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Epinephrine Protects Cancer Cells from Apoptosis via Activation of cAMP-dependent Protein Kinase and BAD Phosphorylation*[S]

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The stress hormone epinephrine is known to elicit multiple systemic effects that include changes in cardiovascular parameters and immune responses. However, information about its direct action on cancer cells is limited. Here we provide evidence that epinephrine reduces sensitivity of cancer cells to apoptosis through interaction with β_2-adrenergic receptors. The antiapoptotic mechanism of epinephrine primarily involves phosphorylation and inactivation of the proapoptotic protein BAD by cAMP-dependent protein kinase. Moreover, BAD phosphorylation was observed at epinephrine concentrations found after acute and chronic psychosocial stress. Antiapoptotic signaling by epinephrine could be one of the mechanisms by which stress promotes tumorigenesis and decreases the efficacy of anti-cancer therapies.

Epinephrine levels are sharply increased in response to acute stress and can be continuously elevated during persistent stress and depression (1, 2). Sustained increases of epinephrine were implicated in pathogenesis of stress-related immunosuppression proposed as the primary mechanism by which stress and depression may increase tumor incidence and promote metastatic growth (2, 3). However, several reports have questioned whether immunosuppression alone is sufficient to explain stress-induced tumor growth, and some studies have found no correlation between stress and cancer (2, 4). Thus, more information about the mechanisms by which stress hormones affect tumors is necessary to resolve the controversy over the connection between stress and cancer. One potential mechanism may involve direct effects of epinephrine on cancer cells.

Cancer cell lines of various origins, including prostate tumors, express β_2-adrenergic receptors (β_2-ARs)[4] that bind epinephrine and norepinephrine (5–7). β_2-ARs belong to superfamily A of seven-transmembrane G protein-coupled receptors (GPCRs) (8). Epinephrine binding leads to activation of GTPase and dissociation of α and $\beta\gamma$ subunits of heterotrimeric G proteins. Depending on the cell context, this may trigger multiple signaling pathways, including the Ras/extracellular signal-regulated kinase, NFκB, and cAMP-dependent protein kinase (PKA) pathways, which regulate diverse cellular responses, such as proliferation, differentiation, secretion, or apoptosis (9).

Since resistance to apoptosis has been implicated in cancer pathogenesis (10), we decided to analyze the effects of the β_2-AR agonist epinephrine on apoptosis in prostate cancer cells. In this paper, we demonstrate that epinephrine reduces sensitivity of prostate cancer cells to apoptosis via β_2-AR/PKA signaling that triggers BAD phosphorylation at S112. This antiapoptotic mechanism operates in the prostate cancer cell lines LNCaP and C4-2 and in the breast cancer cell line MDA-MB231. Our findings suggest that stress may contribute to cancer etiology and therapeutic resistance by decreasing sensitivity of cancer cells to apoptosis.

EXPERIMENTAL PROCEDURES

Cell Lines and Transfection—LNCaP and C4-2 cells were a gift from Leland Chung (Emory University, Atlanta, GA). PC3 and MDA-MB-231 cells were obtained from ATCC. LNCaP were maintained in T-medium supplemented with 5% fetal bovine serum, C4-2 and PC3 cells were maintained in RPMI 1640 with 10% fetal bovine serum, and MDA-MB-231 cells were maintained in Dulbecco's modified Eagle's medium with 10% fetal bovine serum. All cells were kept in 5% CO_2 at 37 °C. Transient transfection was performed at 60–70% confluence using Lipofectamine (Invitrogen) according to the manufacturer's recommendations.

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S3.

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[4] The abbreviations used are: β_2-AR, β_2-adrenergic receptor; PI3K, phosphatidylinositol 3′-kinase; GPCR, G-protein-coupled receptors; CREB, cAMP-responsive element-binding protein; PKA, cAMP-dependent protein kinase; GFP, green fluorescent protein; shRNA, short hairpin RNA; UVB, ultraviolet B; DEVD-afc, Ac-DEVD-7-amido-4-trifluoromethylcoumarin; HA, hemagglutinin.

Antibodies and Other Reagents—Antibodies were from the following sources: androgen receptor, BAD, phospho-specific BAD (Ser^{112}, Ser^{136}, and Ser^{155}), phospho-Akt (Ser^{473} and Thr^{308}), and phospho-CREB (Ser^{133}) from Cell Signaling Technology (Beverly, MA); antibodies to β-actin and anti-FLAG M2 monoclonal antibodies from Sigma; secondary horseradish peroxidase-conjugated antibodies used for Western blots from Amersham Biosciences. Protein G-agarose beads, forskolin, epinephrine, and DEVD-afc were from Calbiochem. All other chemicals and reagents (unless specified) were purchased from Sigma. Tissue culture reagents were purchased from Invitrogen.

Plasmid and DNA Constructs—pcDNA3-HA-BAD and pcDNA3-HA-BADs112/136A constructs were from Robert Datta and Michael Greenberg (Harvard Medical School, Boston, MA); pCMV14-FLAG-CREB was from Gary Kammer (WFUSM, Winston-Salem, NC), EGFP was from Clontech Inc. The PKA inhibitors PKI-GFP and RevAB-GFP have been described previously (11).

shRNA Experiments—For shRNA knockdown experiments of the androgen receptor, a lentiviral vector (pLL3.7) was used with a shRNA insert of annealed oligonucleotides. The androgen receptor DNA target sequence used was 5-T**GCACTGCTACTCTTCAGCA***ttcaagaga***TGCTGAAGAGTAGCAGTG**CTTTTTTC-3 (sequence that corresponds to AR is shown in boldface type; loop sequence is in italic type), and the scrambled sequence was 5-T**GTACTGCAGCCACATTCTC***ttcaagaga***GAGAATGTGGCTGCAGTA**CTTTTTTC-3. HEK 293 cells were transfected with pLL3.7 vector containing either of these sequences in combination with packaging vectors (VSVG, RSV-REV, and pMDL g/p RRE). After 48 h, supernatants were collected from these cells and used to infect LNCaP cells. 48 h after infection, cells were plated for subsequent experiments.

Apoptosis Assays—Prostate cancer LNCaP cells are relatively resistant to apoptosis, even in serum-free culture conditions, due to constitutively active PI3K/Akt signaling. Inhibitors of PI3K reduce Akt activity and induce apoptosis in LNCaP cells, which can be further enhanced by thapsigargin, an inhibitor of the sarcoplasmic/endoplasmic reticulum calcium ATPase (12). For analysis of apoptosis in whole cell populations, cells were serum-starved overnight (16 h) and treated with 50 μM LY294002 and 1 μM thapsigargin. Thapsigargin alone does not induce apoptosis in prostate cancer cells within 24 h (13, 14), but in our experimental conditions, thapsigargin synchronized caspase activation in cells treated with LY294002. This protocol shortened the time and increased the reproducibility of caspase assays and also permitted analysis of apoptosis by time lapse video recording. Apoptosis in whole cell populations was quantified by measuring caspase-3 activity with the fluorogenic substrate Ac-DEVD-7-amido-4-trifluoromethylcoumarin (DEVD-afc) (Bachem) as specified by the manufacturer. Caspase activity in cells treated only with proapoptotic agents was assigned a value of 100.

Apoptosis in a population of transiently transfected GFP-positive cells was measured by time lapse video recording followed by counting the percentage of cells with apoptotic morphology, as described elsewhere (15). At least four randomly chosen fields for each treatment were recorded. A similar methodology was used to video record cells infected with pLL3.7 lentivirus vector that contained androgen receptor shRNA and a GFP expression cassette. Apoptosis in fragmented cells was confirmed by immunofluorescent detection of active caspase 3 and nuclear fragmentation (15) (Fig. S1). Results were confirmed by at least two independent experiments. T-tests (two-tailed distribution, two-sample unequal variance) were used for statistical analysis.

Immunoprecipitation—20 h after transfection, cells were deprived of serum for 3 h, and different treatments were given at this point. Cells were harvested in a cell lysis buffer that contained 20 mM Tris, pH 7.4, 40 mM NaF, 2 mM EDTA, 1 mM EGTA, 1% Triton X-100, 1 μg each of leupeptin, pepstatin, and aprotinin, 1 mM phenylmethylsulfonyl fluoride, 1 mM $NaVO_4$, 50 mM β-glycerophosphate, 40 mM *p*-nitrophenyl phosphate, and 1 mM dithiothreitol. The lysates were cleared of insoluble material by centrifugation at 14,000 × *g* for 10 min at 4 °C. Cell extracts were incubated with 6–8 μg of anti-HA antibodies (12CA5) overnight at 4 °C and with protein A-conjugated beads for another 3 h. Beads were washed three times with cell lysis buffer, and proteins were eluted with an SDS sample buffer for Western blotting analysis.

RESULTS

Epinephrine Protects LNCaP Cells from Apoptosis by Activating β_2-ARs—β_2-ARs that bind epinephrine are abundantly expressed in normal prostate epithelial cells and prostate cancer cells (5, 16). To determine whether epinephrine can regulate apoptosis, we examined its effects on prostate cancer LNCaP cells.

Analysis of apoptosis by measuring caspase activity, poly-(ADP-ribose) polymerase cleavage, and time lapse video recording reveals that maximal apoptosis is observed 6 h after treatment with LY294002 and thapsigargin, whereas concomitant treatment with epinephrine substantially decreases apoptosis (Fig. 1, *A–C*). Epinephrine protected LNCaP cells from apoptosis in the absence of PI3K activity that was judged by continuous inhibition of Akt phosphorylation at Ser^{473} (Fig. 1*B*).

To examine the role of β_2-ARs in epinephrine-induced antiapoptotic signaling, we utilized the highly β_2-AR-selective antagonist ICI118,551 (17). Complete inhibition of the antiapoptotic effect of epinephrine by ICI118,551 suggests that the effects of epinephrine are mediated solely via the β_2-AR (Fig. 1*D*). In contrast, the antiapoptotic effect of forskolin (a direct activator of adenylyl cyclase) was not inhibited by ICI118,551.

Antiapoptotic Effect of Epinephrine Is Mediated via PKA—Epinephrine and forskolin have been previously shown to activate adenylyl cyclase, increase cAMP levels, and activate PKA in LNCaP cells (18). Besides PKA, cAMP can also activate the EPAC/Rap1 pathway (19). Furthermore, in addition to increased cAMP levels, β_2-ARs may trigger other signaling pathways via βγ subunits of G-proteins or β-arrestins. Thus, it is possible that antiapoptotic effects of epinephrine are mediated via PKA-independent signaling by the β_2-ARs (20).

To address the role of PKA in apoptosis, we examined survival effects of epinephrine and forskolin in LNCaP cells transiently expressing a GFP chimera of the PKA inhibitor PKI

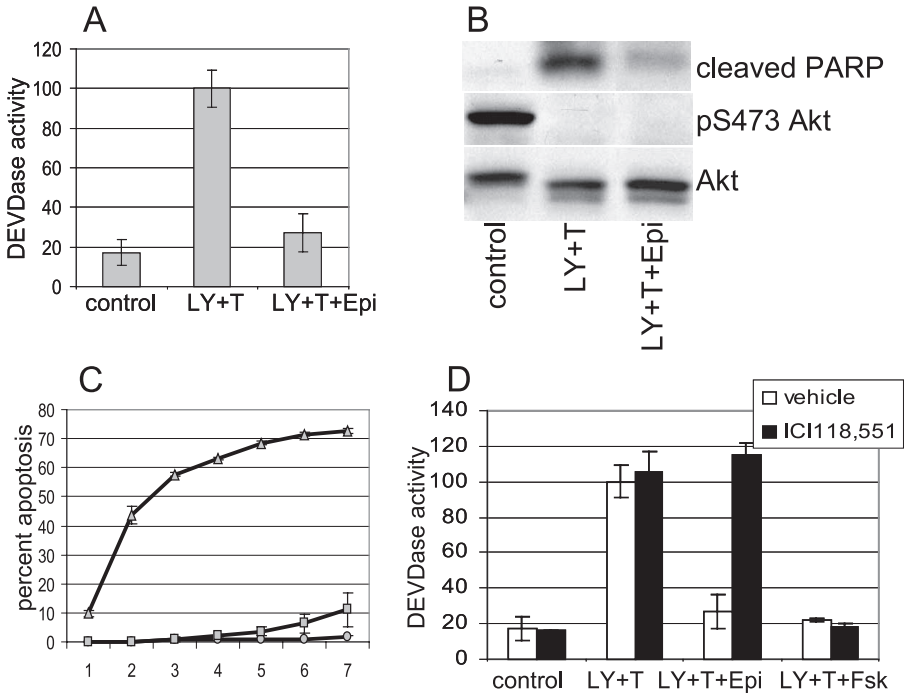

FIGURE 1. **Epinephrine protects LNCaP cells from apoptosis via β_2-adrenergic receptors.** *A*, LNCaP cells were treated with 0.3% Me_2SO (control), 50 μM LY294002, and 1 μM thapsigargin (*LY+T*) followed by 100 nM epinephrine as indicated. After 6 h, cells were collected and lysed, and caspase activity was measured with the fluorogenic substrate DEVD-afc (shown in relative units). *Bars* show the average ± S.D. of duplicate samples from three independent experiments. *B*, cells were treated as in *A* and analyzed by Western blotting with antibodies that recognize cleaved poly(ADP-ribose) polymerase (*PARP*), Akt phosphorylated at Ser^{473}, and total Akt. *C*, cells were treated with vehicle (Me_2SO) (●), LY294002 and thapsigargin (▲), or LY294002, thapsigargin, and epinephrine (■), and apoptosis was followed by time lapse video recording. The percentage of apoptosis was determined by examining at least 300 cells in randomly chosen fields for each treatment. *Error bars* show S.D. between individual fields. *D*, LNCaP cells were treated as in *A* to induce apoptosis and with either 5 μM ICI118,551 (*black bars*) or vehicle (*white bars*) followed by 100 nM epinephrine or 5 μM forskolin as indicated. After 6 h, cells were collected and lysed, and caspase activity was measured with the fluorogenic substrate DEVD-afc (shown in relative units). *Error bars* show the average ± S.D. of three independent experiments.

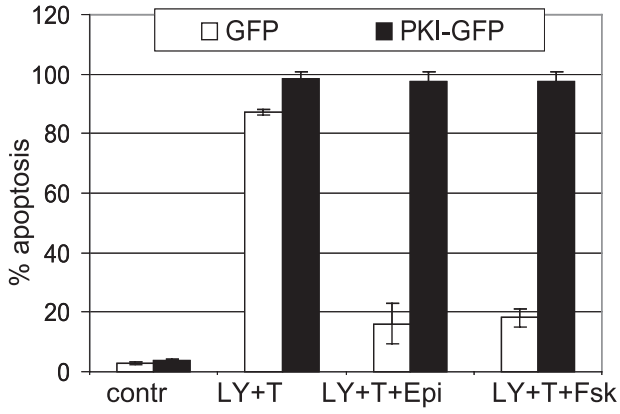

FIGURE 2. **Antiapoptotic effects of epinephrine and forskolin depend on PKA activation.** Cells transfected with GFP (*white bars*) or with PKI-GFP (*black bars*) were treated with 0.3% Me_2SO (*contr*), 50 μM LY294002, and 1 μM thapsigargin (*LY+T*) followed by 100 nM epinephrine (*Epi*) or 5 μM forskolin (*Fsk*) as indicated. Apoptosis was measured by time lapse video recording of GFP-positive cells. Approximately 200 cells were scored for apoptotic morphology (cytoplasmic and nuclear fragmentation). *Bars* show the percentage of apoptosis 6 h after treatments (the time course of apoptosis is shown in Fig. S2). *Error bars* show S.D. between apoptosis in four randomly chosen fields.

(PKI-GFP (11). Time lapse video microscopy was used to determine cumulative cell death in LNCaPs transfected with GFP or PKI-GFP. Both epinephrine and forskolin delayed apoptosis in cells transfected with GFP, but neither agent inhibited apoptosis in cells expressing PKI-GFP (Fig. 2). Inhibition of PKA by PKI-GFP was confirmed by measuring phosphorylation of CREB, a known PKA substrate (21) (Fig. S2*A*). Similar results were obtained in cells expressing a mutant regulatory subunit of PKA (RevAB-GFP) (11) capable of inhibiting PKA activation (Fig. S2*B*). These data indicate that PKA activation is necessary for the antiapoptotic effect of epinephrine.

Antiapoptotic Effect of Epinephrine Partially Depends on Androgen Receptor—Activation of androgen receptor by dihydrotestosterone or the androgen analog R1881 is known to protect prostate cells from apoptosis (22, 23). Because signaling through the $G\alpha_s$/PKA pathway has been shown to transactivate the androgen receptor in a ligand-independent fashion (24, 25), we examined whether AR plays a role in antiapoptotic signaling by epinephrine.

Androgen receptor expression was decreased by an androgen receptor-specific shRNA construct introduced into LNCaP cells using the lentiviral vector pLL3.7 (Fig. 3*A*). Apoptosis in LNCaP cells infected with a lentiviral expression vector bearing androgen receptor shRNA, scrambled shRNA, or with empty vector was assessed by caspase assays. In cells infected with androgen receptor shRNA, the antiapoptotic effect of R1881 was completely suppressed. We observed a slight increase in apoptosis in androgen receptor shRNA infected cells treated with R1881, whereas we observed no significant difference in protection from apoptosis by R1881 in cells infected with empty vector or scrambled shRNA (Fig. 3*B*). Epinephrine reduced apoptosis in cells infected with all lentiviral vectors, although in cells infected with androgen receptor-specific shRNA, we observed a modest but statistically significant reduction of epinephrine-induced survival. Similar results were obtained in experiments where apoptosis was measured by time lapse video recording (Fig. 3*C*).

These results suggest that in our experimental conditions, transactivation of the androgen receptor has only a small contribution to the antiapoptotic effect of epinephrine in LNCaP cells. Therefore, PKA targets that directly regulate apoptosis are primarily responsible for the survival effect of epinephrine.

Epinephrine Inhibits Apoptosis through PKA-dependent BAD Phosphorylation at Ser^{112}—Recently, we demonstrated that BAD phosphorylation plays an important role in apoptosis regulation in LNCaP cells. In intact LNCaP cells, BAD is constitutively phosphorylated at Ser^{75}, which corresponds to Ser^{112} in mouse BAD. Dephosphorylation of BAD in cells treated with the PI3K inhibitor LY294002 precedes the onset of apoptosis in

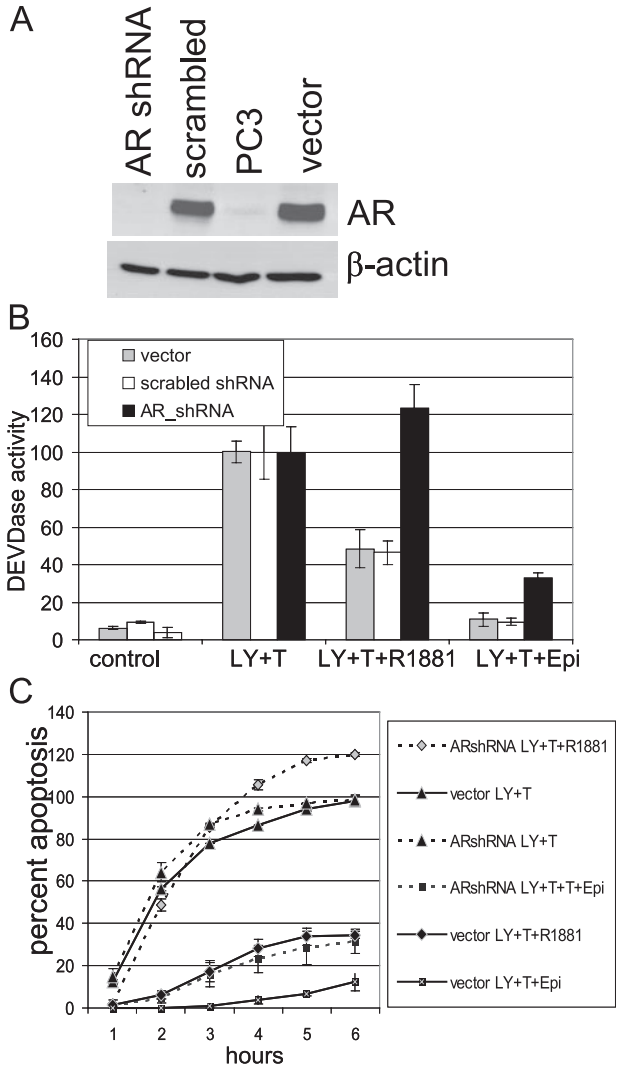

FIGURE 3. **The antiapoptotic effect of epinephrine is partially dependent on androgen receptor expression.** *A*, LNCaP cells were infected with the lentiviral vector pLL3.7 that expresses androgen receptor-specific shRNA (*AR shRNA*), scrambled shRNA, or empty vector. 48 h after infection, cells were lysed and immunoblotted for androgen receptor expression. Androgen receptor-deficient PC3 cells were used as a negative control. Equal loading was confirmed by probing Western blots with antibodies to β-actin. *B*, cells infected with empty vector, scrambled shRNA, or androgen receptor-specific shRNA were incubated 18 h in serum-free medium alone or supplemented with the androgen analog R1881 (10 nM) and treated with combinations of LY294002 (25 μM), thapsigargin (1 μM), and epinephrine (100 nM). 6 h after treatments, cells were collected, and caspase activity was measured with the fluorogenic substrate DEVD-afc (shown in relative units). *Error bars* represent the average ± S.D. of three independent experiments. *C*, cells were infected and treated as in *B*, and apoptosis was followed by time lapse video recording. At least 300 cells in randomly chosen fields were followed for each treatment. *Solid lines*, cells infected with empty vector; *dashed lines*, cells infected with androgen receptor-specific shRNA. *Error bars* show S.D. between apoptosis in individual fields.

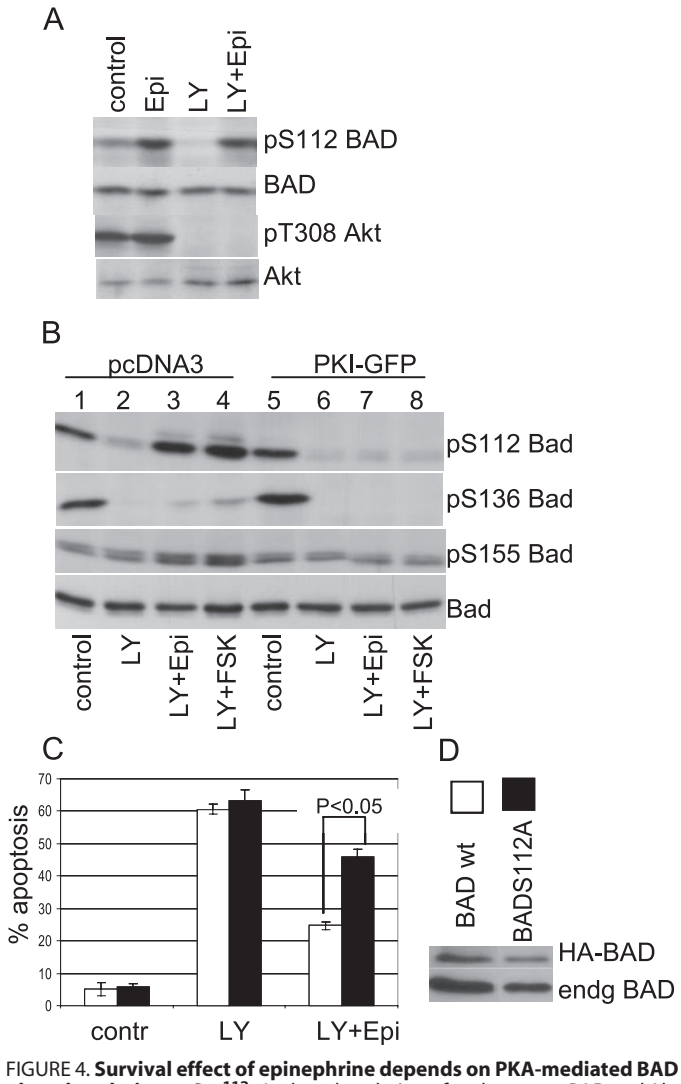

FIGURE 4. **Survival effect of epinephrine depends on PKA-mediated BAD phosphorylation at Ser^{112}.** *A*, phosphorylation of endogenous BAD and Akt in whole cell lysates of LNCaP cells. Cells were maintained in serum-free medium for 3 h and treated with 25 μM LY294002 for 2 h, followed by 100 nM epinephrine. Cell lysates were immunoblotted with phosphospecific antibodies against Ser(P)^{112} BAD or Thr(P)^{308} Akt or antibodies to total BAD or Akt to control equal loading. *B*, LNCaP cells were transfected with HA-BAD mixed with either GFP or PKI-GFP and treated with 0.3% Me_2SO (*control*) or 25 μM LY294002 (*LY*) for 2 h, followed by 300 nM epinephrine (*Epi*) or 5 μM forskolin (*Fsk*) for 1 h where indicated. HA-BAD was immunoprecipitated and immunoblotted with phosphospecific antibodies to Ser(P)^{112}, Ser(P)^{136}, and Ser(P)^{155} or total BAD. *C*, cells were transfected with either wild-type BAD (*white bars*) or BADS112A (*black bars*) mixed with GFP cDNAs (9:1) and treated with LY294002 (*LY*) alone or in combination with epinephrine (*LY+Epi*). Cumulative cell death was measured by time lapse video recording of GFP-positive cells for 6 h. *Bars* show percentage of apoptosis 6 h after treatments (time course of apoptosis is shown in Fig. S3). Each bar was generated by counting at least 350 cells. *Error bars* show S.D. between four randomly selected fields. Difference between apoptosis in cells expressing BADwt and BADS112A and treated with LY294002 plus epinephrine was statistically significant at $p < 0.05$. *D*, expression of HA-BADwt and HA-BADS112A detected by immunoblotting with antibodies against BAD. Endogenous BAD is shown as loading control.

LNCaP cells, whereas knockdown of BAD expression with shRNA inhibits apoptosis (26). Analysis of endogenous BAD phosphorylation with phospho-specific antibodies to Ser(P)^{112} showed that epinephrine increased BAD phosphorylation over basal levels. Treatment with LY294002 decreased BAD phosphorylation, which was restored with concomitant epinephrine treatment. We reasoned that epinephrine-induced BAD phosphorylation was independent of PI3K/Akt signaling, because Akt phosphorylation remained inhibited in cells treated with LY294002 and epinephrine (Figs. 1*B* and 4*A*).

Since PKA activation was connected with the antiapoptotic effects of epinephrine (Fig. 2), we assessed the role of PKA in site-specific BAD phosphorylation by epinephrine. Phospho-BAD-specific antibodies to Ser(P)^{136} and Ser(P)^{155} are not sen-

sitive enough for detecting endogenous BAD phosphorylation. To overcome the lack of sensitivity of phospho-BAD antibodies, recombinant mouse HA-BAD was expressed in LNCaP cells. As shown in Fig. 4*B*, Ser^{112} and Ser^{136} sites in BAD were dephosphorylated in LNCaP cells treated with LY294002, whereas epinephrine restored BAD phosphorylation at Ser^{112} and also modestly increased phosphorylation at Ser^{155} over the level observed in cells with active PI3K/Akt signaling (Fig. 4*B*, *lanes 1–4*). Co-transfection of HA-BAD with the PKA inhibitor PKI-GFP completely abolished BAD phosphorylation by either epinephrine or forskolin at both Ser^{112} and Ser^{155} (Fig. 4*B*, *lanes 5–8*). In contrast, PKA inhibition did not decrease BAD phosphorylation, which depends on constitutive PI3K signaling (Fig. 4, *lanes 1* and *5*).

To determine the role of BAD phosphorylation at Ser^{112} in the antiapoptotic effect of epinephrine, we examined epinephrine-induced survival in cells expressing BADS112A with a mutated phosphorylation site. Wild-type BAD and BADS112A were transiently expressed at comparable levels (Fig. 4*D*). Comparison of cells expressing wild-type BAD and mutant BADS112A showed that the antiapoptotic effect of epinephrine was significantly decreased in cells expressing BADS112A (Figs. 4*C* and S3). Thus, protection of prostate cancer LNCaP cells from apoptosis by epinephrine is predominantly mediated by phosphorylation of the proapoptotic protein BAD at Ser^{112}.

Resting epinephrine concentrations in human and mouse plasma are reportedly below 1 nM. In response to acute stress, epinephrine concentrations may increase to 25 nM (27, 28). To determine a minimal dose of epinephrine that induces BAD phosphorylation and protects from apoptosis, we tested a range of epinephrine concentrations in LNCaP cells. Reductions in apoptosis and increased BAD phosphorylation were observed with 1 nM epinephrine (Fig. 5, *A* and *B*).

To test whether the antiapoptotic effect of epinephrine and BAD phosphorylation occurs in other cancer cell lines, we conducted similar experiments in C4-2 prostate cancer cells and in MDA-MB231 breast cancer cells. C4-2 cells, characterized by increased tumorigenicity (29), exhibited apoptosis comparable with that of LNCaP cells upon treatment with LY294002 and thapsigargin. In contrast, MDA-MB-231 cell lines were more resistant to apoptosis than were LNCaP cells. Therefore, irradiation with UVB, alone or in combination with LY294002, was used to induce apoptosis. As with LNCaP cells, treatment with epinephrine increased BAD phosphorylation and reduced apoptosis in C4-2 and MDA-MB231 cells (Fig. 6).

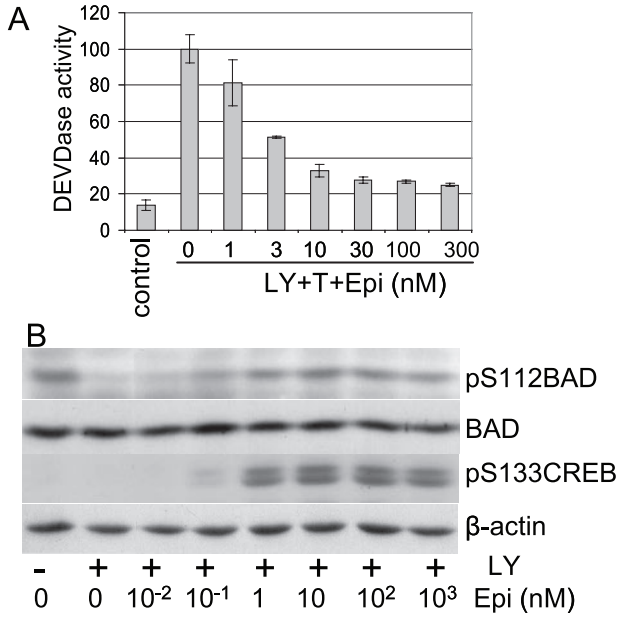

FIGURE 5. **Physiological concentrations of epinephrine protect cells from apoptosis and induce BAD phosphorylation.** *A*, LNCaP cells were serum-starved and treated with 0.3% Me_2SO (*control*), 50 μM LY294002, and 1 μM thapsigargin (*LY+T*) and increasing concentrations of epinephrine (1–300 nM). Cells were collected 6 h after treatments and lysed, and caspase activity was measured using fluorogenic substrate DEVD-afc. *Bars*, average ± S.E. of duplicate samples. Comparable results were observed in three independent experiments. *B*, cells were serum-starved and treated with 50 μM LY294002 for 2 h followed with increasing concentrations of epinephrine (0.01–1000 nM) for 1 h. Then cells were lysed and immunoblotted with antibodies that recognize BAD phosphorylated at Ser^{112} and CREB phosphorylated at Ser^{133}. Antibodies to total BAD and β-actin were used to control equal loading.

DISCUSSION

In the present study, we have shown that epinephrine, a catecholamine secreted by adrenal glands and sympathetic nerve terminals in response to stress, protects prostate and breast cancer cells from apoptosis. Specifically, the antiapoptotic effect of epinephrine is mediated by β_2 adrenergic receptor- and PKA-dependent phosphorylation of BAD at Ser^{112}. This antiapoptotic mechanism can be activated within the range of epinephrine levels observed in response to emotional stress.

β_2-ARs belong to a GPCR superfamily that has been implicated in the pathogenesis of prostate cancer. Several publications report increased expression of GPCRs and GPCR agonists in malignant prostate specimens (30–34). The prevalence of neuroendocrine cells that secrete GPCR ligands in prostatic carcinomas has been correlated with higher grade malignancies and poor prognosis (35, 36). It has also been reported that prostate cancer spreads along nerve projections, which could be a source of neuropeptides that activate GPCR (37).

Recent reports have shown that GPCR agonists can protect prostate cancer cells from apoptosis through PI3K/Akt and NFκB signaling (38, 39). Expression of a G$\beta\gamma$ inhibitor peptide induced apoptosis in PC3 cells, suggesting the existence of survival signaling emanating from G$\beta\gamma$ subunits (40). However, PKA-dependent antiapoptotic signaling downstream from GPCRs is largely unexplored.

PKA has been shown to phosphorylate numerous substrates and elicit a wide range of responses, including changes in gene expression, regulation of vesicle trafficking, transactivation of androgen receptors, and inactivation of the proapoptotic protein BAD (21, 41–43). We observed a modest decrease in the antiapoptotic effect of epinephrine in cells where androgen receptor expression was reduced using a shRNA approach (Fig. 3). Thus, transactivation of androgen receptor by PKA plays a subtle role in the antiapoptotic effect of epinephrine. Subsequent analysis of BAD phosphorylation and apoptosis in cells that express BAD with mutation of S112 demonstrated that antiapoptotic signaling by epinephrine predominantly depends on BAD phosphorylation at Ser^{112} (Fig. 4).

BAD is a proapoptotic protein of the Bcl2 family inactivated by phosphorylation. PKA phosphorylates BAD in tissue culture

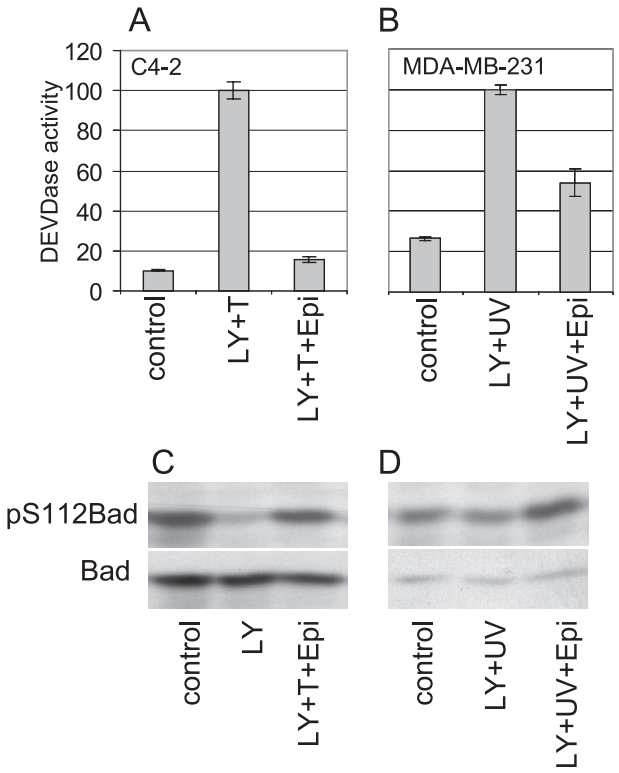

FIGURE 6. **Epinephrine protects from apoptosis and induces BAD phosphorylation in prostate cancer C4-2 and breast cancer MDA-MB231 cells.** C4-2 (*A*) or MDA-MB-231 (*B*) cells were serum-starved for 18 h and treated with 0.3% Me_2SO (*control*), 50 μM LY294002 (*LY*), 1 μM thapsigargin (*T*), 1 min of UV-B (*UV*), and 100 nM epinephrine (*Epi*) as indicated. Cells were lysed 6 h after treatments, and caspase activity was measured using the fluorogenic substrate DEVD-afc. Lysates of C4-2 (*C*) and MDA-MB-231 (*D*) cells were immunoblotted with antibodies that recognize BAD phosphorylated at Ser^{112} and total BAD.

cells at Ser^{112} (42), at Ser^{155} (44), or at both sites (45). BAD phosphorylation at Ser^{112} creates a binding site for the 14-3-3 family of proteins that localize BAD in the cytoplasm and therefore reduces interaction with BclXL in the outer mitochondrial membrane (44).

In LNCaP cells, BAD is constitutively phosphorylated at Ser^{112}, Ser^{136}, and Ser^{155}. Inhibition of PI3K leads to dephosphorylation at Ser^{112} and Ser^{136} but does not significantly change Ser^{155} phosphorylation (Fig. 4*B*). Treatment with epinephrine restored phosphorylation at Ser^{112} and modestly increased phosphorylation at Ser^{155}. Because Ser^{112} phosphorylation inversely correlates with apoptosis, we focused on the analysis of the role of this phosphorylation site in antiapoptotic signaling by epinephrine.

As shown in Fig. 4*C*, the survival effect of epinephrine in cells transiently expressing mutant BADS112A is decreased 2-fold relative to cells expressing wild-type BAD. Phosphorylation of endogenous BAD at Ser^{112} and mutant BADS112A at Ser^{155} may explain the small decrease in apoptosis induced by epinephrine in cells transfected with BADS112A. It is also possible that transactivation of the androgen receptor contributes to the antiapoptotic effect of epinephrine by a mechanism independent from BAD phosphorylation.

Besides BAD, we also observed a correlation between protection from apoptosis and phosphorylation of another PKA substrate, the transcription factor CREB. However, since we did not detect a reduction of epinephrine-induced survival in the presence of translation inhibitor cycloheximide (data not shown), we conclude that CREB-dependent gene expression does not play a substantial role. In summary, our experiments suggest that activation of β_2-ARs followed by PKA-dependent BAD phosphorylation is a major antiapoptotic mechanism activated by epinephrine.

Recently, growth arrest and neuroendocrine differentiation of LNCaP cells in conditions under which PKA is continuously activated have been reported (46). In these studies, treatment with 50 nM epinephrine for 2 days failed to induce significant effects, implying that "stress" levels of epinephrine (1–25 nM) would lead to neuroendocrine differentiation only in combination with other extracellular factors that activate PKA.

Dose-response experiments in Fig. 5 show that 1 nM epinephrine, a concentration observed in blood during chronic stress (28), can induce BAD phosphorylation. If similar dose dependence of stress-induced BAD phosphorylation is observed *in vivo*, then a stressful event may activate antiapoptotic signaling pathway prostate tumors in patients. A recent epidemiological study that demonstrated decreased incidence of prostate cancer in patients who regularly took β-blockers (47) implies that activation of β_2-ARs contributes to prostate cancer development. Given that a significant number of prostate cancer patients experience mood disturbances (48), these results suggest that stress hormones may increase the resistance to therapy of advanced prostate cancers.

Expression of β_2-ARs has been documented in numerous cancer tissues and cell lines. In ovarian cancer cells, epinephrine and norepinephrine stimulated invasion and increased vascular endothelial growth factor production (6, 49). A recent report demonstrated increased angiogenesis and tumor growth induced by stress in an ovarian tumor xenograft model (50). In breast cancer cells, activation of β_2-ARs increased expression of MDR protein, which reduced sensitivity to doxorubicin (51). Protection from apoptosis by epinephrine provides yet another mechanism that may contribute to tumor development and resistance to anti-cancer therapies. Future experiments in animal models are needed to determine the role of epinephrine-induced protection from apoptosis in tumors *in vivo*.

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Mechanisms of Signal Transduction:

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