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Mitigation of tumor necrosis factor alpha cytotoxicity by aurintricarboxylic acid in human peripheral B lymphocytes

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Abstract

The aims of this study were to ascertain whether aurintricarboxylic acid (ATA), an endonuclease inhibitor, known to interfere, with the actions of cytokines such as interferons, is able to antagonize the toxic effects produced by tumor necrosis factor alpha (TNF-α) in human healthy peripheral B lymphocytes and try to elucidate the molecular machinery through which this possible antagonism takes place. Results evidenced that the balance of survival signals of human B lymphocytes in the presence of TNF-α was altered by the interaction of TNF-α with a salicylate compound, ATA. Apoptosis effected by TNF-α alone was suppressed in the presence of ATA, and this effect appeared essentially characterized by: (i) phosphorylation of phosphatidylinositol-3 kinase (PI-3K), influencing in turn protein kinase B/Akt (Akt) and Bad phosphorylation; (ii) nuclear translocation of the nuclear factor kappa B (NF-κB) and (iii) nuclear translocation of protein kinase C zed (PKCζ). Reversal of TNF-α/ATA effects occurred in the presence of the PI-3K specific inhibitors wortmannin or LY294002 in the culture medium and was coincident with inhibition of the translocation of PKCζ in the nucleus, while NF-κB was less affected. These results indicate, therefore, that PI-3K-mediated activation and nuclear transfer of PKCζ might be essential steps of ATA antagonism against TNF-α, suggesting that possible ATA pharmacological applications might be taken into account for staving off systemic or local toxic effects produced by TNF-α.

Keywords: TNF-α; PI-3K; NF-κB; PKCζ

1. Introduction

The critical balance of antiapoptotic vs. proapoptotic cell signals mediated through the TNF-α ligation of TNFR1 is determined by a complex assembly of transducer or adaptor proteins recruited to receptor intracellular domains. Homophilic molecular affiliations engage DD motifs which are intrinsic both to the tail region of TNFR1, and to structural modules of receptor-recruited proteins. The DD motifs provide transitional docking sites that facilitate signal transduction or amplification [1]. In the TNFR1 apoptosis pathway, a Fas-associated DD (FADD) enables recruitment of the zymogen form of caspase 8 [2,3]. The oligomerization and self-cleavage of caspase 8 initiates a caspase cascade, which is promulgated by caspase 9 and procaspases that serially cleave to active forms in a loose downstream hierarchy [4]. Caspase 3 is amongst the most proximate executors of proteolysis [4]. When the antiapoptotic signal of TNFR1 predominates, two molecular pathways lead toward activation of NF-κB. One depends upon TNFR1 recruitment of the TNFR1-associated factor-2 (TRAF2) [5]. This activates a pathway involving the receptor-interacting kinases (RIP), the NF-κB-inducing kinase (NIK) and the inhibitor kappa B (IκB), which restrains NF-κB in a cytosolic complex, and, once phosphorylated, releases NF-κB for nuclear translocation [6]. A second pathway of NF-κB activation involves phosphorylation of PI-3K. This phosphorylates its substrate, the Akt, that can phosphorylate inhibitor kappa B

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Abbreviations: TNF-α, tumor necrosis factor alpha; TNFR1, tumor necrosis factor receptor 1; DD, death domain; NF-κB, nuclear factor kappa B; PI-3K, phosphatidylinositol-3 kinase; Akt, protein kinase B/Akt; PKCζ, protein kinase C zed; ATA, aurintricarboxylic acid.

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kinase (IκK) [7]. In an auxiliary action, Akt phosphorylates the RelA (p65) subunit of NF-κB, and thus enhances the transactivation potential of NF-κB [8]. Previous reports have also outlined a relationship between NF-κB and PKCζ in the signal machinery linked to cell proliferation and survival [9,10]. The atypical PKCζ is ubiquitously expressed and unlike the conventional and novel PKC isoforms, it is not activated by membrane-associated diacylglycerol (DAG) or phorbol esters and generally does not translocate appreciably from cytosol to membrane when activated [11]. ATA, is an endonuclease inhibitor known to alter, in physiologic solution, phosphorylations of a number of intracellular proteins either by direct effects on receptors that transduce signals through the cell surface, or by interfering with the actions of cytokines, such as interferons [12]. Previous studies showed that ATA, dissolved in physiologic media, can alter phosphorylations of a number of intracellular proteins either by stimulation of surface membrane receptors that transduce signals through the cell surface or by interfering with the receptor interactions of a cytokine [13–15]. Since we have recently demonstrated that TNF-α exerts its cytotoxic effects in both human B lymphoma and primary cells [16], the aims of the present study were to investigate whether ATA was able to antagonize the TNF-α toxicity in human healthy peripheral B lymphocytes and eventually highlight the molecular machinery through which this possible antagonism takes place.

2. Methods

2.1. Cell culture and treatment

B lymphocytes were obtained from peripheral blood of healthy consenting donors by using magnetic beads coated with anti-CD 19 mAb (Dynabeads M-450 CD19, Dynal). Only samples exceeding a purity of 95% were used for the experiments. Blood peripheral B lymphocytes were grown in RPMI-1640 medium (Mascia Brunelli) containing 10% fetal calf serum (FCS, Mascia Brunelli), 2 mM L-glutamine, 5 mM HEPES, pH 7.3, and penicillin–streptomycin (50 IU/mL and 50 mg/mL, respectively), at 37° with 5% CO_2. The morphological effects of TNF-α (Boerhinger Mannheim) and ATA (Sigma-Aldrich) alone or in combination were assessed after 16 hr of treatment with 250 nM TNF-α which is a dose proved to be effective in producing apoptosis in human B cells and 100 μM ATA which is a dose reported to be well tolerated by human cells in tissue culture for periods of at least 24 hr and physiologically tolerated in small mammals [15–18], whereas the molecular changes were evaluated following treatments for times up to 30 min (1–5–10–30 min). Since no substantial differences were found among these treatment times, 10-min treatment was chosen as representative of all the time course treatment. When required, cells were pretreated for 20 min with 5 μM wortmannin (Sigma-Aldrich).

2.2. Flow cytometry

The DNA content of cells was determined by flow cytometry. Briefly, the samples were fixed in 70% ethanol for 15 min at 4°; after three washes in PBS, the cells were treated with RNAse 0.5 mg/mL (Sigma-Aldrich) for 15 min at 37°. The cells were harvested by centrifugation and the pellets resuspended in 1 mL of 50 μg/mL propidium iodide (Sigma-Aldrich) in PBS. Analysis was performed by an EPICS-XL flow cytometer (Coulter). Apoptotic cells were located in the hypodiploid region (M1).

2.3. Immunocytochemical detection

Cells were first spun onto coverslips fixed in 4% paraformaldehyde/PBS for 10 min at room temperature, washed twice with PBS, incubated with PBS for additional 15 min to quench the remaining paraformaldehyde, and saturated/permeabilized using Net gel solution (150 mM NaCl, 5 mM EDTA, 50 mM Tris–HCl, pH 7.4, 0.05% NP-40, 0.25% Carrageenan Lambda gelatin, and 0.02% NaN_3) for 30 min room temperature. After two washes with Net gel solution, cells were treated with anti-NF-κB rabbit polyclonal antibody (1:50; Santa Cruz Biotechnology) for 60 min at room temperature. After two washes with Net gel solution, goat anti-rabbit IgG-FITC (1:150; Sigma-Aldrich) was added to the cells and incubated for 45 min at room temperature. After three additional washes (two in Net gel solution and one in PBS), the nuclei were counterstained with propidium iodide (Sigma-Aldrich) and mounted in 1,4-diazabicyclo[2.2.2]octane (DABCO, Sigma-Aldrich)–glycerol–PBS. The immunostaining specificity for the monitored proteins was confirmed by the absence of any reactivity when: (a) secondary antibodies FITC conjugate (1:150; Sigma-Aldrich) and (b) normal rabbit serum (diluted 1:50) were used.

2.4. [^{32}P]-labeling, solubilization of proteins, immunoprecipitation, and immunoblotting

Cells were maintained for 3 hr in phosphate-free Dulbecco's modified Eagle's Medium (D-MEM), labeled with [^{32}P]orthophosphate (Amersham, 100 μCi/mL) for 2 hr, washed and then stimulated with ATA, TNF-α, or with a combination of both drugs for times up to 30 min. Cells were harvested by centrifugation at 4° or processed for nuclear preparation as previously described [19]. Purity of nuclei was tested by measuring both glucose-6-phosphatase activity and analyzing the recovery of the cytoskeletal marker β-tubulin. Whole cells or nuclei were solubilized in lysis buffer (60 mM Tris–HCl, pH 7.8, 150 mM NaCl, 5 mM EDTA, 10% glycerol and protease inhibitors) and normalized at 400 and 250 μg protein, respectively. Whole cell lysates were incubated at 4° for 1 hr with 1 μg of either anti-PI-3K, anti-Akt, and anti-Bad antibodies (all from

Santa Cruz Biotechnology), whereas nuclear lysates were reacted with 1 μg of anti-NF-κB p65 or anti-PKCζ antibodies (Santa Cruz Biotechnology), all antibodies were previously coupled to goat anti-rabbit IgG magnetic beads (Dynal). Immunocomplexes were collected by a magnet, electrophoresed and transferred onto nitrocellulose. The phosphorylation level of the different proteins was assessed either by autoradiography or, when indicated, by reacting the same blot with an anti-phosphotyrosine antibody (PY-99, Santa Cruz Biotechnology). Equal loading of proteins was determined by western blot.

3. Results

3.1. ATA counteracts cytotoxic damage induced by TNF-α

The cytotoxic effect of TNF-α on human peripheral B lymphocytes was monitored by means of flow cytometry investigation after 16 hr of treatment. While treatment with ATA alone did not produce substantial modifications with respect to untreated cells, the association of TNF-α and ATA in the culture medium restored morphological features comparable to those of untreated cells. Remarkably, the flow cytometry analysis demonstrated that TNF-α/ATA treatment significantly reduced the number of cells undergoing apoptosis from 41 to nearly 16% (Fig. 1).

3.2. TNF-α plus ATA activates PI-3K/Akt and down-regulates Bad

The 85 kDa regulatory subunit of PI-3K associates with specific phosphotyrosine residues present either on receptor-associated tyrosine kinases or signal proteins involved in activation of NF-κB [20,21]. Although PI-3K phosphorylation in the cells was not increased above constitutive levels by single TNF-α or ATA treatments, strong phosphorylations of both PI-3K and its substrate Akt were detected in response to the bimolecular combination of TNF-α plus ATA (Fig. 2). These coordinate signals persisted for at least 16 hr (not shown) and drew attention to

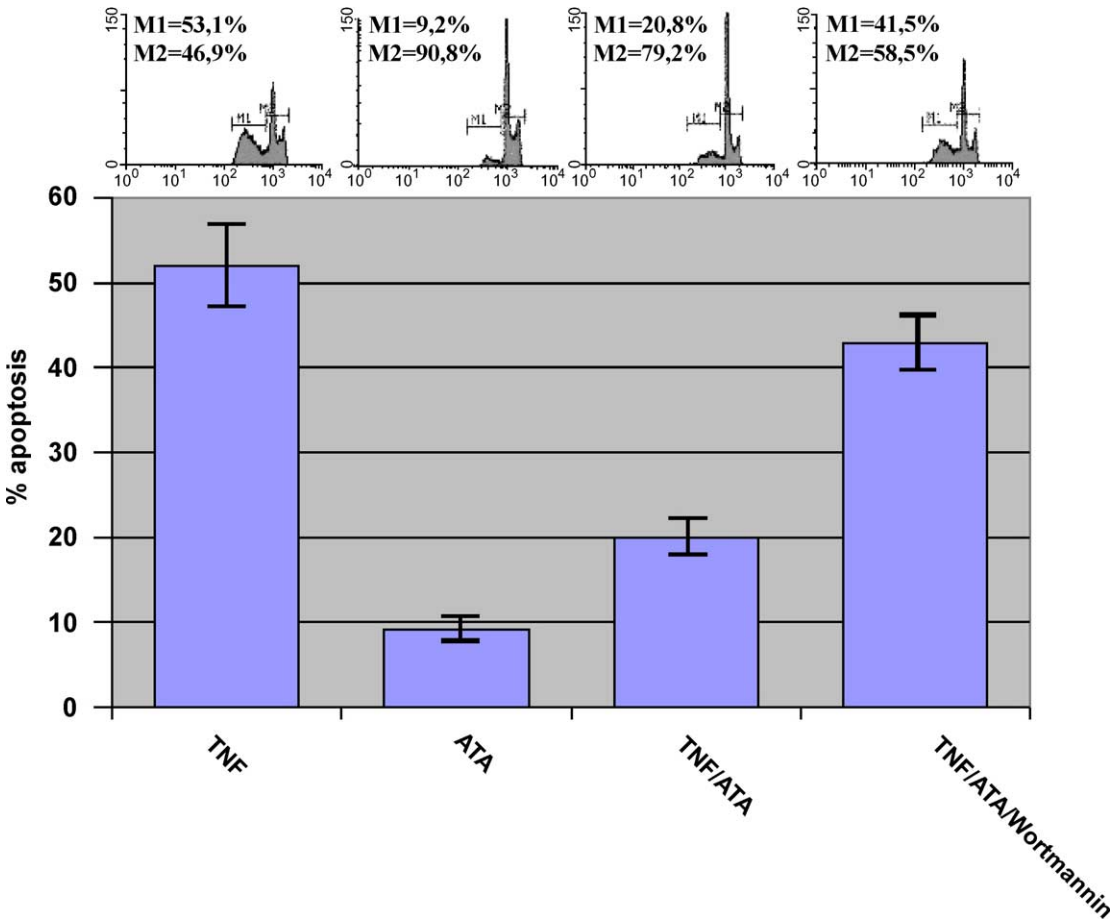

Fig. 1. Flow cytometry investigation of the effect of 16-hr treatment with TNF-α (250 nM) alone or plus ATA (100 μM) on human peripheral B lymphocytes. Samples were fixed in 70% ethanol for 15 min at 4°; after three washes in PBS, the cells were treated with RNAse 0.5 mg/mL for 15 min at 37°. The cells were harvested by centrifugation and the pellets resuspended in 1 mL of 50 μg/mL propidium iodide in PBS. As reported in the figure, TNF-α alone gave rise to a nearly 50% of cells undergoing apoptosis (hypodiploid region, M1). The association of TNF-α and ATA in the culture medium largely mitigated the cytotoxic effect produced by TNF-α alone. Preincubation (20 min) of the cells with the PI-3K specific inhibitor wortmannin (5 μM), or LY294002 (10 μM) prior to TNF-α/ATA strongly impaired their pro-survival effect. M2 region corresponds to nonapoptotic cells. Results are representative of three experiments.

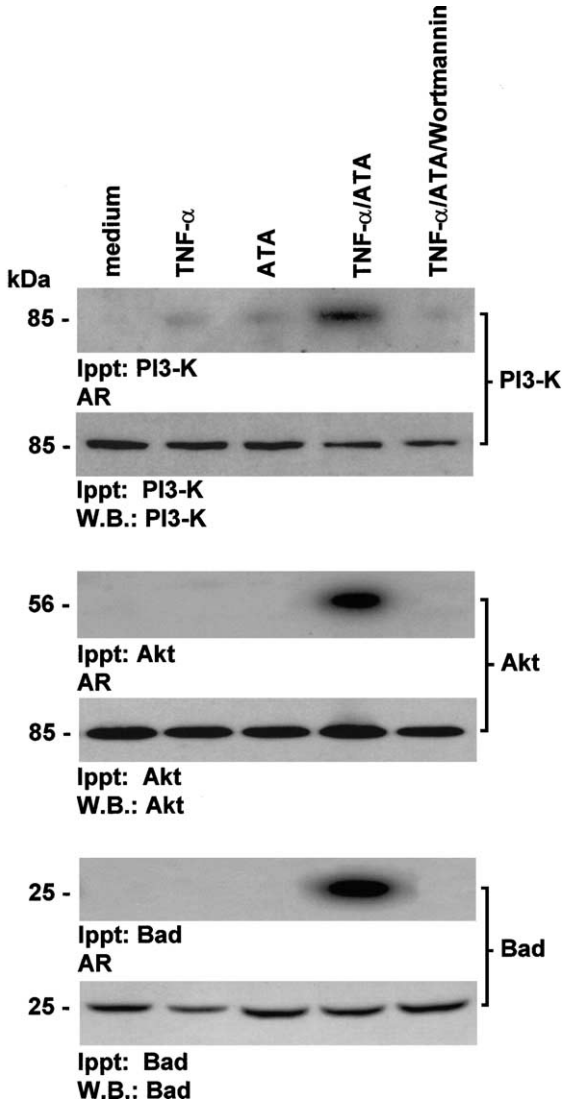

Fig. 2. Activation of PI-3K/Akt and down-regulation of Bad by TNF-α plus ATA. Cells were maintained for 3 hr in phosphate-free Dulbecco's modified Eagle's Medium (D-MEM), labeled with [^{32}P]orthophosphate (100 μCi/mL) for 2 hr, washed, and then stimulated with TNF-α (250 nM), ATA (100 μM), or with a combination of both drugs for times up to 30 min (1–5–10–30 min). Since no substantial differences were found among these treatment times, 10-min treatment was chosen as representative of all the time course treatment. When required, cells were pretreated for 20 min with 5 μM wortmannin. Cells were harvested by centrifugation at 4°. Whole cells were solubilized in lysis buffer (60 mM Tris–HCl, pH 7.8, 150 mM NaCl, 5 mM EDTA, 10% glycerol and protease inhibitors) and normalized at 400 μg protein. Whole cell lysates were incubated at 4° for 1 hr with 1 μg of either anti-PI-3K, anti-Akt, and anti-Bad antibodies, all antibodies were previously coupled to goat anti-rabbit IgG magnetic beads (Dynal). Immunocomplexes were collected by a magnet, electrophoresed and transferred onto nitrocellulose. The phosphorylation level of the different proteins was assessed either by autoradiography or, by reacting the same blot with an anti-phosphotyrosine antibody (PY-99, Santa Cruz Biotechnology). Equal loading of proteins was determined by western blot. Results are representative of three experiments.

Bad as a target of Akt [22]. Experiments in which cells were treated with the bimolecular combination of TNF-α plus ATA also showed that Bad became strongly phosphorylated as compared to basal levels in untreated control cells or samples of cells treated with either agonist alone (Fig. 2). Dependence of the Bad phosphorylation upon the PI-3K/Akt phosphorylation pathway was shown by inhibition with wortmannin (Fig. 2) and LY294002 (not shown).

3.3. TNF-α plus ATA induces translocation and phosphorylation of NF-κB/PKCζ

Western blot analysis of nuclear extracts prepared at the time points as indicated in Section 2, evidenced that in untreated cells, NF-κB was not detectable at nuclear level. A limited nuclear translocation followed treatment with TNF-α alone, but the bimolecular combination of TNF-α plus ATA produced the strongest effect (Fig. 3a). These results were corroborated by the immunocytochemical analysis (Fig. 3b). Since PKCζ is a proximate effector of NF-κB [9], we reprobed the same blots with antibody to PKCζ, that disclosed a positive reaction in the TNF-α plus ATA sample (Fig. 3a) consistent with a nuclear translocation of PKCζ. Addition of the PI-3K inhibitor, wortmannin (or LY294002) in the culture medium completely blocked PKCζ translocation in the nucleus while was less effective for NF-κB (Fig. 3a). Equal loading of nuclear proteins was assessed with antibody to H2b. In order to asses association and phosphorylation level of the two proteins, we performed an autoradiography of nuclear PKCζ immunoprecipitated from cells labeled with ^{32}P and treated with TNF-α, ATA, or TNF-α plus ATA. Results indicated that PKCζ was phosphorylated and that a phosphorylated protein complex of ∼60–70 kDa was associated (Fig. 4, upper panel). By reprobing the blot with an antibody to NF-κB, a positive reaction was evidenced, possibly consistent with the hypothesis of an association of NF-κB with PKCζ (Fig. 4, lower panel).

4. Discussion

The complex and intriguing network through which intracellular signals can regulate the balance between cell death and survival, stirred up, recently, increasing attention in the view of developing possible therapeutic modalities relying on agents able to interfere with the activity of molecules integrated in such a network. The experiments here reported demonstrate that, in human B lymphocytes, a salicylate compound, ATA, when associated with TNF-α, evidently counteracts the cytotoxic effect of TNF-α. ATA is a highly stable aromatic compound (473.4 Da) that is synthesized from a mixture of salicylic acid, formaldehyde, sulfuric acid, and sodium nitrite [23]. In aqueous solutions, it exists primarily as a polyanionic oligomer [23] which is membrane-impermeable. Exclusion from the cytoplasm of diverse cell types originally was demonstrated by radiolabeling [24]. A deduced axiom is that the ATA effect on TNF-α signals must impact at the cell surface receptor level. This reasoning is supported by

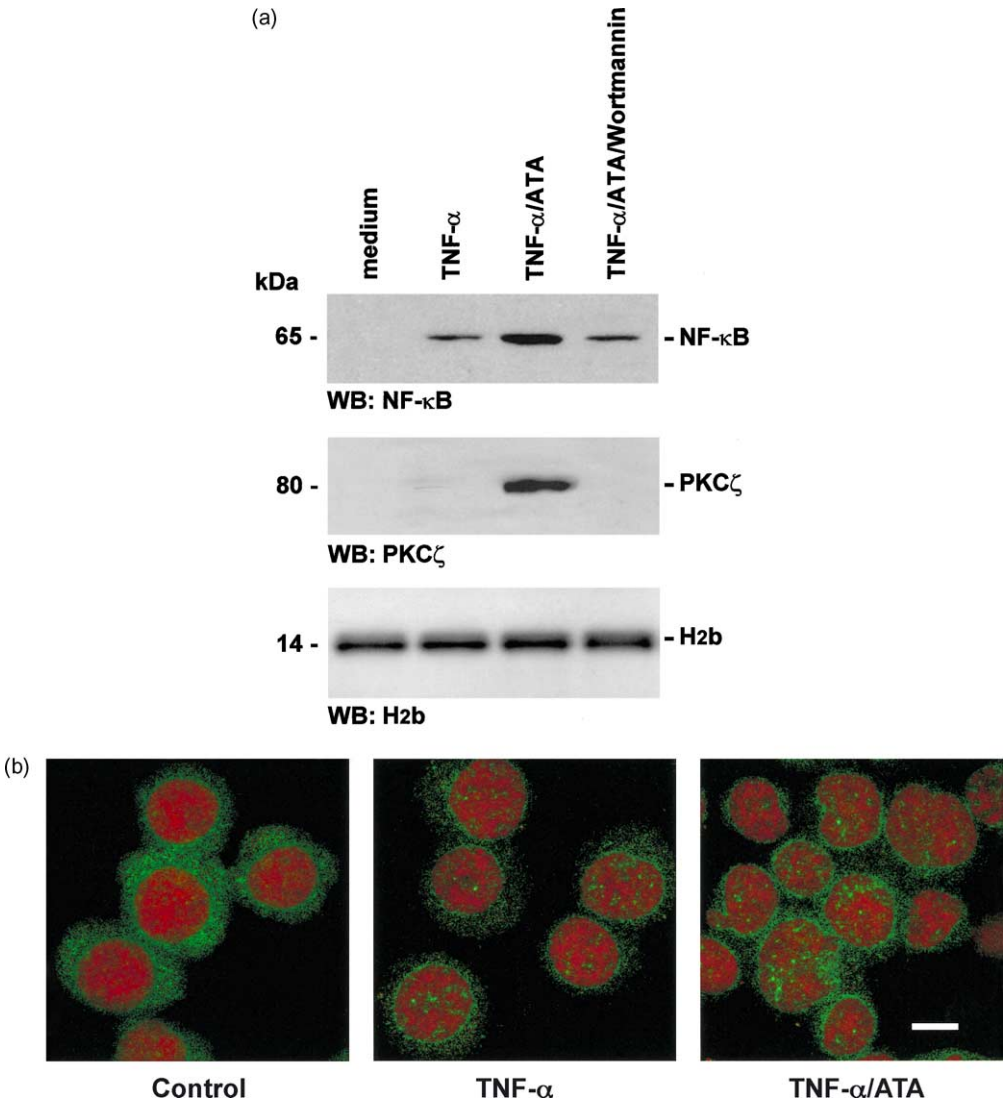

Fig. 3. (a) Detection of NF-κB and PKCζ in nuclei isolated from cells treated concomitantly with TNF-α (250 nM), alone, or with TNF-α plus ATA (100 μM). After cell treatments, nuclei were isolated and solubilized in lysis buffer as reported in Section 2. Ten-minute treatment was chosen as representative of all the time course treatment. Effect of preincubation (20 min) with wortmannin (5 μM) is also shown. Equal loading of nuclear proteins was assessed by reprobing the membrane with antibody to H2b. (b) Immunofluorescence analysis of NF-κB subcellular distribution after treatment of human peripheral B lymphocytes with TNF-α (250 nM), alone or associated to ATA (100 μM). Cells were first spun onto coverslips fixed in 4% paraformaldehyde/PBS for 10 min at room temperature, washed twice with PBS, incubated with PBS for additional 15 min to quench the remaining paraformaldehyde, and saturated/permeabilized using Net gel solution for 30 min at room temperature. After two washes with Net gel solution, cells were treated with anti-NF-κB rabbit polyclonal antibody (1:50) for 60 min at room temperature. After two washes with Net gel solution, goat anti-rabbit IgG-FITC (1:150) was added to the cells and incubated for 45 min at room temperature. After three additional washes (two in Net gel solution and one in PBS), the nuclei were counterstained with propidium iodide and mounted in DABCO–glycerol–PBS. The immunostaining specificity for the monitored proteins was confirmed by the absence of any reactivity when: (a) secondary antibodies FITC conjugate (1:150) and (b) normal rabbit serum (diluted 1:50) were used. In regard to controls, TNF-α alone induced a slight translocation of NF-κB into the nuclei while the associated treatment of TNF-α and ATA produced a stronger translocation of NF-κB.

previous experimental findings in which ATA was found to impede specific receptor functions, including engagement of antibody [25] attachment of an enveloped virus [14], and a synaptic reflex [15]. Previous studies have highlighted the ability of ATA to interfere with antiproliferative activity of interferon alpha through a nonspecific mechanism of competition with the ligand at the receptorial level with the consequent abrogation of the signal [12]. Crystallographic studies of repetitive disaccharide interactions with growth factor receptors have shown that extended nonpeptidyl polymers can alter receptor conformation and facilitate receptor function by charge determined dimerization or multimerization [13]. An attractive hypothetical corollary is that repetitive acidic residues of ATA oligomers might impose analogous changes to TNFR. This does not necessarily imply a common signal pathway for the varied biologic actions of ATA, since observed effects can be relatively selective and dependent upon the specific cell type. For example, ATA was shown to induce phosphorylation of the ErbB4 receptor in human neuroblastoma

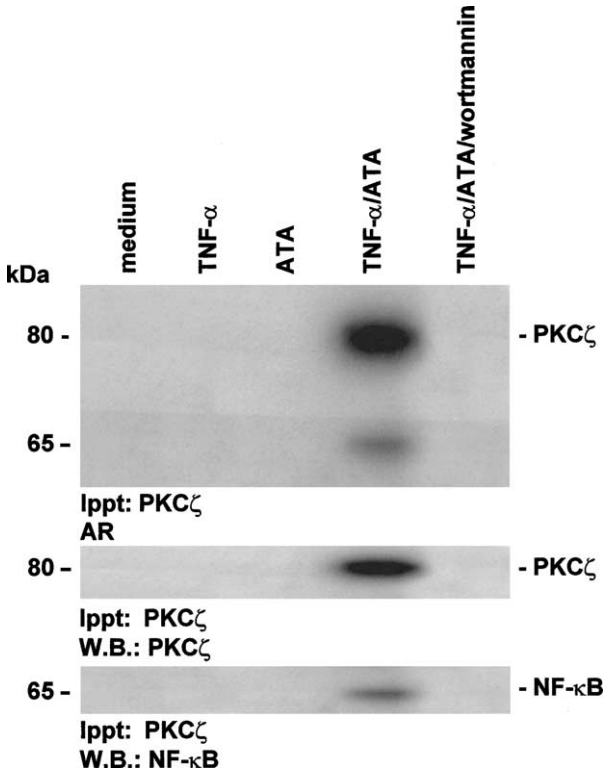

Fig. 4. Phosphorylation and coprecipitation of PKCζ and NF-κB from nuclear lysates. Nuclear lysates were reacted with 1 μg of anti-NF-κB p65 or anti-PKCζ antibodies. All antibodies were previously coupled to goat anti-rabbit IgG magnetic beads (Dynal). Immunocomplexes were collected by a magnet, electrophoresed and transferred onto nitrocellulose. The phosphorylation level of the different proteins was assessed either by autoradiography or by reacting the same blot with an anti-phosphotyrosine antibody (PY-99, Santa Cruz Biotechnology). Equal loading of proteins was determined by western blot. Autoradiography of the original membrane is shown in the upper panel. Identification of proteins was obtained by incubating the same membrane with anti-PKCζ or with anti-NF-κB antibodies (middle and lower panel, respectively). Data are representative of three experiments.

cells [26], while selective tyrosine phosphorylation of Janus tyrosine Kinase 2 (JAK2) kinases and the transcription factor Signal Transducer and Activator of Transcription 5 (STAT5) occurred in rat T cells [27]. One recurrent theme in several different biological contexts has been that ATA phosphorylation-activation of signal molecules tends toward suppression of cell death pathways and sustain of cell survival during stress. In our experiments, the cooperative protective effect of ATA plus TNF-α elicited phosphorylation/activation of PI-3K, Akt and Bad. Downstream of PI-3K/Akt, NF-κB, and PKCζ association and nuclear translocation were observed. Of interest ATA alone was unable to trigger any of the above reported molecules. The 85 kDa subunit of PI-3K can associate with the intracellular region of cytokine receptors [21]. Although mutational analyses have not provided evidence for a direct interaction of PI-3K with TNFR1 [22], present tests with selective inhibitors did confirm that the phosphorylation/activation of PI-3K by TNF-α plus ATA was a determinant of antiapoptotic effects. Nevertheless, a discrete translocation of NF-κB induced by TNF-α alone was observed and was not impaired by PI-3K inhibition. This observation is consistent with reported evidence for a dissociation in molecular events leading to NF-κB translocation vs. RelA (p65) subunit phosphorylation by Akt [28,29]. Physiologic status of the cell is an important determinant of TNF-α action [30,31], but under native conditions the prosurvival NF-κB pathway is preferentially induced [30]. Thus, despite the bias toward apoptosis in our cells, a limited nuclear translocation of NF-κB was detected after TNF-α alone. The much more vigorous translocation induced by TNF-α plus ATA probably did not simply depend upon PI-3K/Akt phosphorylation of the RelA (p65) subunit, since nuclear translocation is not completely prevented by PI-3K inhibitors. Unlikely, PKCζ phosphorylation and translocation to the nucleus appear to be PI-3K/Akt dependent since wortmannin (or LY294002) abrogated this event and restored levels of cell death near to controls. This findings are remarkably interesting in that they indicate different routes for NF-κB nuclear translocation upon treatment with TNF-α alone or following TNF-α/ATA association. Indeed, the discrete nuclear translocation of NF-κB provoked by TNF-α is clearly independent of PI-3K since no activation of this protein was detected following TNF-α. On the other hand, inhibition of PI-3K by wortmannin severely impaired NF-κB nuclear translocation induced by TNF-α/ATA treatment. An additional intriguing finding is the association of NF-κB with phosphorylated PKCζ only following TNF-α/ATA treatment. PKCζ is an atypical PKC which is not activated by DAG or phorbol esters and, once activated, does not translocate from cytosol to membrane [11]. Previous studies on rat adipocytes have suggested that it operates downstream of PI-3K, playing a role in the activation of NF-κB [9,10], while in PC12 cells both are essential signalling elements for mediating rescue from apoptosis. Our results strongly suggest a functional interdependence of these molecules and indicate PKCζ as a possible target molecule in the mechanisms regulating the balance between cell death and survival, thus opening new routes in the establishment of possible therapeutic strategies.

Acknowledgments

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