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Basal levels and patterns of anticancer drug-induced activation of nuclear factor-κB (NF-κB), and its attenuation by tamoxifen, dexamethasone, and curcumin in carcinoma cells☆

Shuang-En Chuang[a,b], Pei-Yen Yeh[b], Yen-Shen Lu[b], Gi-Ming Lai[a], Chao-Ming Liao[b], Ming Gao[b], Ann-Lii Cheng[b,c,*]

[a]*Division of Cancer Research, National Health Research Institutes, Taipei, Taiwan, ROC 11529*
[b]*Cancer Research Center, National Taiwan University College of Medicine, Taipei, Taiwan, ROC 10016*
[c]*Departments of Internal Medicine and Oncology, National Taiwan University Hospital, No. 7, Chung-Shan South Road, Taipei, Taiwan, ROC 10016*

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Abstract

Nuclear factor-κB (NF-κB) has been implicated in the development of drug resistance in cancer cells. We systematically examined the baseline levels of NF-κB activity of representative carcinoma cell lines, and the change of NF-κB activity in response to a challenge with four major anticancer drugs (doxorubicin, 5-fluorouracil, cisplatin, and paclitaxel). We found that the basal level of NF-κB activity was heterogeneous and roughly correlated with drug resistance. When challenged with various drugs, all the cell lines examined responded with a transient activation of NF-κB which then declined to basal level despite variation in the concentration of the agent and the timing of the treatment. In contrast to tumor necrosis factor-α (TNF-α), which activates NF-κB in minutes, NF-κB activation induced by anticancer drugs usually occurred more than 1 hr after stimulation. A gradual increase of total NF-κB and its nuclear translocation, and cytoplasmic translocation of nuclear IκBα and its degradation were involved in this process. In particular, when cells were pretreated with common biologic modulators such as tamoxifen, dexamethasone, and curcumin, the doxorubicin-induced NF-κB activation was attenuated significantly. This inhibition may play a role in sensitizing cancer cells to chemotherapeutic drugs. This study has demonstrated that activation of NF-κB is a general cellular response to anticancer drugs, and the mechanism of activation appears to be distinct from that induced by TNF-α. These observations may have implications for improving the efficacy of systemic chemotherapy for cancer patients.

Keywords: NF-κB; Drug resistance; Tamoxifen; Curcumin; TPA; Steroid

1. Introduction

NF-κB has been implicated in both carcinogenesis and the development of drug resistance in cancer cells [1–3]. While activation of NF-κB may induce apoptosis in certain situations [4–7], most reports suggest that NF-κB mediates survival signals that counteract apoptosis [8–13]. NF-κB-activated expression of genes that inhibit apoptosis, such as A20, IAPs, and TRAFs, is probably involved in the mediation [1,14,15]. Upon activation, NF-κB dissociates from the inhibitory IκBα and translocates from the cytoplasm to the nucleus, where it binds to the promoter elements and transactivates gene expression [16]. Phosphorylation of RelA/p65 at certain serine residues also plays a crucial role in NF-κB trans-activity [17–19]. While intrinsically or constitutively activated NF-κB may be critical in the development of drug resistance in cancer cells, transient, inducible activation of NF-κB may be as important but not as well studied. In fact, drug resistance in cancer cells is increasingly being considered to involve an inducible response to environmental stresses. Prevention of NF-κB activation, therefore, may represent a promising opportunity

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* Corresponding author. Tel.: +886-2-2312-3456x7251; fax: +886-2-2371-1174.
E-mail address: andrew@ha.mc.ntu.edu.tw (A.-L. Cheng).

Abbreviations: ATM, Ataxia Telangiectasia Mutated; EMSA, electrophoresis mobility shift assay; 5-FU, 5-fluorouracil; NF-κB, nuclear factor-κB; PKC, protein kinase C; PMSF, phenylmethylsulfonyl fluoride; TBE, tris–borate EDTA buffer; TNF-α, tumor necrosis factor-α; TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

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for widening therapeutic windows in translational cancer research [13,20].

In this study, we characterized the baseline and the drug-induced activation of NF-κB in a representative panel of cancer cell lines. We also demonstrated that this general activation of NF-κB by anticancer drugs can be attenuated by pretreatment with common biologic modulators. These observations may have implications for refining systemic chemotherapy in the future.

2. Materials and methods

2.1. Cell culture and determination of drug sensitivity by the MTT assay

All cell lines, including Hep-3B (ATCC HB-8064, hepatocellular carcinoma), AGS (ATCC CRL-1739, gastric adenocarcinoma), SiHa (ATCC HTB-35, uterine cervical carcinoma), MCF-7 (ATCC HTB-22, breast carcinoma), NTUB1 (urinary bladder cancer) [21], and H460 (non-small-cell lung cancer) [22], were maintained in Dulbecco's modified Eagle's medium plus 10% fetal bovine serum. The inhibitory effects of various drugs on cell growth were determined by a tetrazolium-based semi-automated colorimetric (MTT) assay [23]. Briefly, cells were plated in 96-well plates at 2–8 $\times$ 10^3 cells/well. After overnight incubation, various concentrations of the drugs were added in triplicate to the wells. After 3 days of culture and continuous drug exposure, when the cells in the drug-free control wells reached 90% confluence, cell numbers were evaluated using the MTT method with an ELISA reader at OD_{492}.

2.2. Preparation of nuclear protein extracts

Cells (2–5 $\times$ 10^7) were harvested, resuspended in 1 mL of buffer A [1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol, 0.2 mM PMSF, 1% Nonidet P-40 (NP-40), 10 mM HEPES, pH 7.9], and incubated for 10 min at 4° for cell disruption. After a low-speed spin at 1500 rpm for 2 min at 4° in a desktop Eppendorf microfuge (model 5417R), the nuclei-containing pellet was resuspended in 0.1 mL of buffer B (25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.2 mM PMSF, 20 mM HEPES, pH 7.9), and incubated for 20 min at 4°. After a high-speed centrifugation at 14,000 rpm for 2 min at 4°, the supernatant was assayed carefully for protein content and then saved in aliquots at −70°.

2.3. EMSA

The double-stranded NF-κB binding oligonucleotides were prepared from two complementary single-stranded oligonucleotides, Prm757 (5′-GATCAGTTGAGGGGACTTTCCCAGGC) and Prm953 (5′-GATCGCCTGGGAAAGTCCCCTCAACT) (the NF-κB binding site is underlined). Both oligonucleotides were PAGE purified. The double-stranded oligonucleotides were end-labeled with [^{32}P]dCTP in the presence of unlabeled dGTP/dATP/dTTP with MMLV reverse transcriptase. The binding reaction was carried out with 2.5 nM ^{32}P-labeled oligonucleotides and 10 μg nuclear extract protein in binding buffer [75 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 4% Ficoll 400, and 0.067 μg/μL poly(dI-dC), 10 mM Tris, pH 7.5] at room temperature for 30 min. The reaction products were separated on a native 5% polyacrylamide gel in a 0.25× TBE running buffer. After electrophoresis, the gel was dried and exposed to X-ray film for autoradiographic analysis. The signal of TNF-α-induced activation of NF-κB was used as a positive control, and super-shift, using an antibody against the RelA/p65 subunit of NF-κB, was used to verify the identity of the NF-κB complex. In the examination of NF-κB activation induced by chemotherapeutic drugs (see Figs. 2 and 3), cells were treated with drugs simultaneously and harvested at the time points indicated. For experiments with biologic modulators (see Fig. 4), cells were pretreated at different time points with tamoxifen, TPA, dexamethasone, or curcumin, then treated with doxorubicin for 3 hr, and harvested at the same time. Ten micrograms of nuclear protein extract was used for each sample.

2.4. Western blot analysis of whole cell and nuclear protein extracts

Methods for preparation of the whole cell protein extracts were as described previously [24]. Briefly, cells (10^7) were rinsed twice with cold PBS in a Petri dish. Cells were disrupted by rocking the dishes at 4° for 20 min in 1 mL of RIPA lysis buffer (150 mM NaCl, 1 mM EGTA, 1% NP-40, 1 mM Na_3VO_4, 1 mM PMSF, 1 μg/mL of aprotinin and leupeptin, 50 mM Tris–HCl, pH 7.4). The homogenates were centrifuged at 4° for 10 min at maximal microfuge speed to remove debris. For western blot analysis, the whole cell lysates, or the nuclear protein extracts prepared as described above, each equivalent to 50 μg total protein, were separated by 12% SDS–PAGE, transferred onto a polyvinylidene difluoride membrane, and probed with specific antibodies against IκBα or the NF-κB p65. Detection was performed with an ECL chemiluminescence kit (Amersham).

3. Results

3.1. Drug sensitivities and basal NF-κB activities in various cell lines

Baseline NF-κB activities varied tremendously among the six epithelial cell lines, with SiHa being the most active, followed by NUTB1, and Hep-3B. AGS, MCF-7, and H460 had no or little activity (Fig. 1). In general, the

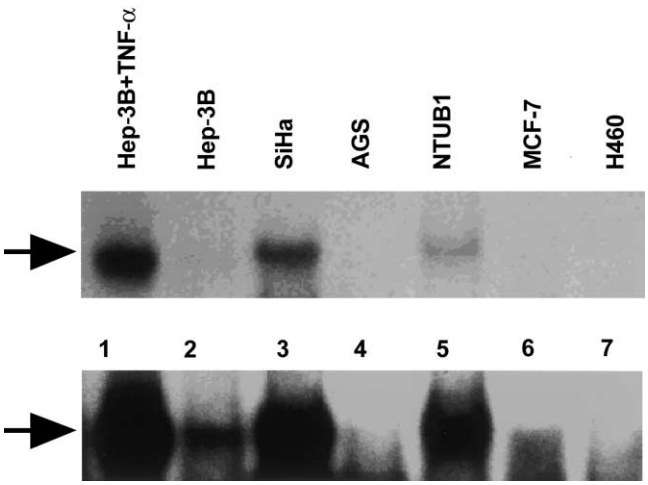

Fig. 1. Baseline levels of NF-κB activity in various epithelial cell lines. NF-κB activity was examined by EMSA. Signals of the activated NF-κB band are indicated with an arrow. TNF-α-induced NF-κB activation was used as a positive control (lane 1). Using an antibody against the RelA/p65 subunit of NF-κB, only the indicated band super-shifted to an upper position, verifying the identity of the NF-κB complex (data not shown). Six epithelial cell lines were used: Hep-3B (hepatocellular), SiHa (cervical), AGS (gastric), NTUB1 (bladder), MCF-7 (breast), and H460 (lung). Upper and lower panels are autoradiographs from the same gel, but the film exposure time was longer in the lower panel in order to allow the faint signal in lane 2 to become visible.

basal level of NF-κB activity was inversely correlated with cellular sensitivity to various drugs as determined by the MTT assay. For example, SiHa cells expressed the highest NF-κB activity and were the most resistant to the four anticancer drugs (doxorubicin, 5-FU, cisplatin, and paclitaxel) among the six epithelial cell lines. The three cell lines without detectable NF-κB activity (AGS, MCF-7, and H460) were more sensitive to the four drugs than the two cell lines (SiHa and NTUB1) that expressed significant NF-κB activity (Table 1).

Table 1
Drug IC_{50} values of carcinoma cell lines

Compound	IC_{50}[a] values of cell lines					
	SiHa	NTUB1	Hep-3B	AGS	MCF-7	H460
Basal NF-κB[b]	72	24	6	Nil	Nil	Nil
Doxorubicin (nM)	200	45	500	32	5	ND[c]
5-FU (μM)	830	16	4	10	2	ND
Cisplatin (μM)	21	3	4	4	8	1
Paclitaxel (nM)	150	34	9	9	1	2

[a] The IC_{50} values (concentration of drug that inhibits cell growth by 50%) are averages of 2–5 experiments.
[b] NF-κB activity (expressed in arbitrary units) was determined by densitometry scanning of the X-ray film of the EMSA.
[c] Not determined.

3.2. Activation of NF-κB by anticancer drugs in Hep-3B, SiHa, and NTUB1 cell lines

Four anticancer drugs with different modes of action, i.e. doxorubicin, 5-FU, cisplatin, and paclitaxel, were tested individually for their effects on NF-κB activation. As shown in Figs. 2 and 3, each of the four drugs activated NF-κB in both the Hep-3B and SiHa cell lines, even though the basal level of NF-κB activity in Hep-3B cells was much lower than in SiHa cells. However, distinct kinetics and patterns of NF-κB activation were observed in different cell lines. While the peak activation in Hep-3B cells occurred at 3 hr post-treatment (Fig. 2), the kinetics of NF-κB activation were drug-dependent in SiHa cells (Fig. 3). In SiHa cells, doxorubicin induced the strongest NF-κB activation, which reached its maximum at around 9 hr and sustained this level for more than 24 hr. The effects of 5-FU peaked within 1.5 hr and faded away quickly. Cisplatin took much longer (12 hr) to activate NF-κB. Activation by paclitaxel showed a biphasic pattern at 1.5 and 9 hr of incubation, and a similar response was also observed in Hep-3B cells (Fig. 2). In the bladder

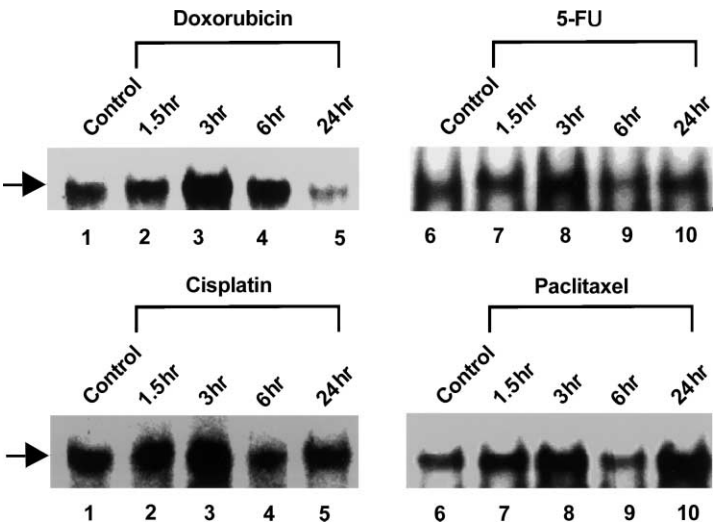

Fig. 2. Time-course of NF-κB activation by anticancer drugs in Hep-3B cells. After the application of drugs, cells were harvested at various time points, as indicated, for EMSA assay. Controls are cells without drug treatment. In this cell line, all four of the drugs induced NF-κB activation that peaked at 3 hr of drug exposure.

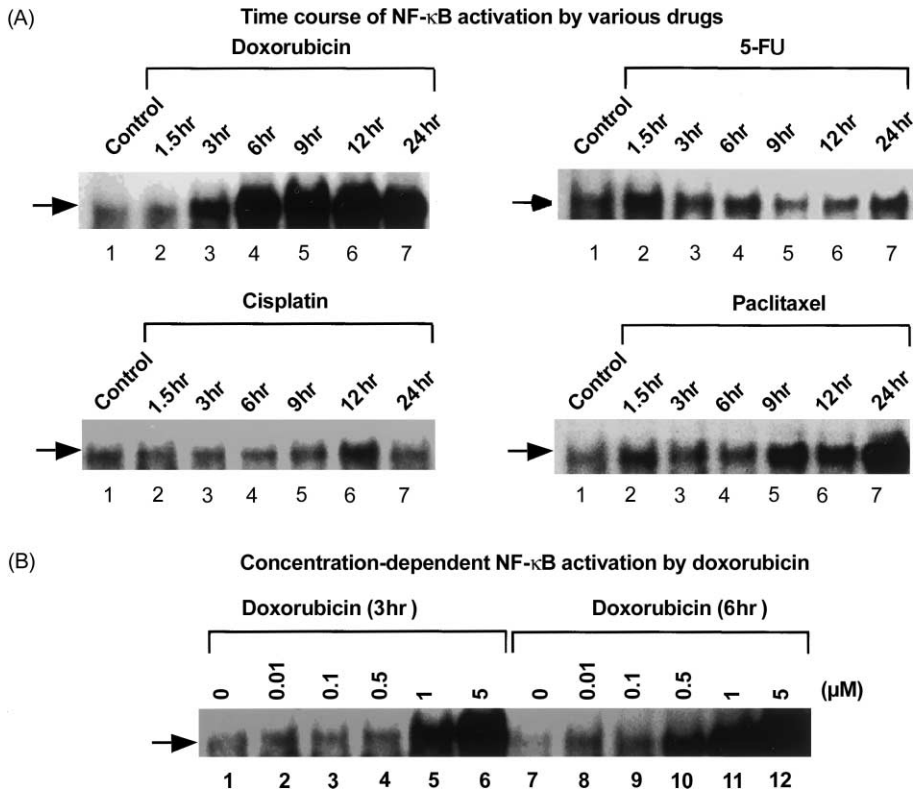

Fig. 3. NF-κB activation by anticancer drugs in SiHa cells. (A) Time course of NF-κB activation. All four of the chemotherapeutic drugs activated NF-κB in a time-dependent manner, with results similar to those observed in Hep-3B, but the duration of activation and the time required to reach the activation peak varied. (B) Concentration-dependent activation of NF-κB by doxorubicin. Cells were treated with various concentrations of drug for either 3 hr (lanes 1–6) or 6 hr (lanes 7–12).

cancer cell line (NTUB1), activation by doxorubicin was evident after 3–6 hr of incubation and decayed thereafter (data not shown), which was similar to the response observed in Hep-3B cells. We used the SiHa cell line and doxorubicin to examine whether a concentration–response relationship exists between the degree of NF-κB activation and the concentration of the drug for either 3 or 6 hr of incubation (Fig. 3B). The results suggest that a concentration threshold exists, since low concentrations of doxorubicin ($\leq$0.1 μM) did not induce NF-κB activation, but higher concentrations resulted in the activation of NF-κB in a concentration-dependent manner. Further, a lower drug concentration (0.5 vs. 1 μM) required a longer doxorubicin incubation time (6 vs. 3 hr) to achieve the same degree of NF-κB activation. Using the NF-κB-luciferase reporter assay system we further confirmed that these increased DNA binding activities were correlated with NF-κB function in a concentration-dependent manner (data not shown).

3.3. Attenuation by common biologic modulators of doxorubicin-induced NF-κB activation in Hep-3B cells

3.3.1. Tamoxifen and TPA

Pretreatment of cells with tamoxifen (Sigma Chemical Co.), an anti-estrogen and PKC inhibitor, for as little as 30 min, resulted in an attenuation of drug-induced NF-κB activation (Fig. 4A, lanes 4–11). This effect was more pronounced if the pretreatments lasted for a longer period of time (12–24 hr, Fig. 4A, lanes 10 and 11). If tamoxifen and doxorubicin were added together, no inhibition was observed (Fig. 4A, lanes 4 and 5). Tamoxifen alone slightly inhibited NF-κB basal activity only when treatment was for a longer duration, e.g. 12 vs. 3 hr (Fig. 4A, lanes 1–3). Treatment with the phorbol ester TPA alone induced a fast and dramatic (peaked in less than 30 min, which is much faster than the four drugs tested) NF-κB activation that decayed gradually within 24 hr (Fig. 4A, lanes 12–15), a kinetic pattern different from that of NF-κB activation mediated by any of the anticancer drugs. When TPA was included during the pretreatment periods with tamoxifen, not only was the inhibitory effect of tamoxifen totally overridden by TPA, but a synergistic NF-κB activation between doxorubicin and TPA was also observed (Fig. 4A, lanes 16–19). This tremendous activation duration was sustained for longer than 24 hr, which was not observed in cells treated with doxorubicin alone or TPA alone. The observed synergism of NF-κB activation between doxorubicin and TPA is likely to reflect distinct pathways through which the two agents exert their effects on NF-κB. While doxorubicin probably conveys its signals inside-out, i.e. causing DNA damage first, and the signal then transferred from the nucleus to the cytoplasm, probably mediated by DNA-dependent protein kinase and/or ATM,

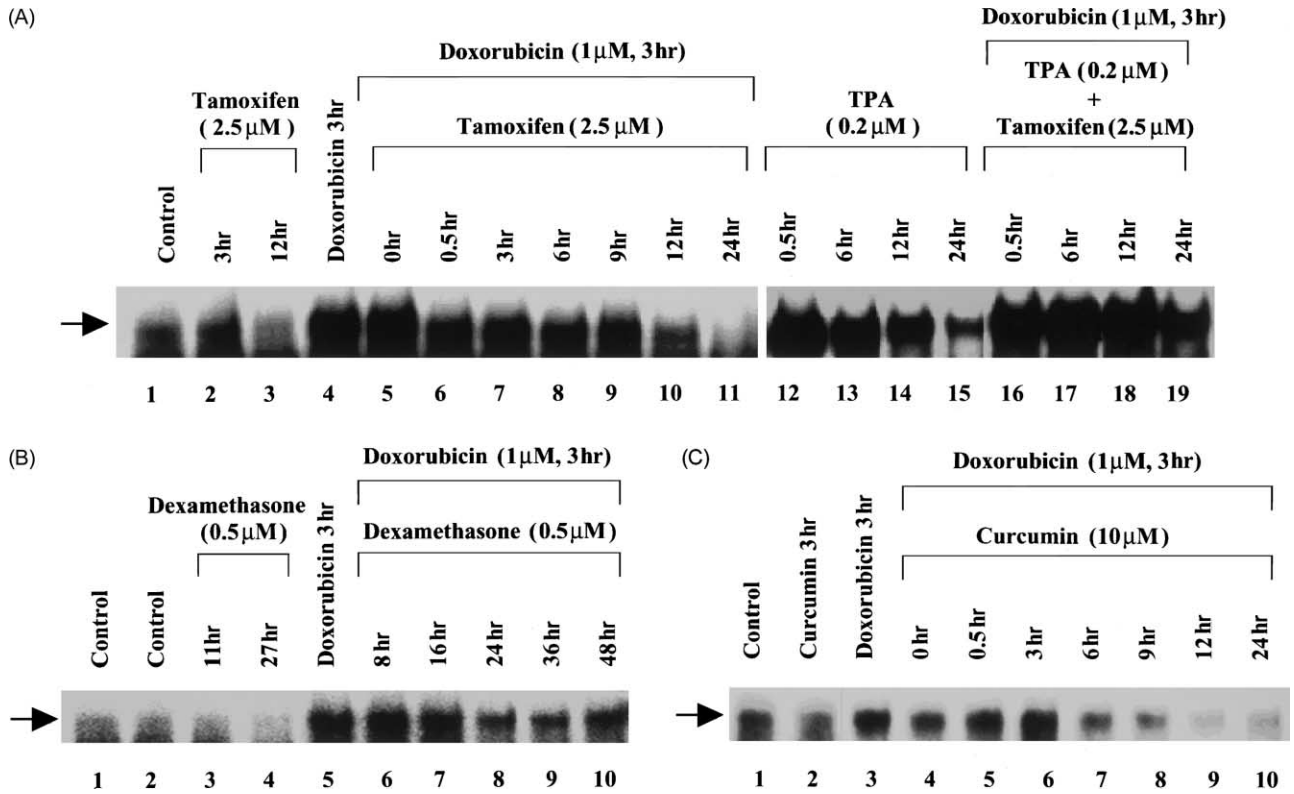

Fig. 4. Effects of biologic modulators on doxorubicin-induced activation of NF-κB in Hep-3B cells. (A) Tamoxifen and TPA. Cells were incubated either with 2.5 μM tamoxifen alone for 3–12 hr (lanes 2 and 3), or with tamoxifen for the intervals indicated, followed by the addition of doxorubicin and further incubation for 3 hr (lanes 5–11). Lane 4: cells treated with doxorubicin alone for 3 hr. Lanes 12–15: cells were incubated with 0.2 μM TPA alone for 0.5 to 24 hr. Lanes 16–19: cells were pretreated with TPA plus tamoxifen for the intervals indicated (0.5 to 24 hr), followed by the addition of doxorubicin and further incubation for 3 hr. (B) Dexamethasone. Cells were either incubated with 0.5 μM dexamethasone alone for 11–27 hr (lanes 3 and 4), treated with 1 μM doxorubicin alone for 3 hr (lane 5), or with dexamethasone for the intervals indicated, followed by the addition of doxorubicin and further incubation for 3 hr (lanes 6–10). (C) Curcumin. Cells were either incubated with 10 μM curcumin alone for 3 hr (lane 2), treated with 1 μM doxorubicin alone for 3 hr (lane 3), or with 10 μM curcumin for the intervals indicated, followed by the addition of doxorubicin and further incubation for 3 hr (lanes 4–10). For all experiments, cells were pretreated with tamoxifen, TPA, dexamethasone, or curcumin, at different time points and then were treated with doxorubicin for 3 hr and harvested at the same time. The same amount of nuclear protein extract (10 μg) was used for each sample. Controls are cells without any treatment. Treatment of cells with tamoxifen, curcumin, or dexamethasone alone for 24 hr at the concentrations indicated did not affect cell viability significantly, as determined by MTT assay and microscopy (data not shown). Hence, inhibition of NF-κB activation by pretreatment with these agents could not have been due to cytotoxicity.

TPA primarily sends its signals outside-in, i.e. from the membrane to the cytoplasm, probably through PKC and/or IKK. The synergism of NF-κB activation was probably the net result of the two signals. However, the exact mechanism remains elusive at the moment.

3.3.2. Dexamethasone

Pretreatment with the steroid dexamethasone also caused a significant inhibition in doxorubicin-induced NF-κB activities in Hep-3B cells (Fig. 4B, lanes 5–10). However, unlike tamoxifen, dexamethasone required a much longer pretreatment period (≥24 hr). Dexamethasone alone inhibited basal NF-κB activity only slightly (Fig. 4B, lanes 1–4).

3.3.3. Curcumin

Pretreatment with the anti-inflammatory and chemopreventive agent curcumin caused a time-dependent inhibition of doxorubicin-induced NF-κB activity (Fig. 4C, lanes 3–10). The time required to inhibit doxorubicin-induced NF-κB activation by curcumin (≥6 hr) was intermediate to that of tamoxifen (0.5 hr) and dexamethasone (≥24 hr). Curcumin alone inhibited basal NF-κB activity only slightly (Fig. 4C, lanes 1 and 2).

3.4. Involvement of nuclear translocation of NF-κB and decline of nuclear IκBα in doxorubicin-induced NF-κB activation

TNF-α-mediated NF-κB activation involves the phosphorylation and rapid degradation of IκBα and subsequent nuclear translocation of the freed NF-κB. To determine whether doxorubicin-induced NF-κB activation also involves this mechanism, western blot analysis was performed with whole cell and nuclear protein extracts. As shown in Fig. 5, in Hep-3B cells, a time-dependent decrease of IκBα levels was observed primarily in the nucleus. NF-κB p65 levels also increased significantly in

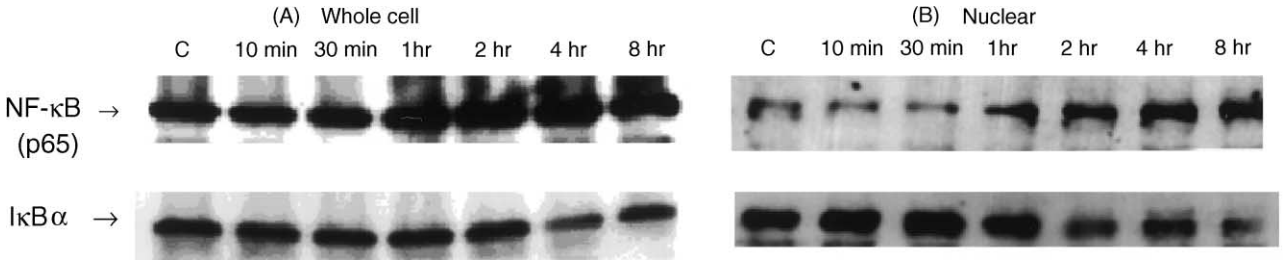

Fig. 5. Doxorubicin-induced nuclear translocation of NF-κB and down-regulation of nuclear IκBα. Hep-3B cells were harvested at various time intervals as indicated (between 10 min and 8 hr) after the application of 1 μM doxorubicin. C: control cells with no treatment. For western blot analysis, the whole cell protein extracts (A) and nuclear protein extracts (B) were prepared from the harvested cells and probed with antibodies specific to NF-κB p65 or IκBα.

the nucleus and only moderately, but consistently, at the whole cell level. The nuclear translocation of NF-κB was found to parallel the nuclear decrease of IκBα. Comparison of the EMSA results (Fig. 2) showed that the nuclear NF-κB level was sustained for a longer period of time than its binding activity. However, in contrast to NF-κB activation by TNF-α, which took place in minutes, doxorubicin-induced activation of NF-κB required about ten times longer, and similar results were also observed in the other cancer cell lines when challenged with other anticancer drugs (data not shown).

4. Discussion

In this study, we demonstrated that NF-κB can be activated by all four of the anticancer drugs in the three cancer cell lines examined. Each of the four anticancer drugs used (doxorubicin, 5-FU, cisplatin, and paclitaxel) possesses distinct modes of action that cause different types of damage to cancer cells, and the three cell lines that we used were of different origins (liver, uterine cervix, and urinary bladder). However, a universal NF-κB activation was observed. These results suggest the existence of a common set of cellular elements that sense the challenge by these drugs as a type of stress and transmit this signal to NF-κB. Although we also found that NF-κB activities were roughly correlated with drug resistance, since NF-κB activation can result in apoptosis in certain systems, cell context may be a crucial determinant of the outcome of NF-κB activation [1].

Although activation of NF-κB was observed in both the hepatocellular carcinoma cell line Hep-3B (Fig. 2) and the cervical carcinoma cell line SiHa (Fig. 3) when treated with each of the four drugs, the kinetics of activation had different patterns. In Hep-3B cells, the NF-κB activation was a transient response to each of the four drugs despite continuous drug exposure, and always peaked at around 3 hr. In contrast, the activation patterns in SiHa cells were different from those of Hep-3B and were also distinct among the four drugs. The mechanisms underlying the different responses of NF-κB among different cells and upon treatment with different drugs remain unclear. Nevertheless, all cytotoxic agent-induced NF-κB activation in this study required more than 1 hr to occur, which is in sharp contrast to TNF-α-induced NF-κB activation, which usually takes place within minutes. Anticancer drug-induced NF-κB activation is likely to be mediated through different pathways. One obvious difference is that while TNF-α transmits its signal inward via TNF receptors on the cell membrane, most anticancer drugs cause damage to nuclear DNA or other cellular targets first and then transmit their signal outward. DNA-dependent protein kinase and ATM have been shown to be responsible for the NF-κB activation induced by DNA damage following exposure to ionizing radiation or to the anticancer drug camptothecin [25,26]. The JNK pathway kinase MEKK1, which responds to various environmental stresses [27,28], may also be involved. Whether these protein kinases are responsible for NF-κB activation by DNA-damaging drugs remains to be clarified. Further, although nuclear accumulation of NF-κB and progressive decline of nuclear IκBα were observed in this study, the decrease of IκBα protein was not significant on the whole cell level during anticancer drug-induced activation of NF-κB. A clear mechanism for this scenario remains elusive. However, two possible explanations seem reasonable: (i) parallel with the nuclear translocation of NF-κB, there might exist a mechanism for the nuclear export of IκBα following drug treatment [29]; and (ii) a mechanism for the nuclear degradation of IκBα might exist. The second explanation is less likely, since the change in total cellular IκBα levels was relatively small. Cytosolic proteosome-mediated IκBα degradation, which is responsible for TNF-α-induced NF-κB activation, might not be involved in drug-induced NF-κB activation since the level of IκBα in whole cells does not change significantly.

Both intrinsic and drug-induced transient activation of NF-κB may play a role in the development of drug resistance in tumor cells. We found that when cancer cells were transiently pretreated with a sublethal concentration of doxorubicin, they became more resistant to doxorubicin upon subsequent challenges [30]. Therefore, attenuation of this NF-κB activation by certain modulators may, at least partially, overcome drug resistance. For example, high-dose tamoxifen has been shown to enhance the therapeutic

efficacy of doxorubicin and etoposide in patients with far-advanced hepatocellular carcinoma [31,32]. We previously proposed that inhibition of the PKC signaling pathway may be one of the mechanisms by which tamoxifen enhances the therapeutic effect of doxorubicin [33]. In this study, we further demonstrated that attenuation of NF-κB activation may represent another mechanism through which tamoxifen exerts its effects (Fig. 4A). It is probable that both PKC and NF-κB are critical determinants of drug resistance of some selected cancers. Another potentially important category of bio-modulators is glucocorticoids. Glucocorticoids are often used for reducing the side-effects of chemotherapy, yet their possible influence on the therapeutic efficacy of cancers has rarely been addressed. Although glucocorticoids can induce apoptosis in many hematological cells, they generally do not have cytotoxic effectiveness in non-hematological solid tumors. However, we previously demonstrated a synergistic cytotoxic effect of dexamethasone and cisplatin in a cervical carcinoma cell line [34]. In this case, the mechanism of action of dexamethasone was most likely the inhibition of NF-κB. As shown in Fig. 4B, preincubation of dexamethasone for more than 24 hr inhibited doxorubicin-induced NF-κB activation. Curcumin is a useful chemopreventive agent against the development of various murine cancers, including cancers of the skin, stomach, colon, and liver [35–37]. With its multiple mechanisms of action, curcumin has been further demonstrated recently to be a biochemical modulator of cytotoxic agents (our unpublished data). The present study has demonstrated that NF-κB might be one of the important targets of curcumin. Our findings that cytotoxic agent-induced NF-κB activation can be influenced by common biologic modulators may have important clinical implications that deserve further investigation.

Acknowledgments

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