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Synergistic suppression of apoptosis in salivary acinar cells by IGF1 and EGF

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Abstract

Tissue homeostasis requires balancing cell proliferation and programmed cell death. IGF1 significantly suppressed etoposide-induced apoptosis, measured by caspase 3 activation and quantitation of cellular subG₁ DNA content, in rat parotid salivary acinar cells (C5). Transduction of C5 cells with an adenovirus expressing a constitutively activated mutant of Akt-suppressed etoposide-induced apoptosis, whereas a kinase-inactive mutant of Akt suppressed the protective effect of IGF1. IGF1 also suppressed apoptosis induced by taxol and brefeldin A. EGF was unable to suppress apoptosis induced by etoposide, but was able to synergize with IGF1 to further suppress caspase 3 activation and DNA cleavage after etoposide treatment. The catalytic activity of Akt was significantly higher following stimulation with both growth factors compared to stimulation with IGF1 or EGF alone. These results suggest that a threshold of activated Akt is required for suppression of apoptosis and the cooperative action of growth factors in regulating salivary gland homeostasis.

Keywords

IGF1; EGF; Akt; protein kinase B; apoptosis; salivary gland

Introduction

Programmed cell death is an important biological process in both development and tissue homeostasis and the signaling pathways that regulate apoptosis have been extensively researched. Two main pathways have been described for the induction of apoptosis: the extrinsic or receptor-mediated pathway, and the intrinsic or mitochondrial pathway. In the extrinsic pathway, the binding of ligands to death receptors activates signaling pathways that lead to programmed cell death.^{1,2} In contrast, the intrinsic pathway is activated by cellular stresses such as irradiation or genotoxic agents.^{3,4} Activation of caspase 3 is the commitment step that leads to cellular death in both pathways.^{5,6} The commitment of a cell to undergo apoptosis can be modified by the balance of pro- and antiapoptotic molecules. Perhaps the best example of this involves the Bcl-2 family of proteins.^{7,8} The balance between the antiapoptotic Bcl-2 family members, such as Bcl-2 and Bcl-x_L, and the proapoptotic family members, such as Bax and Bad, plays a critical role in determining whether a cell will undergo apoptosis.^{9,10}

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A second molecule that can provide a survival signal is the protein kinase Akt, which is also known as protein kinase B (PKB).^{11–13} Activation of Akt occurs following treatment of cells with a variety of growth factors that stimulate proliferation and cell survival,¹⁴ and occurs in a PI3 kinase-dependent manner.¹⁵ Structurally, Akt contains a pleckstrin homology (PH) domain at the amino terminus, a kinase domain, and a carboxyl tail. Stimulation of cells with growth factors results in the activation of PI3 kinase leading to an increase in the concentrations of PI(3,4)P₂ and PI(3,4,5)P₃ in the plasma membrane.¹⁶ The PH domain of Akt binds to the newly generated PIP₂ and PIP₃ resulting in the translocation of Akt from the cytosol to the plasma membrane.¹⁷ Once Akt is translocated to the plasma membrane, it is phosphorylated at threonine³⁰⁸, by phosphatidylinositide-dependent kinase (PDK1), and serine⁴⁷³ by an unknown kinase, leading to its catalytic activation.^{18,19} Activation of Akt has been correlated with the phosphorylation and inactivation of proapoptotic substrates including: procaspase 9,²⁰ proapoptotic Bcl-2 family member Bad,^{21–23} and the transcription factor Forkhead.^{24,25}

Growth factor suppression of apoptosis has been demonstrated in a number of other cell systems. The importance of nerve growth factor in the survival of cultured neuronal cells was one of the first reports describing the role of the PI3 kinase pathway in the prevention of apoptosis.^{26,27} Later studies extended this observation to include IGF1 involvement in neuronal survival.²⁸ In addition to neuronal cells, IGF1 could also protect fibroblasts from apoptosis induced by UV-B light.²⁹ This protection could not be demonstrated with EGF unless the receptor was overexpressed.²⁹ Other reports, in many different cell types, have indicated the effectiveness of some growth factors *versus* others in the suppression of apoptosis.^{27,29} The overexpression of Akt or PI3 kinase molecules also suppressed apoptosis, often at levels greater than that observed with growth factors alone.^{27,29}

Salivary gland function plays an important role in oral health. Saliva aids in digesting food, protects the oral mucosa, and moistens the palate for articulation.³⁰ Salivary gland hypofunction is a principal side effect of chronic usage of prescription drugs particularly in the elderly,³¹ chemotherapy,³² therapeutic irradiation of the head and neck region,³² and systemic diseases such as Sjögren's syndrome.³³ Aberrant apoptosis of salivary acinar cells is hypothesized to be one of the major causes of salivary gland hypofunction related to these various conditions.^{30,32} The salivary glands produce and secrete a number of growth factors into saliva including EGF, NGF, TGF α , TGF β , insulin, IGF1 and IGF2.³⁴ In particular, NGF and EGF have been shown to stimulate the proliferation of salivary acinar cells.³⁵ In the animal model for Sjögren's syndrome, the nonobese diabetic (NOD) mouse, reduced levels of insulin and IGF1 correlated with the onset of clinical symptoms,³⁴ suggesting that adequate IGF1 blood levels are required to maintain the viability of salivary acinar cells.

In this study, we have examined the ability of EGF and IGF1 to induce activation of Akt in salivary acinar cells, and thereby suppress apoptosis induced by a variety of agents. IGF1, but not EGF, was effective in reducing the extent of apoptosis after treatment with etoposide, taxol, or brefeldin A. This same effect could be mimicked by the expression of a constitutively activated mutant of Akt in the same cells. Dual stimulations with both growth factors resulted in increased Akt kinase activity and synergistic suppression of apoptosis. These data suggest that growth factors such as EGF and IGF1 may be important in regulating homeostasis of the salivary gland. Furthermore, EGF and IGF1 may have the potential to temporarily suppress the inadvertent apoptosis in salivary glands that occurs secondary to irradiation of the head and neck, or other chemotherapeutic treatments.

Results

Activation of Akt is induced by EGF and IGF1 in a PI3 kinase-dependent manner

C5 cells were stimulated with 100 ng/ml EGF or 10 ng/ml IGF1 for 0–60 min and the activation of Akt was examined by immunoblotting with an anti-phospho-Akt antibody (phosphoserine⁴⁷³). Maximal stimulation of Akt was demonstrated after 5–15 min of treatment with 100 ng/ml EGF and declined after that time (Figure 1a, lanes 1–6). Stimulation of C5 cells with 10 ng/ml IGF1 induced a more robust activation of Akt, peaking at 15–30 min and remaining elevated for at least 1 h. (Figure 1a, lanes 7–12). The membranes were stripped and immunoblotted with a second antibody directed against total Akt to demonstrate equal loading of the gel (Figure 1a, bottom panel). A longer time course of stimulation examined the extent of Akt activation persistence following IGF1 treatment. As shown in Figure 1b, Akt activation can be readily detected at 15 min, but the first peak appeared at 30 min and then declined slightly. Akt activation continued for at least 8 h and a second maximal peak of Akt activity appeared upon sustained IGF1 stimulation (2–8 h, lanes 7–10), compared to earlier time points (30 min poststimulation, lane 4). The amount of total Akt protein did not appear to change even over long periods of stimulation (Figure 1b, bottom panel). Antibodies specific for the different isoforms of Akt were used to determine which isoforms were activated after IGF1 treatment. Both Akt-1 and Akt-2 were activated after growth factor treatment in C5 parotid cells (data not shown).

As noted in the introduction, previous studies have demonstrated that activation of Akt occurs in a PI3 kinase-dependent manner.^{16,18,19} We have used two compounds that inhibit PI3 kinase, LY294002 and wortmannin, to address this point. Pretreatment of C5 cells with either LY294002 or wortmannin suppressed activation of Akt following IGF1 treatment (Figure 1c, lanes 4 and 10), whereas pretreatment with solvent controls (DMSO or ethanol, Figure 1c, lanes 5 and 11) had no effect on Akt activation after IGF1 stimulation. Membranes were stripped and detected with an anti-Akt antibody to demonstrate that neither LY294002 nor wortmannin altered the level of Akt protein in the cells (Figure 1c, bottom panel).

IGF1 treatment suppressed apoptosis induced by etoposide in rat parotid acinar cells

Previous studies have shown that activation of Akt can suppress apoptosis induced by a variety of different stimuli.^{29,36–38} The data in Figure 1 clearly indicated that IGF1 induced potent, long-lasting activation of Akt, and we examined the ability of IGF1 to suppress apoptosis induced by etoposide in salivary acinar cells. It has been previously demonstrated that etoposide induces apoptosis of the C5 cell line and that this apoptosis was correlated with the activation of JNKs,³⁹ inhibition of basal ERK activation,³⁹ and activation of PKC Δ .⁴⁰ As a marker of apoptosis, we chose to quantitate caspase 3 activation and DNA fragmentation since these changes are detectable at 8 h after treatment of C5 cells with etoposide³⁹ and maximal levels of activated Akt were still present at this time point (Figure 1b, lane 10). Monolayers of C5 cells were either left untreated or pretreated with 10 ng/ml IGF1 for 30 min, then treated with 0.5, 5, or 50 μ M etoposide for 8 h. Etoposide induced the rapid activation of caspase 3 in a dose-dependent manner (Figure 2a) and was significantly higher than starved controls. Pretreatment of C5 cells with 10 ng/ml IGF1 caused a 40–60% decrease in the activation of caspase 3 at all concentrations of etoposide tested, indicating that IGF1 suppressed the induction of apoptosis. The difference in caspase activation between the etoposide-treated and IGF1+etoposide-treated samples was statistically significant at each concentration of etoposide used ($P \leq 0.01$ at 50, 5, and 0.5 μ M by the Student's *t*-test). To confirm the data obtained with the assay for caspase 3, we also quantitated the percent of cells with a subG1 DNA content by flow cytometry. Pretreatment of cells with IGF1 caused a 60% decrease in the number of cells present in the subG1 fraction following treatment with 50 μ M etoposide (Figure 2b). The experiment shown in Figure 2a was repeated and the concentration of IGF1 used in the

pretreatment was varied. A stepwise progression in the suppression of apoptosis with increasing IGF1 concentration was demonstrated. A concentration of 0.1 ng/ml IGF1 did not alter etoposide-induced apoptosis, although concentrations of 1 and 5 ng/ml provided partial suppression of apoptosis (Figure 3a). There was no statistically significant difference between the effects obtained with 10, 20, 50 or 100 ng/ml IGF1 (Figure 3a). Consistent with this result, we did not detect a further increase in the amount of phosphorylated, activated Akt at IGF1 concentrations of 10 ng/ml or higher (Figure 3b).

Suppression of etoposide-induced apoptosis is dependent on Akt

The activation of Akt is dependent upon the activation of PI3 kinase, a point that has been established through the use of chemical inhibitors.^{15,17} Unfortunately, treatment of cells with these inhibitors results in apoptosis, and thus cannot be used to demonstrate the Akt-dependent effects of IGF1 noted above. Therefore, we used recombinant human adenoviruses that express either a kinase-inactive mutant of Akt referred to as KD-Akt, or a constitutively activated mutant of Akt referred to as Myr-Akt. The Myr-Akt mutant was generated by adding the myristoylation sequence from the Src tyrosine kinase to the amino-terminal end of Akt.¹⁷ This change results in the constitutive association of Akt with the plasma membrane, where it is phosphorylated and activated by upstream kinases.¹⁷ Transduction of C5 cells with virus encoding the Myr-Akt mutant resulted in a complete suppression of apoptosis and was more effective in suppressing apoptosis than IGF1 (Figure 4a). Expression of the kinase-inactive mutant of Akt blocked the ability of IGF1 to suppress etoposide-induced activation of caspase 3 (Figure 4a). The kinase-inactive mutant of Akt did not alter the induction of caspase 3 activity by etoposide, nor did the LacZ-encoding adenovirus. A LacZ encoding control virus had no effect upon the ability of IGF1 to suppress apoptosis (Figure 4a). To confirm these data, cell lysates were prepared from duplicate plates of C5 cells transduced with the different adenoviral vectors and treated with etoposide. Immunoblotting of these lysates with anti-caspase 3 antibody revealed that only the Myr-Akt encoding virus was able to inhibit the cleavage of procaspase 3 to active caspase 3 (Figure 4b). Cells transduced with either LacZ or the kinase-inactive mutant of Akt demonstrated the presence of the 20 kDa activated fragment of caspase 3 (Figure 4b).

IGF1 treatment suppressed apoptosis induced by other agents

Although our original studies have focused upon etoposide-induced apoptosis,^{39,40} we have recently demonstrated that other drugs are also able to induce apoptosis in the rat salivary acinar cell line C5. Both taxol and brefeldin A have been demonstrated to induce apoptosis of the C5 cell line.⁴¹ Etoposide is a genotoxin that inhibits DNA repair,⁴² whereas the site of action for taxol is the microtubule network,⁴³ and brefeldin A acts upon the Golgi and trans-Golgi network.⁴⁴ All three are considered to activate the 'intrinsic' apoptotic pathway. To determine whether IGF1 could suppress apoptosis induced by these other agents, C5 cells were treated with varying concentrations of taxol (Figure 5a) and brefeldin A (Figure 5b). As shown in Figure 2, pretreatment of C5 cells with IGF1 caused a 50–60% decrease in the amount of apoptosis as indicated by the activation of caspase 3 (Figure 5). These results were statistically significant at all concentrations of taxol or brefeldin A used. Expression of the kinase-inactive mutant of Akt (KD-Akt) blocked the ability of IGF1 to suppress taxol- or brefeldin A-induced activation of caspase 3 similar to Figure 4 (data not shown). Thus, the effect of IGF1 is not limited to a single apoptotic stimulus.

EGF and IGF1 are able to synergize to enhance protection from apoptosis

As demonstrated in Figure 1, both EGF and IGF1 are able to induce activation of Akt, although the extent of activation varies considerably between these two stimuli. It is not clear whether this difference results from an intrinsic difference in signaling by these two different receptors,

or because of a difference in the number of receptors present on the parotid C5 cell line. Owing to the significant level of protection by the Myr-Akt Adenovirus (Figure 4), we wished to determine whether the extent of Akt activation afforded by these two different growth factors correlated with the amount of protection against apoptosis. Therefore, we examined caspase 3 activation following treatment of C5 cells with 50 μ M etoposide in cells pretreated with either 10 ng/ml IGF1, 100 ng/ml EGF, or a combination of both IGF1 and EGF. Consistent with the data shown above, IGF1 suppressed etoposide-induced activation of caspase 3 (Figure 6a). In contrast, pretreatment of cells with 100 ng/ml EGF had no effect upon the activation of caspase 3 by etoposide, indicating that there was no suppression of apoptosis (Figure 6a). Most interestingly, the pretreatment of C5 cells with a combination of EGF plus IGF1 resulted in an additional 20% repression of caspase 3 activation compared to cells pretreated with IGF1 alone (Figure 6a). The additional suppression of apoptosis by both growth factors was also observed when examined by the extent of DNA fragmentation (Figure 6b) or the extent of caspase 3 cleavage by immunoblotting (Figure 6c). It should be noted that although EGF appeared to decrease the extent of caspase cleavage slightly as determined by immunoblot analysis in Figure 6c, there was no effect on caspase activity detected in the enzymatic assay shown in Figure 6a. This implies that differences exist between these two assays and that the enzymatic assay may more accurately reflect the *in vivo* situation. The data indicated that there were significant differences between the amount of caspase 3 activated in etoposide-treated IGF1-pretreated cells *versus* etoposide-treated EGF- plus IGF1-pretreated cells.

We then confirmed these data in primary parotid acinar cells from FVB mice to demonstrate that this was not unique to established immortalized cells (Figure 6d). A higher concentration of etoposide was used to induce apoptosis of the primary cells (200 μ M etoposide); this concentration was chosen as a result of dose-response and kinetic studies on primary parotid acinar cells from FVB mice (data not shown). Pretreatment of the primary parotid acinar cells with IGF1 caused a 50% decrease in the amount of activated caspase 3, 8 h after treatment of the cells with etoposide (Figure 6d). Pretreatment of the cells with 100 ng/ml EGF did not cause a statistically significant difference in the amount of activated caspase 3 when compared to the etoposide-treated cells (Figure 6d). Similar to the results observed with the C5 cell line, pretreatment with EGF plus IGF1 caused an enhanced suppression, approximately 80%, in the amount of activated caspase 3, 8 h after etoposide treatment in primary parotid cells (Student's *t*-test comparing IGF1+Etop and IGF1+EGF+Etop, $P \leq 0.01$). These data clearly suggest that EGF and IGF1 are able to synergize in the suppression of apoptosis induced by etoposide in salivary acinar cells.

As a means to understand the mechanism underlying the suppression of apoptosis offered by treatment with a combination of both EGF and IGF1 in C5 cells, we examined the activation of Akt kinase activity by measuring the phosphorylation of a substrate peptide. Akt kinase activity was examined at 15 min post-treatment with 10 ng/ml IGF1, 100 ng/ml EGF, or both IGF1 plus EGF, and at 8 h poststimulation with EGF, IGF1 and EGF plus IGF1 (the same time point at which caspase 3 activation was examined). Starved control lysates were used to measure nonspecific background activity and these values were subtracted from the experimental lysates. Akt activity was graphed as average counts per minute (CPM) and standard error bars represent the mean of three independent experiments. At 15 min poststimulation, IGF1-induced activation of Akt was significantly higher than levels induced by EGF (Figure 7a). In contrast, IGF1 and EGF in combination induced strong activation of Akt 15 min poststimulation (Figure 7a). Upon sustained stimulation (8 h) with single growth factors or in combination, the presence of IGF1 was required for Akt activation and significantly higher levels of kinase activity was measured with both growth factors (Figure 7a). EGF and IGF1 appeared to induce equal activation of other signaling enzymes such as ERK, 15 min poststimulation (data not shown). EGF could induce higher Akt activity at longer stimulation time points (Figure 7b), but did not suppress caspase 3 activation and apoptosis

(Figure 6a), perhaps indicating a specific threshold of activated Akt that must be present at the correct time to suppress apoptosis.

Discussion

In this study, we have examined the activation of Akt in an established rat parotid acinar cell line. Both IGF1 and EGF induced the activation of Akt in a dose- and time-dependent manner; however, a more robust activation of Akt was observed following acute treatment of these cells with IGF1 (Figure 1). Akt activation was dependent upon the activation of PI3 kinase as seen in other studies.^{16,18,19} We have also observed persistent activation of Akt after at least 8 h treatment with IGF1.

We have also examined the ability of these growth factors to suppress apoptosis induced by a variety of agents that induce apoptosis by different mechanisms: etoposide, a genotoxin,⁴² taxol, a disrupter of the microtubule network,⁴³ and brefeldin A, a disrupter of the Golgi and trans-Golgi network.⁴⁴ IGF1, but not EGF, was able to suppress apoptosis induced by all three agents (Figures 2, 5, and 6). Surprisingly, in this cell type, the combination of both IGF1 and EGF was able to provide synergistic protection from apoptosis induced by all three agents (Figure 6 and data not shown). Although these studies were conducted using an established rat parotid acinar cell line, we were also able to confirm this observation using primary parotid acinar cells from FVB mice indicating that this observation is not unique to the established cell line, or unique to rat salivary acinar cells.

Akt has been shown to suppress apoptosis, in a number of other cell systems, induced by Fas/FasL,³⁶ growth factor withdrawal,³⁷ anoikis⁴⁵ and DNA damaging agents.³⁸ IGF1 suppression of caspase 3 activation was dependent on Akt since transduction of cells with a recombinant human adenovirus encoding a kinase-inactive mutant of Akt blocked the effect of IGF1 (Figure 4 and data not shown). In contrast, transduction with a control adenovirus encoding LacZ had no effect upon the ability of IGF1 to suppress caspase 3 activation and apoptosis. Transduction of the C5 cells with a recombinant human adenovirus encoding a constitutively activated mutant of Akt (Myr-Akt) completely suppressed caspase 3 cleavage and activation 8 h after etoposide treatment. The extent of apoptotic suppression by Myr-Akt was greater than that achieved with IGF1 alone.

Activation of Akt was determined in early (15 min) and late (8 h) times after stimulation with IGF, EGF, or both growth factors (Figure 7a). There were clear differences in the amount of Akt activated in EGF *versus* IGF1-treated cells in both early and late time points (Figure 7a). The combination of EGF plus IGF1 induced a further increase in the amount of Akt kinase activity as measured by the ability to phosphorylate a specific substrate. EGF induction of significant Akt activity seemed to require a considerable amount of time (2–8 h, Figure 7b lanes 7–10) and may indicate an additional pathway of activation upon sustained stimulation. These results suggest that Akt kinase activity may require a threshold amount that must be present at the proper time in order for the activation of caspase 3, and thus apoptosis, to be suppressed.

The identification of molecules that regulate apoptosis is fundamental to our understanding of tissue homeostasis. As noted above, considerable attention has been placed on the balance between the expression of pro- and antiapoptotic Bcl-2 family members as a determinant in deciding whether a cell will undergo apoptosis.^{9,10} This study provides evidence that activation of Akt is an important survival signal to salivary acinar cells. It has been previously demonstrated that the salivary gland was able to secrete growth factors such as EGF; in fact, EGF was originally isolated from the submandibular salivary gland.⁴⁶ We have demonstrated that although EGF can induce activation of Akt, it does not stimulate sufficient levels of

activated Akt to suppress apoptosis. It is interesting to speculate that the tonic production of EGF by the salivary gland provides a basal level of activated Akt such that extremely low concentrations of growth factors such as IGF1 could provide a potent survival signal in these cells. The synergistic action of EGF may not be limited to IGF1, but could also be true for other paracrine factors such as NGF. The action of EGF and IGF1 may also be important during development of the salivary gland. These possibilities clearly merit investigation in mice that lack expression of Akt1, Akt2, or molecules that lie in the IGF1 signaling pathway.

These studies provide promising evidence for clinical utilization of growth factors in temporarily protecting salivary glands from aberrant apoptosis. Administration of low doses of IGF1 might be sufficient to suppress apoptosis of salivary acinar cells during head and neck irradiation or chemotherapy. We postulate that Akt is a central antiapoptotic component because of its ability to suppress apoptosis induced by a variety of stimuli and may interact with or phosphorylate different substrates to achieve this effect. It is possible that different pathways are targeted depending on the kinetics of Akt activation by different growth factors. Additional studies are required to define Akt substrates activated in the salivary glands, and the role of these molecules in suppressing aberrant apoptosis induced by chemotherapy and irradiation.

Materials and Methods

Cells and cell culture

A previously established and characterized rat parotid acinar cell line (C5) was used in these studies.⁴⁷ These cells demonstrated highly differentiated acinar cell markers and have maintained the β -adrenergic/cyclic AMP signaling pathways.⁴⁷ Culture conditions and medium supplements have been previously described.^{39,40} Cells were plated in 100 mm² Primaria tissue culture dishes (Falcon/Becton Dickinson, Fairlawn, NJ, USA) and used when the cultures were approximately 80% confluent.

Primary parotid acinar cells were prepared from 4 to 5-week-old FVB mice purchased from Taconic Laboratories (Germantown, NY, USA). Animals were maintained in accordance with the University of Colorado Health Sciences Center Laboratory Animal Care guidelines and protocols. Mice were anesthetized with sodium pentobarbital (60 mg/kg) and primary parotid acinar cells were prepared under sterile conditions similar to previously published protocols used in the preparation of primary acinar cells from rat salivary glands.⁴⁸ A 1% vol/vol cell suspension was seeded onto collagen-coated dishes (Falcon/Becton Dickinson) in media similar to that used for the established cell lines with the exception of the FBS content elevated from 2.5 to 10%. Cells reached approximately 80% confluency after 5 days in culture and were utilized immediately in experiments at that time without passage.

Growth factor stimulation

C5 cells were starved for 4 h in serum-free F12/DMEM medium to reduce the background level of activated signaling molecules. The cells were then stimulated with 100 ng/ml EGF or 10 ng/ml IGF1 for the various times indicated in the figures. In dose–response studies, the concentration of EGF used ranged from 10 to 500 ng/ml and the concentrations of IGF1 used ranged from 1 to 100 ng/ml. In some studies, the cells were pretreated with 50 μ M LY294002 (Calbiochem, LaJolla, CA, USA) or DMSO (solvent control) for 5 min, or with 100 nM wortmannin (Calbiochem) or ethanol (solvent control) for 30 min prior to growth factor stimulation.

Induction and quantitation of apoptosis

C5 cells or primary mouse salivary acinar cells were starved overnight and treated with varying doses of etoposide (C5 cells: 0.5–50 μM ; primary mouse salivary acinar cells: 200 μM), taxol (0.05–5 μM), or brefeldin A (0.3–6 μM) with or without prior pretreatment with growth factors. Eight hours after treatment with the apoptotic agent, cells were harvested for analysis of caspase activation, immunoblotting with various specific antibodies, or analysis of DNA fragmentation.³⁹ Pretreatment with 100 ng/ml EGF, 10 ng/ml IGF1, or both was for 30 min before addition of the apoptotic agents. Growth factors were present for the entire 8 h treatment with each apoptotic agent. Etoposide, taxol, and brefeldin A were purchased from Sigma Chemical Company (St. Louis, MO, USA).

Activation of caspase 3 was quantitated using BioMol QuantiZyme Colorimetric Assay kit (BioMol, Plymouth Meeting, PA, USA). The adherent and floating cells were collected from a 100 mm² dish and lysed in caspase lysis buffer supplemented with 0.1% Triton-X, aprotinin (4 $\mu\text{g}/\text{ml}$), Prefebloc (0.5 mg/ml), and leupeptin (2 $\mu\text{g}/\text{ml}$) according to the manufacturer's instructions and previously published reports.^{39,41} Caspase 3 activity in 15 μg of cellular lysate was measured by the cleavage of Ac-DEVD-pNA substrate and absorbance at A_{405} was quantitated in a microtiter plate reader (Molecular Devices, Sunnyvale, CA, USA) at 10-min intervals for 7 h.

For the quantitation of DNA fragmentation, adherent and floating cells were collected from a 100 mm² dish and DNA was isolated as previously described.³⁹ DNA samples (5 μg) were electrophoresed at 100 V on a 1.5% agarose gel for ~2 h. Gels were stained with ethidium bromide and analyzed on UVP Epi Chemi II Darkroom imaging system with Labworks software (Upland, CA, USA).

For quantitation of cells with a subG1 DNA content, the cells in a 100 mm² dish were digested with a trypsin solution for 5–10 min, and the digestion was stopped by the addition of an equal volume of trypsin inhibitor as previously described.⁴¹ A single-cell suspension was made by passing the cells through a progression of syringe needles and cells were stained 5–24 h with 0.3 mg/ml saponin, 25 $\mu\text{g}/\text{ml}$ propidium iodide, 10 mM EDTA, and 5 $\mu\text{g}/\text{ml}$ RNase.^{41,49} Flow cytometric analysis to quantitate the percent of cells in a SubG1 (apoptotic) fraction was performed using a Beckman Coulter XL flow cytometer by the University of Colorado Cancer Center Flow Cytometry Core Facility. Cell cycle modeling was performed with the ModFit software package (Verity House Software, Topsham, ME, USA) to determine the percent of cells in the different parts of the cell cycle.

Immunoblotting and immunoprecipitation

Cells were lysed in RIPA lysis buffer (150 mM NaCl, 50 mM Tris, pH 7.4, 2 mM EGTA, 1% Triton X-100, 0.25% sodium deoxycholate) supplemented with 100 U/ml aprotinin (Pierce Chemical Company) and 1 mM sodium orthovanadate, and the lysates clarified by centrifugation at 4°C for 30 min at 13 000 rpm in a refrigerated Savant microcentrifuge.³⁹ Protein concentrations were determined using the BCA Protein Assay Kit (Pierce Chemical Company). For immunoblotting, 100 μg of clarified whole cellular proteins were resolved on 8, 10 or 15% polyacrylamide gel, transferred to Immobilon membrane (Millipore Corporation, Bedford, MA, USA), and immunoblotted. Enhanced chemiluminescence lighting (ECL-Amersham Pharmacia Biotech, Arlington Heights, IL, USA) was used according to the manufacturer's instructions to detect immunoblotted proteins. Membranes were then stripped as previously described,⁴⁹ reblocked in TBST with 5% nonfat dry milk (Carnation), and detected with a second antibody.

Immunoprecipitation was conducted as previously described.⁴⁹ A 1 mg amount of each whole cell lysate was precleared by the addition of 30 μ l Omnisorb (Calbiochem) for 15 min at 4°C. One microgram of the desired antibody was added to each clarified lysate and incubated for 2 h at 4°C on a rocking platform. The immune complexes were then gathered on Omnisorb (30 μ l added to the lysates for 1 h at 4°C). Immune complexes were washed three times with lysis buffer and resolved by SDS-PAGE, and subjected to immunoblotting as described above. An antiphospho-Akt antibody (antiphosphoserine⁴⁷³) and anti-Akt antibody for immunoblotting were obtained from Cell Signaling Technologies (Beverly, MA, USA). Anticaspase-3 antibody was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Quantitation of caspase 3 cleavage fragments was performed by densometric scanning measurements using a UVP Epi Chemi II Darkroom imaging system with Labworks software. Anti-Akt1, Akt2 and Akt3 antibodies for immunoprecipitation were obtained by Upstate Biotechnology (Lake Placid, NY, USA). Antiaactive MAPK (pERK) and total ERK antibodies were purchased from Promega Corporation (Madison, WI, USA). The activation of Akt kinase activity was quantitated using a radioactive Akt kinase assay kit (Upstate Biotechnology) using 300 μ g of whole cell lysates according to the manufacture's instructions.

Transduction of cells with recombinant human adenoviruses

Recombinant Adenoviruses expressing a constitutively active mutant of Akt-1 (Ad-Myr-Akt) or a kinase inactive mutant of Akt (Ad-KD) were generated by overlap recombination.⁵⁰ Dr. Jerome Schaack, University of Colorado Health Sciences Center, kindly provided the Adenovirus expressing Lac Z (Ad-LacZ).⁵¹ Recombinant viruses were propagated as previously described.^{41,50} In all experiments, the C5 cells were transduced with the desired recombinant adenoviruses at equal multiplicity of infection. Transductions were allowed to proceed for 1 h in media lacking fetal calf serum before replacing the medium with complete media containing fetal calf serum. Cells were treated 18 h with post-transduction 50 μ M etoposide for eight hours as described above. Alternatively, cells transduced with Ad-KD or Ad-LacZ were pretreated with 10 ng/ml IGF1 for 30 min, followed by etoposide treatment as described above. Expression of Akt Adenoviruses was confirmed by immunoblotting with an antibody against the HA epitope (Roche Molecular Biochemicals, Indianapolis, IN, USA) 18 h after transduction.

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Abbreviations

DMSO	dimethylsulfoxide
EGF	epidermal growth factor
ERK	extracellular regulated kinase
IGF1	insulin-like growth factor 1
PDK1	phosphatidylinositide-dependent kinase

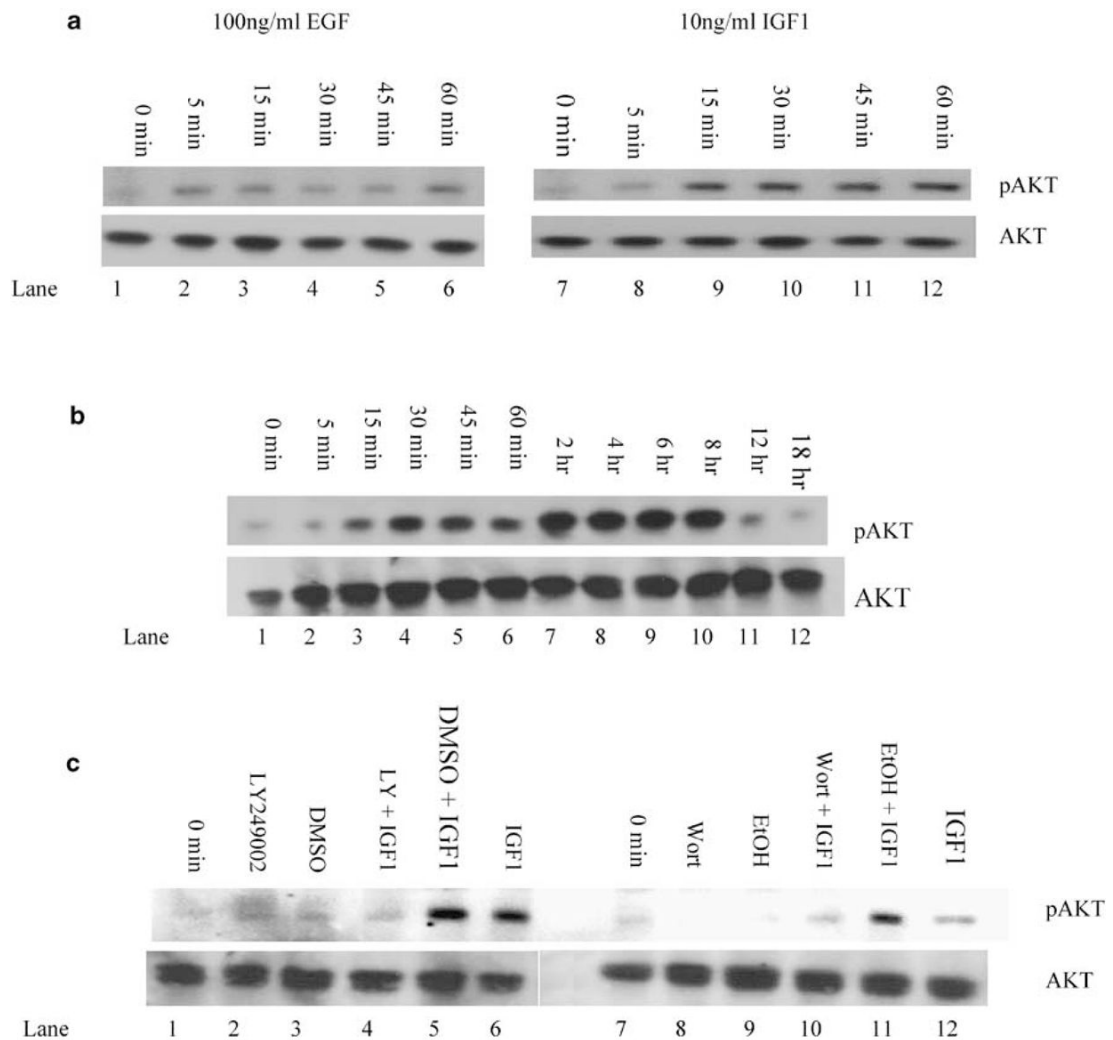
PI3 kinase phosphatidylinositol-3' kinase
 PKB protein kinase B

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**Figure 1.**

Activation of Akt by IGF1 and EGF. Subconfluent monolayers of C5 parotid cells were stimulated with either 10 ng/ml IGF1 or 100 ng/ml EGF for various times. Whole cell lysates were prepared as described in the Materials and Methods section, and 100 μ g of the protein lysates were electrophoresed on an 8% SDS-PAGE gel, and immunoblotted with an antibody that detects phospho-Akt (phosphoserine⁴⁷³). Membranes were stripped and immunoblotted with a total Akt antibody to confirm equal loading of lanes (bottom panel in each figure). Blots are a representative example of three independent experiments. In **(a)** C5 cells were stimulated with 100 ng/ml EGF for 0–60 min (lanes 1–6) or 10 ng/ml IGF-1 (lanes 7–12). In **(b)** C5 cells were stimulated with 10 ng/ml IGF1 for 0–18 h and immunoblotted as described for **(a)**. In **(c)** C5 cells were pretreated with either 50 mM LY294002 (lanes 2 and 4), DMSO (lanes 3 and 5; solvent control for LY290042), 100 nM wortmannin (lanes 8 and 10), or ethanol (lanes 9 and 11; the solvent control for wortmannin) prior to stimulation of the cells with 10 ng/ml IGF-1 for 30 min prior to lysis. Lysates were prepared and immunoblotted as described in **(a)**. The positions of phosphorylated Akt (pAKT) and AKT are indicated on the right side of each panel

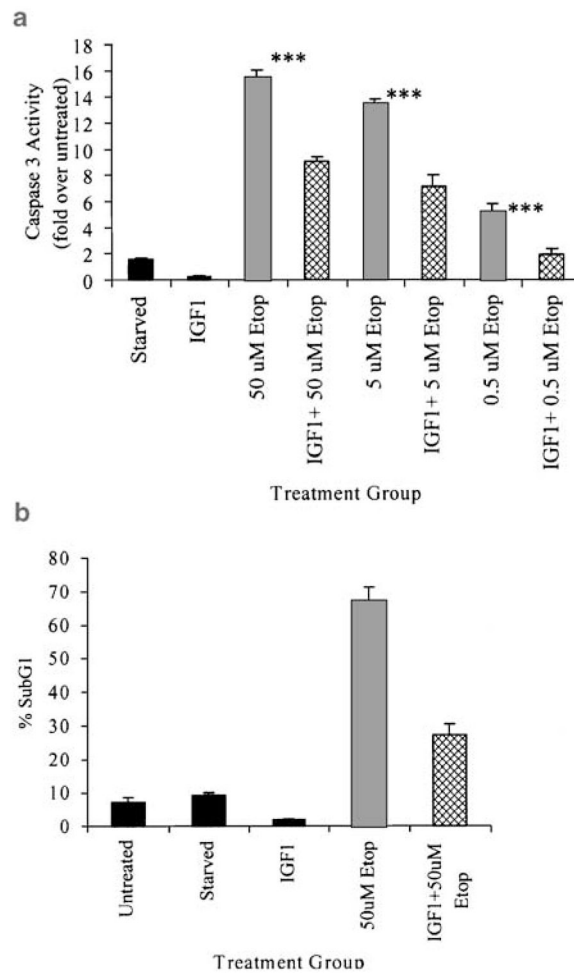


Figure 2.

IGF1 suppresses etoposide-induced apoptosis. C5 cells were either treated with 10 ng/ml IGF1 or left untreated in the presence of increasing concentrations of etoposide (0.5, 5 or 50 μ M). In (a) all adherent and nonadherent cells were collected after 8 h treatment with etoposide, and lysed in caspase lysis buffer (BioMol QuantiZyme Colormetric Assay kit). In all, 15 μ g of cell lysate was used to analyze the level of enzyme activity for each sample in quadruplicate. The ability of the enzyme to cleave a chromogenic substrate was read at A_{405} every 10 min for 7 h and the fold increase relative to untreated control cells is plotted. Student's *t*-test *P* values were calculated using Microsoft Excel comparing two different treatment groups from six independent biological experiments ($P \leq 0.000001$ at 50 μ M, $P \leq 0.0002$ at 5 μ M, and $P \leq 0.006$ at 0.5 μ M). In (b) duplicate treated plates to those analyzed in panel (a) were analyzed for the induction of apoptosis by quantitation of the SubG1 (apoptotic) fraction of cells by flow cytometry as described in the materials and methods section. All adherent and floating cells were collected for analysis. Data were analyzed with the ModFit software package to determine the percent of cells in the different parts of the cell cycle and graphed as an average percentage of cells in the SubG1 fraction from three independent experiments

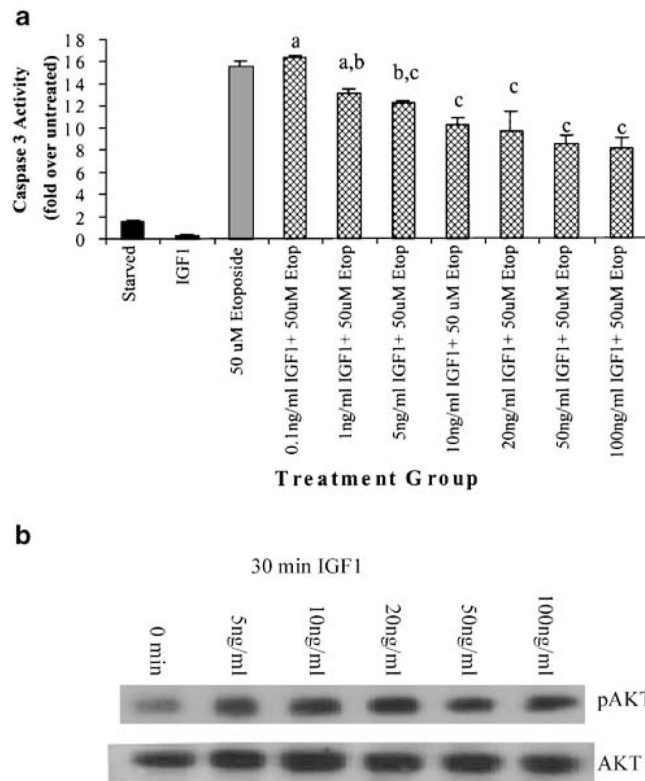


Figure 3. Suppression of etoposide-induced apoptosis is IGF1 dose dependent. In order to confirm that IGF1 was responsible for the suppression of etoposide-induced apoptosis, dose-response curves were determined. In (a) C5 cells were prestimulated with varying doses of IGF1 for 30 min and then treated with etoposide for 8 h as described in Figure 2a. The activation of caspase 3 was used as an indication of the induction of apoptosis from six independent biological experiments. In (b) C5 cells were stimulated with varying doses of IGF1 for 30 min and whole cell lysates were immunoblotted to determine Akt activation as described in Figure 1. Blot is a representative example of three independent experiments

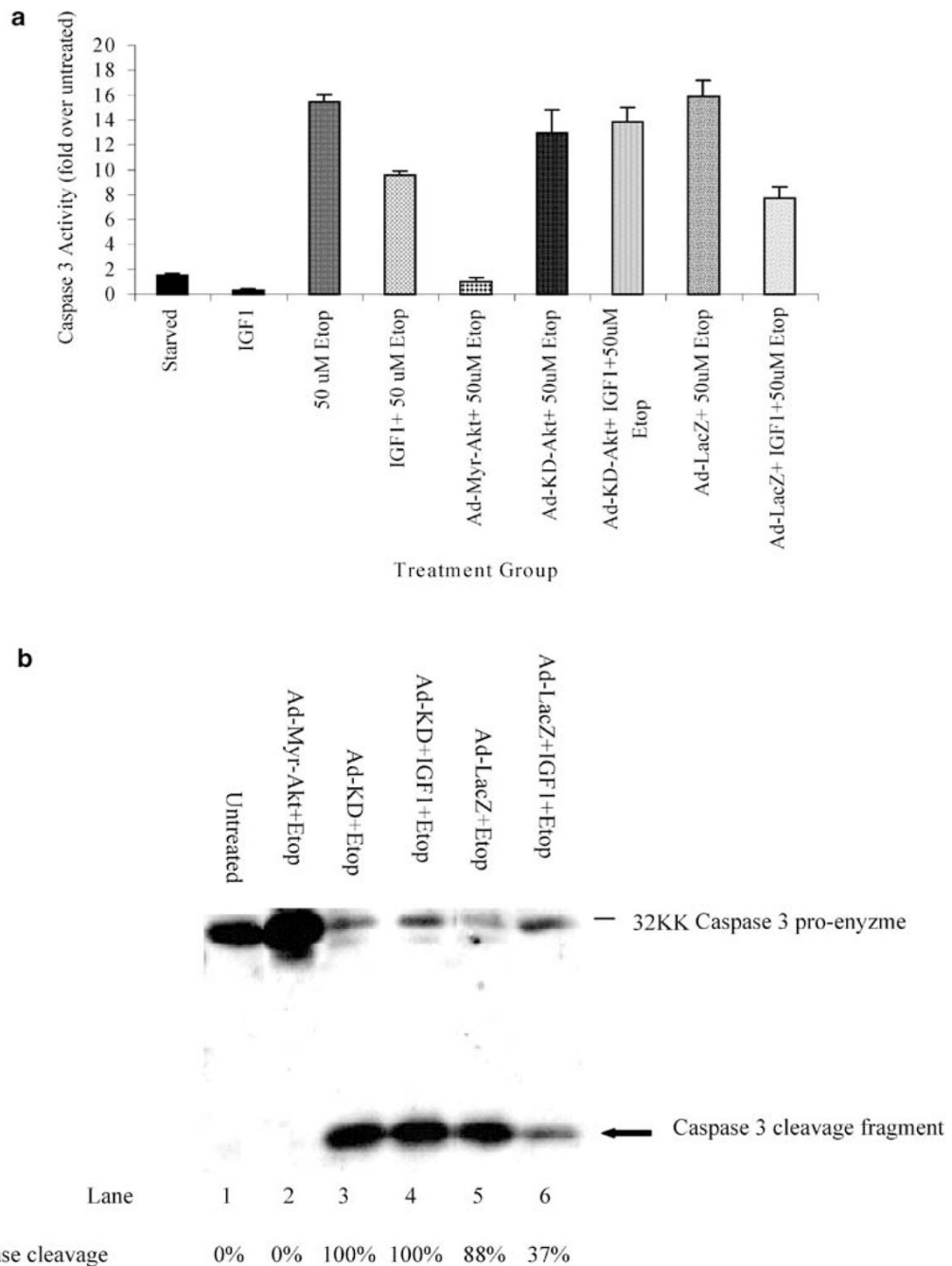


Figure 4.

IGF1 suppression of etoposide-induced apoptosis is dependent on Akt. To determine the role of Akt in providing IGF1-dependent suppression of etoposide-induced apoptosis, C5 cells were transduced with recombinant adenoviruses expressing either a constitutively activated mutant of Akt (Myr-Akt), a kinase inactive mutant of Akt (KD-Akt), or an LacZ-encoding control adenovirus. Cells were treated 18 h after transduction with 50 μ M etoposide for 8 h. In (a) the activation of caspase 3 was examined as described in Figure 2a. Graph depicts the average caspase kinetic activity over untreated controls from four independent experiments. In (b) duplicate plates of cells were lysed and analyzed by immunoblotting for the amount of cleaved activated caspase 3. Quantitation of caspase 3 cleavage fragments was performed by

densometric scanning measurements using a UVP Epi Chemi II Darkroom imaging system with Labworks software. Densometric scans of three blots from independent experiments were averaged and the Ad-KD+etoposide (lane 3) was arbitrarily chosen as 100%. In both cases, the adherent and nonadherent floating cells were collected and analyzed as a pool. Blot is a representative example of three independent experiments

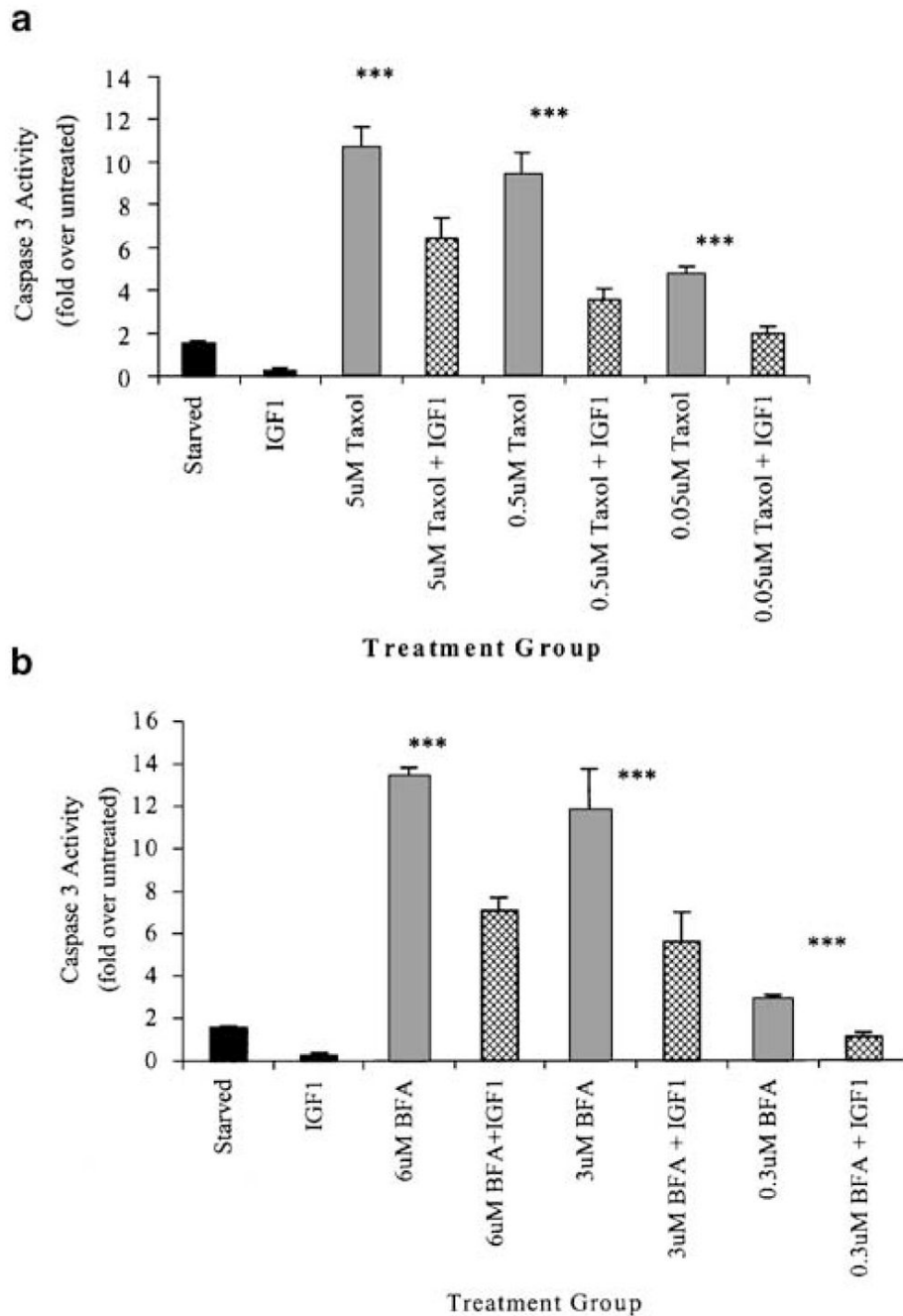
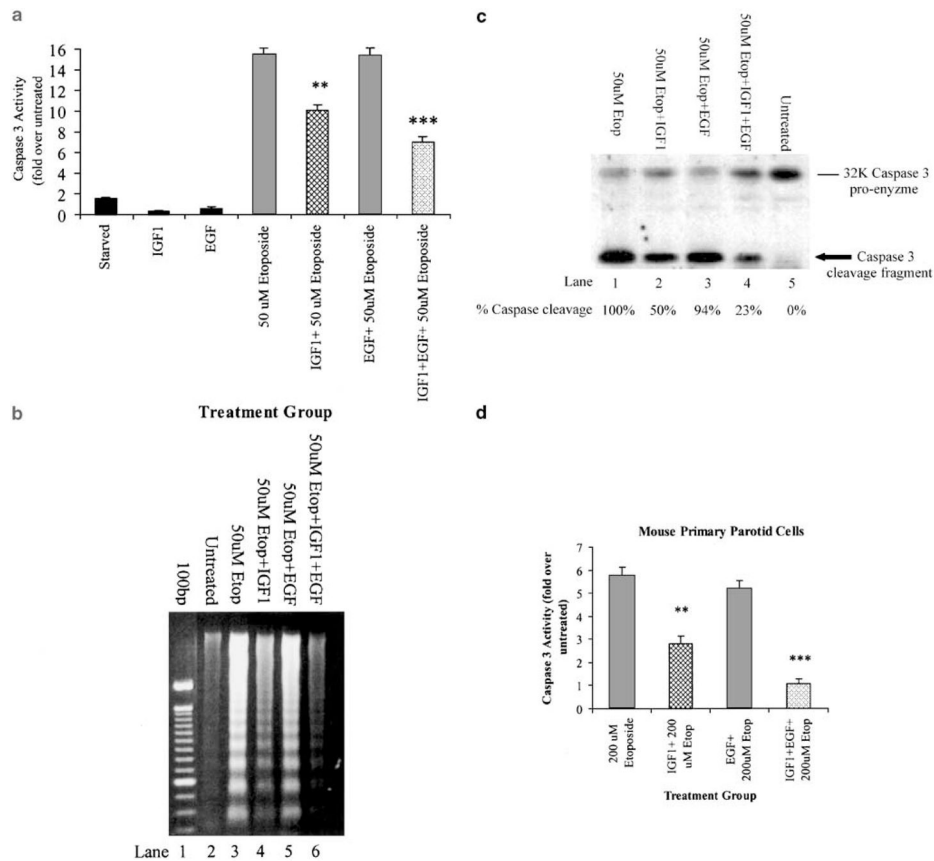


Figure 5.

IGF1 is able to suppress apoptosis induced by other agents. C5 cells were either pretreated with 10 ng/ml IGF1 for 30 min or left untreated. The cells were then treated in (a) with increasing concentrations of taxol (0.05, 0.5, or 5 μ M); or in (b) with increasing concentrations of brefeldin A (0.3, 3, or 6 μ M). In both cases, all adherent and nonadherent cells were collected after 8 h treatment with the indicated agents, and the activation of caspase analyzed as described in Figure 2a. Student's *t*-test *P* values were calculated in Microsoft Excel program comparing two different treatment groups from five independent biological experiments (Taxol: $P \leq 0.0004$ at 5 μ M, $P \leq 0.0006$ at 0.5 μ M, and $P \leq 0.002$ at 0.05 μ M; Brefeldin A: $P \leq 0.00001$ at 6 μ M, $P \leq 0.04$ at 3 μ M, and $P \leq 0.0007$ at 0.3 μ M)

**Figure 6.**

EGF synergizes with IGF1 to suppress etoposide-induced apoptosis. C5 cells were pretreated with 10 ng/ml IGF1, or 100 ng/ml EGF, or 10 ng/ml IGF1 plus 100 ng/ml EGF prior to treatment with 50 μ M etoposide for 8 h. In (a) the activation of caspase 3 was determined as described in Figure 2a and plotted as fold increase relative to untreated control cells. Student's *t*-test *P* values were calculated in Microsoft Excel program comparing two different treatment groups from six independent biological experiments (***) denotes IGF1+50 μ M Etoposide versus IGF1+EGF+50 μ M etoposide, $P \leq 0.007$). In (b) the fragmentation of chromosomal DNA was used to confirm the activation of caspase 3 shown in panel (a). In (c) duplicate treated plates were analyzed for the cleavage of caspase 3 by immunoblotting with a caspase 3 antibody. The amount of caspase 3 cleavage was quantitated using the UVP Epi Chemi II Darkroom imaging system with Labworks software. Densitometric scans of three blots from independent experiments were averaged and the etoposide treatment (lane 1) was arbitrarily chosen as 100%. Blot is a representative example of three independent experiments. Student's *t*-test *P* values were calculated in Microsoft Excel program comparing IGF1+50 μ M etoposide versus IGF1+EGF+50 μ M etoposide, $P \leq 0.039$. In (d) primary parotid acinar cells were isolated from FVB mice as described in the Materials and Methods section. After five days in culture, cells were left untreated, pretreated with 10 ng/ml IGF1, 100 ng/ml EGF, or 10 ng/ml IGF1 plus 100 ng/ml EGF for 30 min similar to that (a). Apoptosis was induced with 200 μ M etoposide (this concentration was determined in preliminary studies with primary parotid cells) for 8 h. Caspase 3 activation was determined as previously described in Figure 2a, and values are plotted as fold over untreated controls. Student's *t*-test *P* values were calculated in Microsoft Excel program comparing two different treatment groups from three independent biological experiments (** denotes 200 μ M etoposide versus IGF1+200 μ M etoposide, $P \leq 0.004$; *** denotes IGF1+200 μ M etoposide versus IGF1+EGF+200 μ M etoposide, $P \leq 0.01$). In the

experiments shown, both the adherent and nonadherent floating cells were collected and analyzed as a pool

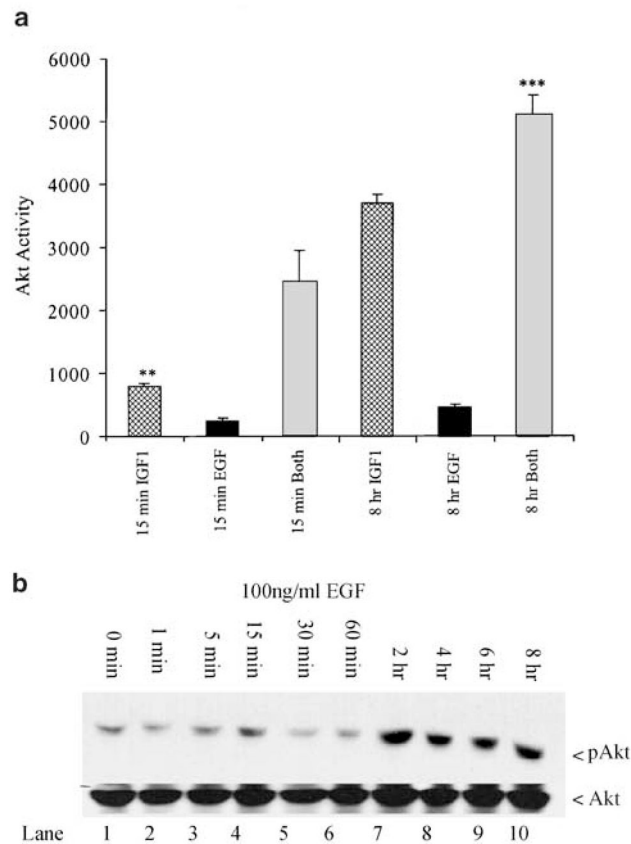


Figure 7.

IGF1 and EGF induce a synergistic increase in Akt activation. C5 cells were stimulated with either 10 ng/ml IGF1 or 100 ng/ml EGF or both to examine activation of signaling pathways. In (a) whole cell lysates from stimulated cells were prepared as described in the Materials and Methods section. Total Akt was immunoprecipitated from whole cell lysates (300 μ g) and used in an immune complex protein kinase assay containing 32 P [γ - 32 P]ATP and a specific substrate peptide (RPRAATF). The incorporation of 32 P into the substrate peptide was determined by liquid scintillation counting and is plotted as average counts per minute (CPM). Starved control lysates from individual experiments (activity range: 2650–2810 cpm) were used to measure nonspecific background activity and these values were subtracted from the experimental lysates. An aliquot of precipitated Akt was electrophoresed on a SDS–PAGE gel to ensure equal amounts of total Akt enzyme in each reaction (data not shown). Student's *t*-test *P* values were calculated in Microsoft Excel program comparing two different treatment groups from three independent experiments (** denotes 15 min IGF1 *versus* 15 min EGF, $P \leq 0.005$; 15 min IGF1 *versus* 15 min IGF1+EGF, $P \leq 0.027$; *** denotes 8 h IGF1 *versus* 8 h IGF1+EGF, $P \leq 0.01$) In (b) C5 cells were stimulated with 100 ng/ml EGF for 0–8 h and whole cell lysates were prepared as above. Protein lysates were electrophoresed on an 8% SDS–PAGE gel, and immunoblotted with a phospho-Akt antibody (serine⁴⁷³) as described in Figure 1. Membranes were stripped and immunoblotted with a total Akt antibody to confirm equal loading of lanes. Blot is a representative example of three independent experiments