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PI-3-Kinase/NF-κB Mediated Response of Jurkat T Leukemic Cells to Two Different Chemotherapeutic Drugs, Etoposide and TRAIL

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Abstract Jurkat T leukemic cells respond to Etoposide, antineoplastic agent which targets the DNA unwinding enzyme, Topoisomerase II, and TNF-Related-Apoptosis-Inducing-Ligand (TRAIL), 34 kDa transmembrane protein, which displays minimal or no toxicity on normal cells and tissues, not only disclosing the occurrence of apoptosis but also a kind of resistance. A similar rate of viability upon the exposure to these two drugs up to 24 h has been evidenced, followed by the occurrence of a rescue process against TRAIL, not performed against Etoposide, along with an higher number of dead cells upon Etoposide exposure, in comparison with TRAIL treatment. These preliminary results let us to speculate on the possible involvement of PI-3-kinase in TRAIL resistance disclosed by surviving cells (20%), may be phosphorylating Akt-1 and, in parallel, IκB α on both serine and tyrosine residues. On the other hand, in Etoposide Jurkat exposed cells Ser 32-36 phosphorylation of IκB α is not sufficient to overbalance the apoptotic fate of the cells, since Bax increase, IAP decrease, and caspase-3 activation determine the persistence of the apoptotic state along with the occurrence of cell death by necrosis. Thus, the existence of a balance between apoptotic and rescue response in 20% of cells surviving to TRAIL suggests the possibility of pushing it in favor of cell death in order to improve the yield of pharmacological strategies. J. Cell. Biochem. 93: 301–311, 2004.

Key words: PI-3-kinase; NF-κB; drug resistance; Jurkat

A large number of drugs are currently used in acute T leukemia cells therapeutical strategies and many of them kill malignant cells by inducing apoptosis, each one with a specific mechanism of action, but, sometimes, drug resistance is disclosed by these cells [Bishop, 1997]. Among these, Etoposide and Tumor necrosis factor Related Apoptosis Inducing Ligand (TRAIL) are largely investigated [Wen et al., 2000]. Etoposide belongs to a new class of antineoplastic agents which targets the DNA unwinding enzyme Topoisomerase II [Liu and Chen, 1994; Frolich-Ammon and Osheroff, 1995; Hande, 1998]. DNA Topoisomerases are nuclear enzymes, which make transient DNA strand breaks, essential for DNA replication, transcription, chromosomal segregation and DNA recombination. They are present in all cells as type I, which makes single strand cuts in DNA and type II, which cuts and passes double stranded DNA. Etoposide is an analogue of 4-demethylepipodophyllin benzylidene glucoside (DEPBG) isolated in 1966 by a series of aldehyde condensation products of the unpurified root of the Indian Podophyllum plant. Since 1973 it has demonstrated antineoplastic activity against acute myeloid leukemia (AML), Hodgkin's disease, non Hodgkin's lymphoma and lung, gastric, breast, and ovarian cancer. Concerning its mechanism of action, although Etoposide can alter microtubule assembly, it causes dose-dependent single strand and double strand DNA breaks, when incubated with cells

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and, when removed, DNA breakage is quickly repaired. It does not block the catalytic function of DNA Topoisomerase II, but, rather, poisons this enzyme by increasing the steady-state concentration of their covalent DNA cleavage complexes. This action converts Topoisomerase into physiological toxins which introduce high levels of transient protein-associated breaks in the genome of treated cells. When these permanent DNA breaks are present at a sufficient concentration they trigger a series of events which ultimately culminate in cell cycle arrest and death by apoptosis [Bhalla et al., 2000].

Tumor necrosis factor Related Apoptosis Inducing Ligand (TRAIL), also called Apo-2 ligand (Apo-2 L), transmembrane protein of 34 kDa produced by bacteria, is a member of the TNF family, which induces apoptosis in various tumor cell lines, displaying minimal or no toxicity on normal cells and tissues [Walczak et al., 1999; Almason and Ashkenazi, 2003; Mouzakiti and Packham, 2003]. Apo-2 L binds to several members of the TNF receptor family, i.e. death receptors (DRs) 4 and 5, and anti-apoptotic decoy receptors (DcRs) 1 and 2 and OPG. DR4 and DR5 contain a cytoplasmic region consisting of a stretch of 80 aminoacids, designated the death domain (DD) responsible for transducing the death signal. DcR1 is linked to the plasma membrane by a glycophosphatidylinositol moiety and lacks signaling activity, DcR2 has a truncated, non-functional DD, while OPG is a soluble, more distantly related receptor capable of binding to Apo 2L/TRAIL, although the physiological significance of its interaction with this ligand is unclear.

Even though both Etoposide and TRAIL induce the occurrence of apoptosis in leukemic lines by a cell extrinsic and intrinsic pathway [Milani et al., 2003; Di Pietro et al., 2004], at the same time, also a kind of resistance is displayed by these cells against apoptotic stimuli. Thus, here we try to elucidate some of the signaling mechanisms underlying at the basis of such resistance in order to point out a possible molecular target to circumvent such resistance and to improve the therapeutic effects. In particular, PI-3-kinase signaling related pathways have been evaluated in Jurkat T leukemic cells exposed to Etoposide and TRAIL. Etoposide concentration (1 μM) has been chosen as the dose useful to induce loss of reproductive ability in leukemic cells [Di Pietro et al., 2004], as far as TRAIL concentration (100 ng/ml) has been chosen basing on literature which showed a good apoptotic response in Jurkat cells, determined by Annexin V staining followed by flow cytometry [Wen et al., 2000]. In order to check the specificity of PI-3-kinase response, Wortmannin and Ly 294002 have been added in culture, as specific inhibitors [Cantrell, 2001; Vlahos et al., 2004].

MATERIALS AND METHODS

Reagents

TRAIL was produced in bacteria, purified by chromatography on Ni^{++} affinity resin and used at the final concentration of 100 ng/ml [Zamai et al., 2000].

Etoposide (Sigma-Aldrich, St. Louis, MO) was used at the final concentration of 1 μM.

The specific inhibitors of PI-3-kinase Ly 294002 and Wortmannin (Sigma-Aldrich) were added in culture at the concentration of 10 μM and 100 nM respectively.

Cell Culture and Drug Treatments

Jurkat T leukemic cells were grown in suspension in HEPES buffered RPMI 1640 (Gibco Laboratories Life Techonologies, Grand Island, NY) supplemented with 10% FCS, 5 mM glutamine, penicillin/streptomycin (50 IU/ml and 50 μg/ml, respectively) in a humidified atmosphere plus 5% CO_2. Cells were treated with 1 μM Etoposide for 1 h or with 100 ng/ml TRAIL for 30 min. When required, cells were pretreated with 100 nM Wortmannin for 1 h or with 10 μM Ly 294002 for 45 min. After Etoposide incubation, cells were reseeded in fresh RPMI. For immunofluorescence microscopy, samples were treated with 100 ng/ml TRAIL for 3 and 24 h.

TRAIL's and Etoposide's effects on cell viability were assessed by Trypan Blue dye exclusion test and followed up to 96 h.

Evaluation of Apoptosis by Flow Cytometry

Samples containing $2–5 \times 10^5$ cells were harvested by centrifugation at 200*g* for 10 min at 4°C, fixed in 70% cold ethanol for at least 1 h at 4°C, and treated as previously detailed [Zauli et al., 1997]. Analysis of propidium iodide (PI) fluorescence was performed with an EPICS Coulter flow cytometer (Instrumentation Laboratory Co., Lexington, MA) with FL2 detector in a linear mode using the Expo 32 analysis software (Beckman Coulter Co., Miami,

FL). For each sample, 10,000–20,000 events were collected. Multicycle sotfware (Phoenix Flow Systems Inc., San Diego, CA) was used for cell cycle phases analysis. For quantitative evaluation of apoptosis, subdiploid (less than 2 n) DNA content was calculated as described [Secchiero et al., 1998] and expressed as percentage of apoptotic versus non apoptotic cells, regardless of the specific cell-cycle phase. The evaluation of necrotic cells and debris was done by gating the specific population according to forward scatter (FS) and side scatter (SS) parameters.

Isolation of Nuclei

Cells, resuspended in 10 mM Tris-HCl pH 7.4, 10 mM NaCl, 2 mM $MgCl_2$, 0.6% Triton X-100, 1.0 mM PMSF, 1 μg/ml leupeptin and aprotinin, were incubated at room temperature for 2 min, then cooled on ice for 5 min. After five passages through a 22-gauge needle, $MgCl_2$ concentration was adjusted to 5 mM. Nuclei were obtained by centrifuging the suspension at 1,200*g* for 15 min and cytoplasmic fractions consisted of the postnuclear supernatants. Nuclei were then harvested in RIPA buffer (1× PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 10 mg/ml PMSF, 100 mM sodium orthovanadate, 1 μg/ml leupeptin, and aprotinin).

Western Blot Analysis

Whole cell and nuclear proteins (30 μg) were SDS–PAGE separated and transferred to nitrocellulose filters. Immunoblot analysis was performed with various primary antibodies: anti-PI-3K (p85α), anti-p-Tyr, anti-Akt-1 (p60) anti-p-Akt-1, anti-IκBα and anti-p-Ser-32-IκBα, anti-Bax, anti-Bcl2, anti-c-IAP (Santa Cruz Biotechnology, CA), anti cleaved caspase-3 (Cellular Signalling, New England Biolabs, Hitchin, UK).

Primary antibodies were then visualized with horseradish-peroxidase coupled anti-rabbit or anti-mouse immunoglobulins (Santa Cruz Biotechnology, Santa Cruz, CA), by using the enhanced chemiluminescence Western Blotting detection system (Amersham Intl., Buckinghamshire, UK). Each membrane was probed with anti-β-actin (Sigma-Aldrich) to show that each electrophoretic lane was loaded with equal amount of proteins or with anti-tubulin to verify nuclear purification. When required, blots were stripped of bound antibodies by incubating membranes in 100 mM glycine pH 3 at room temperature, blocked, and reprobed with other primary and secondary antibodies.

Immunofluorescence Microscopy

Jurkat T cells (1×10^5) were cytocentrifuged, fixed in 4% paraformaldehyde phosphate-buffered saline, and permeabilized with NET GEL (150 mM NaCl, 5 mM EDTA, 50 mM Tris-Cl pH 7.4, 0.05% NP-40, 0.02% Na azide, 0.25% gelatin type 4 BLOOM 60 Sigma-Aldrich) for 30 min. Immunolabelling was performed in the presence of 5 μg/ml rabbit cleaved caspase-3 polyclonal antibody (Cellular Signalling, New England Biolabs) diluted in NET GEL for 1 h. Slides were washed in NET GEL and reacted for 45 min with fluorescein FITC-conjugated anti-rabbit IgG antibody (Boheringer Mannheim, Indianapolis, IN) diluted 1:100 in NET GEL. After several washes in NET GEL and PBS, slides were mounted in glycerol–DABCO containing 5 μg/ml DAPI (4-6,diamidino-2-phenylindol) to counterstain nuclei. Internal controls, performed omitting the primary antibody, did not disclose any FITC staining. The observations were carried out by means of a LEICA Light Microscope equipped with a Coolsnap Videocamera for acquiring computerized images.

Image Processing and Statistics

Densitometric values of Western blotting analysis of each protein, expressed as Integrated Optical Intensity (IOI) were estimated in a CHEMIDOC XRS System by the QuantiOne 1-D analysis software (BIORAD, Richmond, CA). Values obtained have been normalized based on densitometric values of internal beta actin. Data were analyzed using the two tailed, two sample *T*-test. Results were expressed as mean ± SD. Values of $P < 0.005$ were considered significant.

RESULTS

Since both Etoposide and TRAIL can induce apoptosis, possibly at a different rate, we firstly evaluated cell viability and apoptotic fraction. Trypan blue dye, which counterstains dead cells, revealed that Etoposide and TRAIL induced a substantial reduction on viability in Jurkat T leukemic cells, persisting up to 96 h upon Etoposide exposure, while the cells surviving 48 h after TRAIL exposure carried out a rescue strategy persisting up to 96 h (Fig. 1).

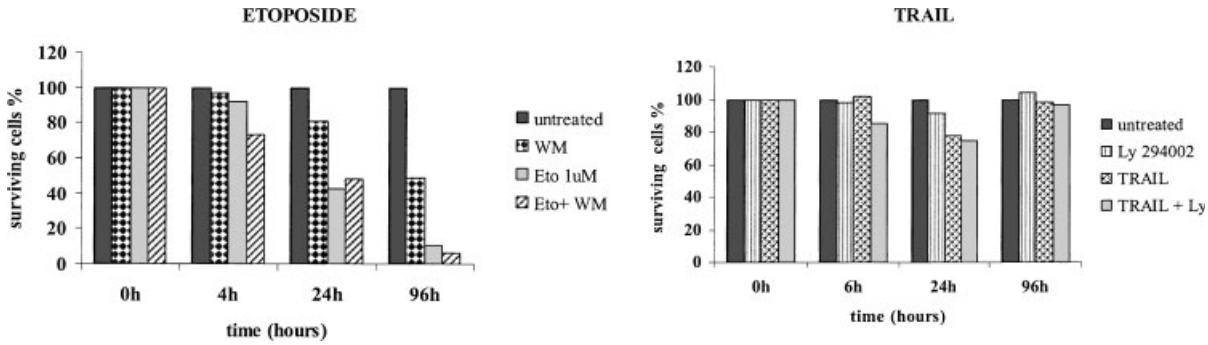

Fig. 1. Effect of Etoposide and TRAIL on cell viability of Jurkat cells determined by Trypan blue dye exclusion test. Results express the mean percentage ± SD of three different experiments.

Flow cytometry analysis of apoptotic, necrotic, and debris fraction, performed 24 h after different stimuli, evidenced a stronger response of Jurkat to TRAIL, comparable to that obtained upon Etoposide + Wortmannin combined treatment. In addition, the highest percentage of necrotic cells and debris was detected upon TRAIL + LY combined treatment and, to Wortmannin lesser extent, upon Etoposide + WM combined treatment (Table I).

Due to the fact that the sensitivity of these cells to Etoposide and TRAIL has been largely investigated [Wen et al., 2000; Di Pietro et al., 2004], and, in addition, preliminary results obtained in the presence of specific inhibitors have suggested a possible involvement of PI-3-kinase signaling related pathways in the occurrence of such response [O' Gorman et al., 2000], we have chosen to study some of the molecular mechanisms at the basis of the drug resistance focusing the attention on the expression and activation of PI-3-kinase, well-known enzyme involved in many cellular processes such as mitogenesis [Zhou and Hershensin, 2003], differentiation [Zhu et al., 2003], neoplastic transformation, apoptosis [Chang et al., 2003], and pharmacological response [Yu et al., 2003]. PI-3-kinase expression and activation, documented by Tyrosine phosphorylation in Etoposide treated cells and nuclear translocation in TRAIL exposed cells, increased upon the two different treatments, reduced in the presence of specific inhibitors (Fig. 2). Moreover, in order to characterize the downstream effect of such activation, Akt expression and activation have been then evaluated. While Jurkat exposed to Etoposide did not show any modification in Akt expression but a reduced phosphorylation on Thr 308, TRAIL treated cells disclosed Akt increased expression and phosphorylation on Ser 473, reduced in the presence of Ly 294002 (Fig. 3). Since this inhibitor partially blocked Akt activation on Ser 473, evidence for PI-3-kinase activity [Alessi et al., 1996; O'Gorman et al., 2001], an increased PI-3-kinase activity determined by TRAIL exposure can be hypothesized.

TABLE I. Evaluation of Apoptosis, Necrosis, and Debris by Flow-Cytometry 24 h After Exposure of Jurkat Cells to Etoposide (1 μM) and TRAIL (100 ng/ml)

	Apoptosis (%)	Necrosis (%)
Untreated	3 ± 0.9	4 ± 0.3
Wortmannin	4 ± 1.0	4 ± 0.2
Ly 294002	1 ± 0.7	2 ± 0.1
Etoposide	5 ± 1.3	5 ± 0.4
Etoposide + Wm	9 ± 1.5	9 ± 0.7
TRAIL	11 ± 1.5	7 ± 0.3
TRAIL + Ly	3 ± 0.8	12 ± 1.0

Values are expressed as mean % of three different experiments ± SD.

In addition, the resistance to TRAIL disclosed by these cells led us to evaluate the possible NF-κB involvement in the occurrence of such a process [Turco et al., 2004]. NF-κB expression did not modify in the two experimental conditions (not shown), while Iκ-Bα activation increased upon Etoposide and TRAIL exposure (Fig. 4).

In Eto-treated Jurkat cells, IAP protein, inhibitor of caspase-3, regulated by Iκ-Bα phosphorylation, decreased, Bax level increased along with caspase-3 activation (Fig. 5), suggesting the occurrence of the apoptotic state, even though the number of apoptotic cells is not so high but a large amount of necrotic cells could be represented. Concerning TRAIL treated cells, Iκ-Bα nuclear translocation was evidenced, reduced in the presence of Ly 294002 (Fig. 4). In particular, when phosphorylation

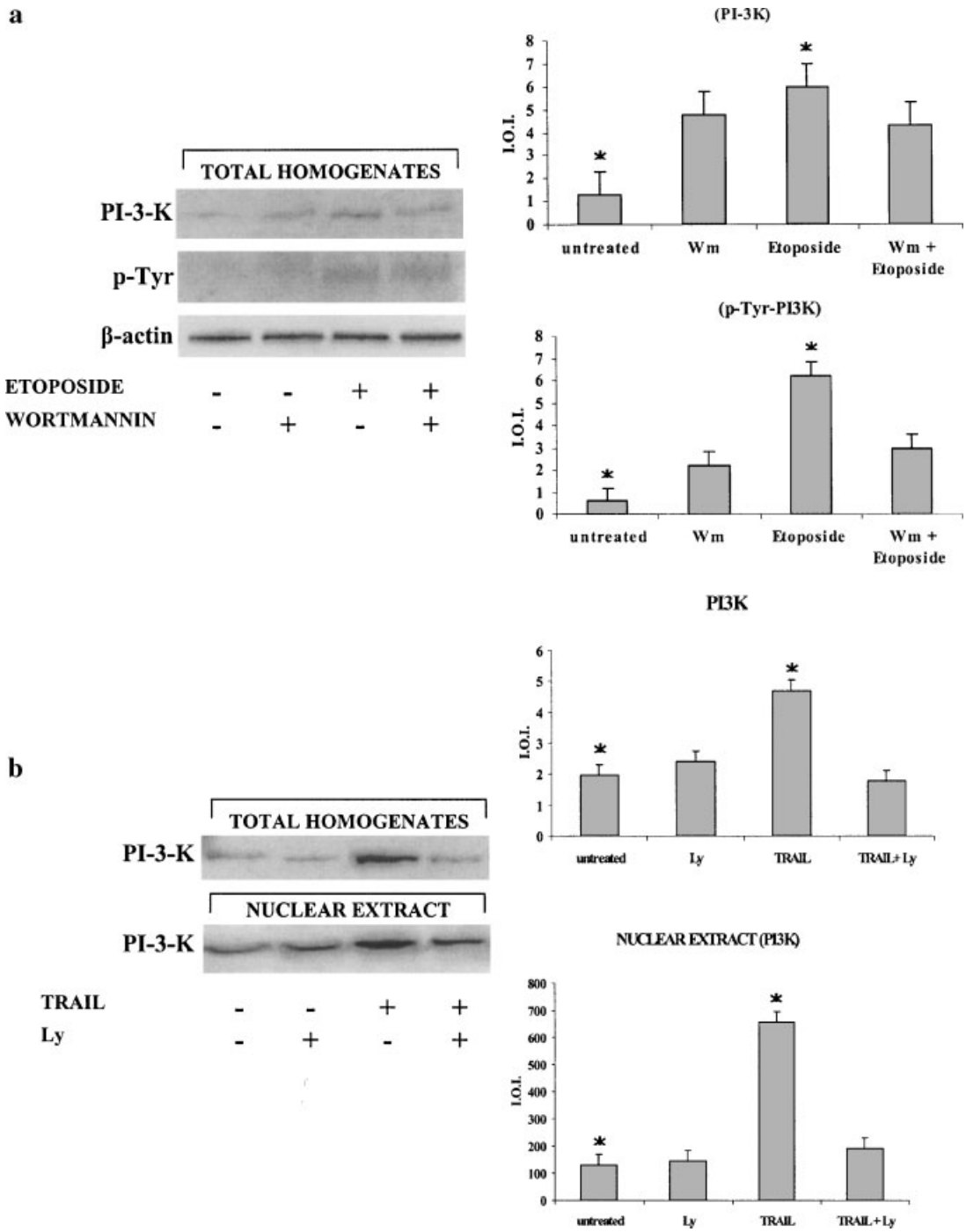

Fig. 2. Western blotting analysis of PI-3-kinase expression and activation in Jurkat cells exposed to Etoposide (**a**) and TRAIL (**b**). PI-3-kinase activation has been detected in Jurkat cells exposed to Etoposide by stripping PI-3-kinase antibody and reincubating the nitrocellulose in the presence of p-Tyr, polyclonal antibody which recognizes proteins phosphorylated on tyrosine residues. Samples have been normalized as shown by goat β actin polyclonal antibody incubation and protein levels have been quantified by densitometric analysis. Results are representative of three different experiments ± SD. Untreated vs Etoposide: $P < 0.005$. In TRAIL exposed Jurkat cells, PI-3-kinase activation has been determined by evaluating nuclear translocation of such molecule, since this is recognized as reasonable evidence of activation. Each electrophoretic lane has been loaded with equal amount of proteic lysate and protein levels have been quantified by densitometric analysis. Results are representative of three different experiments ± SD. Untreated vs TRAIL: $P < 0.005$.

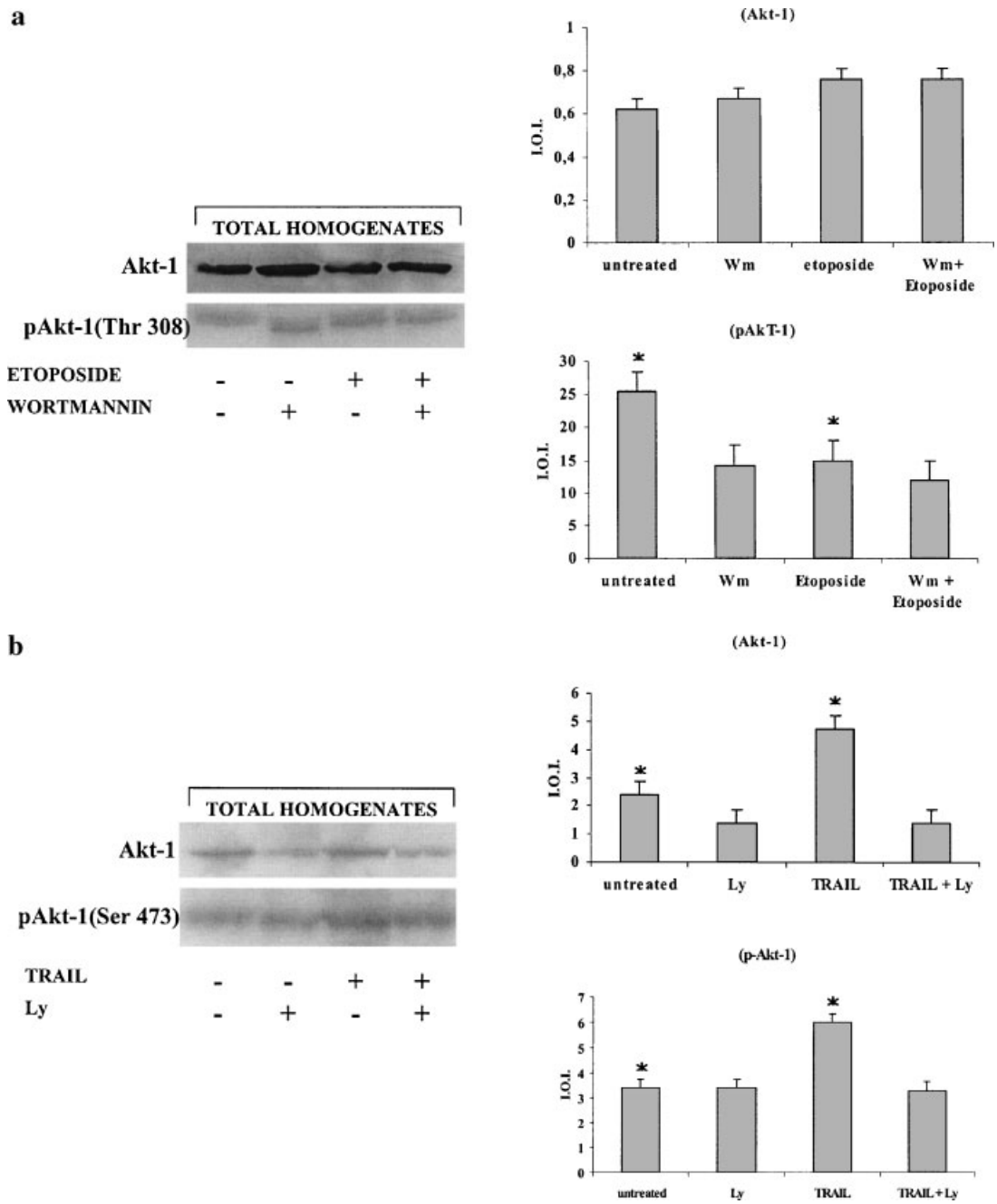

Fig. 3. Western blotting analysis of AKT-1 expression and phosphorylation in Jurkat cells exposed to Etoposide (**a**) and TRAIL (**b**). AKT-1 activation has been detected by stripping AKT-1 monoclonal antibody and reincubating the nitrocellulose in the presence of p-AKT-1 polyclonal antibody, which recognizes the protein phosphorylated on Ser 473. Each electrophoretic lane has been loaded with equal amount of proteic lysate and protein levels have been quantified by densitometric analysis. Results are representative of three different experiments ± SD. Untreated vs. Etoposide and untreated vs. TRAIL: $P < 0.005$.

site levels have been evaluated, TRAIL has been found to induce a substantial phosphorylation both at Ser 32-36, which gives rise to degradation of the molecule, and at Tyr 42, which does not lead to degradation of the inhibitor and stabilizes Iκ-Bα [Traenckner et al., 1995; Beraud et al., 1999]. In parallel, caspase-3 activation, detected by immunofluorescence analysis, increased up to 6 h after treatment, when apoptotic cells are already detectable, returning to basal level at 24 h (Fig. 6) concomitantly to recovery of proliferation.

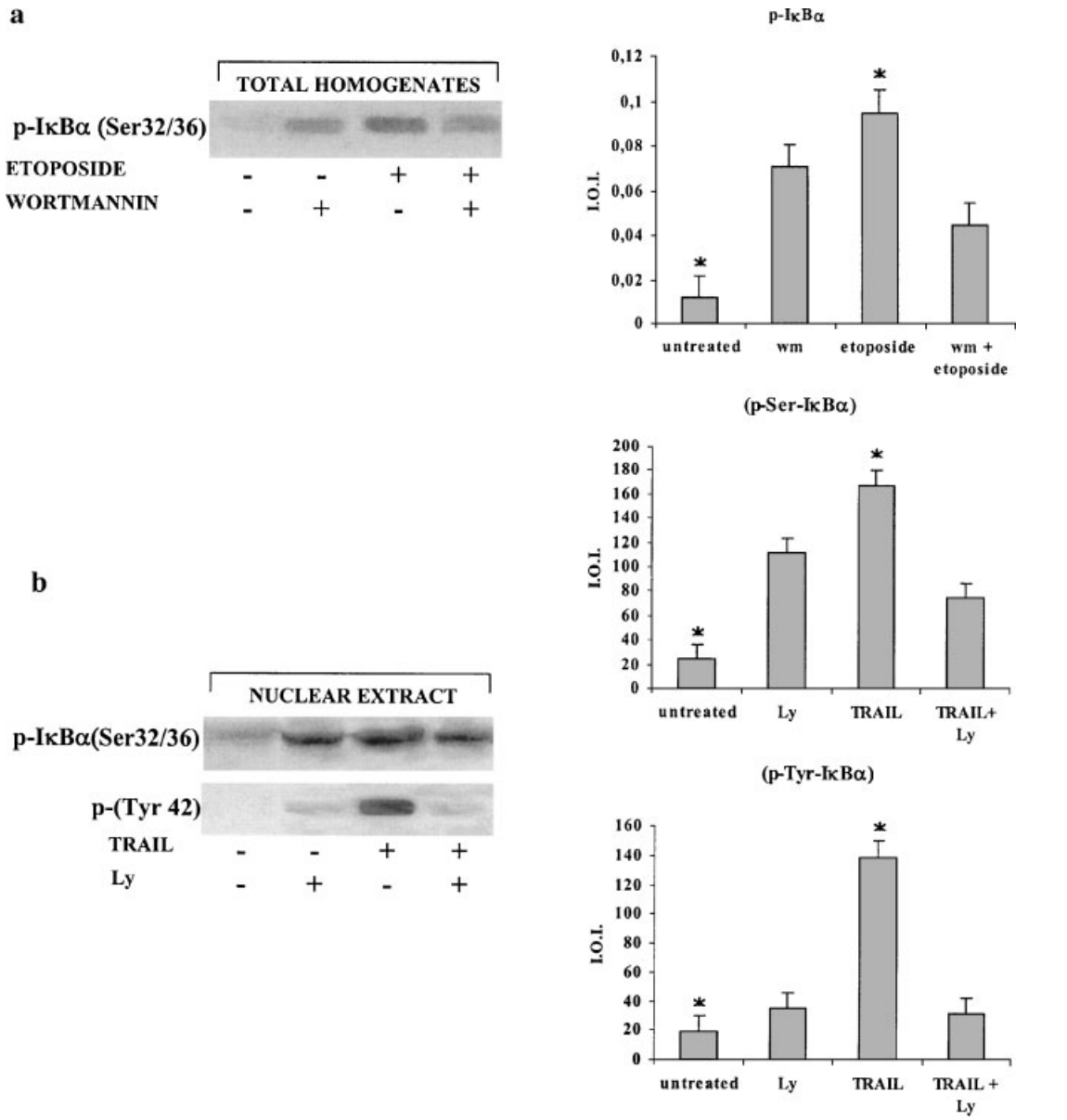

Fig. 4. Western blotting analysis of IκBα activation in Jurkat cells exposed to Etoposide (**a**) and TRAIL (**b**). IκBα activation has been detected by incubating the nitrocellulose in the presence of p-Ser-32-36 IκBα monoclonal antibody and p-Tyr polyclonal antibody which recognizes proteins phosphorylated on Tyrosine residues. Each electrophoretic lane has been loaded with equal amount of proteic lysate and protein levels have been quantified by densitometric analysis. Results are representative of three different experiments ± SD. Untreated vs. Etoposide and untreated vs. TRAIL: $P < 0.005$.

DISCUSSION

Jurkat T leukemic cells have been chosen for their sensitivity to Etoposide [Fujino et al., 2002] and TRAIL [Jang et al., 2003]. These cells respond to these two drugs disclosing a similar rate of viability up to 24 h, followed by the occurrence of a rescue process against TRAIL, not performed against Etoposide. Instead, the number of dead cells was higher in Etoposide treated cells, up to 4 h, persisting until 24 h, in comparison with TRAIL treated cells, considered at the same time intervals. Interestingly, in both experimental conditions, when Wortmannin or Ly were added in culture, the number of dead cells increased allowing to speculate on the apoptotic or anti-apoptotic role played by PI-3-kinase, as elsewhere already suggested [Zhu et al., 2003]. Moreover, previous studies have shown that PI-3-kinase plays a

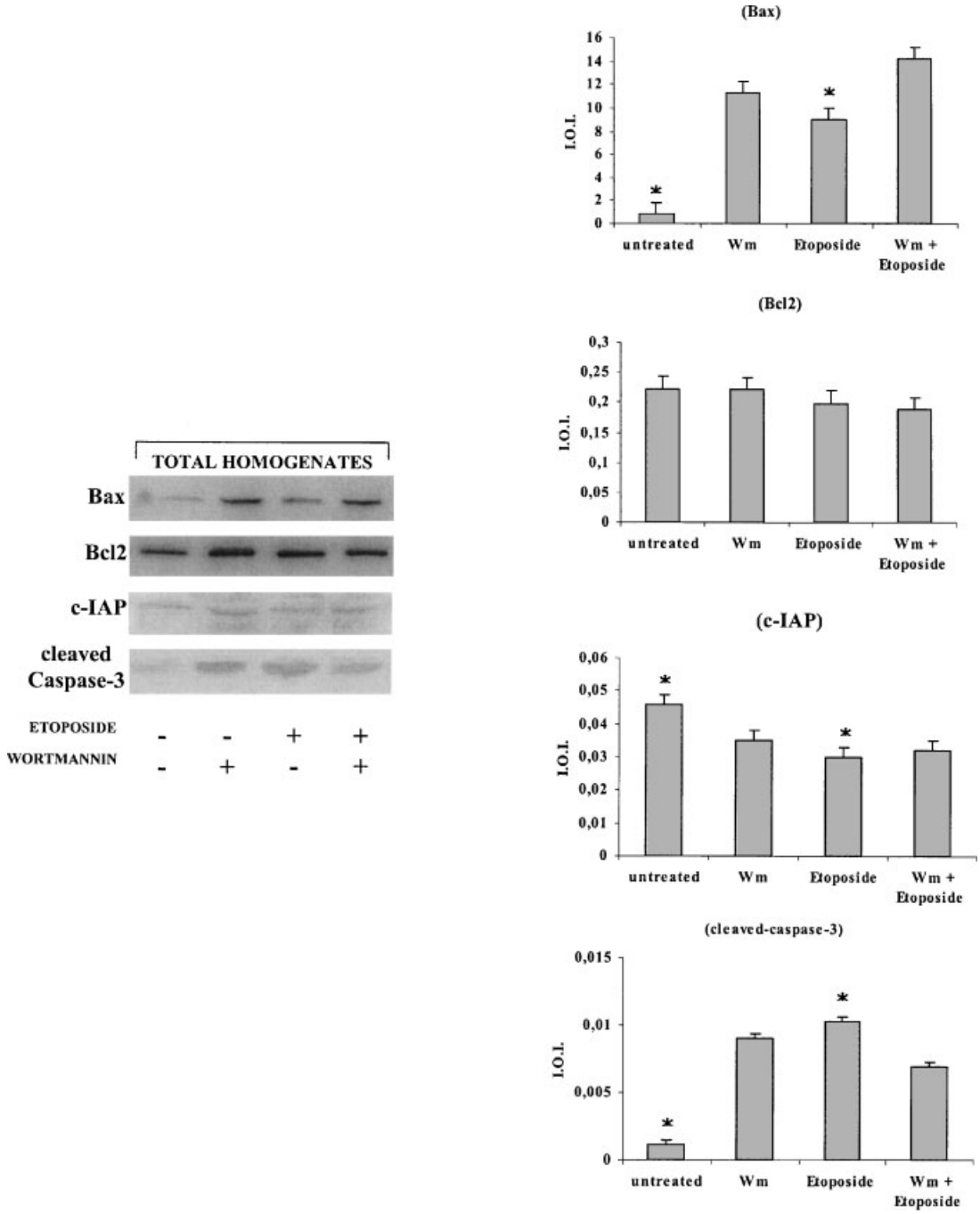

Fig. 5. Western Blotting analysis of Bax, Bcl2, IAP, and cleaved caspase-3 expression in Jurkat cells exposed to Etoposide. Each electrophoretic lane has been loaded with equal amount of protein lysates and protein levels have been quantified by densitometric analysis. Results are representative of three different experiments ± SD. Untreated vs. Etoposide: $P < 0.005$.

critical role in phosphorylating and transactivating the IκB α subunit in response to different agents such as pervanadate [Bhalla et al., 2000] and TNF α [Wang and Baldwin, 1998; Ozes et al., 1999].

In addition, the increased phosphorylation of Akt-1 on serine 473 upon TRAIL exposure, and its inhibition in the presence of Ly, is paralleled by IκB α phosphorylation at Ser 32 and 36 and nuclear translocation. The kinases

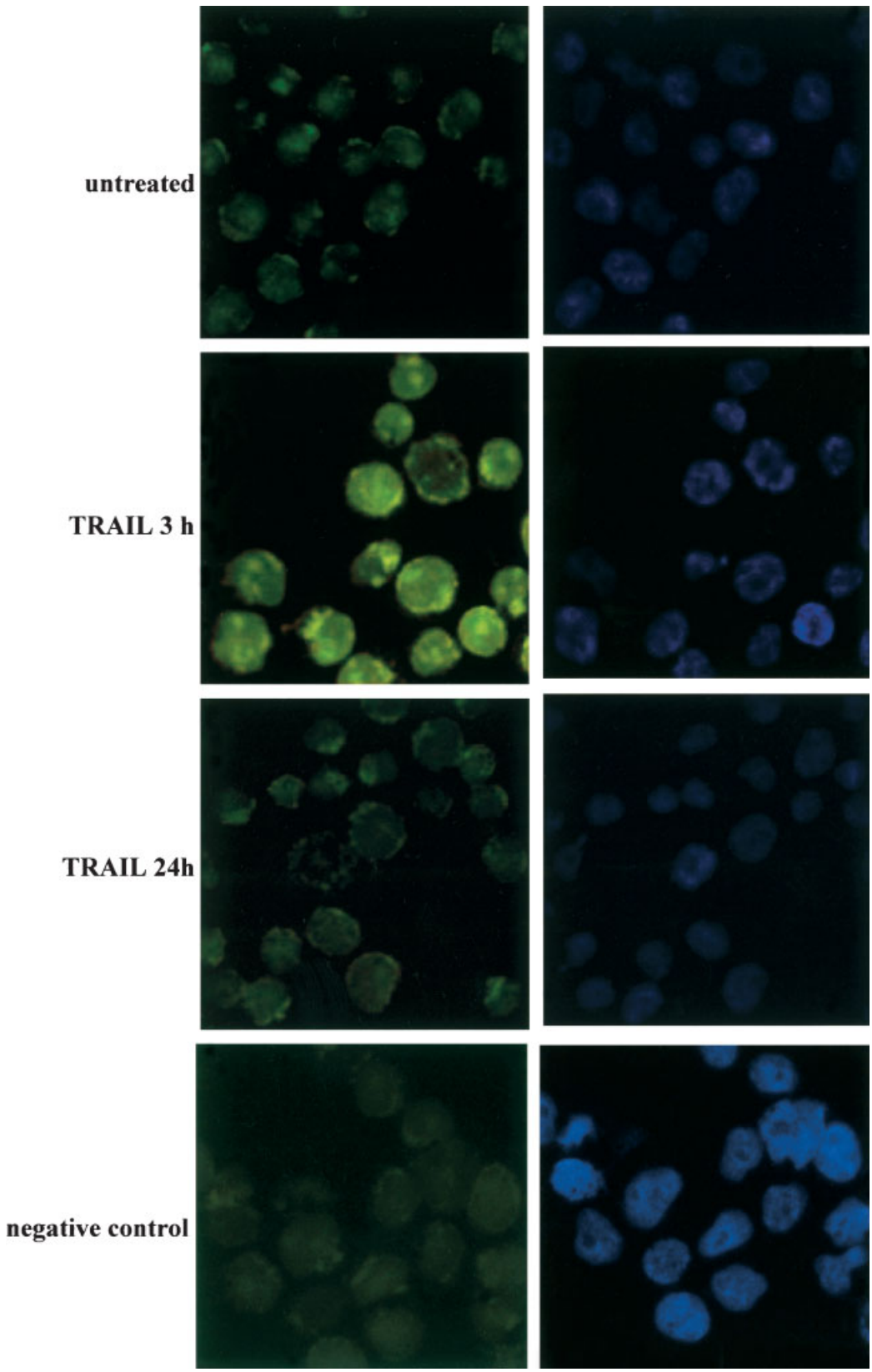

Fig. 6. Immunofluorescence detection of caspase-3 cleaved fragment in Jurkat cells exposed to TRAIL. Negative control has been performed by omitting the primary antibody. Green fluorescence detects caspase-3 cleaved fragment expression (**left column**). Blue fluorescence of DAPI (4-6, diamino-2-phenyl-indol) counterstains nuclei (**right column**). Magnification: 40×.

determining such phosphorylation have been recognized as IKK α and β, and are serine/threonine kinases [DiDonato et al., 1997], which determine degradation of the IKB inhibitor through the proteasome pathway. IκB α, moreover, can be also activated by a tyrosine phosphorylation pathway, which, unlike serine phosphorylation, does not lead to the degradation of the inhibitor through the proteasome pathway, but triggers dissociation of IKB from NF-κB [Traenckner et al., 1995]. With regard to this fact and considered that IκB α is also phosphorylated on Tyr 42, it could be suggested that phosphorylation by PI-3-kinase may directly cause the release of IKB α from NF-κB complex, as elsewhere suggested in other experimental models [Milani et al., 2003]. Alternatively, PI-3-kinase, namely, p85 α subunit, may recruit the tyrosine phosphorylated IκB α, when it is released, as a function of its natural role. In any case the possibility that other IκB proteins can play a role in transducing signals evoked by TRAIL cannot be excluded. All in all these two different phosphorylative pathways found out in TRAIL exposed cells can support the attempt of Jurkat to counteract TRAIL cytotoxic effect, while in Etoposide exposed cells Ser32-36 phosphorylation of IKB α is not sufficient to overbalance the fate of the cells since Bax increase, IAP decrease, and caspase-3 activation determine the persistence of the apoptotic state along with the cell death by necrosis.

Moreover the fact that, unlike Etoposide exposed cells, TRAIL treated cells try to circumvent apoptotic stimulus, may be by activating this signal transduction system, make us to suggest the existence of a balance between apoptotic and rescue response in that 20% of surviving cells and the possibility of pushing it in favor of cell death in order to improve the yeild of pharmacological strategies.

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