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Research Article

Phorbol ester up-regulates aldose reductase expression in A549 cells: a potential role for aldose reductase in cell cycle modulation

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Abstract. Over-expression of aldose reductase (AR) has been observed in many cancer cells. To clarify the role of AR in tumor cells, we investigated the pathways mediating expression of the AR gene induced by 12-O-tetradecanoylphorbol-13-acetate (TPA), a potent tumor promoter. In A549 human lung adenocarcinoma cells, TPA elicited a dose- and time-dependent increase in AR mRNA level with an elevated enzyme activity. The TPA-induced increase in mRNA level and promoter activity of the AR gene was significantly attenuated in the presence of an inhibitor of protein kinase C, tyrosine kinase, or nuclear factor κB (NF-κB). TPA augmented the NF-κB-dependent gene transcription, indicating the involvement of NF-κB in this regulation. Accumulation of TPA-treated cells in S phase was almost completely abolished in the presence of ethyl 1-benzyl-3-hydroxy-2(5H)-oxopyrrole-4-carboxylate, an AR inhibitor. Taken together, TPA augmented the promoter activity of the AR gene via the activation of protein kinase and NF-κB. The inhibition of AR may assist in the chemotherapy of malignant tumors by suppressing the rapid growth of cancer cells.

Key words. Aldose reductase; cancer; nuclear factor κB; phorbol ester; protein kinase C.

Aldose reductase (AR; EC 1.1.1.21), which is a member of the NADPH-dependent aldo-keto reductase (AKR) family [1], catalyzes the reduction of the aldehyde form of glucose to sorbitol. Sorbitol is subsequently converted to fructose by NAD^+-dependent sorbitol dehydrogenase. This is the so-called polyol pathway, an alternative route of glucose metabolism. AR has been studied for its involvement in the pathogenesis of various diabetic complications [2, 3]. However, the physiological function of AR has not been fully elucidated. AR exhibits broad substrate specificity for a variety of aldehydes including catecholamine and steroid metabolites [3]. The enzyme also catalyzes the reduction of methylglyoxal, a toxic aldehyde product of glucose [4]. Along with methylglyoxal, such reactive aldehydes with an exogenous origin or produced during lipid peroxidation, such as acrolein [5] and 4-hydroxy-2,3-trans-nonenal [6], are also good substrates for AR. The enzyme may therefore serve as an extrahepatic detoxification enzyme against endogenous and xenobiotic aldehydes in various tissues or cells. The over-expression of the gene encoding AR was reported to increase tolerance against methylglyoxal in *Saccharomyces cerevisiae* [7].

The AKR family including AR is over-expressed in a human hepatocellular carcinoma (HCC) and a chemically

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induced rat hepatoma. They were identified as tumor-associated or cancer-related proteins [8, 9]. Although the function of AR in the cancer cells is unclear, AR was suggested to detoxify the reactive aldehyde compounds produced by the augmented metabolism of fast-growing carcinoma cells [10]. In line with such a postulation, the reduced efficacy of chemotherapeutic agents used to treat breast cancer was associated with increased AR expression and the ensuing accumulation of intracellular sorbitol [11]. Furthermore, the over-expression of AR induced by hypertonicity in HepG2 cells, a stable line of human hepatocellular carcinoma cells, made the cells more resistant to daunorubicin, an anti-cancer agent [12]. The suppression of AR activity in HeLa cells by ethyl 1-benzyl-3-hydroxy-2(5H)-oxopyrrole-4-carboxylate (EBPC), an AR inhibitor, enhanced the sensitivity of the cells against genotoxic chemotherapeutic agents such as doxorubicin and cisplatin [13]. Although the molecular mechanism(s) underlying the resistance of cancer cells to the chemotherapeutic agents is unclear, AR appears to activate the signaling pathway(s) and regulate the expression of genes essential to the survival or growth of cancer cells such as nuclear factor κB (NF-κB) and adhesion molecules [14, 15].

We previously demonstrated that reactive oxygen species (ROS) and a p38 mitogen-activated protein (MAP) kinase pathway were involved in methylglyoxal-induced AR gene expression in rat vascular smooth muscle cells [16]. We also reported that the epidermal growth factor receptor-extracellular signal-regulated kinase (ERK) pathway was the major signaling pathway involved in the induction of AR gene expression by hydrogen peroxide in A7r5 cells, a rat vascular smooth muscle cell line [17]. On the other hand, the regulatory mechanisms of AR gene expression and the role of AR in tumor cells are not fully understood. Since 12-O-tetradecanoylphorbol-13-acetate (TPA) is a potent tumor promoter, we elucidated the signaling pathway(s) involved in the TPA-induced up-regulation of AR gene expression, and the role of AR in A549 human lung adenocarcinoma cells. We report here that the activation of protein kinase C (PKC) and tyrosine kinase by TPA augmented the promoter activity of the AR gene via NF-κB, and the inhibition of AR activity abolished the accumulation of TPA-treated cells in the S phase.

Materials and methods

Materials

Leupeptin, cycloheximide, pepstatin A, phenylmethanesulfonyl fluoride (PMSF), TPA, actinomycin D, pyrrolidinedithiocarbamate (PDTC), aprotinin, MG-132, and the antibody for actin (0.7 mg/ml, no. A 2066) were obtained from Sigma-Aldrich (St. Louis, MO). EBPC was purchased from Tocris Cookson (Avonmouth, UK). AG 18, genistein, and bisindolylmaleimide I were purchased from Calbiochem. (La Jolla, Calif.). Penicillin, streptomycin, trypsin-EDTA, and fetal bovine serum were obtained from Invitrogen (Carlsbad, Calif.). Dulbecco's modified Eagle's medium (DMEM) was purchased from Biowhittaker (Walkersville, Md). Rabbit polyclonal antibodies specific to NF-κB p65 (0.2 mg/ml, C-20), goat anti-rabbit IgG-HRP antibody (0.4 mg/ml), and goat anti-mouse IgG-HRP antibody (0.4 mg/ml) were purchased from Santa Cruz Biotechnology (Santa Cruz, Calif.). The rabbit polyclonal antibody specific to IκB-α (no. 9242) and mouse monoclonal antibody against phosphorylated IκB-α (Ser 32/36, no. 9246) were obtained from Cell Signaling (Beverly, Mass.). [α-^{32}P]dCTP (3000 Ci/mmol) and [γ-^{32}P]dATP (3000 Ci/mmol) were purchased from ICN Pharmaceuticals (Irvine, Calif.). The luciferase assay system, the β-galactosidase enzyme assay system, the Gel shift assay system, and the NF-κB consensus oligonucleotide were obtained from Promega Co. (Madison, Wis.). SuperFect transfection reagent was obtained from Qiagen (Valencia, Calif,). NE-PER nuclear and cytoplasmic extraction reagents was purchased from Pierce Biotechnology (Rockford, Ill.). Other reagents were of the highest grade available.

Cell culture

The A549 human lung adenocarcinoma cell line was obtained from ATCC (Manassas, Va.). The cells were maintained in DMEM containing 100 U/ml penicillin and 100 µg/ml streptomycin, supplemented with 10% heat-inactivated fetal bovine serum at 37 °C under an atmosphere of 95% air and 5% CO_2. The A549 cells (1×10^6 cells) were seeded in 100-mm dishes and cultured for 2–3 days. When the cells reached confluence, they were harvested by trypsinization for subculture and the following experiments. The cells were preincubated overnight prior to being treated with various reagents.

Northern blot analysis

A549 cells (5×10^5 cells in a 60-mm dish), which were pretreated with or without 40 µg/ml cycloheximide, 4 µM actinomycin D, 1 µM bisindolylmaleimide I, or various concentrations of genistein or PDTC, were exposed to 20 nM TPA for the indicated times. The total RNA was isolated by extraction with acid guanidium thiocyanate-phenol-chloroform as described elsewhere [18]. Aliquots of 5 µg of the total RNA, heated-denatured at 65 °C for 15 min in a gel running buffer (40 mM MOPS, 10 mM sodium acetate, and 1 mM EDTA, pH 7.0) containing 50% formamide were electrophoresed on 1% agarose gels containing 2.2 M formaldehyde. The size-fractionated RNA was transferred to Hybond-N$^+$ nylon membranes (Amersham Biosciences, Amersham UK) and hybridized with a ^{32}P-labeled human AR cDNA probe at 68 °C in QuikHyb solution (Stratagene, La Jolla, Calif.), as described previously [16].

Assay for AR activity

The cells treated with 20 nM TPA for the indicated times were washed with ice-cold PBS (pH 7.4) and harvested by scraping. The cell suspensions were homogenized using a glass Dounce homogenizer in a 20 mM sodium phosphate buffer (pH 7.0) containing 2 mM dithiothreitol (DTT), 5 μM leupeptin, 2 μM pepstatin, and 1 μM aprotinin, and 20 μM PMSF. After centrifuging the homogenate for 10 min at 2000 g, the supernatant fraction was used for the enzyme assay. The activity of AR was determined in reaction mixtures containing 0.1 M sodium phosphate buffer (pH 6.2), 150 μM NADPH, 10 mM DL-glyceraldehyde, and the enzyme solution in a total volume of 1 ml, as described previously [19].

Preparation of nuclear, cytoplasmic, and total cell extracts

The cellular compartment was extracted using a minor modification of the procedure described previously [20]. Briefly, cells treated with 20 nM TPA for the indicated time were lysed in buffer A [10 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 0.5 mM DTT, 5 μM leupeptin, 2 μM pepstatin A, 1 μM aprotinin, and 20 μM PMSF] by repeated freezing and thawing. The nuclear and cytoplasmic fractions were separated by centrifugation at 1000 g. The supernatant, a cytosolic extract, was obtained by further centrifugation at 10,000 g in a microcentrifuge for 15 min. The supernatant fraction of the pellets resuspended in buffer B [10 mM Tris·Cl (pH 7.5), 0.5% deoxycholate, 1% Nonidet P-40, 5 mM EDTA, 0.5 mM DTT, 5 μM leupeptin, 2 μM pepstatin A, 1 μM aprotinin, and 20 μM PMSF] containing the nuclear proteins was collected after being centrifuged at 10,000 g for 20 min. The total cell extracts were isolated by pretreating the cells with or without 10 μM MG-132 for 60 min and treated with 20 nM TPA for the indicated times, which was followed by and lysing in a PRO-PREP Protein Extraction Solution (iNtRON Biotechnology, Seoul, Korea) for 90 min at –20 °C. The cell lysates were centrifuged at 10,000 g for 20 min, and the supernatants (total cell extracts) were collected. Protein concentration was determined by the Bradford method using bovine serum albumin as the standard.

Western blot analysis

An aliquot of the protein was subjected to SDS-polyacrylamide gel electrophoresis and transferred onto Hybond-P^+ polyvinylidene difluoride membrane (Amersham Biosciences). The membranes, which were blocked with 5% nonfat milk in Tris-buffered saline (TBS) containing 0.1% Tween-20 for 2 h at room temperature, were reacted with the appropriate antibody in TBS containing 1% bovine serum albumin (BSA) and 0.05% Tween-20 overnight at 4 °C. The AR protein was detected with monoclonal anti-human AR antibody [21]. The resulting membranes were then incubated with peroxidase-conjugated goat anti-rabbit or anti-mouse antibody (1:3000) for 2 h at room temperature. After washing in TBS containing 0.1% BSA and 0.1% Tween-20, the immunoreactive bands were detected using an ECL detection system (Amersham Biosciences).

Preparation of nuclear extracts and the electrophoretic mobility shift assay

A549 cells (2.5×10^6 cells in a 100-mm dish), which had been pretreated with or without 300 μM genistein, 1 μM bisindolylmaleimide I, 50 μM PDTC, or 300 μM AG18 (tyrphostin A23) for 30 min were exposed to 20 nM TPA for 1 h. The nuclear extracts were isolated with NE-PER nuclear and cytoplasmic extraction reagents. A DNA probe was prepared by end-labeling an oligonucleotide containing the NF-κB consensus sequence (5′-AGT TGA GGG GAC TTT CCC AGG C-3′) with [γ-^{32}P]dATP. The nuclear proteins (5 μg) were incubated in a total volume of 10 μl for 30 min at room temperature in a binding reaction buffer containing 50 mM Tris (pH 7.5), 250 mM NaCl, 2.5 mM DTT, 2.5 mM EDTA, 5 mM $MgCl_2$, 20% glycerol, 0.25 mg/ml poly(dI-dC), and 115,000 cpm ^{32}P-labeled oligonucleotide. The protein-DNA complexes were resolved in 4% nondenaturing polyacrylamide gels, and detected by autoradiography.

Reporter assay

The AR promoter-luc construct, a 3.2-kb fragment of the 5′-flanking region of the mouse AR gene placed upstream of the pGL2 luciferase reporter vector [22], was used to analyze the promoter activity. A plasmid, pNFκB-Luc (Stratagene), was used to evaluate the transactivation of NF-κB. The cells were plated in six-well plates at a density of 1×10^5 cells per well 18–24 h prior to the transfection. The cells were then cotransfected with 1.5 μg of the reporter construct and 0.5 μg of the SV40 β-galactosidase expression vector (pSV β-Gal vector, Promega) using a SuperFect reagent (Qiagen, Valencia, Calif.). After incubation for 18–24 h, the cells pretreated with or without 300 μM genistein, 1 μM bisindolylmaleimide I, or 50 μM PDTC for 30 min were exposed to 20 nM TPA for 24 h and lysed in a luciferase reporter lysis buffer (Promega). An aliquot of the total lysates was used to determine the luciferase activity with Microlumat Plus LB96V (EG&G Berthold, Bad Wildbad, Germany). The variations in transfection efficiency were normalized to the β-galactosidase activity determined by the enzyme assay system according to the manufacturer's protocol (Promega).

Flow cytometry analysis

The A549 cells were plated at a density of 5×10^5 in 100-mm dishes. After an overnight incubation, the cells in fresh media were treated with 50 μM EBPC for 30 min. TPA was then added to the medium, and the cells were further incubated for 24 h. The cells were then trypsinized and collected by centrifugation at 1000 rpm for 5 min.

The cell pellets were washed twice with PBS and resuspended in PBS containing 0.05 µg/ml propidium iodide, 10 mM Tris (pH 8.0), 1 mM NaCl, 0.1% NP-40, and 0.7 µg/ml RNase A. The cell suspension was analyzed using a FACScaliber Flow Cytometer.

Statistical analysis

A Student's *t* test was used to compare the means. All the data are expressed as a mean ± SE.

Results

TPA induced expression of the AR gene and increased enzyme activity in A549 cells

Exposure of A549 cells to TPA increased the expression of the AR transcript. The TPA-induced expression of AR mRNA was concentration and time dependent. Maximum levels were obtained after 24 h of exposure with 10–100 nM TPA (fig. 1A). When the cells were treated with 20 nM TPA, an increase in AR mRNA level was detected at 12 h, and reached a maximum at 72 h (fig. 1B). The activity of AR in TPA-treated cells was measured to determine if the increased AR mRNA led to an elevation in enzyme activity. A significant increase in AR activity was detected after 24 h, which continued for up to 72 h of incubation with TPA (fig. 2A). An elevated level of AR protein was also demonstrated by Western blot analysis in cells treated with TPA for 72 h (fig. 2B). The TPA-induced increase in AR mRNA was thus followed by an increase in enzyme activity and protein level.

Cycloheximide and actinomycin D abolished TPA-induced AR gene expression

To examine the mechanisms involved in the TPA-induced increase in the AR mRNA level, the effects of actinomycin D, an RNA synthesis inhibitor, and cycloheximide, a protein synthesis inhibitor on TPA-induced AR gene expression were evaluated (fig. 3). A549 cells constitutively expressed a moderate level of AR mRNA. The induction of AR mRNA by TPA was completely abolished in the presence of either actinomycin D or cycloheximide, while they did not affect the basal AR expression level.

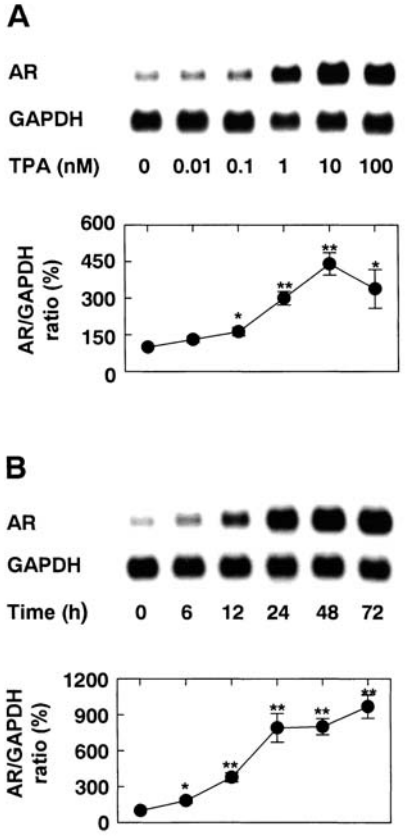

Figure 1. Induction of AR mRNA by TPA in A549 cells incubated with various TPA concentrations for 24 h (*A*) or with 20 nM TPA for the indicated times (*B*). Total RNA was extracted and subjected to Northern blot analysis, as described in Materials and methods. The intensity of the bands was quantified by an image analyzer, and plotted as the percentage of AR to GAPDH mRNA ratio. The vertical bars represent the SE (n = 3); *p < 0.05, **p < 0.01, compared with the control.

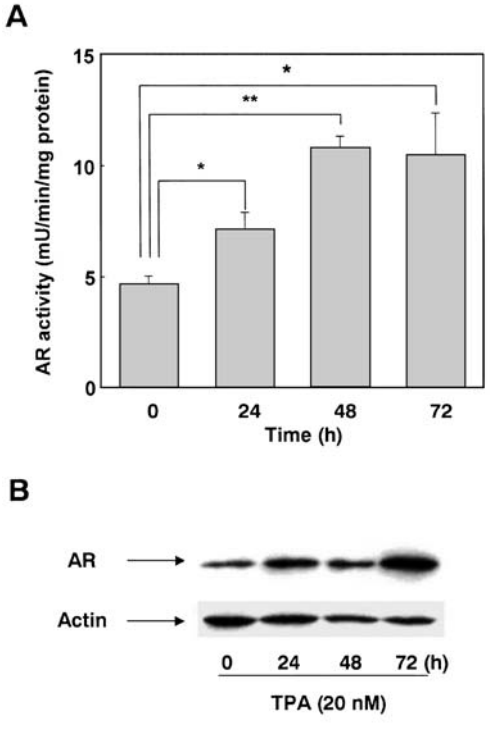

Figure 2. Effects of TPA on AR activity and protein expression in A549 cells. (*A*) The enzyme activity of AR in the supernatant fraction of cells treated with 20 nM TPA was measured spectrophotometrically as described in Materials and methods. The bars represent the means ± SE (n = 3); *p < 0.05, **p < 0.01, compared with the control. (*B*) Protein levels of AR in A549 cells treated with 20 nM TPA. AR protein in the supernatant fraction was analyzed by Western blot with monoclonal anti-human AR antibody as described in Materials and methods.

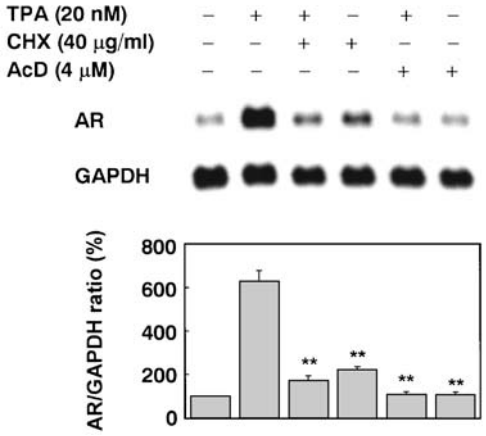

Figure 3. Effects of actinomycin D and cycloheximide on TPA-induced AR mRNA expression. A549 cells were incubated with 20 nM TPA for 24 h in the presence or absence of cycloheximide (CHX; 40 μg/ml) or actinomycin D (AcD; 4 μM). The bars represent the means ± SE (n = 3); **p < 0.01, compared with the TPA-only group.

These results indicated that the de novo synthesis of mRNA as well as the de novo synthesis of the protein(s) that act on the AR gene promoter are essential for the induction of the AR mRNA observed in the TPA-treated A549 cells.

Protein kinase inhibitors attenuated TPA-induced AR gene expression

A549 cells were pretreated with 1 μM bisindolylmaleimide I, a PKC inhibitor, to determine if activation of PKC by TPA indeed elicits AR gene expression. As shown in figure 4A, TPA-induced expression of AR mRNA was significantly attenuated in the cells pretreated with the inhibitor. These results suggested the involvement of PKC in the induction of AR mRNA by TPA.

The effects of genistein, a tyrosine kinase inhibitor, were also investigated. When the cells were pretreated with genistein for 30 min, the TPA-induced increase in the AR mRNA level was suppressed in a dose-dependent manner (fig. 4B), indicating that the tyrosine kinase-mediated signaling pathway(s) may be involved in the TPA-induced up-regulation of AR gene expression.

Involvement of NF-κB in TPA-induced AR gene expression

Among the putative recognition sequences depicted in the analyses of the promoter region of the AR gene, the osmotic response element (ORE) appears to be essential for the enhanced AR expression after exposure to tumor necrosis factor-α [23]. Since ORE differs from the NF-κB binding sequence by a single base pair, this study investigated the involvement of NF-κB in the induction of the AR gene by TPA. When the cells were pretreated with 10–100 μM PDTC, an anti-oxidant known to be an NF-κB inhibitor, the increased AR mRNA induced by TPA was

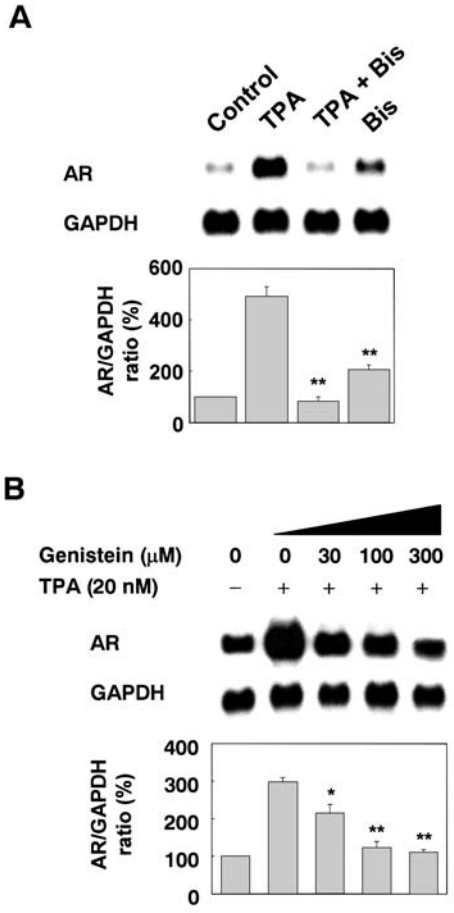

Figure 4. Effects of bisindolylmaleimide I and genistein on TPA-induced AR mRNA expression. Cells pretreated with 1 μM bisindolylmaleimide I (Bis) (*A*) or various concentrations of genistein (*B*) for 30 min were incubated with 20 nM TPA for 24 h. Bars represent the means ± SE (n = 3); *p < 0.05, **p < 0.01, compared with the TPA-only group.

suppressed in a concentration-dependent manner, suggesting the possible involvement of NF-κB in TPA-induced AR expression (fig. 5A). In the cytosolic fraction and nuclear extract of the TPA-treated cells, the levels of p65 constituting NF-κB were examined by Western blotting. As shown in figure 5B, p65 was rapidly translocated from the cytosol to the nuclear compartment in the stimulated cells, and persisted there for 24 h after the TPA treatment. The level of IκB-α in the cytosol was determined because the amount of NF-κB protein released to translocate to the nucleus is proportional to the inactivation of IκB. Along with the rapid increase in phosphorylated IκB-α by the TPA treatment, the level of IκB-α rapidly declined, but was restored after 24 h (fig. 5C).

TPA increased the promoter activity of the AR gene via NF-κB

To further clarify the signal transduction pathway in TPA-induced AR expression, transient transfections were per-

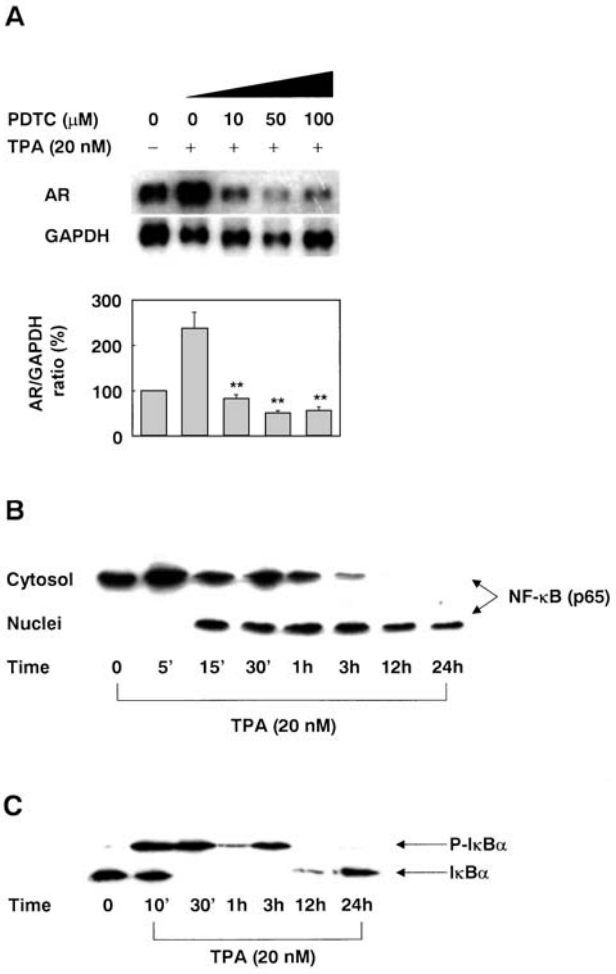

Figure 5. Involvement of NF-κB in the TPA-induced AR gene expression. (*A*) Cells pretreated with various PDTC concentrations were incubated with 20 nM TPA for 24 h. Bars represent the means ± SE (n = 3); **p < 0.01, compared with the TPA-only group. (*B*) Cells incubated with 20 nM TPA for the indicated times were fractionated into cytosolic and nuclear fractions, and subjected to SDS-PAGE. Translocation of the NF-κB (p65) protein was analyzed by Western blot analysis using p65-specific antibodies as described in Materials and methods. (*C*) Phosphorylated IκBα and cytosolic IκBα were detected using phosphor-IκB-α or IκB-α-specific antibodies.

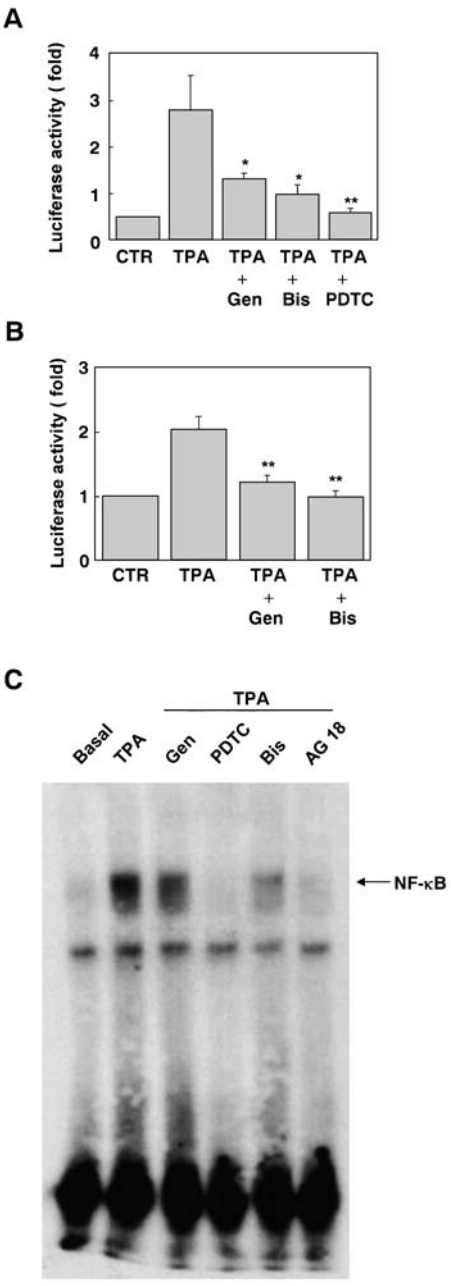

Figure 6. Effects of various inhibitors on the TPA-induced increase in AR promoter activity and on the transactivation of NF-κB. Cells transfected with the promoter-luciferase construct mARP3.2, containing the –3.2-kb upstream region of the mouse AR gene, were incubated with 20 nM TPA for 24 h in the presence or absence of 300 μM genistein (Gen), 1 μM bisindolylmaleimide I (Bis), or 50 μM PDTC (*A*). The luciferase activity was measured as described in Materials and methods. Cells transfected with a plasmid pNFκB-Luc were incubated with 20 nM TPA for 24 h in the presence or absence of 300 μM genistein (Gen) or 1 μM bisindolylmaleimide I (Bis) (*B*). The results were normalized using the β-galactosidase activity and are expressed as the means ± SE (n = 3); *p < 0.05, **p < 0.01, compared with the TPA-only group. CTR, control. (*C*) NF-κB-DNA-binding activity in the nuclear extracts was determined using an electrophoretic mobility shift assay. Cells were incubated with 20 nM TPA for 1 h in the presence or absence of 300 μM genistein (Gen), 50 μM PDTC, 1 μM bisindolylmaleimide I (Bis), or 300 μM tyrphostin A23 (AG18).

formed using the promoter-luciferase construct mARP3.2 containing a –3.2-kb upstream region of the mouse AR gene [22]. In the cells treated with TPA, a threefold increase in AR promoter activity was demonstrated, indicating that TPA increased the AR mRNA level by enhancing the transcriptional activity of the AR promoter. The increased promoter activity by TPA was significantly suppressed in the presence of 1 μM bisindolylmaleimide I, 100 μM genistein, or 50 μM PDTC (fig. 6A).

A reporter vector containing tandem repeats of the NF-κB binding elements was used for the promoter assay. At 18–24 h after transfection with the vector, the A549 cells were stimulated with TPA for 24 h in the absence or presence of various inhibitors. As shown in figure 6B, an ap-

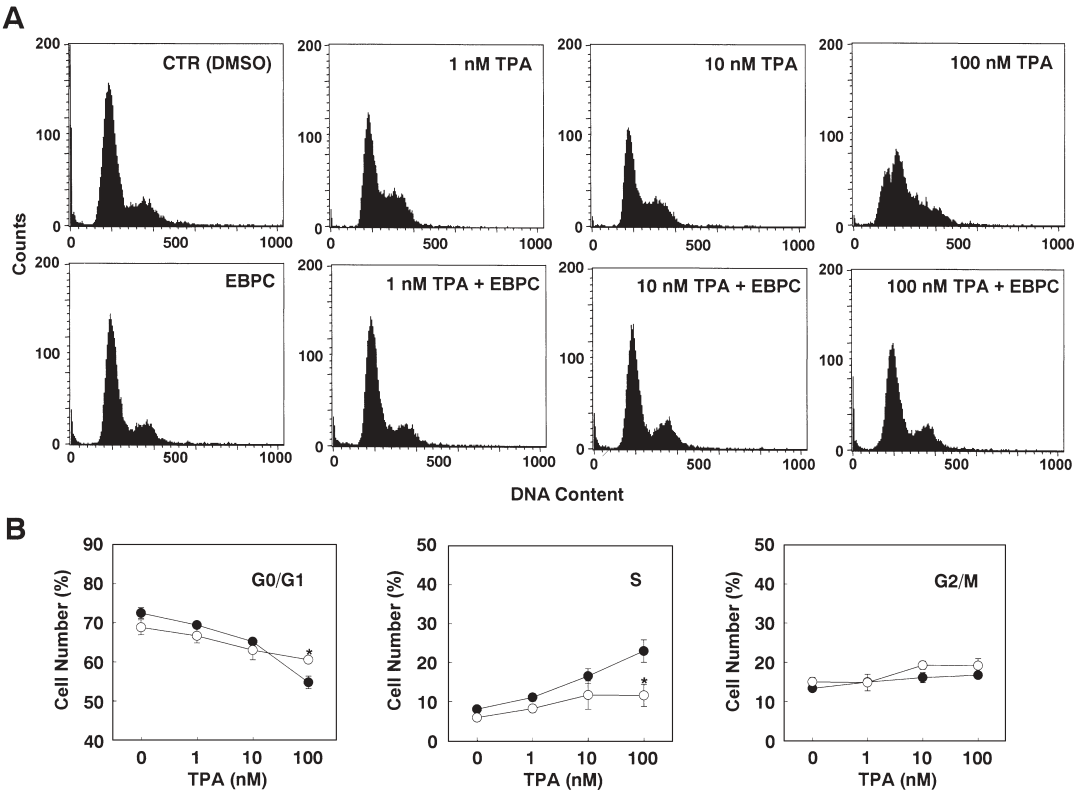

Figure 7. Effects of TPA and AR inhibition on cell cycle of the A549 cells. (*A*) Cells were treated with various TPA concentrations in the presence or absence of 50 μM EBPC for 24 h, stained with propium iodide, and analyzed by flow cytometry. DNA content is presented as the relative fluorescence. The cells in the G_0/G_1 phase represent the first peak, and those in S phase are represented in the area between the G_0/G_1 and G_2/M phase (the second peak). The data show representative histograms from three experiments. (*B*) Plot of the cell cycle distribution in A549 cells exposed to TPA alone (closed circles), and TPA with 50 μM EBPC (open circles). The vertical bars represent the SE (n = 3). *$p < 0.05$ compared with the corresponding TPA-treated cells.

proximately twofold increase in luciferase activity was observed in the TPA-treated cells. Bisindolylmaleimide I as well as genistein significantly suppressed the TPA-induced increase in the promoter activity, suggesting that transactivation of the AR gene by NF-κB is dependent on protein kinases. When the effects of these inhibitors on NF-κB-specific DNA binding were examined, the increased NF-κB binding activity in the TPA-treated cells was attenuated in the presence of PDTC, bisindolylmaleimide I, or tyrosine kinase inhibitors (fig. 6C). The band indicated as NF-κB was not detected in the presence of a 100-fold molar excess of the unlabeled oligonucleotide (data not shown).

Inhibition of AR suppressed the growth of A549 cells treated with TPA

This study investigated the effect of AR inhibition on cell cycle progression to evaluate the role of the increased AR activity in TPA-treated A549 cells. The DNA content analysis showed that the number of cells accumulated in the G_0/G_1 phase dose-dependently declined following 24 h of TPA treatment (fig. 7). In contrast, the proportion of cells entering the S phase was increased in a dose-dependent manner. EBPC, an AR inhibitor, suppressed the TPA-induced decrease in the proportion of cells in the G_0/G_1 phase. Notably, the increased entry of the TPA-treated cells into the S phase was almost completely abolished by EBPC. This suggests that the TPA-induced increase in AR activity plays a key role in the cell cycle progression of A549 cells.

Discussion

This study showed that a tumor promoter agent, TPA, up-regulated the expression of the AR gene via NF-κB in A549 human lung adenocarcinoma cells. The TPA-induced increase in the mRNA and promoter activity of the AR gene was attenuated in the presence of bisindolmaleimide I, a PKC inhibitor, indicating that activation of PKC is an obligatory event in TPA-mediated AR expression. Although prolonged exposure to TPA down-regulates PKC activity [24], the downstream signaling of PKC might remain elevated for some time. Indeed, the TPA-mediated increase in the AR mRNA level remained elevated for up to 72 h after TPA stimulation in A549

cells. TPA is known to elicit oxidative stress by stimulating NADPH oxidase in nonphagocyte cells as well as in phagocytes [25]. Our previous study performed on rat vascular smooth muscle cells showed that the elimination of ROS suppressed induction of AR mRNA [16, 17, 26]. In A549 cells, however, *N*-acetyl-L-cysteine, a thiol antioxidant [27], did not affect the expression of AR mRNA induced by TPA (data not shown).

Pretreatment of A549 cells with genistein, a tyrosine kinase inhibitor, blocked the effects of TPA on the expression and promoter activity of the AR gene. There is considerable evidence suggesting the activation of tyrosine kinase by TPA, even though the mechanism is still unknown. In A549 cells, TPA was shown to activate tyrosine kinase, and both PKC and tyrosine kinase stimulate the downstream NF-κB pathway leading to increased expression of intracellular adhesion molecule-1 [28, 29]. The activation of p44/42 MAP kinase (ERK), p38, or JNK was not involved in this event. On the other hand, our previous studies using rat vascular smooth muscle cells demonstrated that the p38 and ERK pathways participate in the methylglyoxal- and hydrogen peroxide-induced up-regulation of AR gene expression [16, 17]. When the effects of the inhibitors of p38 (SB203580) and MEK1 (PD98059) were examined, neither inhibitor affected the expression or the promoter activity of the AR gene in the A549 cells (data not shown). Accordingly, the major signaling cascade mediating the induction of the AR gene appears to vary with different stimuli and the cell lineage used in the experiments. In A549 cells, tyrosine kinase might be the link between the activation of PKC and induction of AR gene expression by TPA.

NF-κB was suggested to play a key role in the TPA-induced expression of the AR gene. An analysis of the promoter region of the AR gene depicted the putative recognition sequences for a variety of transcriptional factors [22]. Among them, the ORE appears to be essential for enhanced AR expression after exposure to tumor necrosis factor-α [23]. Since ORE differs from the NF-κB binding sequence by one base pair, we investigated the involvement of NF-κB in the induction of the AR gene by TPA. PDTC, an NF-κB inhibitor, significantly suppressed the induction of AR mRNA in a dose-dependent manner. Furthermore, PDTC almost completely abolished the TPA-induced increase in AR promoter activity. The transactivation of NF-κB in response to TPA paralleled the translocation of p65 to the nucleus. TPA treatment led to the rapid phosphorylation and ensuing loss of the IκB-α protein. These findings, which were also observed in other cells exposed to cytokines [28, 30], support a previous report showing that the phosphorylation of IκB-α by the activation of IκB kinases precedes the degradation of IκB-α [31]. TPA also stimulated NF-κB DNA-protein binding, which was suppressed by the inhibitors of PKC and tyrosine kinase. This suggests that activation of PKC and tyrosine kinase elicits the increased expression of the AR gene via NF-κB.

Of particular interest is the possibility that the augmented expression of AR induced by TPA might participate in the regulation of cell cycle progression. The inhibition of AR activity by EBPC almost completely abolished the S phase accumulation of A549 cells treated with TPA. The alterations in the cell cycle distribution, the arrest in the G_0/G_1 phase with the reduced number of cells entering the S phase observed in the EBPC-treated cells, suggest the involvement of AR in cell cycle progression and the growth of A549 cells. The association of AR with cell growth has been documented in several studies. In rat astrocytes, the level of AR protein was increased after being stimulated with mitogens such as basic fibroblast growth factor (bFGF), acidic fibroblast growth factor (aFGF), or epidermal growth factor [32]. In NIH 3T3 cells, aFGF and serum increased AR mRNA levels [33]. Stimulation with serum or thrombin also induced AR mRNA in cultured human vascular smooth muscle cells (VSMCs) [34]. Inhibition of AR activity attenuated the proliferation of VSMC elicited by serum, bFGF, thrombin, or 4-hydroxy-trans-2-nonenal [35]. Therefore, the augmented expression of AR might promote the proliferation of various cells under diverse physiological or pathological conditions.

The link between the increased AR activity and cell growth has yet to be determined. In cancer cells, up-regulation of AR was observed not only in chemically induced rat hepatoma cells [36], but also in human hepatocellular carcinoma cells including HepG2, Huh 7, Bel-7404, and HCC-M cells [37–39]. AR has been speculated to contribute to the detoxification of toxic aldehydes and modulate the survival of cells exposed to various noxious stimuli [16, 26, 40, 41]. Increased expression of the AR gene has been reported to reduce the efficacy of chemotherapeutic agents [11, 12], and suppression of AR activity enhanced the sensitivity of these cells against doxorubicin and cisplatin [13]. Such a cytoprotective role of AR could be attributed to the broad substrate specificity of this enzyme not only for the aldehyde form of glucose, but also for the xenobiotic or endogenously generated reactive aldehydes. The present study demonstrated that the inhibition of AR activity elicits an alteration in the cell cycle in cancer cells, which endorses the concept that AR facilitates tumor growth by augmenting the metabolism of the reactive products generated during rapid cell proliferation. In this context, these findings add further impetus to the use of AR inhibitors to alleviate the rapid growth of cancer cells, and to minimize resistance against various chemotherapeutic agents.

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