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Mechanisms of Energy Restriction: Effects of Corticosterone on Cell Growth, Cell Cycle Machinery, and Apoptosis[1]

Weiqin Jiang, Zongjian Zhu, Neehar Bhatia, Rajesh Agarwal, and Henry J. Thompson[2]

Centers for Nutrition in the Prevention of Disease [W. J., Z. Z., H. J. T.] and Cancer Causation and Prevention [N. B.], AMC Cancer Research Center, Denver, Colorado 80214, and School of Pharmacy, University of Colorado Health Sciences Center, Denver, Colorado 80262 [R. A.]

ABSTRACT

The restriction of energy intake has documented beneficial effects on numerous diseases including cancer, yet the mechanism(s) that accounts for these effects is unknown. Recently, we showed that the inhibitory activity against mammary carcinogenesis mediated by energy restriction (ER) is accompanied by an increase in the secretion of adrenal cortical steroids. However, ER caused a concomitant reduction in circulating levels of insulin-like growth factor-1, which also may be involved in inhibiting carcinogenesis. To determine what cellular and molecular effects may be because of corticosterone *per se*, detailed mechanistic studies were performed *in vitro* using a mouse mammary hyperplastic cell line (TM10). The following questions were addressed: (*a*) is corticosterone-mediated growth inhibition accounted for by disruption of cell cycle machinery; (*b*) is growth inhibition accompanied by the induction of apoptosis; and (*c*) is growth inhibition reversible? At doses of corticosterone (50–200 μM for 24–72 h) that resulted in inhibition (up to 76%; $P < 0.001$) of growth, a dose- and time-dependent G_1 arrest in cell cycle progression was observed. In the studies analyzing cell cycle regulatory molecules, corticosterone treatment of cells resulted in a strong induction (up to ~10-fold over control; $P < 0.01$) of KIP1/P27 together with a decrease (up to 98%; $P < 0.01$) in cyclin-dependent kinase 4 (CDK4) and cyclin D1 protein levels. Cells treated with corticosterone also showed an increased binding (up to 2.6-fold over control; $P < 0.01$) of KIP1/P27 with CDK4, together with a strong decrease (up to 89%; $P < 0.01$) in the kinase activity of the CDK4-cyclin D1 complex. Treatment of cells with KIP1/P27 antisense oligonucleotides reversed the growth inhibitory effects of corticosterone. Treatment of cells with RU 486, a glucocorticoid receptor blocker, reversed the effects of corticosterone on cell growth and KIP/P27 protein levels suggesting the involvement of the glucocorticoid receptor in accounting for these effects. Additional studies assessing the biological fate of cells after corticosterone treatment showed that corticosterone exerted reversible growth inhibitory effects with limited apoptotic cell death. Together, these findings show a reversible cytostatic effect of corticosterone via perturbations in cell cycle regulators causing a G_1 arrest in the absence of increased levels of apoptosis. These data provide evidence for a role of corticosterone on some but not all of the cellular activities associated with ER-mediated inhibition of mammary carcinogenesis.

INTRODUCTION

ER[3] has been shown to exert profound inhibitory effects on carcinogenesis at several organ sites including mammary gland, colon, liver, and skin (1–4). An accumulating amount of evidence points to a specific effect of ER on various growth factors, oncogenes, and tumor suppresser genes that are involved in the carcinogenic process (1, 5–8). ER has been shown to decrease cell proliferation and increase apoptotic cell death (9, 10). Recent studies have also documented an effect of ER on KIP1/P27 induction and a decrease in cyclin D1 expression in mammary tumors (11). Likewise, dietary feeding of corticosterone was able to increase KIP1/P27 expression in mammary epithelial cells in rats suggesting that ER leads to an increased level of cortical steroid that alters cell cycle machinery in mammary epithelium resulting in an inhibition of mammary carcinogenesis (12). Indeed, our recent studies have shown that urinary levels of immunoreactive cortical steroids were directly related to the inhibition of mammary carcinogenesis by ER (1) and that the dietary administration of corticosterone to *ad libitum* fed, adrenal intact animals also inhibited mammary carcinogenesis.[4] Nonetheless, we have shown recently that coincident with the up-regulation of cortical steroid levels in plasma, ER causes a reduction in circulating levels of IGF-1,[5] an effect also observed in animals administered supplemental dietary corticosterone.[4] Hence, *in vivo* it is likely that it will be difficult to dissociate the effect of increased levels of corticosterone *per se* from effects exerted on other hormones such as IGF-1. This situation prompted the use of an *in vitro* model to complement *in vivo* investigations. In this paper, we report effects on a mouse mammary hyperplastic epithelial cell line, TM10, which was exposed to levels of corticosterone that inhibited cell growth in the absence of cytotoxicity. This cell line was used because it has been reported that ER may preferentially block the progression of mammary hyperplasias to carcinoma (1). The data obtained, showing a cytostatic effect of corticosterone via perturbations in cell cycle regulators causing a G_1 arrest, provide additional evidence for a role of cortical steroid hormones on some but not all of the cellular activities associated with ER-mediated inhibition of mammary carcinogenesis.

MATERIALS AND METHODS

Chemicals. The following materials were purchased from commercial sources: DMEM and F-12 medium, Triton X-100, corticosterone, mifepristone (RU-486), glutaraldehyde, crystal violet, and propidium iodide (Sigma, St. Louis, MO); adult bovine serum (Gemini Bioproducts, Calabasas, CA); insulin and epidermal growth factor (Intergen, Purchase, NY); gentamicin reagent solution (Life Technologies, Inc., Grand Island, NY); anticyclin D1, anti-KIP1/P27, and anti-CDK4 antibodies (Neomarkers, Inc., Fremont, CA); goat anti-mouse immunoglobulin-horseradish peroxidase-conjugated secondary antibodies, Rb-GST fusion protein and protein A/G PLUS-agarose beads (Santa Cruz Corp., Santa Cruz, CA); Kip1/p27 antisense oligonucleotides (TriLink BioTechnologies, Inc., San Diego, CA); GSV cytofectin (Glen Research, Sterling, VA); and ECL detection system (Amersham Corp., Arlington Heights, IL).

Cell Culture. The mouse mammary hyperplastic epithelial TM10 cell line was obtained from the laboratory of Medina *et al.* (13). Cells were grown at 37°C in a humidified incubator with 5% CO_2 in DMEM/F-12 medium (1:1, v/v) containing 2% adult bovine serum, 10 μg/ml insulin, 5 ng/ml epidermal growth factor, and 5 μg/ml gentamicin.

Cell Growth Assay. The effect of corticosterone on cell growth was determined by evaluating the number of adherent cells as described previously

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[2] To whom requests for reprints should be addressed, at the Center for Nutrition in the Prevention of Disease, AMC Cancer Research Center, 1600 Pierce Street, Denver, CO 80214. Phone: (303) 239-3463; Fax: (303) 239-3443; E-mail: thompsonh@amc.org.

[3] The abbreviations used are: ER, energy restriction; CKI, cyclin-dependent kinase inhibitor; CDK, cyclin-dependent kinase; Rb, retinoblastoma; ECL, enhanced chemiluminescence; IGF, insulin-like growth factor; FACS, fluorescence-activated cell sorter.

[4] Z. Zhu, W. Jiang, and H. J. Thompson. Mechanisms by which ER inhibits mammary carcinogenesis: effects of corticosterone, submitted for publication.

[5] Z. Zhu, W. Jiang, and H. J. Thompson. Effects of ER and repletion on transformed cell deletion and potential anticancer hormonal mediators: Corticosterone, IGF-1, and leptin, submitted for publication.

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(14, 15). Briefly, TM10 cells were plated at 1×10^3 cells per well in flat-bottomed 96-well plates in 100-μl of culture medium under the culture conditions detailed above. After 24 h, cells were fed with fresh medium and treated with ethanol alone (0.5%, v/v) or corticosterone at doses of 50, 100, or 200 μM dissolved in ethanol (0.5% final volume in medium). The cultures were fed with fresh medium with the ethanol alone or the same concentrations of corticosterone every other day up to the end of the experiment, and each treatment and time point was replicated in eight wells. At days 1 to 3 after these treatments, cells were fixed with 1% glutaraldehyde, replaced with PBS and stored at 4°C. At the end of an experiment, all of the plates were stained with crystal violet. After dissolving the crystal violet in 70% ethanol, the absorbance was determined at 590 nm using a SPECTRA MAX PLUS Microplate Spectrophotometer System (Molecular Devices, Sunnyvale, CA).

In another study assessing whether the cell growth inhibitory effects of corticosterone were reversible, TM10 cells were treated with ethanol or various doses of corticosterone (50, 100, or 200 μM) as detailed above. After 72 h of these treatments, cell growth was determined by cell counting. At this point, in separate dishes, after 72 h of these treatments with corticosterone, cultures were washed three times with medium to remove corticosterone, and cells were then grown in fresh medium without corticosterone for another 24, 48, or 72 h. Cell number was determined at these time periods using a hemocytometer.

Cell Cycle Distribution Analyses. Logarithmically growing semiconfluent TM10 cells were treated with 0.5% ethanol (final concentration) or 50, 100, or 200 μM doses of corticosterone in the same volume of ethanol for 24 and 48 h, and thereafter cell pellets were collected. The nuclei were stained with propidium iodide using a procedure described by Krishan (16) and subjected to FACS analysis at the University of Colorado Health Sciences Center Flow Cytometry Core Facility.

Assessment of IGF-1 Levels in Cell Culture Medium. Logarithmically growing semiconfluent cultures of TM10 cells were treated with ethanol or 50, 100, or 200 μM doses of corticosterone in ethanol for 24, 48, and 72 h as detailed above. The medium was collected at the end of these treatments and stored at −80°C until analysis. IGF-1 in the concentrated medium was assessed using an ELISA kit according to the manufacturer's directions (Diagnostic Systems Laboratories, Inc., Webster, TX).

Immunoprecipitation and Western Blotting. Logarithmically growing semiconfluent cultures of TM10 cells were treated with ethanol or 50, 100, or 200 μM doses of corticosterone in ethanol for 24, 48, and 72 h as detailed above. The medium was aspirated at the end of these treatments, and the cells were quickly washed two times with cold PBS. A 0.3-ml aliquot of lysis buffer [10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 0.2 mM sodium orthovanadate, 0.2 mM phenylmethylsulfonyl fluoride, 0.5% NP-40, and 0.2 unit/ml aprotinin] was then added per plate. After bathing in lysis buffer for 15 min on ice, the cells were scraped from the plate; the mixture of buffer and cells was transferred to microfuge tubes and left in ice for an additional 15 min. The lysates were collected by centrifugation for 15 min in a tabletop centrifuge at 4°C, and protein concentration in the clear supernatants was determined by the Bio-Rad protein assay (Bio-Rad, Hercules, CA).

Western blotting was performed as described before (17). Briefly, 40 μg of protein lysate per sample was denatured with SDS-PAGE sample buffer [63 mM Tris-HCl (pH 6.8), 10% glycerol, 2% SDS, 0.0025% bromphenol blue, and 5% 2-mercaptoenthanal], subjected to SDS-PAGE on 12% gel, and the protein bands blotted onto a membrane. The levels of KIP1/P27, CDK4, and cyclin D1 were determined using specific primary antibodies, followed by treatment with the appropriate peroxidase-conjugated secondary antibody and visualized by the ECL detection system.

Immunoprecipitation was performed as described before (17). Briefly, 200 μg of protein lysate per sample was mixed with 2 μg of anti-KIP1/P27 antibody and 25 μl of protein A/G PLUS-agarose beads, and incubated overnight at 4°C on a rocker platform. On the next day, beads were collected by centrifugation and washed four times with lysis buffer. The immunoprecipitated KIP1/P27 was denatured with the 2× SDS-PAGE sample buffer and subjected to 12% SDS-PAGE gel followed by Western blotting. The level of CDK4 bound to KIP1/P27 was determined by specific primary antibody to CDK4 followed by peroxidase-conjugated antimouse secondary antibody and visualization by the ECL detection system.

Kinase Assay. Cyclin D1- and CDK4-associated kinase activity was determined using Rb-GST fusion protein as substrate as described earlier (17, 18) with some modifications. Briefly, ethanol vehicle- or corticosterone-treated TM10 cells were lysed in Rb lysis buffer [50 mM HEPES-KOH (pH 7.5), containing 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 1 mM DTT, 0.1% Tween 20, 10% glycerol, 80 mM β-glycerophosphate, 1 mM sodium fluoride, 0.1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml leupeptin and aprotinin], and using anticyclin D1 or anti-CDK4 antibody (2 μg) and protein A/G PLUS-agarose beads (20 μl), specific proteins were immunoprecipitated from 200 μg of protein lysate per sample as described above. Beads were washed three times with Rb lysis buffer and then once with Rb kinase assay buffer [50 mM HEPES-KOH (pH 7.5), containing 2.5 mM EGTA, 10 mM β-glycerophosphate, 1 mM sodium fluoride, 0.1 mM sodium orthovanadate, 10 mM $MgCl_2$, and 1 mM DTT]. Phosphorylation of Rb was measured by incubating the beads with 40 μl of radiolabeled Rb kinase solution [0.25 μl (2 μg) of Rb-GST fusion protein, 0.5 μl of [γ-^{32}P]ATP, 0.5 μl of 0.1 mM ATP, and 38.75 μl of Rb kinase buffer] for 30 min at 37°C. The reaction was stopped by boiling the samples in SDS sample buffer for 5 min. The samples were analyzed by 12% SDS-PAGE, and the gel was dried and subjected to autoradiography.

Unless specified otherwise, in each case (Western blotting, immunoprecipitation and Western blotting, and kinase assays), only representative gel data are shown from three independent studies. In each study, bands were quantitated by scanning the film with ScanJet (Hewlett Packard, Palo Alto, CA), and the intensity of the bands was analyzed by using the Image-Pro Plus software (Media Cybernetics, Silver Spring, MD). The quantification bars shown in each case are mean ± SE of three independent studies.

Cell Death Studies. For the studies assessing the effect of corticosterone on cell death, TM10 cells were plated at 1×10^4 cells/60-mm plate as described above. After 24 h, cells were fed with fresh medium and treated with ethanol or various doses of corticosterone (50, 100, or 200 μM) under identical conditions as detailed above. The cultures were fed with fresh medium with ethanol or the same doses of corticosterone every other day up to the end of the experiment. Each treatment and time point had three plates. After 1–3 days of corticosterone treatments, cells were trypsinized and collected. Trypan blue dye exclusion was used to determine cell viability.

To determine apoptotic and necrotic cell death, cells were stained with annexin V and propidium iodide using an apoptosis assay kit (Molecular Probes, Eugene, OR). Briefly, after the above treatments, the collected cells were washed with cold PBS, and 1×10^6 cells were incubated in 100 μl annexin-binding buffer containing 5 μl of annexin V and 1 μl of the 100 μg/ml propidium iodide for 15 min at room temperature. After the incubation period, 400 μl of annexin-binding buffer was added to each sample, mixed gently, and the samples were kept on ice for counting the stained cells by flow cytometry.

Transfection of Kip1/p27 Antisense. The oligonucleotides were purchased from TriLink BioTechnologies, Inc. (San Diego, CA). The antisense oligonucleotide sequence used was 5′-UGG CUC UCC UGC GCC-3′ (targets bp 306–320 of murine Kip1) as described previously (19). For the cytofectin procedure, 10 nM oligonucleotides were mixed with GSV cytofectin (2.5 μg/ml; Ref. 19; Glen Research, Sterling, VA) in serum-free medium and incubated for 10 min at 37°C to make an oligonucleotide-cytofectin solution. Logarithmically growing semiconfluent TM10 were cultured in the oligonucleotide-cytofectin solution in complete medium for 24 h. Eighty-five to 90% of exposed cells took up and concentrated the oligonucleotide in the cell nucleus as reflected by uptake of 6-carboxyfluorescein-labeled oligonucleotides determined by fluorescence microscopy.

Blockage of the Glucocorticoid Receptor by RU-486. For the studies assessing the effect of glucocorticoid receptor antagonist, RU-486, and/or corticosterone on cell growth and P27, TM10 cells were treated with either ethanol or 0.1 μM RU-486 and/or 200 μM corticosterone dissolved in ethanol under identical conditions as detailed above. Each treatment and time point had three plates. After 2 days of treatments, cell number was determined by cell counting using a hemocytometer, and P27 levels in cell lysate were determined by Western blotting as described above.

Statistical Analyses. Differences in the number of TM10 cells after exposure to corticosterone at different doses and time points were evaluated by ANOVA (20). Post hoc comparisons among treatment conditions were made using the Bonferroni multiple-range test (20). Data derived from Western blot analyses represent semiquantitative estimates of the amount of a specific protein that is present in a cell extract. This fact was taken into account in the statistical evaluation of the data. The data displayed in the graphs are reported

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as means ± SE of the ratio (experimental:control) of the actual scanning units derived from the densitometric analysis of each Western blot. All of the values are the means of three independent experiments. For statistical analyses the actual scanning density data derived from the analysis of the Western blots using Image Pro Plus were first ranked. This approach is particularly suitable for semiquantitative measurements that are collected as continuously distributed data. This approach has the advantage of maintaining the relative relationships among data being compared without giving undue weight to outlying results. The ranked data were then subjected to multivariate ANOVA (20). Statistically, this is a robust approach that takes into account both the fact that levels and/or activities of proteins in a molecular pathway may not vary independently from one another, as well as the issues that exist when multiple comparisons are being made on a particular set of data.

RESULTS

Effects of Corticosterone on Cell Growth and Cell Cycle Distribution. In initial range finding experiments, doses of corticosterone ≤25 μM had no effect on cell viability or growth (data not shown). As indicated in Fig. 1*A*, exposure of TM10 cells to higher concentrations of corticosterone for 24, 48, and 72 h resulted in a statistically significant reduction in cell growth ($P < 0.001$). Compared with the ethanol control, the magnitude of the reduction was 8, 14, and 25% after 24 h; 43, 51, and 66% after 48 h; and 50, 57, and 76% after 72 h of corticosterone treatments at 50, 100, and 200 μM doses, respectively (Fig. 1*A*). As shown in Fig. 1*B*, corticosterone treatment resulted in an insignificant decrease in cell viability measured by trypan blue dye exclusion. Compared with the ethanol-treated control, the percentage of nonviable cells in the total counted cells at 50–200 μM corticosterone was 0.25–1.75% higher after 24 h, 2.5–4.0% higher after 48 h exposure, and 1.5–5.75% higher after 72 h of exposure.

To determine whether cell growth inhibition observed in response to corticosterone treatment was associated with cell cycle arrest, the distribution of cells in different phases of cell cycle was assessed after 24 and 48 h of treatment with 50, 100, and 200 μM doses of corticosterone. As shown in Fig. 1, *C* and *D*, FACS analysis of ethanol control and corticosterone-treated TM10 cells clearly indicated a dose-dependent G_1 arrest after 24 h of corticosterone treatment, and an additional increase in the G_1 population after 48 h of corticosterone treatment at the same doses. The increase in the G_1 population was accompanied by a decrease of cells in the S and G_2-M phases (Fig. 1, *C* and *D*). The cell population in the G_1 phase increased with increasing dose of corticosterone, 50, 53, 60, and 66% after 24 h ($P < 0.01$; Fig. 1*C*); and 67, 89, 94, and 95% after 48 h ($P < 0.01$; Fig. 1*D*) of corticosterone treatments at 0 (ethanol), 50, 100, and 200 μM doses, respectively.

Effect of Corticosterone on Levels of IGF-1 in Medium. Levels of IGF-1 in prepared medium were low (10 ng/ml) and could only be detected in medium that was concentrated. Given that cells in culture often secrete IGF-1 into the medium, the effects of corticosterone on levels of IGF-1 in conditioned medium were assessed. Corticosterone had no effect of levels of IGF-1 in conditioned medium (data not shown).

Effect of Corticosterone on Cell Cycle Regulatory Molecules. Alterations were assessed in cell cycle regulators that might account for the observed inhibition of cell growth and the induction of G_1 arrest in cell cycle progression by corticosterone. On the basis of our published *in vivo* studies showing ER results in lower levels of cyclin D1 and increased levels of KIP1/P27 in rat mammary epithelial cells and tumors (11), attention was focused on the expression of cyclin D1, CDK4, and KIP1/P27, molecules that are also associated with the G_1 phase of the cell cycle (21–23). As shown in Fig. 2, corticosterone treatment resulted in a time-dependent decrease in levels of CDK4 and cyclin D1. Compared with ethanol controls, the reduction in CDK4 protein levels was 12, 45, and 92% ($P < 0.01$; Fig. 2*A*) and in cyclin D1 was 59, 98, and 84% ($P < 0.01$; Fig. 2*B*) after 24, 48, and 72 h of corticosterone treatment at the 200 μM dose, respectively. As shown in Fig. 3, compared with ethanol treated controls, treatment of cells with 50, 100, or 200 μM doses of corticosterone resulted in a significant increase in the levels of KIP1/P27 ($P < 0.01$). In the dose-response study, 48 h of corticosterone treatment at 50, 100, or

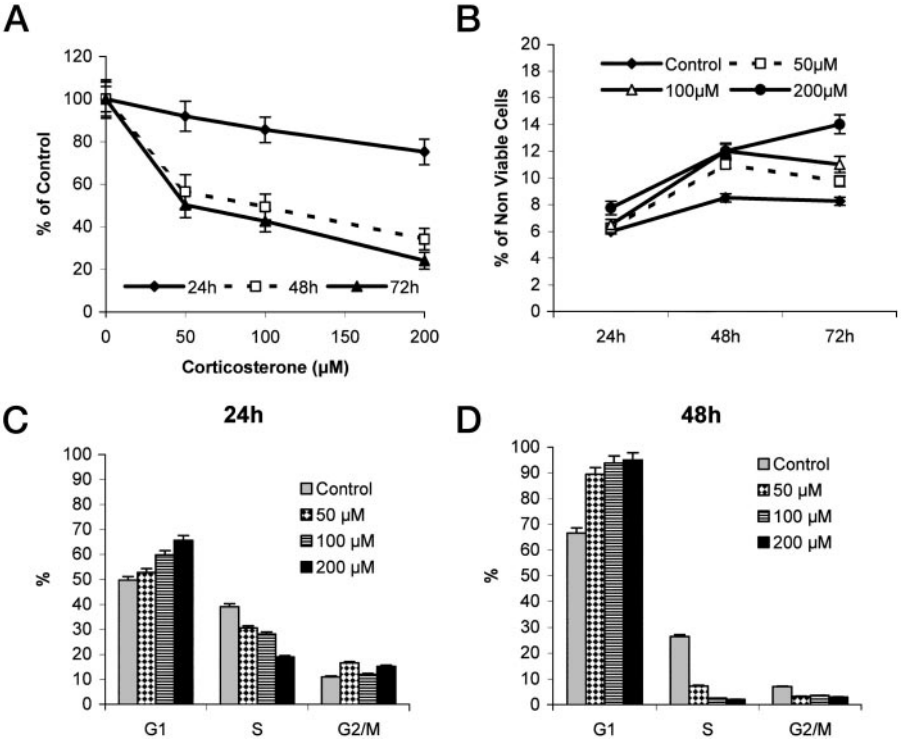

Fig. 1. Effect of corticosterone on cell growth, cell death and cell cycle distribution of TM10 cells. *A*, logarithmically growing semiconfluent cells cultured in 96-well plates were treated with ethanol vehicle alone or corticosterone in ethanol at a concentration of 50, 100, or 200 μM for 24, 48, or 72 h, and the cell growth was determined by crystal violet staining as described in the "Materials and Methods." Data are expressed as the percentage of vehicle-treated control cells. Each data point is a mean of eight replicates. *B*, logarithmically growing semiconfluent cells cultured in 60-mm plate were treated with ethanol vehicle alone or corticosterone in ethanol at a concentration of 50, 100, or 200 μM for 24, 48, or 72 h. Cell viability, expressed as the percentage of nonviable cells, was determined using phase-contrast microscopy after cells were stained with trypan blue as described in "Materials and Methods." Each data point is a mean of triplicate determinations. *C* and *D*, logarithmically growing semiconfluent cells were treated with ethanol vehicle alone or corticosterone in ethanol at a concentration of 50, 100, or 200 μM for 24 (*C*) or 48 h (*D*) as described in "Materials and Methods." Cell cycle distribution was determined by FACS analysis. The data are shown as the percentage of cells in G_1, S, and G_2/M phases. Each *bar* is a mean of triplicate measurements; *bars*, ±SE.

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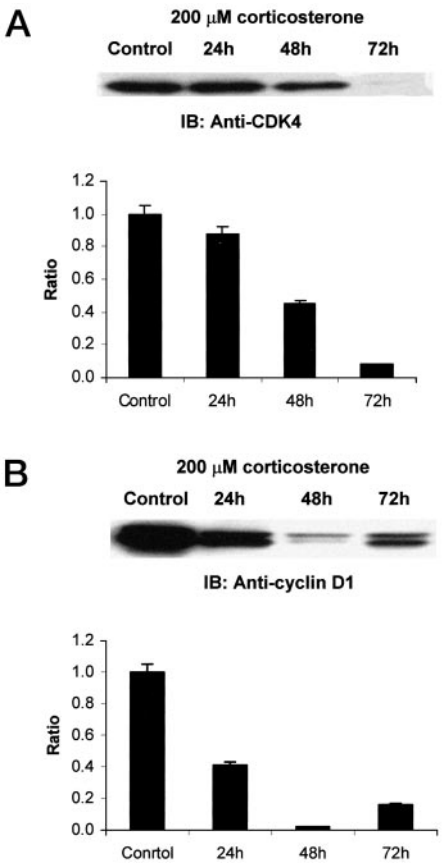

Fig. 2. Effect of corticosterone on levels of CDK4 and cyclin D1 proteins in TM10 cells. Logarithmically growing semiconfluent cells were treated with ethanol vehicle alone or 200 μM doses of corticosterone in ethanol for 24, 48, or 72 h and the levels of CDK4 (*A*) or cyclin D1 (*B*) proteins in cell lysates were determined by Western blotting as detailed in "Materials and Methods." These data are representative of the results from three independent experiments. The data are expressed as the ratio of the actual scanning density units observed in the treated *versus* the control cultures; values are mean. *IB*, Western immunoblot; *bars*, ±SE.

200 μM doses resulted in 4.1, 6.4, and 9.9-fold increase ($P < 0.01$) in KIP1/P27 protein levels as compared with vehicle control, respectively (Fig. 3*A*). Similarly, in the time-response studies, corticosterone treatment at the 200 μM dose for 24, 48, or 72 h resulted in no increase or a 2.5- or 3.9-fold increase ($P < 0.01$) in KIP1/P27 levels as compared with control, respectively (Fig. 3*B*).

KIP1/P27 Binding with CDK4, and CDK4- and Cyclin D1-associated Kinase Activity. Because a significant dose- and time-dependent increase in KIP1/P27 was observed after corticosterone treatment, it was next determined whether up-regulation of this CKI exerted an effect on its binding with G_1 regulator CDK4 as well as on the kinase activity of CDK4 and associated cyclin D1. As shown by data in Fig. 4, *A* and *B*, treatment of TM10 cells with corticosterone for 48 h resulted in a significant decrease, in a dose-dependent manner, of CKD4- (62, 67, and 69% reduction; $P < 0.01$) and cyclin D1- (63, 74, 89% reduction; $P < 0.01$) associated kinase activity. In other studies, as shown in Fig. 4*C*, the decrease in CDK4 and cyclin D1 kinase activity was found to be associated with a significant increase ($P < 0.01$) in the binding of KIP1/P27 to CDK4. The increase started at the 100 μM dose (1.8-fold increase) of corticosterone and was 2.6-fold at the 200 μM dose (Fig. 4*C*).

Effects of Kip1/p27 Antisense Oligonucleotide Transfection on Cell Growth and KIP1/P27 Production. The effect of antisense transfection on cell growth was determined at 24 h after corticosterone treatment. As shown in Fig. 5*A*, corticosterone-induced inhibition of cell growth was reversed by the transfection of Kip1/p27 antisense oligonucleotides. To determine whether the reversal was associated with depletion of KIP1/P27 protein, the levels of KIP1/P27 protein were determined at 24 and 48 h of corticosterone treatment by Western blotting. As shown in Fig. 5*B*, the production of KIP1/P27 protein was blocked by transfection of Kip1/p27 antisense oligonucleotides regardless of corticosterone treatment. Compared with TM10 cells without transfection of Kip1/p27 antisense, the levels of KIP1/P27 protein in TM10 cells transfected with Kip1/p27 antisense were reduced by 77% and 79% at 24 and 48 h after treatment with 200 μM corticosterone, respectively. As a positive control, the levels of KIP1/P27 protein in serum-deprived TM10 cells with or without transfection of Kip1/p27 antisense were also examined (Fig. 5*B*). Compared with TM10 cells without Kip1/p27 antisense transfection, the production of KIP1/P27 protein in Kip1/p27 antisense-transfected TM10 cells was decreased by 87%.

Effects of RU-486 and/or Corticosterone on Cell Growth and P27. To determine whether the cytostatic effects of corticosterone were mediated through the glucocorticoid receptor, a glucocorticoid receptor antagonist, RU-486, was used. As shown in Fig. 6*A*, RU-486 abolished the growth inhibitory activity of corticosterone. Moreover, the increase in cellular levels of P27 that was induced by corticosterone was blocked by concomitant treatment of cells with RU-486 (Fig. 6*B*).

Induction of Apoptosis. On the basis of the data showing that corticosterone inhibited TM10 cell growth, experiments were conducted to determine whether growth inhibition was associated with the induction of either apoptotic or necrotic cell death. This was done by counting annexin-V and propidium iodide-stained cells using FACS analysis. As shown in Fig. 7, *A* and *B*, corticosterone treatment of TM10 cells resulted in only mild apoptotic cell death and no

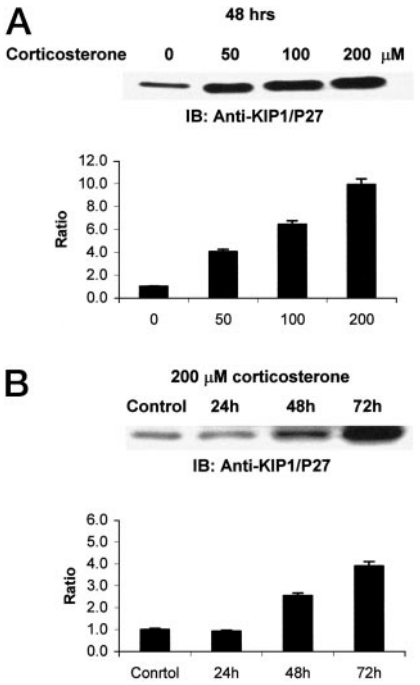

Fig. 3. Effect of corticosterone on levels of KIP1/P27 protein in TM10 cells. Logarithmically growing semiconfluent cells were treated with ethanol vehicle alone or corticosterone in ethanol at a concentration of 50, 100, or 200 μM for 48 h (*A*), or corticosterone at concentration of 200 μM in ethanol for 24, 48, or 72 h (*B*) as described in "Materials and Methods." The levels of KIP1/P27 protein in cell lysates were determined by Western blotting. These data are representative of the results from three independent experiments. The data are expressed as the ratio of the actual scanning density units observed in the treated *versus* the control cultures; values are means. *IB*, Western immunoblot; *bars*, ±SE.

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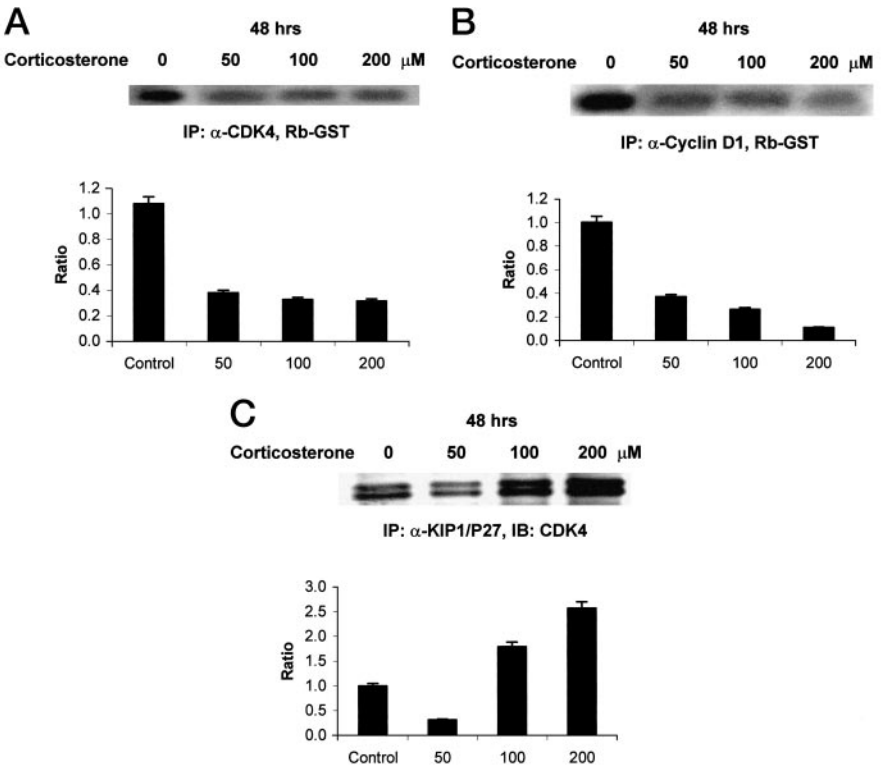

Fig. 4. Effect of corticosterone on CDK4- and cyclin D1-associated kinase activity, and binding of KIP1/P27 to CDK4 in TM10 cells. Logarithmically growing semiconfluent cells were treated with ethanol vehicle alone or corticosterone in ethanol at a concentration of 50, 100, or 200 μM for 48 h. At the end of these treatments, CDK4- (*A*) and cyclin D1- (*B*) associated kinase activity, and binding of KIP1/P27 to CDK4 (*C*) in the cell lysates were determined as described in "Materials and Methods." These data are representative of the results of three independent experiments. The data are expressed as the ratio of the actual scanning density units observed in the treated *versus* the control cultures; values are means. *IP,* immunoprecipitation; *IB,* Western immunoblot; *bars,* ±SE.

necrotic cell death. Together, these observations suggested that corticosterone treatment of TM10 cells does not result in cytotoxicity via either an apoptotic or necrotic cell death mechanism.

Reversibility of Cell Growth Inhibition. These findings led to the question, "what is the fate of growth-arrested cells?" To answer this question, TM10 cells were treated for 72 h with different doses of corticosterone, and thereafter, corticosterone was removed from the medium and the fold change in cell number per day was monitored for another 72 h. As shown in Fig. 8*A* and as anticipated, compared with ethanol control, treatment of cells for 72 h with corticosterone resulted in 26, 35, and 54% inhibition of cell growth at 50, 100, or 200 μM doses, respectively. However, when the fold change in cell number per day data were analyzed for 24, 48, and 72 h after the removal of the corticosterone from the medium, as shown by data in Fig. 8*B*, there was no noticeable difference between different treatments *versus* ethanol control. Together, these data suggest that the growth inhibitory effect of corticosterone is cytostatic and that the cytostatic effect is reversible when the agent is removed.

DISCUSSION

Our laboratory has reported that ER inhibits the progression of premalignant mammary gland hyperplasias to carcinomas and that the inhibitory activity of ER against mammary carcinomas is correlated with increased adrenal cortical steroid secretion (1). These findings prompted us to determine whether effects on mammary cancer comparable with those induced by ER could be achieved via increasing plasma cortical steroid levels by dietary administration of corticosterone; inhibition of carcinogenesis was observed. However, because both ER and dietary corticosterone were observed to induce a concomitant decrease in plasma levels of IGF-1,[4,5] it is likely that dissociating *in vivo* the effects of corticosterone *per se* on cellular and molecular mechanisms from those attributable to IGF-1 will be difficult. Hence, the *in vitro* studies reported in this paper were initiated.

Glucocorticoids such as corticosterone in rodents and cortisol in humans are important regulatory molecules that govern metabolism and development (24–28). Glucocorticoids have been shown to be potent antiproliferative agents in many cell types (29, 30), and are also known to induce a G_1 arrest and programmed death of several leukemia cell lines (31–33). Whereas these diverse effects of glucocorticoid hormones have been well documented, their mechanisms remain unclear. Hence three questions were investigated: (*a*) is corticosterone-mediated growth inhibition of a mammary hyperplastic epithelial cell line *in vitro* accounted for by disruption of cell cycle machinery; (*b*) is growth inhibition accompanied by the induction of apoptosis; and (*c*) is growth inhibition reversible? Because we were unaware of other work in this or similar mammary hyperplastic cell lines in which effects of corticosterone had been investigated, initial range finding studies were conducted to determine the concentrations of corticosterone in cell culture medium that would be growth inhibitory. Surprisingly, levels of corticosterone in the range of 3.25–25 μM had little effect on this cell line under conditions in which the culture medium was supplemented with levels of serum and growth factors routinely used to maintain these cells. This may be because of altered sensitivity to corticosterone when cells are grown on a plastic substratum but is clearly not because of the absence of a functional glucocorticoid receptor, because as shown in Fig. 6, growth inhibition by corticosterone blocked the glucocorticoid receptor antagonist RU-486. Interestingly, as shown in Fig. 1, *A* and *B*, levels of corticosterone between 50 and 200 μM were dose-dependently growth inhibitory without affecting cell viability. Consequently, all of the subsequent studies were done at these concentrations of corticosterone recognizing that whereas these concentrations are considerably higher than physiological levels of exposure observed under ER, our goal was to determine, using an *in vitro* model system, what cellular and molecular events were induced in mammary epithelial cells that were growth inhibited by noncytotoxic doses of corticosterone in the presence of amounts of serum and growth factors normally used to maintain this cell line.

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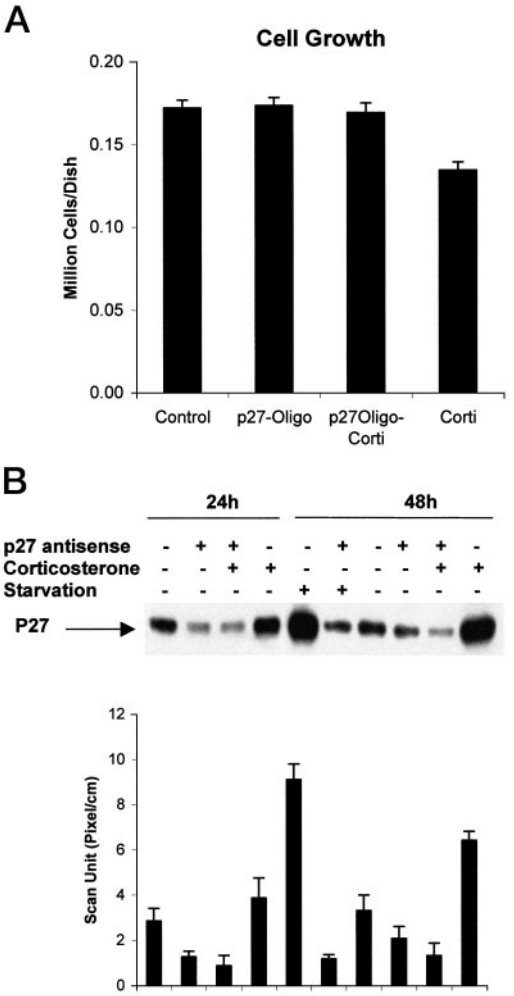

Fig. 5. Effects of Kip1/p27 antisense transfection on cell growth and KIP1/P27 production in TM10 cells. Logarithmically growing semiconfluent cells were cultured with or without p27 antisense oligonucleotides (p27-Oligo) for 24 h and exposed to ethanol vehicle alone or 200 μM corticosterone in ethanol as described in "Materials and Methods." Cell growth at 24 h of corticosterone (*Corti*) exposure was determined by counting cell number (*A*), and levels of P27 protein at 24 or 48 h of corticosterone exposure and 48 h of serum starvation (positive control) were determined by Western blotting (*B*) as described in "Materials and Methods." These data are representative of the results of three independent experiments. Values are means; *bars*, ±SE.

As shown in Fig. 1, *C* and *D*, corticosterone induced a dose- and time-dependent inhibition in the G_1 phase of the cell cycle. This observation is consistent with our previous report that ER induces inhibition of cell proliferation *in vivo* (9). Moreover, it indicates that it is possible to achieve this effect in the absence of concomitant changes in exposure to extracellular growth factors such as IGF-1 because corticosterone treatment had no effect on levels of IGF-1 in the conditioned medium obtained from treated cells. The observation that corticosterone induced a G_1 arrest prompted us to focus our investigation on cell cycle regulatory molecules involved in progression through early G_1. This decision was made because our *in vivo* data published previously provided immunohistochemical evidence that ER or dietary administration of corticosterone was associated with a reduction in the percentage of mammary epithelial cells that stained positive for the cyclin D1 (11). Because cell cycle progression is regulated by several different CDKs that form catalytic complexes with their respective cyclin partner (22, 23), this work concentrated on the levels and activity of CDK4 that is a primary catalytic partner of cyclin D1 and that initiates the phosphorylation of Rb during early G_1 (21–23, 34). As shown in Fig. 2, *A* and *B*, levels of both CDK4 and cyclin D1 were markedly down-regulated with exposure to increasing concentrations of corticosterone. Thus, we predicted that the level of kinase activity of this complex also would be lower in corticosterone-treated cells. As shown in Fig. 4, *A* and *B*, kinase activity of the CDK4-cyclin D1 complex immunoprecipitated with anti-CDK4 or anticyclin D1 was markedly reduced. The magnitude of these effects was sufficient to account, at least in part, for the arrest of the cell cycle as demonstrated in Fig. 1, *C* and *D*. This finding also is consistent with evidence that activation of the glucocorticoid receptor results in growth arrest with associated changes in the regulation of the CDK4-cyclin D1 catalytic complex (35)

Active cyclin-CDK complexes can be inactivated by binding with CKIs. Our previous *in vivo* work indicated that CKIs of the Cip/Kip family but not the inhibitor of cyclin-dependent kinase family were regulated by ER or dietary corticosterone administration (11, 12). Specifically, the results of immunohistochemical analyses showed that a larger percentage of mammary epithelial cells stained positive for KIP1/P27 in animals subjected to ER or fed corticosterone in the diet (11, 12). As shown in Fig. 3, *A* and *B*, levels of KIP1/P27 protein were markedly induced by corticosterone treatment thus demonstrat-

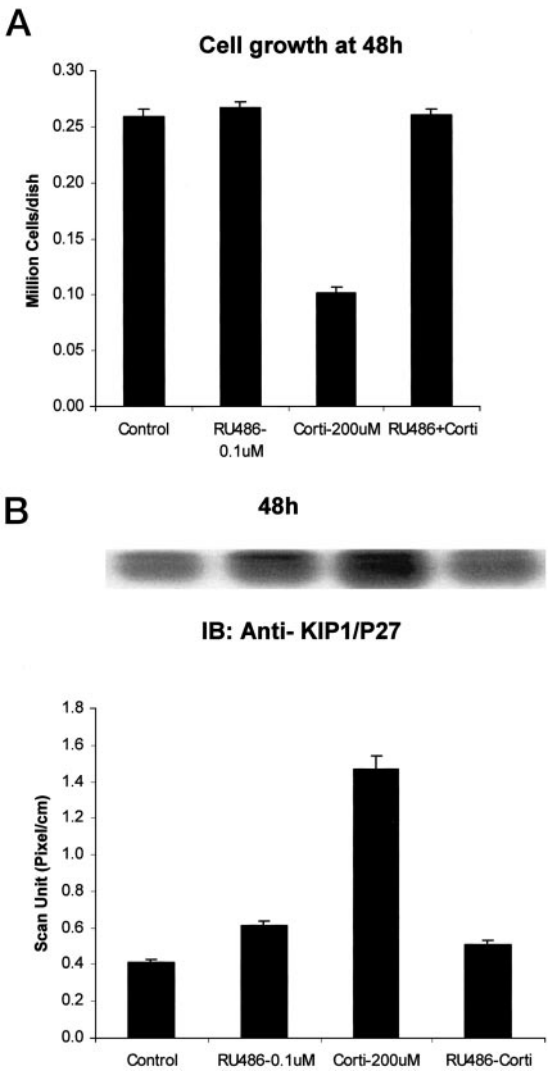

Fig. 6. Blockage of glucocorticoid receptor by RU-486. Logarithmically growing semiconfluent cells were treated with ethanol vehicle (*control*), 0.1 μM RU-486 (*RU486–0.1uM*), 200 μM corticosterone (*Corti-200uM*), or 0.1 μM RU-486 plus 200 μM corticosterone (*RU486+Corti*) in ethanol for 48 h. The cell number was determined by cell counting (*A*), and P27 level was determined by Western blot (*B*) as described in "Materials and Methods." In each case, the data are mean of three measurements; *bars*, ±SE.

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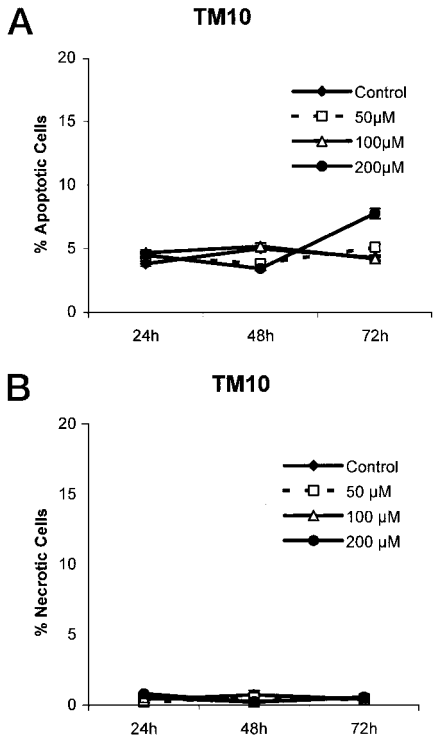

Fig. 7. Effect of corticosterone on apoptosis and necrosis of TM10 cells. Logarithmically growing semiconfluent cells were treated with ethanol vehicle alone or corticosterone in ethanol at a concentration of 50, 100, or 200 μM for 24, 48, or 72 h as described in "Materials and Methods." Apoptotic cell death was determined by FACS analysis (*A*) after staining with annexin V; and necrotic cell death was determined by FACS analysis (*B*) after staining with propidium iodide as described in "Materials and Methods." Each data point is mean of triplicate measurements; *bars*, ±SE.

ing that this effect can be achieved in the absence of a concomitant change in extracellular exposure to growth factors such as IGF-1. The fact that this effect of corticosterone was blocked by concomitant treatment of cells with RU-486 (Fig. 6*B*) is consistent with published reports that activation of the glucocorticoid receptor causes an increase in cellular levels of KIP1/P27 (35). Whereas some work indicates that CDK2 is the primary target of the inhibitory activity KIP1/P27 (36), other reports show that KIP1/P27 inhibits many CDKs and that at high levels of expression of KIP1/P27 the activity of the CDK4-cyclin D1 complex is inhibited (37, 38). As shown in Fig. 4*C*, in the presence of elevated levels of KIP1/P27 induced by corticosterone treatment, KIP1/P27 binding to the cyclin D1-CDK4 complex was increased. This observation is consistent with the lower kinase activity reported in Fig. 4, *A* and *B*, and shows that the up-regulation of this CKI by corticosterone does result in increased binding to a cyclin-CDK complex.

In pursuing the effects of corticosterone on cell cycle regulation, we judged that it was unlikely that additional investigation of the effects in elevated KIP1/P27 on the activity of other kinase complexes, such as cyclin E-CDK2, would provide significant additional insight in the *in vitro* model used in this study. The rationale underlying this decision was that there was little reason to expect that levels of cyclin E or CDK2, or the activity of this complex would be above basal levels in corticosterone-treated cells given the marked down-regulation of the kinase activity of the CDK4-cyclin D1 complex. Therefore, it was decided to determine the effects on cell growth inhibition of blocking corticosterone induction of KIP1/P27 using a Kip1/p27 antisense strategy. As shown in Fig. 5, treatment with Kip1/p27 antisense oligonucleotides blocked the growth-inhibitory activity of corticosterone treatment and was associated with depletion of cellular levels of KIP1/P27. These findings indicate that induction of KIP1/P27 is essential to growth inhibition by corticosterone. However, because in other systems corticosterone has been reported to exert independent effects on cell cycle progression via the cyclin D1-CDK4 catalytic complex (35), the ability of Kip1/p27 antisense treatment to fully reverse growth inhibition was unanticipated and requires additional investigations, using other model systems and experimental approaches, to define the mechanisms that account for this effect.

The second question addressed in this study was whether corticosterone would induce mammary epithelial cells to undergo apoptosis. On the basis of our *in vivo* observations of the effects of ER or corticosterone on apoptosis induction in mammary epithelial cells (9, 12), and the reported effects of glucocorticoids on apoptosis induction in other cell types, we predicted that growth inhibition reported in Fig. 1 would be accompanied by induction of apoptosis and possibly secondary necrosis. However, as shown in Fig. 7, such effects were not observed. This finding was unexpected, and it implies that the *in vivo* effects of ER or corticosterone administration on apoptosis are more likely to be because of the reduction in circulating levels of IGF-1 or some other survival factor(s) that these treatments modulate concomitantly with the change in plasma corticosterone. Thus, it appears that cortical steroids *in vitro* mimic some but not all of the effects observed *in vivo* in response to either ER or dietary administration of corticosterone.

The third question investigated in this study was whether the growth inhibitory activity of corticosterone was reversible. Again, based on *in vivo* evidence and the expectation that corticosterone would induce apoptosis, we hypothesized that growth inhibition

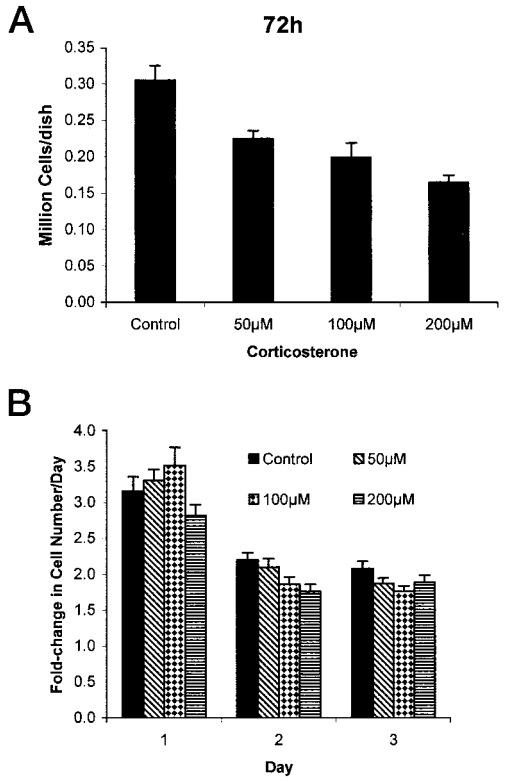

Fig. 8. Reversible effect of corticosterone on growth of TM10 cells. Logarithmically growing semiconfluent cells were treated with ethanol vehicle alone or corticosterone in ethanol at a concentration of 50, 100, or 200 μM for 72 h (*A*), after identical treatments for 72 h cells were washed three times with medium to remove corticosterone, and grown in fresh medium without corticosterone for another 24, 48, and 72 h (*B*). The cell number was determined by cell counting and fold change in cell number per day was calculated as described in "Materials and Methods." In each case, the data are mean of three measurements; *bars*, ±SE.

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would be permanent. However, as summarized in Fig. 8, growth inhibition was clearly reversible. Thus, the profound growth inhibitory effects of corticosterone are cytostatic. This implies that, at least from the viewpoint of the action of cortical steroids, the effects of ER *in vivo* would be reversible. However, given the concomitant effects of ER or dietary administration of corticosterone on other hormones and growth factors, this issue can only be answered via *in vivo* investigation.

In summary, the results of the present study are consistent with the findings of *in vivo* studies in which decreased cyclin D1 and increased KIP1/P27 were found in mammary epithelial cells and tumors from animals subjected to ER or administered corticosterone in the diet (11, 12). In the absence of effects on extracellular levels of growth factors like IGF-1, corticosterone inhibited mammary epithelial cell growth and arrested cells in the G_1 phase of the cell cycle. These effects were likely because of an increase in intracellular levels of KIP1/P27 with a concomitant reduction in the kinase activity of the CDK4-cyclin D1 catalytic complex. However, unlike the *in vivo* effects of ER or dietary corticosterone, corticosterone treatment *in vitro* failed to induce apoptosis. The fact that cell growth inhibition was reversible is consistent with the lack of induction of apoptosis and indicates the need to determine *in vivo* whether the protective effects of ER against mammary carcinogenesis require sustained restriction of energy intake for inhibitory activity to be maintained.

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Mechanisms of Energy Restriction: Effects of Corticosterone on Cell Growth, Cell Cycle Machinery, and Apoptosis

Weiqin Jiang, Zongjian Zhu, Neehar Bhatia, et al.

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