FEBS Letters 584 (2010) 3492–3498

journal homepage: www.FEBSLetters.org

Elevation of cyclic AMP causes an imbalance between NF-κB and p53 in NALM-6 cells treated by doxorubicin

Majid Safa [a,1], Hamid Zand [b,*,1], Kazem Mousavizadeh [c], Ahmad Kazemi [a], Masoumeh Bakhshayesh [d], Parisa Hayat [d]

[a] Department of Hematology, Faculty of Allied Medicine, Iran University of Medical Sciences, Tehran, Iran
[b] National Nutrition and Food Technology Research Institute, Faculty of Nutrition Sciences and Food Technology, Shahid Beheshti University of Medical Sciences, Tehran, Iran
[c] Department of Basic Sciences and Oncopathology Research Center and Cellular and Molecular Research Center, Iran University of Medical Sciences, Tehran, Iran
[d] Cellular and Molecular Research Center, Iran University of Medical Sciences, Tehran, Iran

ARTICLE INFO

Article history:
Received 21 April 2010
Revised 30 June 2010
Accepted 4 July 2010
Available online 15 July 2010

Edited by Varda Rotter

Keywords:
cAMP
DNA damage
IκB
NF-κB
p53

ABSTRACT

We previously showed that cAMP can inhibit DNA damage-induced wild type p53 accumulation in human pre-B NALM-6 cells, leading to a profound reduction of their apoptotic response. Here, we provide evidence for the potentiation of DNA damage-induced NF-κB activation by cAMP. We found that inhibition of NF-κB activation prevents the inhibitory effect of cAMP on doxorubicin-induced apoptosis. Moreover, cAMP exerts its inhibitory effect on doxorubicin-induced apoptosis in a PKA-independent manner. The present study also shows that elevation of cAMP prolongs the phosphorylation of IκB and subsequent activation of NF-κB in doxorubicin treated NALM-6 cells in a proteasome-dependent manner. Taken together, our results demonstrate that cAMP abrogates the balance between apoptotic and antiapoptotic transcription factors that are hallmarks of DNA damage signaling.

1. Introduction

The sensitivity of tumor cells to apoptosis induced by DNA damaging agents depends on the balance between apoptotic and anti-apoptotic signals. Many cellular stimuli such as DNA damage result in the induction of both the tumor suppressor p53 and NF-κB [1]. In contrast to activation of p53, which is associated with the induction of apoptosis, stimulation of NF-κB has been shown to promote resistance to apoptosis [2,3]. However, in some other situations, NF-κB appears to be proapoptotic [4]. We and others have recently shown that elevation of cAMP reduces p53 accumulation and apoptosis induced by DNA damage through an uncertain mechanism [5,6]. In this study, we investigated the effect of cAMP on DNA damage-induced NF-κB activation.

DNA damage can be detected by the sensors of DNA lesions, which in turn recruit two major kinases capable of transducing DNA damage signals, ataxia telangiectasia mutated (ATM), ataxia telangiectasia and Rad3 related (ATR). In response to DNA damage, ATM phosphorylates a number of downstream effectors proteins and kinases such as the checkpoint kinases1 (Chk1) and Chk2, Mdm2 and p53, which regulate cell-cycle arrest, DNA repair, cell senescence and apoptosis [7]. It has been demonstrated that ATM is also essential for NF-κB activation by multiple genotoxic agents that induce DNA double strand breaks (DSBs), including ionizing radiation (IR), and the topoisomerase I and II inhibitors [8,9]. NF-κB essential modulator (NEMO), the regulatory subunit of I kappa B kinases (IKKs) complex, enters the nucleus and associates with the DSB-activated ATM after the induction of DNA damage. The subsequent ATM-dependent phosphorylation of NEMO at serine 85 is critical for further NEMO modification by ubiquitin, NEMO nuclear export and activation of cytoplasmic IKK complex [10].

The present study analyzed the capacity of cAMP increasing agents to modulate the toxicity of DNA topoisomerase II inhibitor (doxorubicin) in human Pre-B NALM-6 cells. These cells possess two characteristics important for regulation of apoptosis induced by DNA damage, namely a constituent NF-κB expression and wild type p53. Exploration of the cross-talk processes that link signaling systems in defined cell types is crucial to our understanding of cell function in health and disease and thus developing new diagnostic methodologies and therapeutics. The present study offers novel processes that provide connections between DNA damage signaling and cAMP pathways.

* Corresponding author. Address: Department of Basic Medical Sciences, National Nutrition and Food Technology Research Institute, Faculty of Nutrition Sciences and Food Technology, Shahid Beheshti University Medical Sciences, P.O. Box # 19395-4741, Tehran, Iran. Fax: +98 21 22360657.
E-mail addresses: hamid_zand@sbmu.ac.ir, hamidzand@gmail.com (H. Zand).
[1] These two authors contributed equally to this work.

0014-5793/$36.00
doi:10.1016/j.febslet.2010.07.009

2. Materials and methods

2.1. Reagents and antibodies

Doxorubicin, forskolin, 3-isobutyl-1-methylxanthine (IBMX), MG-132, H89, Bay 11-7082 and 3-(4,5-methylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) were purchased from Sigma (Sigma; St. Louis, MO). 8-CPT-cAMP was obtained from Santa Cruz Biotechnology (Santa Cruz Biotechnology, CA). All primary antibodies were obtained from Santa Cruz Biotechnology except caspase-3 (8G10) and cleaved PARP (Asp214), which were obtained from Cell Signaling Technology (Beverly, MA).

2.2. Cell culture

NALM-6 (human pre-B ALL) cells were grown in suspension in RPMI medium supplemented with 2 mM L-glutamine, 10% FBS, 100 units/ml penicillin and 100 lg/ml streptomycin.

2.3. Detection of cell death by MTT colorimetric assay

The cell viability was determined using MTT assay [11]. Briefly, NALM-6 cells were seeded at 5000/well in flat-bottom 96-well culture plates. The cells were preincubated with or without Bay 11-7082 for 30 min before treatment with forskolin and IBMX for 30 min. Then they were treated with several concentrations of doxorubicin and harvested after 24 h. After removing of the medium, the cells were incubated with MTT solution (5 mg/ml in PBS) for 4 h and the resulting formazan was solubilized with DMSO (100 μl). The absorption was measured at 570 nm (620 nm as a reference) in an ELISA reader.

2.4. Apoptosis assay

To explore the effect of elevated cAMP on doxorubicin-induced cell death, the cells were subjected to apoptosis analysis. Briefly, the cells were seeded into 12-well cell culture plates and treated with Bay 11-7082 (4 μM) for 30 min prior to addition of forskolin (50 μM) and IBMX (100 μM) or 8-CPT-cAMP (200 μM). After 30 min, the cells were incubated with doxorubicin (0.5 μM) for 16 h and then collected. The cell aliquots were washed with PBS and 10^6 cells were stained using Annexin V-FLOUS-Staining Kit (Roche, Germany) according to the manufacturer's instructions. The percentage of apoptotic cells was quantified using Becton–Dickinson FACS. Annexin V-positive and PI-negative cells were considered to be in the early apoptotic phase and those having positive staining both for Annexin V and PI were deemed to undergo late apoptosis or necrosis.

2.5. Nuclear fragmentation

The preparation of nuclear extracts was performed using the Nuclear Extract Kit (Active Motif, Carlsbad, CA). NALM-6 cells (4×10^6) were washed with 1 ml ice-cold PBS/phosphatase inhibitors, lysed in 250 μl hypotonic buffer and then centrifuged at 14 000×*g* for 30 s at 4 °C. The cell pellets were resuspended in 30 μl complete lysis buffer and centrifuged at 14 000×*g* for 10 min at 4 °C. Finally, the supernatants (nuclear fraction) were saved.

2.6. NF-κB DNA-binding assay

The DNA-binding activity of NF-κB was quantified in the NALM-6 cells by TransAM™ NF-κB p65 Transcription Factor Assay Kit (Active Motif) according to the manufacturer's instructions. Briefly, equal amounts of the nuclear extracts were incubated in 96-well plates coated with immobilized oligonucleotide (5′-AGTTGAGGGGACTTTCCCAGGC-3′) containing a consensus (5′-GGGACTTTCC-3′) binding site for the p65 subunit of NF-κB. NF-κB binding to the target oligonucleotide was detected by incubation with primary antibody specific for p65 subunit and HRP-conjugated secondary antibody. For quantification of NF-κB activity, the optical densities were measured at 450 nm by a microplate reader.

2.7. Western blot analysis

The cells were lysed in RIPA buffer (50 mM Tris [pH 7.5], 150 mM NaCl, 1% NP-40, 0.1% SDS, 0.5 mM EDTA, 10 mM NaF, 5 mM b-glycerophosphate, 0.1 mM Na3VO4, 0.2 mM phenylmethylsulfonyl fluoride [PMSF], 10 mg/ml leupeptin and 0.5% aprotinin) and the protein concentrations were determined using the Bradford method (Bio-Rad). Equal amounts of the proteins were separated on a 10% SDS–PAGE and subsequently transferred into a nitrocellulose membrane (Amersham Pharmacia Biotech) using a semidry transfer cell (Bio-Rad). The proteins were detected using appropriate primary antibodies and the enhanced chemi-luminescence detection system (ECL Plus, Amersham Pharmacia Biotech) according to the manufacturer's protocol.

2.8. Statistical analysis

SPSS 12 software was used to perform statistical analyses. The significance of differences between the experimental variables was determined by the use of two tailed Student's *t*-test. A probability level of $P > 0.05$ was considered as statistically significant.

3. Results

3.1. Inhibition of NF-κB activation prevents the antiapoptotic effect of cAMP in NALM-6 cells treated by doxorubicin

To reveal the effect of cAMP on NF-κB signaling, we first examined the effects of cAMP on the apoptosis induced by doxorubicin in the presence of Bay 11-7082, an inhibitor of IκB phosphorylation and NF-κB activation. As shown in Fig. 1A, the MTT assay analysis showed that the cAMP increasing agents markedly reduce doxorubicin-induced cell death, however, exposure of the cells to Bay 11-7082 (4 μM) dramatically reverses the inhibitory effect of 8-CPT-cAMP (200 μM), forskolin (50 μM) and IBMX (100 μM) on the cytotoxicity of doxorubicin. These observations indicate that cAMP may positively regulate the activation of NF-κB.

To address whether attenuation of doxorubicin-induced apoptosis by cAMP can be prevented by NF-κB inhibition in NALM-6, we performed Annexin V staining of the NALM-6 cells exposed to doxorubicin (0.5 μM) in the presence or absence of both cAMP and Bay 11-7082 (4 μM). As shown in Fig. 1B, the percentage of annexin V-positive cells in the cultures of NALM-6 cells cotreated with doxorubicin and cAMP is profoundly lower than that of the doxorubicin treated cells after 16 h, however, inhibition of NF-κB using Bay 11-7082 (4 μM) completely restores early apoptotic features of doxorubicin even more than doxorubicin alone.

A previous study from this laboratory demonstrated that p53 was induced 2-4 h after treatment with 0.5 μM doxorubicin, however, elevation of cAMP 30 min prior to doxorubicin exposure attenuated p53 accumulation and DNA damage- induced apoptosis. The results of the present study also showed that attenuation of p53 phosphorylation at Ser^{15} and its subsequent accumulation persisted even after 18 h treatment with doxorubicin in the presence of cAMP in the NALM-6 cells (Fig. 1C). The p21

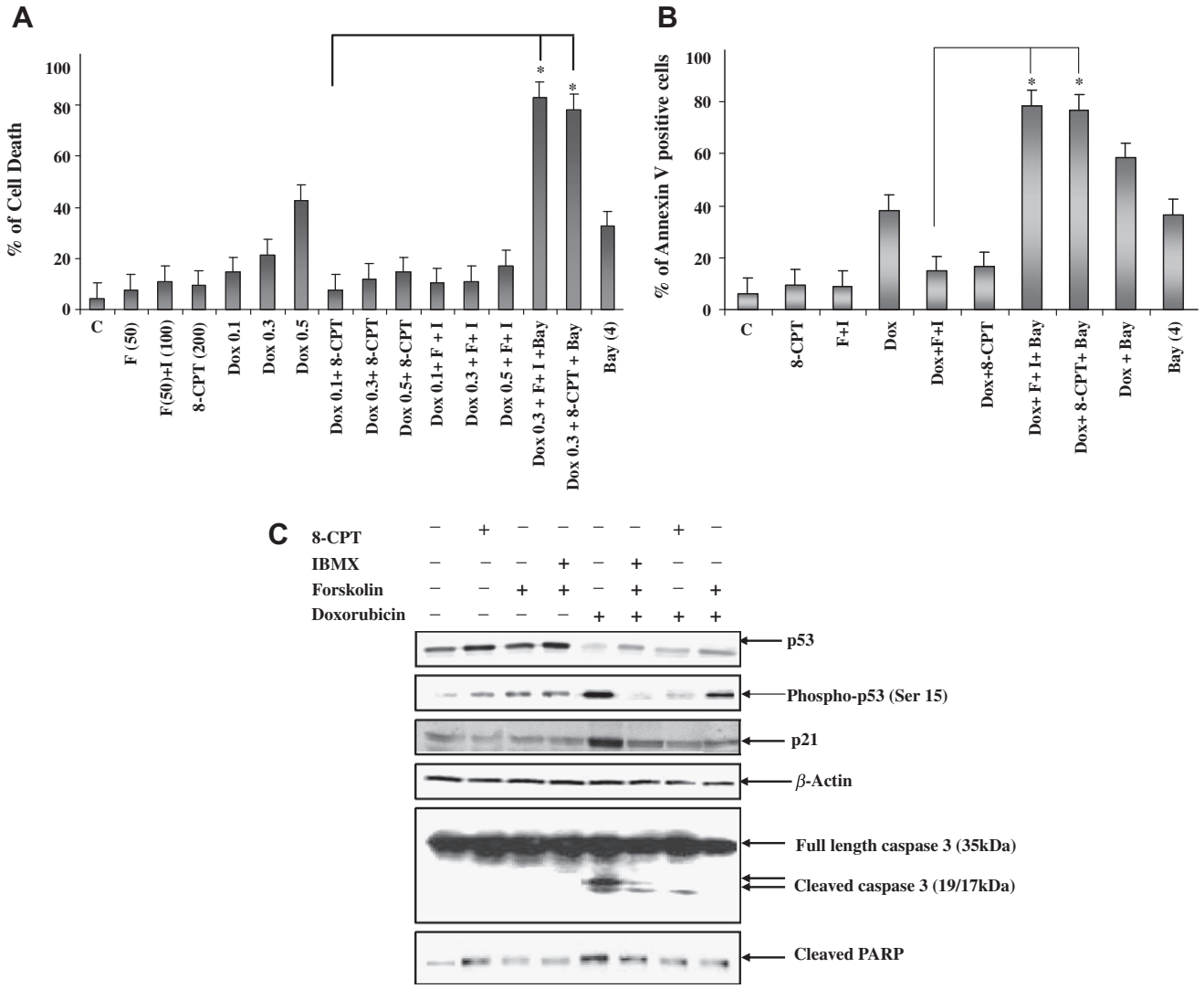

Fig. 1. Inhibition of NF-κB activation reduces the antiapoptotic effects of cAMP in the NALM-6 cells exposed to doxorubicin. (A) NALM-6 cells were preincubated with or without Bay 11-7082 (Bay, 4 μM) for 30 min before treatment with forskolin (F, 50 μM), IBMX (I, 100 μM) or 8-CPT-cAMP (8-CPT 200 μM) for 30 min. The cells were then exposed to various concentrations of doxorubicin (Dox), harvested at 24 h post doxorubicin exposure and analyzed by MTT assay as indicated in the Section 2. Each data point presented is an average of the measurements from 5 wells. The values represented as mean ± S.E. ($n = 3$; $^{*}P < 0.05$; $P < 0.01$ relative to the cells treated with doxorubicin only). (B) The NALM-6 cells were treated with Bay 11-7082 (4 μM) for 30 min prior to addition of forskolin (50 μM) and IBMX (100 μM) or 8-CPT-cAMP (200 μM). After 30 min, the cells were incubated with doxorubicin (0.5 μM) for 16 h and then assayed for cell death using Annexin V staining. The percentage of Annexin V-positive cells was analyzed by FACS. Each value represented the average of three independent experiments ± S.E. ($n = 3$; $^{*}P < 0.05$; $P < 0.01$ relative to the cells treated with doxorubicin only). (C) The inhibitory effect of cAMP on p53 and downstream signaling. Immunoblot analysis was performed using specific antibodies, to investigate the effect of cAMP increasing agents on the level of p53, Phospho-p53 (Ser^{15}), p21 and cleavage of caspase-3 and PARP induced by doxorubicin,. The NALM-6 cells ($0.5–0.8 \times 10^6$) were treated by doxorubicin (0.5 μM) for 16 h in the presence and absence of forskolin (F), IBMX (I) and 8-CPT-cAMP (8-CPT). Equal amounts of the cell lysates were subjected to Western blot analysis using the indicated antibodies. An anti-actin antibody was used to ensure equal loading and quality of the protein extracts.

cyclin-dependent kinase inhibitor, which has been shown to be a direct target of p53 and a component of the DNA damage-induced G1 arrest response, was also reduced in the NALM-6 cells treated by doxorubicin in the presences of forskolin, IBMX and 8-CPT-cAMP (Fig. 1C). Western blot analysis indicated that activation and cleavage of caspase-3 in apoptosis induced by doxorubicin was blocked by the elevation of intracellular cAMP. Activation of caspase-3 leads to the cleavage of a number of proteins, one of which is Poly (ADP-ribose) polymerase (PARP) [12]. These results were also confirmed by observation of PARP cleavage during the apoptosis induced by doxorubicin in the presence or absence of cAMP (Fig. 1C). Taken together, these results show that elevation of cAMP decreases p53-mediated apoptosis and inhibition of NF-κB blocks the inhibitory effect of cAMP on the apoptosis induced by doxorubicin.

3.2. The inhibitory effect of cAMP on doxorubicin-induced apoptosis is not exerted through cAMP dependent protein kinase (PKA)

In the eukaryotic cells, PKA is a key enzyme in signal transduction, representing the main target of the second messenger cAMP. Therefore, we wished to investigate whether inhibition of PKA by H89 affected the inhibitory effect of cAMP on the apoptosis induced by doxorubicin. To do so, the NALM-6 cells were preincubated with or without H89 (30 μM) for 30 min before treatment with forskolin and IBMX for 30 min. The cells were then exposed to doxorubicin, harvested after 16 h and then analyzed for Annexin V uptake by FACS. To confirm the inhibition of PKA by H89, we assessed the effect of H89 on the extent of cAMP responsive element binding (CREB) phosphorylation, as a recognized target of PKA [13], by immunoblot analysis. To this end, the NALM-6 cells

were preincubated with H89 30 min before forskolin and IBMX treatment. The cell were then treated by doxorubicin for 30 min, harvested and subjected to immunoblot analysis with phospho-specific antibodies against CREB phosphorylated at Ser[133]. As can be seen in Fig. 2A, preincubation of the cells with forskolin and IBMX leads to a significant inhibition of the apoptosis induced by doxorubicin. In contrast, pretreatment of the cells with 30 μM H89 could not prevent the inhibitory effect of forskolin and IBMX on the doxorubicin-induced apoptosis. As showed in Fig. 2B, preincubation of the NALM-6 cells by 30 μM H89 completely prevents the forskolin/IBMX-induced CREB phosphorylation. These results indicate that H89 (30 μM) could inhibit PKA function in these experiments.

3.3. Elevation of intracellular cAMP prolong doxorubicin-induced phosphorylation and subsequent degradation of IκB

Almost all signals that lead to activation of NF-κB, converge on the activation of a high molecular weight complex that contains a serine-specific IκB kinase (IKKs). Activation of IKKs, in turn, leads to site-specific phosphorylation of IκB and its degradation by the ubiquitin–proteasome pathway allowing nuclear translocation of NF-κB. The DNA damage-induced phosphorylation of IκB at Ser[32] and Ser[36] has been proposed to contribute to promote NF-κB activation [14]. This together with our present finding that the inhibition of IκB phosphorylation prevents the inhibitory effect of cAMP on doxorubicin-induced apoptosis, suggest that phosphorylation of IκB has major role in the antiapoptotic effect of cAMP. Therefore, we wished to investigate whether cAMP enhances doxorubicin-induced NF-κB DNA-binding by positive regulation of IκB phosphorylation and its subsequent degradation after DNA damage. To this end, the NALM-6 cells were treated with doxorubicin in the presence or absence of forskolin and IBMX, harvested at several intervals up to 24 h and then subjected to western blotting by the antibodies directed toward IκB phosphorylated at Ser[32] and IκBα. As shown in Fig. 3, phosphorylation of IκB was induced in the absence of cAMP by 2 h after doxorubicin incubation, reached its maximum level within 6 h and progressively declined by the endpoint of the experiment at 24 h. Preincubation of the NALM-6 cells with forskolin and IBMX prior to doxorubicin exposure caused an alteration in the phosphorylation pattern of IκB. While, in the presence of cAMP, the phosphorylation of IκB was induced after a lag time of about 4 h and triggered by 6 h after DNA damage-induced by doxorubicin. Then it reached its maximum level within 8 h and remained relatively constant at this level up to 24 h. Moreover, we showed that in spite of the early inhibition of degradation, doxorubicin-induced degradation of IκB was prolonged in compare to the NALM-6 cells treated by doxorubicin alone (Fig. 3, the middle panel).

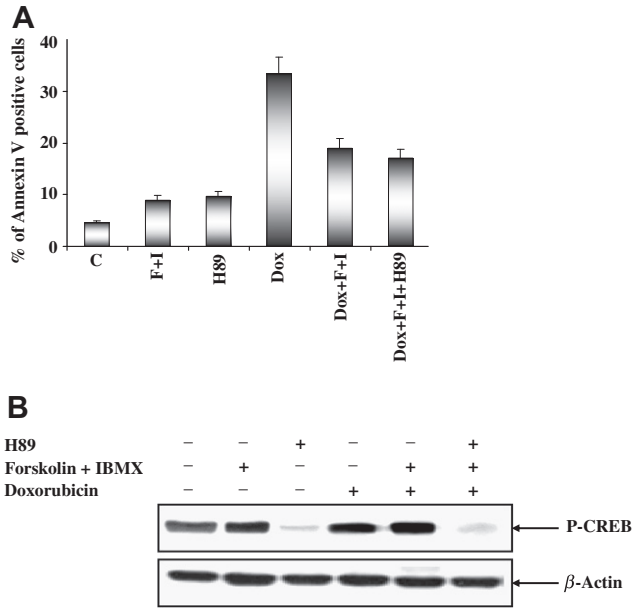

Fig. 2. cAMP exerts its effect on doxorubicin-induced apoptosis through a PKA-independent mechanism. (A) The NALM-6 cells were treated with H89 (30 μM) for 30 min prior to the addition of IBMX and forskolin. After 30 min, the cells were exposed to doxorubicin (0.5 μM) and incubated for an additional 16 h. The cells were then assayed for cell death using Annexin V staining. The percentage of Annexin V-positive cells was analyzed by FACS. Each value represents the average of three independent experiments ± S.E. (B) The NALM-6 cells were treated with H89 (30 μM) for 30 min prior to the addition of IBMX and forskolin. After 30 min, the cells were exposed to doxorubicin and incubated for an additional 30 min. The whole cell lysates were then prepared and analyzed by immunoblotting with the p-CREB (Ser[133]) and anti-actin antibodies as positive control. The immunoblot shows one representative experiment out of three independent experiments.

3.4. cAMP prolongs IκB degradation and NF-κB activation induced by doxorubicin in a proteasome-dependent manner

NF-κB activation induced by DNA damage pathway involves the rapid phosphorylation of IκBα at Ser[32] and Ser[36] by IKKs and subsequent ubiquitin- induced degradation by 26S proteasome. Therefore, to further characterize the mechanism by which cAMP utilizes proteasome to exert its stimulatory effect on NF-κB, we examined the effect of cAMP on IκB level in the presence of the proteasome inhibitor, MG-132, by western immunoblot analysis. As shown in Fig. 4, preincubation of NALM-6 cells with MG-132 (20 μM) prevented the stimulatory effect of forskolin and IBMX on the doxorubicin- induced degradation of IκB. As already mentioned, in the absence of proteasome inhibitor (MG-132), cAMP prolongs phosphorylation and subsequent catabolism of IκB-induced by doxorubicin. Our finding also showed that preincubation with MG-132 caused a slightly increase in phosphorylation of IκBα at Ser[32] (Fig. 4, the middle panel). These observations indicate that phosphorylation of IκBα is not affected by inhibition of proteasome.

3.5. In spite of early inhibition, the cAMP increasing agents potentiate NF-κB DNA-binding activity induced by doxorubicin

Since our results showed that cAMP increasing agents prolong IκB phosphorylation induced by doxorubicin, therefore, we wished to verify whether NF-κB DNA-binding activity also is potentiated by elevation of cAMP in the NALM-6 cells treated by doxorubicin using NF-κB activity assay kit. For this purpose, we examined the effect of forskolin and IBMX on DNA damage- induced NF-κB activity in the presence or absence of Bay 11-7082. As indicated in Fig. 5A, the DNA-binding of NF-κB increased rapidly and reached its maximum level within 4–8 h following the doxorubicin treatment. NF-κB activity then gradually decreased up to 24 h. However, in the presence of forskolin and IBMX, we observed a delay in NF-κB/p65 DNA-binding until 4–6 h of doxorubicin treatment followed by an immediate increase in NF-κB activity up to 24 h. In the presence of Bay 11-7082, cAMP showed no effect on NF-κB activity during the DNA damage.

We then examined whether cAMP affected proapoptotic genes regulated by NF-κB. To this end, prior to doxorubicin incubation in absence or presence of forskolin and IBMX, the NALM-6 cells were treated by Bay 11-7082 to inhibit NF-κB activity. The cells were harvested for 24 h after doxorubicin incubation and then analyzed for the expression of XIAP and Bcl2 levels in the NALM-6 cells by western blot. As shown in Fig. 5B, cAMP induced XIAP and Bcl2 expression in the cells treated by doxorubicin in a NF-κB dependent manner. Inhibition of NF-κB activity using Bay 11-7082

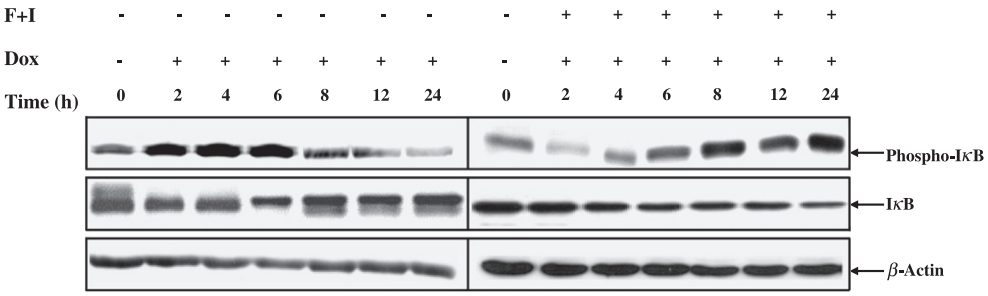

Fig. 3. In spite of early delay, cAMP potentiates IκB phosphorylation and subsequent degradation in the NALM-6 cells incubated by doxorubicin. The NALM-6 cells were preincubated with or without forskolin (F) and IBMX (I) 30 min before treatment with 0.5 μM doxorubicin. The cells were then harvested at the indicated times after doxorubicin exposure and subjected to immunoblot analysis using IκB antibody and phospho-specific antibody against IκB phosphorylated at Ser^{32}. As a loading control, β-actin levels were determined (the bottom panel). One representative experiment of three is shown.

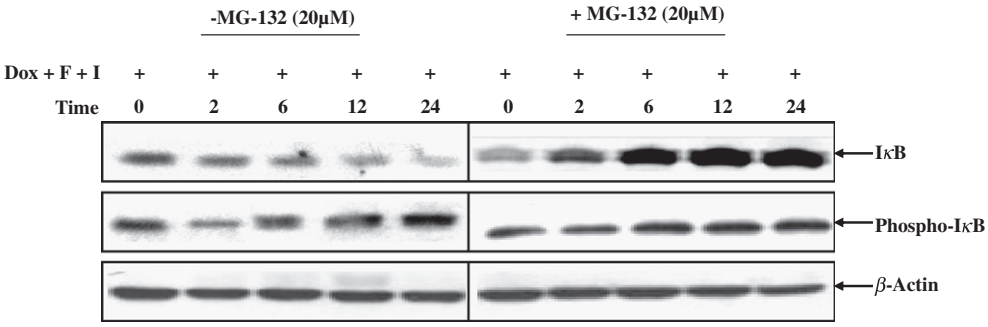

Fig. 4. Stimulation of IκBα degradation induced by cAMP and doxorubicin is blocked by proteasome inhibitors. The NALM-6 cells were preincubated with or without MG-132 for 4 h before treatment with forskolin (F) and IBMX (I) for 30 min. The cells were then treated with doxorubicin, harvested at the indicated times after the doxorubicin exposure, and subjected to immunoblot analysis using IκBα antibody and phospho-specific antibody against phosphorylated form of IκB at Ser^{32}. As a loading control, β-actin levels were determined (the bottom panel). One representative experiment of three is shown.

declined the expression of both proapoptotic proteins in the cells cotreated by doxorubicin and cAMP.

Taken together, these results demonstrate that elevation of intracellular cAMP prolongs NF-κB activity induced by doxorubicin in NALM-6 cells.

4. Discussion

In the case of massive DNA damage, the affected cells can be destroyed by initiating apoptosis. The transcription factor p53, also known as the "guardian of the genome", has a key function in the activation of programmed cell death [2]. But it is not always successful in switching on the apoptotic program. NF-κB often opposes the function of p53 and its activation triggers a survival program that protects the damaged cells from destruction [15]. Activation of NF-κB following genotoxic stress provides an opportunity for DNA-damage repair and ensures cell survival. The activation of this program by NF-κB is considered to be one of the possible causes for the resistance of tumor cells to chemo- or radiation-therapy [16].

Recently, we reported that elevation of cAMP, prior to DNA damage, decreases phosphorylation of key sites in p53, which are pivotal for the release of p53 from Hdm2, translocation into nucleus and subsequent p53-induced apoptosis [10]. Surprisingly, in the present study, our observations showed that PKA, as the major target of cAMP, did not mediate the inhibitory effect of cAMP on the apoptosis induced by doxorubicin. This study also provided evidence, for the first time, that doxorubicin-induced NF-κB activation was prolonged by elevation of cAMP in the NALM-6 human pre-B cells that express wild type p53. This potentiation of NF-κB activation occurs through the enhancement of IκB phosphorylation and its subsequent degradation in a proteasome-dependent manner. Our results also showed that inhibition of NF-κB activation using Bay 11-7082 profoundly reverses the inhibitory effect of cAMP on the doxorubicin-induced apoptosis. Further, based on our findings, although cAMP targets both p53 and NF-κB signalings triggered by doxorubicin in the NALM-6 cells to inhibit apoptosis, NF-κB still retains its key position as a chemoresistance factor.

Several lines of evidence indicate that NF-κB has antiapoptotic role during the DNA damage and inhibition of constitutive and that DNA damage-induced NF-κB activation enhances apoptosis induced by DNA damaging agents [17,18]. Although there is some evidence indicating that the transcriptional activity of NF-κB is stimulated upon phosphorylation of its p65 subunit by PKA and modulation of its interaction with CBP/p300 [19], other studies have shown that cAMP/PKA inhibits NF-κB-mediated transcription in human monocytic cells and endothelial cells [20]. Our results using PKA inhibitor, H89, did not support this idea that PKA mediates antiapoptotic effect of cAMP in the apoptosis induced by doxorubicin. We also observed that the inhibition of PKA by H89 had no effect on IκB degradation induced by doxorubicin (data not shown). We observed that doxorubicin-induced phosphorylation of IκB and subsequent activation of NF-κB were initiated with a delay of 2–4 h in the presence of cAMP. It can be attributed to the pleiotropic effect of cAMP and multiple targets of this second messenger.

It has been shown that Epac has also been implicated in regulating of various cellular processes, notably playing an antiapoptotic role in B-chronic lymphocytic leukaemia cells (B-CLL) [21] and T-ALL cells [22]. However, selective agonist of Epac had no inhibitory effect on the apoptosis induced by doxorubicin in the NALM-6 cells (unpublished data). These results seem to raise a possible role for the third target of cAMP, i.e. cyclic nucleotide-gated (CNG) cation channels [23]. Whether Ca^{+2} as a cation which can influx through

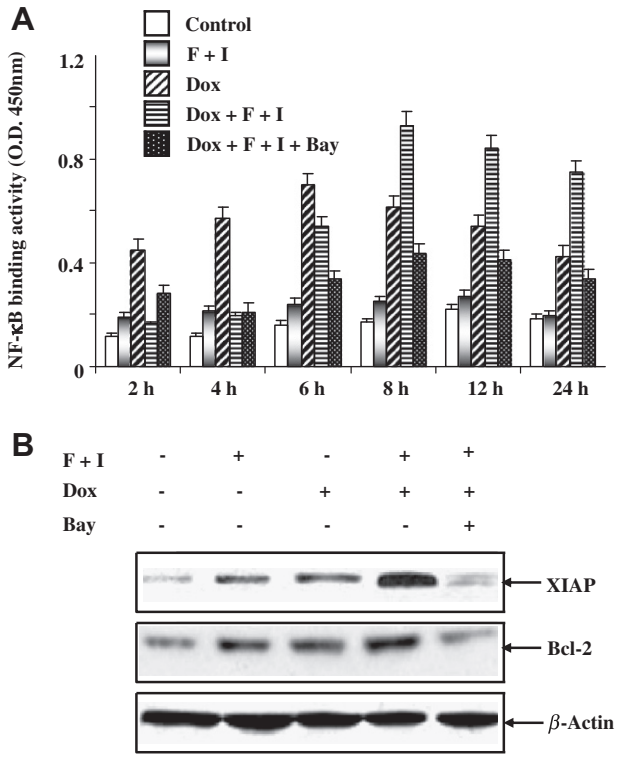

Fig. 5. cAMP increases the induction of NF-κB DNA-binding activity by doxorubicin. (A) The NALM-6 cells were treated with or without NF-κB inhibitor, Bay 11-7082 (4 μM) for 30 min before treatment with forskolin (F) and IBMX (I) for 30 min. The cells were then incubated with doxorubicin and harvested at the indicated times after doxorubicin incubation. Then the nuclear fraction was separated using Nuclear Extraction Kit. NF-κB activity was quantified by enzyme-linked immunosorbent assay using the TransAM NF-B p65 Transcription Factor Assay Kit, according to the manufacturer's instructions. Values represented as mean ± S.E. (B) The NALM-6 cells were treated with Bay 11-7082 (4 μM) for 30 min prior to the addition of forskolin and IBMX (F + I). After 30 min, the cells were exposed to doxorubicin (0.5 μM) and incubated for an additional 24 h. The cells were then harvested and subjected to immunoblot analysis using specific antibodies against XIAP and Bcl2. An anti-actin antibody was used to ensure equal loading and quality of the protein extracts. These are the representative data of more than two experiments.

CNG channel, is mediator of cAMP in the inhibition of apoptosis induced by DNA damage, or whether Ca^{+2} dependent cysteine proteases, calpains, are the main mediators of degradation of p53 and IκB, is still debated. Although, inhibition of proteasome using MG-132 completely prevented the inhibitory effect of cAMP on doxorubicin-induced NF-κB activation, it has been shown that MG-132 can also inhibit calpains activity [24]. It has been suggested that p53 and IκB are substrates for degradation by calcium dependent calpains [25,26]. Further investigations are in progress in our lab to study the role of CNG and intracellular calcium in DNA damage.

Since our findings indicated that cAMP downregulated doxorubicin-induced p53 and potentiates NF-κB activation, a question emerges: how much is the contribution of p53 destabilization and NF-κB activation in the inhibitory effect of cAMP in doxorubicin-induced apoptosis. It has been shown that p53 and NF-κB RelA activated by genotoxic stress mutually suppress each other's ability to activate transcription, most likely through competition for transcriptional coactivators such as CBP or p300 [27,28]. Therefore, the inhibition of p53 function and the secondary activation of NF-κB are among the possible mechanisms responsible for the inhibitory action cAMP on DNA damage-induced apoptosis. Recently, Naderi et al. demonstrated that inhibitory effect of cAMP on gamma-radiation-induced apoptosis is p53 dependent in human pre-B Reh cells [5]. Although, these results indicate that functional p53 might be required for the inhibitory effect of cAMP on DNA damage-induced apoptosis, our results showed that potentiation of doxorubicin-induced NF-κB activation by cAMP is also important in the inhibitory effect of cAMP on the apoptosis induced by doxorubicin. These results further support the notion that NF-κB activation has a major role in regulation of DNA damage pathway.

We preincubated the cells with pharmacological agents to elevate intracellular cAMP in NALM-6 cells, although these results can be also generalized under in vivo conditions of tumor cells. In most of tumor cells cyclooxygenase-2 (COX-2) is overexpressed. Also prostaglandins (PGs) such as PGE2 are produced at heightened levels in these tumors. PGE2 exerts its biological action through elevation of cAMP in a paracrine manner [29]. Therefore, this survival action of cAMP seems to be very important for malignant cells in vivo.

5. Conclusion

Given the importance of NF-κB in immune-cell function, it is not all that surprising that it is involved in the development of such cancers. The present study demonstrated that cAMP increasing agents inhibit p53 accumulation and prolong NF-κB activation during the DNA damage inflicted by doxorubicin. Our results also showed that cAMP can be considered as a survival factor for DNA damaging agents. The complexity of these signaling systems may require additional studies to resolve the connections that serve to integrate cellular responses through these pathways.

Acknowledgement

This study was financially supported by the Vice Chancellor of Research, Iran University of Medical Sciences, Tehran, Iran.

References

[1] Yang, C.R., Wilson-Van Patten, C., Planchon, S.M., Wuerzberger-Davis, S.M., Davis, T.W., Cuthill, S., Miyamoto, S. and Boothman, D.A. (2000) Coordinate modulation of Sp1, NF-kappa B, and p53 in confluent human malignant melanoma cells after ionizing radiation. FASEB 14, 379.
[2] Colman, M.S., Afshari, C.A. and Barrett, J.C. (2000) Regulation of p53 stability and activity in response to genotoxic stress. Mutat. Res. 462, 179–188.
[3] Luo, J.L., Kamata, H. and Karin, M. (2005) IKK/NF-kappaB signaling: balancing life and death – a new approach to cancer therapy. J. Clin. Invest. 115, 2625–2632.
[4] Kaltschmidt, B., Kaltschmidt, C., Hofmann, T.G., Hehner, S.P., Droge, W. and Schmitz, M.L. (2000) The pro- or anti-apoptotic function of NF-kappaB is determined by the nature of the apoptotic stimulus. Eur. J. Biochem. 267, 3828–3835.
[5] Naderi, E.H., Findley, H.W., Ruud, E., Blomhoff, H.K. and Naderi, S. (2009) Activation of cAMP signaling inhibits DNA damage-induced apoptosis in BCP-ALL cells through abrogation of p53 accumulation. Blood 114, 608–618.
[6] Safa, M., Kazemi, A., Zand, H., Azarkeivan, A., Zaker, F. and Hayat, P. (2009) Inhibitory role of cAMP on doxorubicin-induced apoptosis in pre-B ALL cells through dephosphorylation of p53 serine residues. Apoptosis 1, 1.
[7] Piret, B., Schoonbroodt, S. and Piette, J. (1999) The ATM protein is required for sustained activation of NF-kappaB following DNA damage. Oncogene 18, 2261–2271.
[8] Wu, C.J., Conze, D.B., Li, T., Srinivasula, S.M. and Ashwell, J.D. (2006) Sensing of Lys 63-linked polyubiquitination by NEMO is a key event in NF-kappaB activation [corrected]. Nat. Cell Biol. 8, 398–406.
[9] Wuerzberger-Davis, S.M., Nakamura, Y., Seufzer, B.J. and Miyamoto, S. (2007) NF-kappaB activation by combinations of NEMO SUMOylation and ATM activation stresses in the absence of DNA damage. Oncogene 26, 641–651.
[10] Huang, T.T., Wuerzberger-Davis, S.M., Wu, Z.H. and Miyamoto, S. (2003) Sequential modification of NEMO/IKKgamma by SUMO-1 and ubiquitin mediates NF-kappaB activation by genotoxic stress. Cell 115, 565–576.
[11] Mosmann, T. (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Methods 65, 55–63.
[12] Boulakia, C.A., Chen, G., Ng, F.W., Teodoro, J.G., Branton, P.E., Nicholson, D.W., Poirier, G.G. and Shore, G.C. (1996) Bcl-2 and adenovirus E1B 19 kDA protein prevent E1A-induced processing of CPP32 and cleavage of poly(ADP-ribose) polymerase. Oncogene 12, 529–535.

[13] Richards, J.P., Bachinger, H.P., Goodman, R.H. and Brennan, R.G. (1996) Analysis of the structural properties of cAMP-responsive element-binding protein (CREB) and phosphorylated CREB. J. Biol. Chem. 271, 13716–13723.
[14] Bender, K., Gottlicher, M., Whiteside, S., Rahmsdorf, H.J. and Herrlich, P. (1998) Sequential DNA damage-independent and -dependent activation of NF-[kappa]B by UV. EMBO J. 17, 5170–5181.
[15] Perkins, N.D. (2004) NF-kappaB: tumor promoter or suppressor? Trends Cell. Biol. 14, 64–69.
[16] Salvatore, C., Camarda, G., Maggi, C.A., Goso, C., Manzini, S. and Binaschi, M. (2005) NF-kappaB activation contributes to anthracycline resistance pathway in human ovarian carcinoma cell line A2780. Int. J. Oncol. 27, 799–806.
[17] Brooks, C.L. and Gu, W. (2003) Ubiquitination, phosphorylation and acetylation: the molecular basis for p53 regulation. Curr. Opin. Cell. Biol. 15, 164–171.
[18] Braun, T., Carvalho, G., Fabre, C., Grosjean, J., Fenaux, P. and Kroemer, G. (2006) Targeting NF-kappaB in hematologic malignancies. Cell Death Differ. 13, 748–758.
[19] Zhong, H., May, M.J., Jimi, E. and Ghosh, S. (2002) The phosphorylation status of nuclear NF-kappa B determines its association with CBP/p300 or HDAC-1. Mol. Cell 9, 625–636.
[20] Ollivier, V., Parry, G.C., Cobb, R.R., de Prost, D. and Mackman, N. (1996) Elevated cyclic AMP inhibits NF-kappaB-mediated transcription in human monocytic cells and endothelial cells. J. Biol. Chem. 271, 20828–20835.
[21] Tiwari, S., Felekkis, K., Moon, E.Y., Flies, A., Sherr, D.H. and Lerner, A. (2004) Among circulating hematopoietic cells, B-CLL uniquely expresses functional EPAC1, but EPAC1-mediated Rap1 activation does not account for PDE4 inhibitor-induced apoptosis. Blood 103, 2661–2667.
[22] Ji, Z., Mei, F.C., Johnson, B.H., Thompson, E.B. and Cheng, X. (2007) Protein kinase A, not Epac, suppresses hedgehog activity and regulates glucocorticoid sensitivity in acute lymphoblastic leukemia cells. J. Biol. Chem. 282, 37370–37377.
[23] Rochais, F., Vandecasteele, G., Lefebvre, F., Lugnier, C., Lum, H., Mazet, J.L., Cooper, D.M. and Fischmeister, R. (2004) Negative feedback exerted by cAMP-dependent protein kinase and cAMP phosphodiesterase on subsarcolemmal cAMP signals in intact cardiac myocytes: an in vivo study using adenovirus-mediated expression of CNG channels. J. Biol. Chem. 279, 52095–52105.
[24] Mailhes, J.B., Hilliard, C., Lowery, M. and London, S.N. (2002) MG-132, an inhibitor of proteasomes and calpains, induced inhibition of oocyte maturation and aneuploidy in mouse oocytes. Cell Chromosome 1, 2.
[25] Kubbutat, M.H. and Vousden, K.H. (1997) Proteolytic cleavage of human p53 by calpain: a potential regulator of protein stability. Mol. Cell. Biol. 17, 460–468.
[26] Pianetti, S., Arsura, M., Romieu-Mourez, R., Coffey, R.J. and Sonenshein, G.E. (2001) Her-2/neu overexpression induces NF-kappaB via a PI3-kinase/Akt pathway involving calpain-mediated degradation of IkappaB-alpha that can be inhibited by the tumor suppressor PTEN. Oncogene 20, 1287–1299.
[27] Gurova, K.V. et al. (2005) Small molecules that reactivate p53 in renal cell carcinoma reveal a NF-kappaB-dependent mechanism of p53 suppression in tumors. Proc. Natl. Acad. Sci. USA 102, 17448–17453.
[28] Ikeda, A. et al. (2000) P300/CBP-dependent and -independent transcriptional interference between NF-kappaB RelA and p53. Biochem. Biophys. Res. Commun. 272, 375–379.
[29] Wang, M.T., Honn, K.V. and Nie, D. (2007) Cyclooxygenases, prostanoids, and tumor progression. Cancer Metastasis Rev. 26, 525–534.