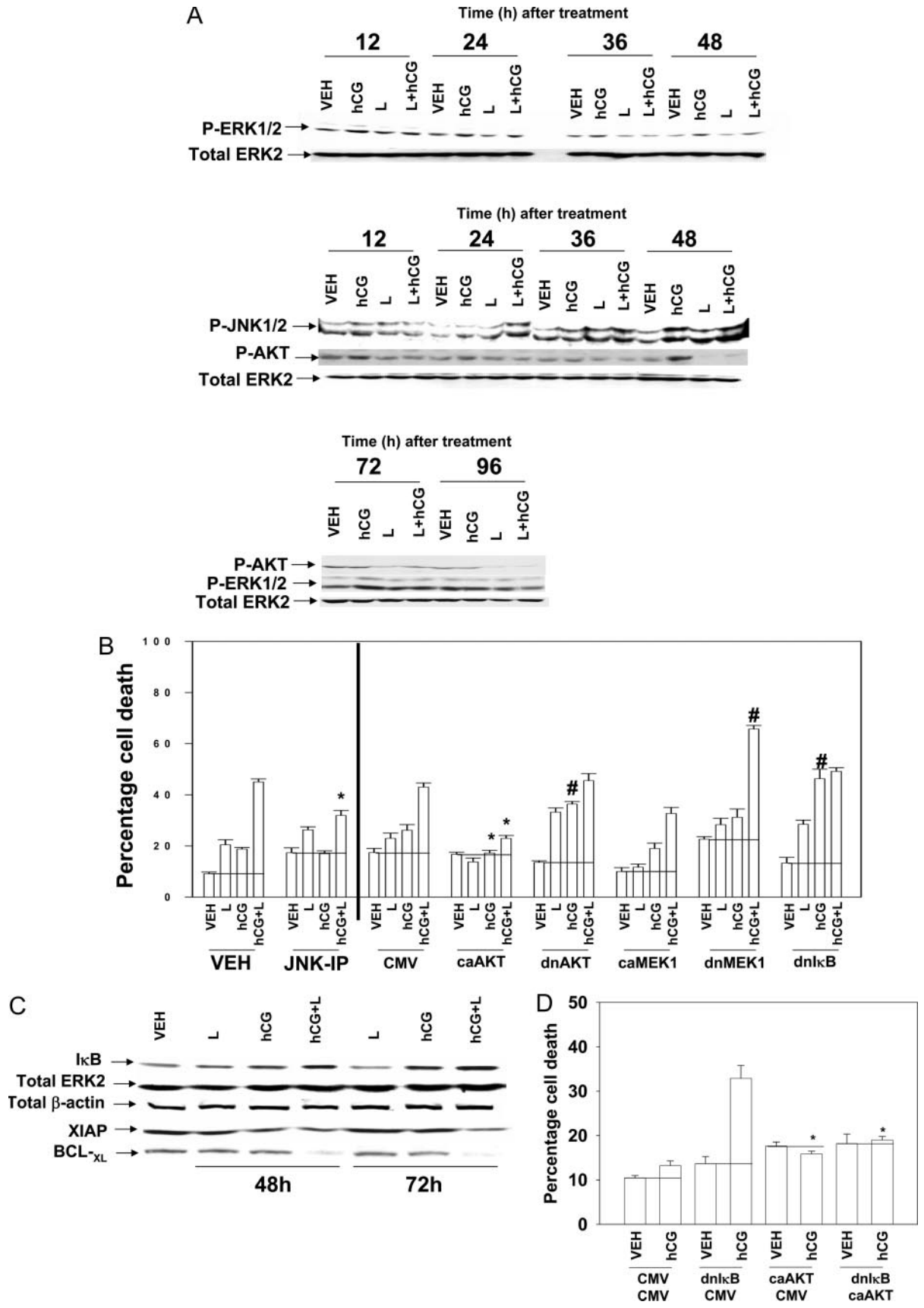


Fig. 8. Lovastatin and hCG treatment causes inactivation of AKT and JNK1/2-dependent cell killing. A, LNCaP cells were cultured as described under *Materials and Methods*. Twenty-four hours after plating, cells were pretreated with vehicle (DMSO) or with the HMG CoA reductase inhibitor lovastatin (L; 0.6 μM), followed 30 min later by treatment with vehicle (PBS) or with 2 mU/ml hCG. At the indicated times after treatment (12–96 h), cells were isolated, lysed in SDS-PAGE sample buffer, and equal protein concentrations were subjected to SDS-PAGE followed by immunoblotting

a $G\alpha_i$-coupled GPCR, followed by MEK1/2 activation, the action of metalloprotease(s), and cleavage of a poorly defined ERBB receptor-activating ligand that was not heparin-binding-EGF, TGFα, or heregulin. Radiation and hCG treatment profoundly increased PARP1 activation in a greater than additive manner, an effect that was dependent on ERBB1 and MEK1 signaling. Inhibition of either pan-caspase function or AIF function reduced the lethality of hCG and radiation exposure, each by approximately 50%, whereas inhibition of PARP1 function abolished hCG and radiation exposure lethality. Inhibition of MEK1/2 or JNK1/2 suppressed the lethal interaction between hCG and radiation, whereas inhibition of AKT promoted cell killing. At times 6 to 12 h after hCG exposure, when autocrine growth factor-induced ERBB1 activation had subsided, and hCG no longer acted as a radiosensitizer (A. Yacoub and P. Dent, unpublished observations). Treatment with low concentrations of the protein kinase C modulator bryostatin 1 at 1 to 2 nM caused ERBB1 activation in LNCaP cells, but it did not recapitulate the effect of hCG exposure, arguing that LH and ERBB1 receptor signaling is required to hyperactivate PARP1 (A. Yacoub and P. Dent, unpublished observations).

We noted that hCG and radiation combined to promote a modest additional activation of JNK1/2 compared with either agent alone; however, this activation was necessary for the cytotoxic death response of LNCaP cells. Together, these findings argue that hCG and radiation interact to promote ERBB1/ERK1/2- and JNK1/2-dependent hyperactivation of PARP1, which leads to caspase-dependent and caspase-independent death processes in prostate cancer cells.

HMG CoA reductase inhibitors (statins) have been proposed for several years as potential cancer therapeutics, and modest evidence exists in humans suggesting that individuals who are prescribed statins to lower plasma cholesterol levels for >4 years also have a slightly lower incidence of developing cancer (Graaf et al., 2004; Friis et al., 2005). However, many in vitro studies using statins as cancer therapeutics, either as a single agent or in combination with toxic drugs, have not successfully translated into the clinic, possibly because relatively nonphysiological doses of the statin drug, $\gg$1 μM, were often used to manipulate the biology/signaling responses of tumor cells in vitro and in animals (Horiguchi et al., 2004; Wu et al., 2004). In the present study, we used lovastatin concentrations of 0.1 to 0.6 μM, which are achievable (free) in human plasma for up to 120 h without adverse toxicity (Thibault et al., 1996; Holstein et al., 2006). At these concentrations, in LNCaP cells growing in vitro, the total expression and SDS-PAGE mobility of a previously validated geranylgeranylated protein (Rho A) was not altered by lovastatin treatment as a single agent over a 72-h time course, suggesting that that our effects on global protein geranylgeranylation per se were relatively modest. However, in cells exposed to lovastatin and hCG, the total protein expression of Rho A was reduced 12 to 48 h after exposure, which correlated with the induction of cell killing by hCG and lovastatin. Despite not observing significant changes in the SDS-PAGE mobility/processing of Rho A, we noted that treatment of cells with GGPP abolished the lethal interaction between hCG and lovastatin, which argues that a reduction in protein geranylgeranylation was causal in the promotion of hCG lethality by lovastatin.

Treatment of prostate cancer cells with hCG promoted initial activation of ERK1/2 and JNK1/2 (0–6 h), followed by a later activation of ERK1/2, JNK1/2, and AKT (12–72 h). Inhibition of AKT activation by use of either lovastatin or by expression of dominant-negative AKT enhanced hCG lethality, whereas expression of constitutively active AKT blocked the lethality of hCG, lovastatin, and hCG and lovastatin in combination. The reduction in Rho A protein levels ~48 h after lovastatin and hCG treatment correlated with the reduction in AKT activity in cells. Expression of activated MEK1 suppressed, and expression of dominant-negative MEK1 enhanced, the lethality of hCG and lovastatin treatment. These findings argue that ERK1/2 was a parallel secondary protective pathway against hCG and lovastatin treatment, when AKT function was suppressed. JNK1/2 activation has been linked by many groups to cell killing and inhibition of JNK1/2 activation suppressed hCG and lovastatin lethality, which correlated with reduced release of cytochrome *c* into the cytosol, arguing that the intrinsic caspase pathway was causal in cell killing: in agreement with this hypothesis, expression of dominant-negative caspase-9 or treatment with the inhibitor LEHD, but not the caspase-8 inhibitor IETD, confirmed the mitochondrial intrinsic pathway as a key mediator in hCG and lovastatin lethality (A. Yacoub and P. Dent, unpublished observations).

Mitochondrial function in cancer cells has frequently been linked to altered expression and/or function of pro- and antiapoptotic factors (e.g., BAX and $BCL\text{-}_{XL}$), with the balance of activity/expression of these factors determining cell survival (e.g., references in Dent et al., 2003a,b; Mayo et al., 2003; Wu et al., 2004; Li et al., 2005). Elevated levels of

to determine the phosphorylation status (activity status) of ERK1/2, JNK1/2, and AKT (S473) and the expression level of ERK2 protein. Data are from a representative study (n = 3). B, LNCaP cells were cultured as described under *Materials and Methods*. Twelve hours after plating, cells were infected with either empty vector virus (CMV) or with viruses to express-dominant negative AKT, dominant-negative MEK1, dominant-negative IkB, constitutively active AKT, or constitutively active MEK1 at an m.o.i. of 25. In other parallel plates of cells, 24 h after plating, cells are pretreated 30 min before any additional manipulation with either vehicle (DMSO) or a JNK-IP at 10 μM. All cells were then treated with vehicle (DMSO) or with the HMG CoA reductase inhibitor lovastatin (L; 0.6 μM), followed 30 min later by treatment with vehicle (PBS) or with 2 mU/ml hCG. Cells were isolated 96 h after hCG treatment by trypsinization, the attached and floating cells were combined, and cell viability was determined by trypan blue exclusion assays using a light microscope (*, $p < 0.05$ value less than corresponding vehicle/irradiated + vehicle value; #, $p < 0.05$ value greater than corresponding vehicle/irradiated + vehicle value; n = 3/data point ± S.E.M. from two studies). C, LNCaP cells were cultured as described under *Materials and Methods*. Twenty-four hours after plating, cells were pretreated with vehicle (DMSO) or with the HMG CoA reductase inhibitor lovastatin (L; 0.6 μM), followed 30 min later by treatment with vehicle (PBS) or with 2 mU/ml hCG. At the indicated times after treatment (48 and 96 h), cells were isolated, lysed in SDS-PAGE sample buffer, and equal protein concentrations were subjected to SDS-PAGE followed by immunoblotting to determine the expression of IκB, XIAP, and $BCL\text{-}_{XL}$ and the total expression of ERK2 protein. Data are from a representative study (n = 3). D, LNCaP cells were cultured as described under *Materials and Methods*. Twelve hours after plating, cells were infected with either empty vector virus (CMV) or with viruses to express dominant-negative IkB and/or constitutively active AKT each at an m.o.i. of 25, as indicated. The total m.o.i. for virus infection was 50. All cells were then treated with vehicle (DMSO) or with the HMG CoA reductase inhibitor lovastatin (L; 0.6 μM), followed 30 min later by treatment with vehicle (PBS) or with 2 mU/ml hCG. Cells were isolated 96 h after hCG treatment by trypsinization, the attached and floating cells were combined, and cell viability was determined by trypan blue exclusion assays using a light microscope (*, $p < 0.05$ value less than corresponding vehicle/irradiated + vehicle value; n = 3/data point ± S.E.M. from two studies).

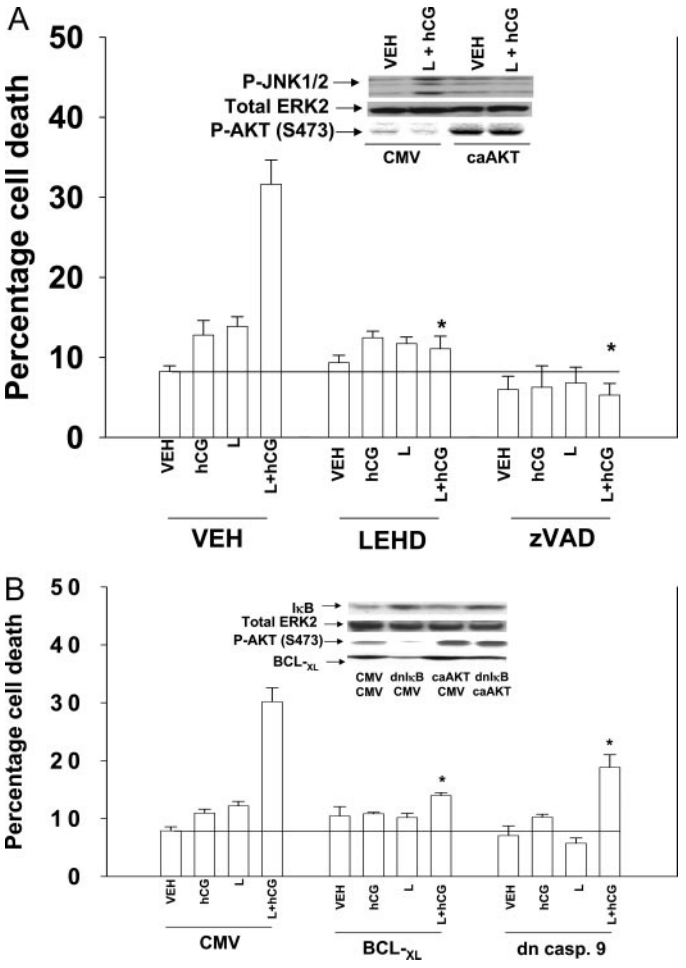


Fig. 9. Loss of BCL-$_{XL}$ expression and activation of JNK1/2 as a result of AKT inactivation promote cell death via the intrinsic caspase pathway. A, LNCaP cells were cultured as described under *Materials and Methods*. Twenty-three hours after plating, cells were treated with either VEH (DMSO), with the caspase-9 inhibitor LEHD at 50 μM, or with the pan-caspase inhibitor zVAD at 50 μM. Twenty-four hours after plating, cells were pretreated with vehicle (DMSO) or with the HMG CoA reductase inhibitor lovastatin (L; 0.6 μM), followed 30 min later by treatment with vehicle (PBS) or with 2 mU/ml hCG. Cells were resupplemented with caspase inhibitors every 24 h. Cells were isolated 96 h after hCG treatment by trypsinization, the attached and floating cells were combined, and cell viability was determined by trypan blue exclusion assays using a light microscope (*, $p < 0.05$ value less than corresponding vehicle/irradiated + vehicle value; $n = 3$/data point ± S.E.M. from two studies). Inset, LNCaP cells were cultured as described under *Materials and Methods*. Twelve hours after plating, cells were infected with either empty vector virus (CMV) or with a virus to express constitutively active AKT at an m.o.i. of 25. Cells were then treated with vehicle (DMSO) or with the HMG CoA reductase inhibitor lovastatin (L; 0.6 μM), followed 30 min later by treatment with vehicle (PBS) or with 2 mU/ml hCG. Cells were isolated 48 h after hCG treatment by lysis in SDS-PAGE sample buffer, and equal protein concentrations were subjected to SDS-PAGE followed by immunoblotting to determine the phosphorylation status (activity status) of JNK1/2 and AKT (S473) and the expression level of ERK2 protein. B, LNCaP cells were cultured as described under *Materials and Methods*. Twelve hours after plating, cells were infected with either empty vector virus (CMV) or with viruses to express dominant-negative caspase-9 or to overexpress BCL-$_{XL}$ at an m.o.i. of 25. Twenty-four hours after plating, cells were then treated with vehicle (DMSO) or with the HMG CoA reductase inhibitor lovastatin (L; 0.6 μM), followed 30 min later by treatment with vehicle (PBS) or with 2 mU/ml hCG. Cells were isolated 96 h after hCG treatment by trypsinization, the attached and floating cells were combined, and cell viability was determine by trypan blue exclusion assays using a light microscope (*, $p < 0.05$ value less than corresponding vehicle/irradiated + vehicle value; $n = 3$/data point ± S.E.M. from two studies). Inset, LNCaP cells were cultured as described under *Materials and Methods*. Twelve hours after plating, cells were infected with either empty vector virus (CMV) or with viruses to express dominant-negative IkB and/or constitutively active AKT each at an m.o.i. of 25. The total m.o.i. for virus infection was 50. All cells were then treated with vehicle (DMSO). Cells were isolated 48 h after infection, lysed in SDS-PAGE sample buffer, and equal protein concentrations were subjected to SDS-PAGE followed by immunoblotting to determine the expression of IκB and BCL-$_{XL}$, the phosphorylation of AKT (S473), and the total expression of ERK2 protein. Data are from a representative study ($n = 3$).

signaling by both the PI3K-AKT pathway and the transcription factor NF-κB have been linked in cancer cells to increased expression of antiapoptotic proteins (e.g., BCL-$_{XL}$ and XIAP), whose actions tend to shift the balance of mitochondrial function toward cell survival. Combined exposure of cells to hCG and lovastatin increased the expression of IκB, which would tend to inhibit NF-κB function and expression of dominant-negative IκB enhanced the lethality of hCG, which correlated with reduced expression of BCL-$_{XL}$ and of XIAP.

Based on prior studies by others and our own findings (Mayo et al., 2003; van de Donk et al., 2003; Wu et al., 2004; Li et al., 2005), we initially hypothesized that lovastatin-induced inhibition of AKT function and increased expression of IκB were linked in a simple linear pathway to the observed reduction in BCL-$_{XL}$ and XIAP levels; i.e., reduced AKT activity caused elevated expression of dominant-negative IκB, which suppressed NF-κB activity and resulted in reduced expression of BCL-$_{XL}$. However, basal levels of NF-κB activity were very low in LNCaP cells and not significantly altered under any of our treatment conditions. Furthermore, expression of constitutively active AKT circumvented the ability of dominant-negative IκB to enhance hCG lethality and restored BCL-$_{XL}$ expression to near basal levels, even when dominant-negative IκB was expressed. Constitutive overexpression of BCL-$_{XL}$ blocked lovastatin and hCG-induced cell killing. Together, these findings argue that signaling by AKT and by NF-κB represent two parallel pathways, which protect LNCaP prostate cancer cells from the toxic effects of hCG and lovastatin treatment. Our findings suggest that AKT signaling represents a primary upstream cell survival pathway in LNCaP cells exposed to hCG and lovastatin, in comparison with secondary survival signaling by NF-κB. It is probable, however, that in other prostate cancer cell types, where NF-κB is more active, that NF-κB signaling may play an equal or a more important role in maintaining cell viability after lovastatin and hCG exposure.

ERBB receptor signaling has been noted to play a key role in the normal development of the prostate and can play a growth promoting role in prostatic hyperplasia and prostate cancer (Scher et al., 1995; De Miguel et al., 1999). Evidence also exists that expression of hCG in prostate cancer correlates with a poor clinical outcome and that ERBB1 and LH receptor signaling processes in granulosa cells can be linked in terms of receptor expression and receptor desensitization effects (Styne, 1991; Sheaff et al., 1996). Thus, it is possible that endogenous levels of hCG in prostate cancer cells may act to promote cell growth by activating ERBB1 through a paracrine mechanism. Although activation of ERBB1 has been shown to promote cell growth in multiple nontransformed and transformed cell types, prolonged/intense activation of ERBB1 and other growth factor receptors has also been linked to differentiation and cytotoxic responses in a

variety of transformed cell types (Gulli et al., 1996; Reinehr et al., 2005; Zhao et al., 2006). For example, in the A431 squamous carcinoma line, low levels of ERBB1 activation promote cell growth; higher levels promote growth arrest with modest levels of cell killing with high levels of receptor activation causing profound cell death.

In our study, we noted that both hCG and radiation as single agents caused increased phosphorylation of ERBB1 at multiple sites, with one site, ERBB1 Tyr845, being phosphorylated 30 min after exposure only under conditions where cells were simultaneously treated with both agents: The relative changes in the levels of ERBB1 Tyr845 phosphorylation and of PARP1 ADP-ribosylation correlated more closely than the levels of ERBB1 Tyr1173 or Tyr1068 phosphorylation did with PARP1 activity. Studies by other groups have argued that ERBB1 Tyr845 phosphorylation is dependent upon Src family nonreceptor tyrosine kinases, and in A431 cells stimulated by toxic concentrations of EGF, Src-dependent phosphorylation of ERBB1 plays an important role in the earliest steps in cell killing (Biscardi et al., 1999; Sato et al., 2003; Boerner et al., 2005). Reinehr et al. (2005) have argued that c-Yes phosphorylation of ERBB1 can play an important role in ERBB1-mediated cell death signaling in primary hepatocytes. These findings suggest that increased ERBB1 Tyr845 phosphorylation may play a role in radiosensitization by hCG.

However, other data generated during our analyses has argued against ERBB1 Tyr845 as being causal in the radiosensitization effect. We and others have noted that ERBB1 tyrosine phosphorylation at Tyr845 is relatively insensitive to inhibition by the ERBB1 kinase domain inhibitor AG-1478 (Reinehr et al., 2005; A. Yacoub and P. Dent, unpublished observations), and enhanced ERBB1 Tyr845 phosphorylation has not been linked to changes in MEK1/2 activity (Boerner et al., 2005). Inhibition of ERBB1 using AG-1478 significantly reduced PARP1 activation and also protected cells from hCG and radiation exposure-induced killing. PARP1 ADP ribosylation/PARP1 activity has been linked in one study, using rat astrocytes cultured for 30 days, to EGF exposure, and hyperactivation of PARP1 has been shown by several laboratories to deplete cellular NAD^+ and ATP stores (Spina Purrello et al., 2002; Xu et al., 2005). Thus, activation of ERBB1, including phosphorylation of the receptor at multiple sites, is likely to play a role in the pathway(s) leading to profound intense PARP1 signaling. Studies outside the scope of the present study will be required to fully delineate the pathway downstream of ERBB1 linking this receptor to the regulation of PARP1.

Radiotherapy, external beam and/or brachytherapy, is a primary mode of treatment for prostate cancer (Moyad et al., 2005). One site of dose-limiting toxicity for prostate cancer radiotherapy is the sigmoid colon, and a variety of techniques, including intensity modulated radiotherapy and brachytherapy, have been used to target tumor and spare normal tissue (De Meerleer et al., 2004; Morton, 2005). Our findings suggest that hCG and lovastatin could be used to enhance the toxic effects of radiotherapy in prostate cancer, and further studies will be required to determine whether our in vitro analyses translate into an animal model system.

Acknowledgments

This article is dedicated to "Oilcan" in recognition of his being 5 years out, disease-free.

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