Amino Acids (2004) 27: 207–214
DOI 10.1007/s00726-004-0101-9

Amino Acids
Printed in Austria

Polyamine depletion inhibits etoposide-induced NF-κB activation in transformed mouse fibroblasts

B. Tantini[1], **C. Pignatti**[1], **M. Fattori**[1], **E. Fiumana**[1], **A. Facchini**[1], **C. Stefanelli**[1], **C. M. Caldarera**[1], **A. E. Pegg**[2], and **F. Flamigni**[1]

[1] Department of Biochemistry "G. Moruzzi", University of Bologna, Bologna, Italy
[2] Department of Cellular and Molecular Physiology, Pennsylvania State University College of Medicine, The Milton S. Hershey, Pennsylvania, U.S.A.

Received February 28, 2004
Accepted April 27, 2004
Published online September 22, 2004;

Summary. In a previous research, we have shown that adequate levels of polyamines are required in transformed mouse fibroblasts for the correlated activations of MAPK subtypes (ERK and JNK) and caspases induced by etoposide and leading to apoptosis. We report now that the treatment of fibroblasts with etoposide also elicited a progressive and sustained increase of NF-κB activation. The DNA binding activity of p65 NF-κB subunit was increased up to approximately 4-fold and was accompanied by enhancement of p65 phosphorylation. A two days pre-treatment of fibroblasts with α-difluoromethylornithine (DFMO), which caused polyamine depletion, provoked a slight activating effect when given alone, but markedly inhibited the etoposide-induced increases in p65 DNA binding and phosphorylation. The NF-κB inhibiting effect of DFMO was prevented by the addition of exogenous putrescine, which restored the intracellular content of polyamines. Selective inhibitors of the etoposide-stimulated MAPK subtypes also reduced NF-κB activation. Moreover, pharmacological NF-κB inhibition reduced the increase in caspase activity and cell death elicited by etoposide, suggesting that NF-κB is involved in signaling to apoptosis. The results of the present study, together with our previous findings, suggest that polyamines play a permissive role in the pathways triggered by etoposide and leading to cell death of fibroblasts, by supporting the activation of MAPKs, NF-κB and caspases.

Keywords: Apoptosis – Difluoromethylornithine – Etoposide – MAPK – NF-κB – Polyamines

Abbreviations: NF-κB, nuclear factor κB; IKK, I-κB kinase; DFMO, α-difluoromethylornithine; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; JNK, Jun N-terminal kinase; AcDEVD-AMC, acetyl-Asp-Glu-Val-Asp-amino-4-methylcoumarin

1 Introduction

Nuclear factor κB (NF-κB) is a key transcription factor in the immune and inflammatory responses, and in the control of cell growth and survival (Ghosh and Karin, 2002). Although NF-κB is more often regarded as a pro-survival factor, several reports indicate it can participate even in apoptotic pathways (Barkett and Gilmore, 1999). NF-κB can stimulate the expression of either anti-apoptotic or pro-apoptotic genes and the final outcome may be context sensitive and cell-type specific.

Five members of the NF-κB/Rel family have been described in mammalian cells, however the most prevalent DNA-binding form of NF-κB is a heterodimer of the p50 and p65 (Rel A) subunits (Barkett and Gilmore, 1999; Schmitz et al., 2001; Ghosh and Karin, 2002; Vermeulen et al., 2002). In unstimulated cells, NF-κB is largely (but not completely) located in the cytoplasm and bound to an inhibitory molecule of the I-κB family. The canonical mechanism of NF-κB activation entails the rapid phosphorylation of I-κB by the I-κB kinase (IKK) complex (Ghosh and Karin, 2002; Li et al., 2002). The subsequent ubiquitination and degradation of the inhibitor by the 26S proteasome allows NF-κB to accumulate in the nucleus and bind to specific κB elements in target genes. However, a further mechanism of NF-κB activation is emerging that is I-κB independent and involves the phosphorylation of NF-κB subunits, which may affect various functions of NF-κB including DNA binding and transcriptional competence (Schmitz et al., 2001; Vermeulen et al., 2002). In particular, the p65 subunit can be phosphorylated at various serine residues. Phosphorylation of Ser-536 in the transactivation domain-1 can be accomplished by the IKK complex and results important for NF-κB activation elicited by various stimuli. Thus, it has been

reported quite recently (Jiang et al., 2003) that NF-κB can be activated by lymphotoxin β receptor signalling without altering the level of the phosphorylation of I-κB and the nuclear localization of p65, but by inducing the phosphorylation of p65 at Ser-536. Control of NF-κB and I-κB phosphorylation may involve several signalling pathways in different cells, including those utilizing members of the mitogen-activated protein kinase (MAPK) family, such as extracellular signal-regulated kinases (ERK), Jun N-terminal kinases (JNK) and p38 MAPKs (Schulze-Osthoff et al., 1997; Vermeulen et al., 2002).

Another class of molecules correlated to both cell growth and cell death is represented by the polyamines, putrescine, spermidine and spermine (Pegg et al., 1995; Monti et al., 1999; Thomas and Thomas, 2001). The concentration of these organic polycations in cells can be finely modulated by enzymatic and transport systems. We have found that inhibition of polyamine biosynthesis by α-difluoromethylornithine (DFMO) can sensitize transformed mouse fibroblasts to apoptosis or protect them depending on the death trigger employed (Stefanelli et al., 2001), thus hinting at the importance of the pathway engaged. Interestingly, some studies suggest the existence of a cross-talk between polyamine and NF-κB pathways (Shah et al., 1999, 2001; Li et al., 2001; Pfeffer et al., 2001; Tantini et al., 2002). Li et al. (2001) have proposed that polyamine depletion by DFMO modifies the susceptibility of intestinal epithelial cells to apoptosis by inducing NF-κB activation. On the other hand, another group (Shah et al., 1999, 2001) reported that addition of polyamines increased NF-κB DNA binding activity in cell-free extracts and in intact breast cancer cells. In order to explore the mechanisms underlying the polyamine requirement for apoptosis induced in transformed fibroblasts by etoposide (Stefanelli et al., 2001), a drug used in cancer chemotherapy, we have examined the role of polyamines in the signalling pathways stimulated by etoposide. Previously (Stefanelli et al., 2002), we reported evidence that polyamine depletion by DFMO blocked a sustained activation of the MAPKs, ERK and JNK. This, in turn, appeared to be necessary for the activation of caspases, the proteases that execute the apoptotic program (Shi, 2002).

Since the NF-κB pathway can be activated by MAPKs (Schulze-Osthoff et al., 1997; Chen et al., 2001; Vermeulen et al., 2002) and by etoposide itself (Kasibhatla et al., 1998; Watanabe et al., 2000; Tabata et al., 2001; Spalding et al., 2002) in some cell systems, we have investigated in the present study whether NF-κB may play a role in the impaired response to etoposide provoked by polyamine depletion in transformed fibroblasts.

2 Materials and methods

2.1 Materials and cell cultures

DFMO was kindly provided by Ilex Oncology, Inc. PD98059 and Bay 11-7082 were from Alexis, SP600125 was purchased from Tocris. Etoposide, putrescine, *N*-carbobenzoxy-Leu-Leu-Leucinal (MG-132), acetyl-Asp-Glu-Val-Asp-amino-4-methylcoumarin (AcDEVD-AMC) and all other biochemical reagents were products of Sigma Chemical Company. Rabbit polyclonal antibodies anti p50 and p65 NF-κB, phospho-p65 NF-κB (Ser 536) and phospho-IκB-α (Ser 32) were from Cell Signaling Technology, whereas rabbit polyclonal antibodies against IκB-α were from Santa Cruz Biotechnology, Inc.

Fibroblasts from normal mice were derived from 14-day-old male embryos and immortalized by transfection with a plasmid expressing SV40 large and small T antigens, and grown as described (Mackintosh and Pegg, 2000; Stefanelli et al., 2001). In all the experiments, cells were cultured for 2 days and then treated with etoposide, in the presence or absence of different inhibitors, for the time indicated. To obtain polyamine depletion, the medium was added with 100 μM DFMO from the seeding (Stefanelli et al., 2002). Thus, DFMO was still present during etoposide treatment. Cell viability was evaluated by trypan blue exclusion, with the percentage of cell death being defined as the percentage of cells that included the dye. The data presented as Table 1 and bar graphs were analysed for statistical significance ($p < 0.05$) by unpaired t-test.

2.2 Nuclear cell extract preparation and NF-κB DNA binding activity

Nuclear cell extracts were prepared by employing a kit from Active Motif. At the end of the incubations, the cells were washed, collected in ice-cold PBS in the presence of phosphatase inhibitors and centrifuged at 500 rpm for 5 min. The pellets were then resuspended in a hypotonic buffer, treated with detergent and centrifuged at 14,000 g for 30 sec. After collection of cytoplasmic fraction, the nuclei were lysed and nuclear proteins solubilized in the lysis buffer in the presence of the protease inhibitors cocktail.

The binding of NF-κB to DNA was measured in nuclear extracts with a specific TransAM^{TM}NF-κB p65 assay kit from Active Motif, according to manufacturers' instructions. This assay is based on the use of multi-well plates coated with a cold oligonucleotide containing the consensus binding site for NF-κB. Nuclear proteins (20 μg) were added to each well and incubated for 1 h to allow the binding of NF-κB to this oligonucleotide. The presence of the DNA-bound transcription factor was then detected by a primary antibody that recognizes an epitope on p65 only when NF-κB is activated and bound to its target DNA. After addition of a secondary antibody conjugated to horseradish peroxidase, the results were quantified by spectrophotometry. The specificity of the assay was checked by adding wild-type or mutated consensus oligonucleotide.

2.3 Western blotting

The determinations of the levels and phosphorylation of p65 and p50 NF-κB or IκB-α were performed by Western blotting, essentially as described (Flamigni et al., 1999), using specific primary antibodies. Immunoreactive bands were visualized by chemiluminescence and quantified by intensitometric software QScan from Biosoft. In some experiments NF-κB levels were assessed in nuclear extracts.

2.4 Polyamine analysis

Polyamines were separated and quantified in acidic cellular extracts by HPLC after derivatization with dansyl chloride (Stefanelli et al., 2001, 2002). Polyamine content is expressed as nmol/mg of protein.

2.5 Caspase activity

The activity of caspase enzymes was measured by the cleavage of the fluorogenic peptide substrate AcDEVD-AMC, that represents a substrate for caspase 3 and other members of the caspase family, during a 15 min at 37°C as previously described (Stefanelli et al., 1998). Caspase activity is expressed as unit/mg of protein, where one unit is defined as the amount of enzyme activity cleaving 1.0 nmol of substrate per minute.

2.6 TUNEL assay

The terminal deoxynucleotidyl tranferase-mediated dUTP nick end-labelling (TUNEL) assay was performed using a colorimetric reagent kit (TiterTACS from Trevigen, Inc), which allows quantitative labelling of the 3′-OH ends of fragmented DNA in cultured cells. Briefly, fibroblasts were fixed, washed in PBS and labelled according to manufacturer's instructions. After washing the samples with PBS, colorimetric reaction was allowed to occur and absorbance was read at 450 nm on a microplate reader (Victor^2, Perkin Elmer).

3 Results

3.1 Effect of etoposide and polyamine depletion on NF-κB activation

In order to address the question of the role of polyamines in etoposide-triggered death signalling, mouse fibroblasts were incubated for two days with 100 μM DFMO, an irreversible inhibitor of ornithine decarboxylase (ODC), the key enzyme of polyamine biosynthesis, before being treated with etoposide. Table 1 shows that the 48 h treatment with DFMO reduced putrescine content to undetectable levels and spermidine by about 80%, whereas spermine content was not affected, as usually reported for this drug (Thomas and Thomas, 2001). The depletion or strong reduction in putrescine and spermidine level provoked by DFMO, was completely prevented by co-administration of 100 μM putrescine.

Table 1. Effect of DFMO on polyamine content in mouse fibroblasts

Treatment	Polyamine content (nmol/mg)		
	Putrescine	Spermidine	Spermine
Control	3.4 ± 1.0	24.0 ± 3.9	8.1 ± 1.7
DFMO	<0.4*	4.9 ± 1.6*	9.0 ± 1.2
DFMO + Putrescine	5.4 ± 0.8	26.1 ± 2.8	7.7 ± 1.0

Cells were grown for 48 h after plating without any addition (Control), in the presence of 100 μM DFMO or in the presence of 100 μM DFMO plus 100 μM putrescine. Then cells were harvested and assayed for polyamine content

* $p < 0.05$ vs. control cells. Data represent means ± SEM of three determinations

To investigate possible effects of etoposide and polyamines on NF-κB activation, etoposide (20 μM) was added to both non-treated and DFMO treated cells, then the DNA binding activity of NF-κB was measured by using a sensitive and p65 directed ELISA-based assay (Fig. 1A). The treatment with etoposide resulted in a progressive increase of DNA binding activity of the p65 NF-κB subunit (up to about 4-fold after 16 h). DFMO pretreatment provoked a small increase in basal p65 DNA binding (i.e. in the absence of etoposide), but prevented the etoposide-induced increase almost completely (Fig. 1A and B). The effect of DFMO is polyamine-specific, since addition of 100 μM putrescine to DFMO-treated fibroblasts, which restored polyamine content, allowed the enhancement of p65 DNA binding by etoposide (Fig. 1B).

Phosphorylation of p65 NF-κB subunit is emerging as an important mechanism of NF-κB activation (Schmitz et al., 2001; Vermeulen et al., 2002; Li et al., 2002; Jiang et al.,

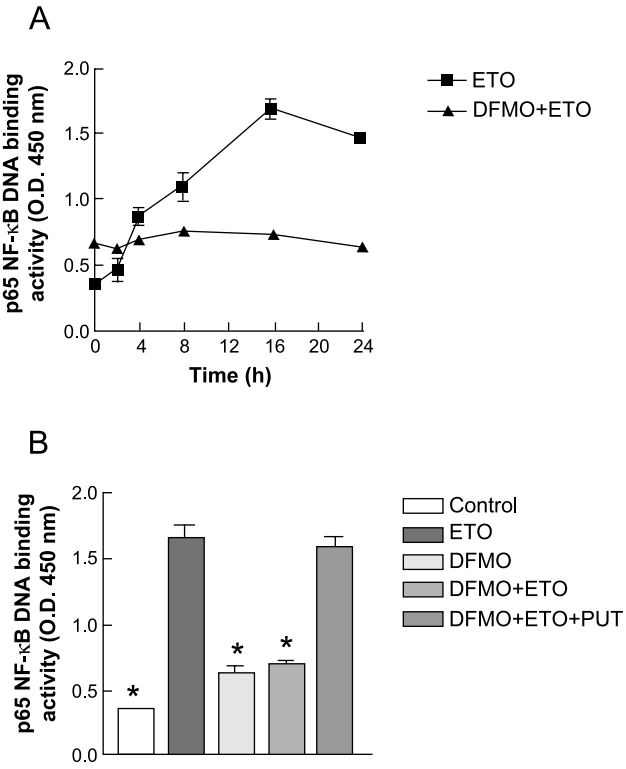

Fig. 1. Effect of etoposide and polyamine depletion on NF-κB DNA binding activity in mouse fibroblasts. **A** Mouse fibroblasts, grown for 48 h after plating in the presence or absence of 100 μM DFMO, were then treated with 20 μM etoposide for the times indicated. **B** The cells, grown for 48 h after plating in the presence or absence of 100 μM DFMO or 100 μM DFMO plus 100 μM putrescine, were then treated with 20 μM etoposide for 16 h. Cells were collected and nuclear extracts were analyzed for p65 NF-κB DNA binding activity. Data are means ± SEM (N = 3); * $p < 0.05$ vs. etoposide-treated cells. *ETO*, etoposide; *PUT*, putrescine

2003). Therefore, we have measured the level of phosphorylated p65 in etoposide- and DFMO-treated fibroblasts by immunoblotting using an anti-phospho-p65 (Ser-536)-specific antibody (Fig. 2). Figure 2A shows how the amount of phosphorylated p65 increased progressively after etoposide up to 8 h of treatment. DFMO pre-treatment reduced this increase and putrescine addition prevented the DFMO effect (Fig. 2B). Figure 2B also shows that these changes were not accompanied by significant modifications in the total amount of p65 (as well as of p50), ruling out effects on the expression of these NF-κB subunits.

We have then investigated whether the changes in NF-κB DNA binding activity provoked by etoposide and DFMO could be brought about via regulation of I-κB degradation and NF-κB nuclear translocation.

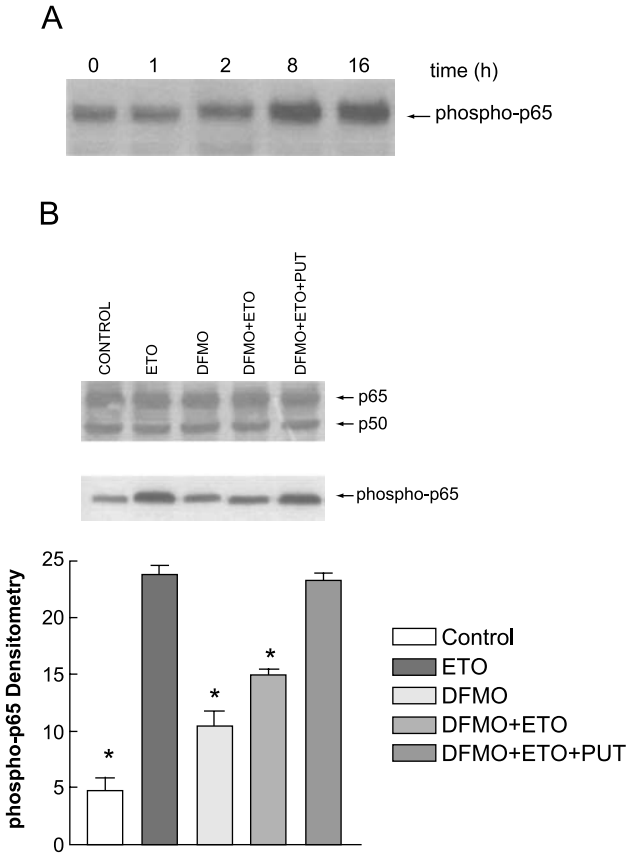

Fig. 2. Effect of etoposide and polyamine depletion on the phosphorylation of p65 NF-κB subunit in mouse fibroblasts. **A** Mouse fibroblasts were grown for 48 h and then incubated with 20 μM etoposide for the times indicated. **B** Mouse fibroblasts, grown for 48 h after plating in the presence or absence of 100 μM DFMO or 100 μM DFMO plus 100 μM putrescine, were then treated with 20 μM etoposide for 8 h. Cell extracts were analysed by Western Blotting using specific antibodies for total p50 and p65 or phosphorylated p65 NF-κB subunits. In the case of phosphorylated p65, quantification of the bands by densitometry is also shown (**B**, bottom). Data are means $\pm$ SEM (N = 3); * $p < 0.05$ vs. etoposide-treated cells. *ETO*, etoposide; *PUT*, putrescine

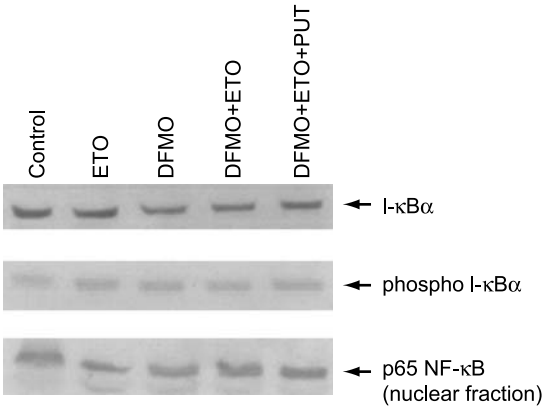

Fig. 3. Effect of etoposide and polyamine depletion on the levels of I-κBα, phosphorylated I-κBα and nuclear p65 NF-κB in mouse fibroblasts. Mouse fibroblasts were grown for 48 h after plating in the presence or absence of 100 μM DFMO or 100 μM DFMO plus 100 μM putrescine and then treated with 20 μM etoposide for 8 h. Cell extracts were analysed by Western Blotting using specific antibodies for total I-κBα, phosphorylated I-κBα or total nuclear p65 NF-κB. The results shown are representative of three experiments

We could detect the presence of I-κBα in control cells even in the phosphorylated form, but were unable to demonstrate any reproducible or marked modulation in the amounts of total and phosphorylated I-κBα after treatment with etoposide (Fig. 3). The I-κBα band disappeared only at a late time (24 h) (not shown). The level of p65 NF-κB in the nuclear fraction was also not changed following etoposide (Fig. 3). Likewise, DFMO pre-treatment had no significant effect on the amount of I-κBα and nuclear p65 NF-κB. It should be noted that treatment with 5 μM MG-132, a proteasome inhibitor (Stefanelli et al., 1998) widely used to inhibit the I-κB pathway, reduced the etoposide-induced increase in NF-κB DNA binding activity by about 50% (not shown). In conclusion, these data suggest that a steady-state I-κB-dependent control of NF-κB operates in this experimental system, but apparently, it does not represent a major mechanism by which etoposide and DFMO affect NF-κB activation.

3.2 Effect of kinase inhibitors on etoposide-induced NF-κB activation and apoptosis

We have previously demonstrated that etoposide provoked a marked activation of the ERK and JNK subtypes of MAPKs in the current model of fibroblasts, whereas another family member, p38 MAPK, was hardly affected (Stefanelli et al., 2002). Thus, selective inhibitors of ERK and JNK pathways were utilized to assess a possible role

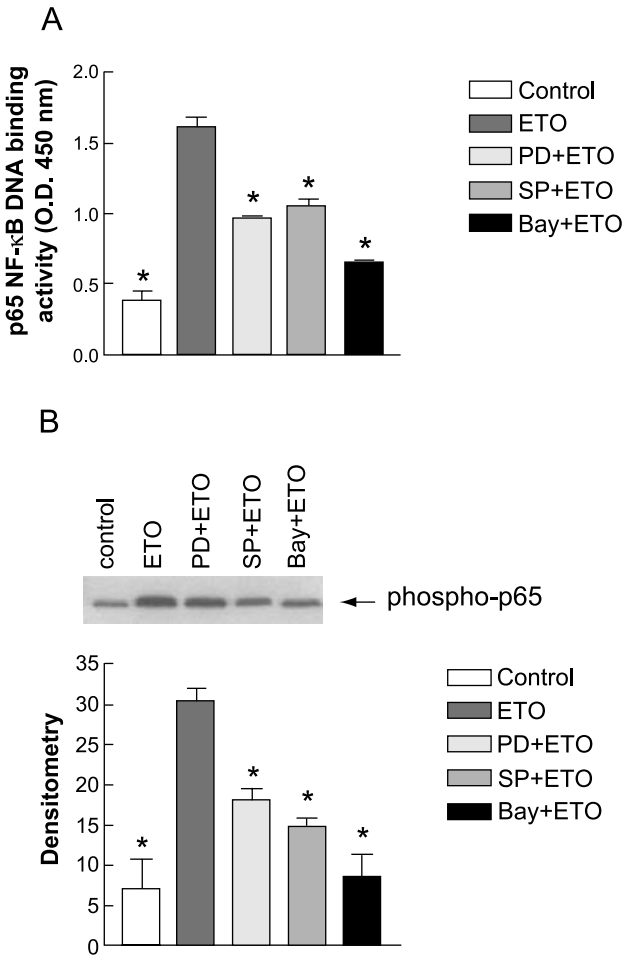

Fig. 4. Effect of kinase inhibitors on etoposide-induced NF-κB activation in mouse fibroblasts. **A** Mouse fibroblasts, pretreated with 50 μM PD98059 or 10 μM SP600125 for 30 min, or with 10 μM Bay 11-7082 for 1 h, were then incubated with 20 μM etoposide for 16 h. Cells were collected and nuclear extracts were analysed for p65 NF-κB DNA binding activity. **B** Mouse fibroblasts were treated as described above. After the incubation with etoposide for 8 h, cells were collected and the extracts were analysed by Western Blotting using specific antibodies for phosphorylated p65 NF-κB. At the bottom, quantification of the bands by densitometry is shown. Data are means ± SEM (N = 3); * $p < 0.05$ vs. etoposide-treated cells. *ETO*, etoposide

of these MAPK subtypes in NF-κB activation. Figure 4A shows that PD98059, widely used to dissect the ERK pathway, and SP600125, a new developed JNK inhibitor (Bennett et al., 2001) partially prevented the increase in NF-κB DNA binding activity caused by etoposide, at the doses usually utilized to specifically block these pathways (Bennett et al., 2001; Stefanelli et al., 2002). PD98059 and SP600125 also reduced the phosphorylation of p65 at Ser-536 (Fig. 4B). In addition, pre-treatment with Bay 11-0782, an irreversible and selective IKK inhibitor (Pierce et al., 1997; Huang et al., 2002), markedly inhibited both p65 DNA binding activity and p65 phosphorylation (Fig. 4), suggesting that both events are dependent on IKK activity. The amount of total p65 (and p50) was not significantly changed by all these chemical inhibitors (not shown).

Etoposide treatment induces cell death of transformed fibroblasts sustained by a progressive caspase activation (Stefanelli et al., 2001, 2002). In order to ascertain whether the NF-κB pathway is implicated in the etoposide-induced apoptosis, the effect of Bay 11-0782 on the induction of caspase activity and cell death was examined. As shown in Fig. 5, Bay 11-0782 reduced the increase in caspase activity and cell death that followed etoposide treatment. The extent of DNA strand fragmentation was also decreased, as judged by a quantitative TUNEL assay. The proteasome inhibitor MG-132 also reduced the apoptosis-related effects elicited by etoposide. Neither Bay 11-0782 nor MG-132 modified caspase activity when given alone and only slightly increased the extent of cell death and fragmented DNA.

4 Discussion

Etoposide is regarded as a ''slow-kinetic'' NF-κB activating agent with respect to classical stimuli, such as the pro-inflammatory cytokines, tumor necrosis factor or interleukin-1 (Huang et al., 2002). Accordingly, we have found in the present study that etoposide can induce an NF-κB activation that requires several hours to reach relatively high levels, but persisted at least up to 24 h, as judged by a sensitive ELISA-based assay of the p65 DNA binding activity. A picture of increasing complexity is emerging from recent investigations on the control of NF-κB and includes multiple phosphorylation of NF-κB subunits (Schmitz et al., 2001; Vermeulen et al., 2002). Our findings indicate a dissociation between the control of nuclear translocation of NF-κB and its DNA binding activity. Koul (2001) found that expression of PTEN inhibited cytokine-induced DNA binding of NF-κB without interfering with the I-κB pathway and nuclear translocation of NF-κB, but rather by preventing the phosphorylation of NF-κB subunits. In fact, phosphorylation of either p50 or p65 can reportedly increase their affinity for DNA (Hayashi et al., 1993; Naumann and Scheidereit, 1994; Koul et al., 2001). According to the current study, an important mechanism of NF-κB activation by etoposide may be a progressive enhancement of the phosphorylation of p65 at Ser-536. Signal-induced p65 phosphorylation appears to favour κB-dependent gene expression by augmenting the transcriptional capacity of NF-κB and the recruitment of co-activators, such as

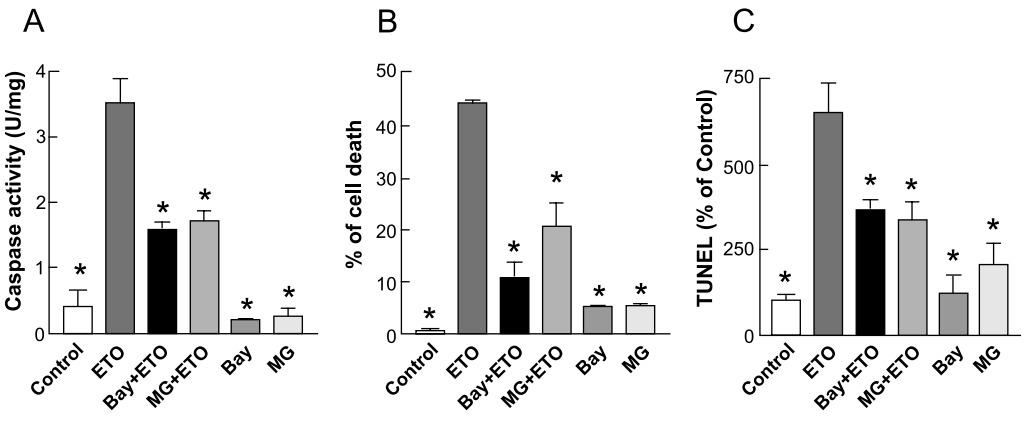

Fig. 5. Effect of NF-κB inhibition on caspase activity and cell death of mouse fibroblasts treated with etoposide. Mouse fibroblasts, pretreated with 10 μM Bay 11-7082 for 1 h or 5 μM MG-132 for 30 min were incubated in the presence or absence of 20 μM etoposide for 24 h. Then cells were analysed for caspase activity (**A**), for cell viability by trypan blue exclusion (**B**), and for DNA strand break fragmentation by TUNEL assay (**C**). Data are means ± SEM (N = 3); * $p < 0.05$ vs. etoposide-treated cells. *ETO*, etoposide

CBP/p300, rather than its nuclear localization (Schmitz et al., 2001; Vermeulen et al., 2002; Li et al., 2002; Jiang et al., 2003). Ser-536, located in the transactivation domain-1, is a major inducible phosphorylation site of p65. However, although not examined, phosphorylation of NF-κB subunits at other sites may also be increased following etoposide.

The experiments with Bay 11-0782 suggest a key role for the IKK complex in the control of both p65 phosphorylation and p65 DNA binding. The IKK complex can control multiple mechanisms of NF-κB activation (Schmitz et al., 2001; Vermeulen et al., 2002) and represents a convergence point for most NF-κB activating stimuli, including etoposide (Huang et al., 2002). MAPKs can stimulate NF-κB through IKK-dependent or independent pathways (Schulze-Osthoff et al., 1997; Chen and Lin, 2001; Vermeulen et al., 2002). We have reported that etoposide elicited a marked and sustained activation of the ERK and JNK members of MAPK family in fibroblasts (Stefanelli et al., 2002). The experiments with selective, pharmacological inhibitors of MAPK pathways indicate that the ERK and JNK subtypes can contribute to NF-κB activation in this model.

Polyamine depletion by DFMO largely prevented the etoposide-induced increases in p65 DNA binding and phosphorylation. Since DFMO can block the activation of MAPKs elicited by etoposide (Stefanelli et al., 2002), it can be hypothesized that the NF-κB inhibiting actions of DFMO may be due, at least in part, to its effects on the MAPKs. The ability of polyamines to affect transcription factors through indirect effects on signalling pathways has been suggested (Bacharach et al., 2001). However, other modes of action of polyamines on NF-κB cannot be excluded. Polyamines are flexible, polycationic molecules that can bind to nucleic acids and proteins and directly affect DNA-protein interactions (Thomas and Thomas, 2001). In accordance with this notion, Shah et al. (Shah et al., 1999, 2001) demonstrated that addition of polyamines at millimolar concentrations to cellular extracts of breast cancer cells can favour the binding of NF-κB to its specific response element. The same group reported that addition of spermine to intact cells facilitated the formation of NF-κB complexes with DNA and the co-activator CBP/p300, as well as the NF-κB driven transcription in transfection experiments (Shah et al., 1999, 2001). However, two studies that examined the effects of the treatment of intestinal epithelial cells with DFMO (Li et al., 2001; Pfeffer et al., 2001), found that it stimulated the formation of NF-κB DNA complexes, at least in part through the I-κB pathway and NF-κB nuclear translocation. In partial agreement with these findings, we found a small, but reproducible increase of basal p65 NF-κB binding activity when DFMO was administered alone.

It has been proposed that NF-κB is a mediator in the etoposide-induced apoptosis of some cell types (Kasibhatla et al., 1998; Watanabe et al., 2000; Spalding et al., 2002). Experiments with Bay 11-0782, which has proved to be an effective inhibitor of multiple aspects of NF-κB activation in this study, suggest that NF-κB can actually contribute to caspase activation and cell death induced by etoposide in fibroblasts. The use of MG-132, which inhibits NF-κB by a different mechanism, supports this hypothesis. Since MAPK family members, i.e. ERK and JNK, are also implicated in the etoposide-induced caspase activation in transformed mouse fibroblasts

(Stefanelli et al., 2002), the present data suggest that NF-κB may cooperate with these MAPKs to induce apoptosis and in part mediate their action.

Polyamines are multifunctional cellular components absolutely required for cell proliferation, and inhibition of their biosynthesis represents a pharmacological tool in cell growth control (Pegg et al., 1995). Recently polyamines have been implicated in cell survival and apoptosis with complex and apparently contradictory results, which may be in part concentration and serum-dependent (Thomas and Thomas, 2001). It appears that excessive polyamine levels directly trigger apoptosis (Stefanelli et al., 1998), but, on the other hand, polyamine depletion can sensitize or protect cells exposed to death triggers, depending on the pathways engaged by the death stimulus (Stefanelli et al., 2001; Li et al., 2001). Li et al. (2001) suggested that the altered susceptibility to apoptosis induced by DFMO in intestinal epithelial cells may be mediated by NF-κB activation, on the basis of experiments with chemicals that disrupt the NF-κB function. This hypothesis does not seem extendible to the current model. In any case, in DFMO pre-treated fibroblasts the impaired response to etoposide in terms of caspase activation and cell death (Stefanelli et al., 2001, 2002) was not restored by the simultaneous addition of Bay 11-0782 (not shown). Finally, it should also be considered that polyamines can regulate the mechanisms of protein synthesis through the regulation of the activity of the eukaryotic initiation factor 5A, which is involved in the triggering of apoptosis in tumor cells and can be an additional target downstream ODC for the induction of apoptotic death in tumor cells (Caraglia et al., 2000, 2003).

In conclusion, the results of the present study and the previous ones (Stefanelli et al., 2001, 2002) altogether suggest that polyamines play a permissive role in the pathways triggered by etoposide and leading to cell death of fibroblasts, by supporting the activation of MAPKs, NF-κB and caspases.

Acknowledgements

This research was supported by grants from Italian MIUR (ex 60% and 40%) and from Compagnia di San Paolo, Torino, Italy.

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Authors' address: Benedetta Tantini, Department of Biochemistry "G. Moruzzi", University of Bologna, Via Irnerio, 48, 40126 Bologna, Italy, Fax: + 39 051 2091224, E-mail: benedetta.tantini@unibo.it