BIOCHEMICAL PHARMACOLOGY 75 (2008) 2183–2191

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Geldanamycin inhibits tyrosine phosphorylation-dependent NF-κB activation

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ARTICLE INFO

Article history:
Received 1 February 2008
Accepted 14 March 2008

Keywords:
Geldanamycin
Hsp90
c-Src
NF-κB
IκBα
Pervanadate

ABSTRACT

Hsp90 is a protein chaperone regulating the stability and activity of many signalling molecules. The requirement of Hsp90 activity in the NF-κB pathway has been recently reported by several authors using the Hsp90 ATPase inhibitor geldanamycin (GA), an anti-tumor drug. Hsp90 inhibition blocks the synthesis and activation of the IKK complex, the major kinases complex responsible for IκBα phosphorylation on serine 32 and 36, a key step for its degradation and the nuclear translocation of NF-κB. However, the effect of GA on other IκBα kinases, including tyrosine kinases, is unknown. In the present study, we investigated the effect of GA on NF-κB activation induced by sodium pervanadate (PV), a tyrosine phosphatase inhibitor triggering c-Src-mediated tyrosine phosphorylation of IκBα. We report for the first time that GA inhibits PV-induced IκBα tyrosine phosphorylation and degradation. Using an *in vitro* kinase assay, we demonstrated that GA inhibits the activity of c-Src as an IκBα tyrosine kinase, but not its cellular expression. As a result, GA blocked PV-induced NF-κB DNA-binding activity on an exogenous κB element and on the endogenous *iκbα* promoter, thereby inhibiting *iκbα* transcription. Finally, we demonstrated that, despite NF-κB inhibition, pre-treatment with GA does not potentiate PV-induced apoptosis. We conclude that c-Src requires Hsp90 for its tyrosine kinase activity, and its inhibition by GA blocks c-Src-dependent signalling pathways, such as NF-κB activation induced by sodium pervanadate. The effect of GA on PV-induced apoptosis is discussed in the light of recent publications in the literature.

1. Introduction

The transcription factor NF-κB regulates the expression of numerous genes involved in immune and inflammatory responses, cellular proliferation, differentiation and cell survival [1]. It consists of homo- or heterodimers of a group of five proteins: p50/p105, p52/p100, p65 (RelA), RelB and c-Rel. In unstimulated cells, NF-κB is sequestered in the cytoplasm in an inactive form through its association with a member of an inhibitory family of which the most characterized is IκBα [2].

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Abbreviations: Hsp90, heat shock protein 90; NF-κB, nuclear factor-κB; IκB, inhibitor of κB; IKK, IκB kinase; NEMO, NF-κB essential modulator; TNFα, tumor necrosis factor α; IL-1β, interleukin-1β; ELKS, glutamic acid (E), leucine (L), lysine (K), and serine (S); PV, sodium pervanadate.

0006-2952/$ – see front matter
doi:10.1016/j.bcp.2008.03.009

Pro-inflammatory cytokines (like TNF-α or IL-1β) induce the classical pathway of NF-κB activation, leading to IκBα phosphorylation on Ser-32 and -36 by the cytoplasmic IκB kinase (IKK) complex, which consists of the scaffold protein NEMO/IKKγ and the IKKα and IKKβ kinases [3]. The phosphorylated IκBα is then polyubiquitinated and degraded through the proteasome pathway, making NF-κB free to translocate into the nucleus to regulate the expression of many target genes, like *iκbα* [4]. Consequently, the newly synthesized IκBα binds to nuclear NF-κB, removes it from its DNA-binding sites and transports it out of the cytosol [3]. An atypical mechanism of NF-κB activation, taking place upon cellular stimulation by oxidants like sodium pervanadate, hypoxia/reoxygenation and, in some cell types, hydrogen peroxide has been described [5–8]. This pathway leads to IκBα phosphorylation on Tyr42 independently of IKK activation [5–7,9]. More recently, it has also been described that epidermal growth factor (EGF) and ciliary neurotrophic factor (CNTF) induce tyrosine phosphorylation of IκBα in lung carcinoma cell lines [10] and in neurons [11], respectively. In HeLa cells, the tyrosine kinase c-Src has been reported to be responsible for tyrosine phosphorylation of IκBα induced by pervanadate (PV) or hypoxia/reoxygenation [7].

The molecular chaperone Hsp90, a heat shock protein of 90 kDa, is one of the most abundant cytosolic proteins in eukaryotic cells. Hsp90 and its co-chaperones control the biogenesis, stability, activity and folding of a number of signalling molecules, including many kinases and transcription factors [12–14]. Hsp90 clients play important roles in the regulation of cell growth, apoptosis and oncogenesis but its mechanism of action is not yet well known [15]. The main inhibitor of Hsp90 function is geldanamycin (GA), an anti-tumor drug [13,16,17]. GA belongs to the benzoquinoid ansamycin antibiotics and was isolated from *Streptomyces hygroscopicus* [18]. Through its ability to reverse cellular transformation induced by the viral protein v-Src, GA proved to have an anti-tumor activity [19]. Despite the potent anti-cancer activity of GA in cell culture, difficulties in clinical trials were encountered due to its high hepatotoxicity in human tumor models [20]. The GA analogue 17 AAG (17-allylamino-17-demethoxy-geldanamycin) induces less hepatotoxicity, exerts similar anti-tumor activity and could enter Phase I clinical trials [21,22]. Another derivative, 17 DMAG (17-dimethylaminoethylamino-17-demethoxy-geldanamycin) proved to be more soluble than 17AAG and thus more pharmaceutically practicable [23]. Hsp90 requires ATP binding and hydrolysis to maintain its function and thus the activation of client proteins. This ATPase activity triggers a conformational change in the molecule, switching it between a closed and relaxed state [16,24–26]. The N-terminal domain of Hsp90 is the binding site for GA and for ATP. GA directly binds to Hsp90, inhibits the ATPase activity and destabilizes the Hsp90-multi-chaperone complexes, resulting in the degradation of client proteins *via* an ubiquitin-proteasome dependent pathway [13,16,17,27]. Recently, Hsp90 has been found to be a novel regulator of the IKK complex [28–32]. Indeed, Scheidereit and co-workers have shown that Hsp90 is a component of NF-κB signalling and is involved in IKK activation [29,32]. Hsp90 and its co-chaperone Cdc37 have been found to associate stoichiometrically with the IKK complex by binding to the IKKα and IKKβ kinase domains, and GA disrupts this association [28]. When Hsp90 function is inhibited, degradation of IKK subunits seems to be proteasome-dependent but a recent study demonstrated that IKKs can also be selectively degraded by autophagy [13,16,29,30]. On the other hand, a growing list of kinases, including Src family tyrosine kinases, are known to exist as heterocomplexes with Cdc37/Hsp90, allowing their stabilization and activation [12,13,33]. Previous works carried out in yeast have revealed that Hsp90 is necessary for the correct folding, maturation, stability and activity of v-Src kinase, the virally encoded counterpart of c-Src [17,34]. In human cells, this mutated protein is also more susceptible to GA-induced degradation *via* the ubiquitin-proteasome pathway than c-Src [16,35]. Despite earlier works having reported that c-Src binds Hsp90 *in vitro* [36], studies on the effect of Hsp90 inhibitors on c-Src activity are still discrepant. Bijlmakers and Marsh have reported that Hsp90 is necessary for normal cellular synthesis of c-Src but that GA does not affect total level of c-Src protein [35]. The same result was obtained in neuroblastoma or myoblast cell lines [37,38]. On the contrary, An et al. reported that prolonged exposure of PC3 cells to GA induce a decrease of c-Src expression [39]. The effect of GA could be thus cell-type dependent. Given that the precise role of Hsp90 on c-Src activity and expression is poorly understood and subject of debates in the literature, we wanted to explore here the effects of GA on the c-Src-dependent NF-κB pathway, *i.e.*, those inducing IκBα tyrosine phosphorylation upon PV stimulation. Using HeLa and Jurkat cell lines, we demonstrate that GA induces a significant reduction of IκBα tyrosine phosphorylation after PV stimulation. An *in vitro* kinase assay also revealed that c-Src-mediated IκBα tyrosine phosphorylation is GA-sensitive, but cellular c-Src expression was not affected by GA. In a second time, we demonstrated that GA blocked PV-induced NF-κB DNA-binding activity on an exogenous κB element and on the endogenous *iκbα* promoter, thereby inhibiting *iκbα* transcription. Finally, we report that pre-treatment with GA does not potentiate PV-induced apoptosis. We conclude that (i) Hsp90 activity is necessary for PV-induced NF-κB activation, (ii) c-Src requires Hsp90 for its activity, not for its synthesis and (iii) the cytotoxicity of PV is not enhanced by GA addition. This result is discussed in the light of recent publications in the literature.

2. Materials and methods

2.1. Cell culture and reagents

HeLa cells were cultured in EMEM with 10% (v/v) foetal bovine serum and glutamine (Biowhittaker, Petit Rechain, Belgium). Jurkat cells were cultured in RPMI 1640 medium (Biowhittaker, Petit Rechain, Belgium) supplemented with 10% (v/v) foetal bovine serum. GA was used at a final concentration of 0.5 μM (Sigma, St. Louis, MI, USA) and TNF-α at 200 U/mL (Roche, Mannheim, Germany). Sodium pervanadate (PV) was freshly prepared before each experiment as previously described [6] and used at a final concentration of 200 μM.

2.2. Antibodies

Monoclonal anti-IκBα used for Western blotting was kindly provided by C. Dagermont (France). Polyclonal anti-IκBα, IKKγ and c-Src used for immunoprecipitations and monoclonal anti-IKKβ, IKKγ and c-Src used for Western blotting were from Santa Cruz Biotechnology (CA, USA). Anti-IκBα phosphorylated on Ser32 and -36 and anti-phosphotyrosine antibodies were from Cell Signaling Technology (Netherlands). Anti-IKKα was from BD Pharmingen (BD Biosciences, CA, USA).

2.3. Western blotting and electrophoretic mobility shift assay (EMSA)

Cytoplasmic extracts were analyzed by Western blotting as described [40]. Nuclear extracts were analyzed by EMSA as previously described [40], using a ^{32}P-labeled oligonucleotide probe (5′-GGTTACAAGGGACTTTCCGCTG-3′; Eurogentec, Liège, Belgium) corresponding to the κB site of the HIV-1 LTR.

2.4. Immunoprecipitation assays

After treatments, HeLa cells were lysed for 10 min on ice in RIPA buffer (50 mM Tris–HCl, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.25% sodium deoxycholate, 1 mM PMSF and proteases inhibitors (complete, Roche)). Jurkat cells were lysed for 15 min on ice in whole cell extraction buffer (25 mM HEPES–KOH, 150 mM NaCl, 0.5% Triton X-100, 10% glycerol, 1 mM DTT, 1 mM Na_3VO_4, 25 mM β-glycerophosphate, 1 mM NaF and proteases inhibitors (complete, Roche)). Lysates containing total proteins were incubated with 2 μg/mL of antibodies 2 h at 4 °C. Immunocomplex were precipitated using protein G-PLUS-Agarose beads overnight at 4 °C. Beads were then washed, boiled for 3 min and fractionated in SDS-PAGE.

2.5. In vitro kinase assays

For IKK kinase assays, IKK complex was precipitated using an antibody against NEMO/IKKγ as described in immunoprecipitation assays. Immunoprecipitates were then resuspended in 30 μL of kinase buffer (50 mM Tris–HCl pH 8, 100 mM NaCl, 2 mM $MgCl_2$, 1 mM DTT, 1 mM NaF, 1 mM Na_3VO_4, 25 mM β-glycerophosphate, 10 mM NPP, and proteases inhibitors (complete, Roche)) supplemented with ATP (1 mM) in the presence of wild-type glutathione S-transferase (GST)-IκBα$_{1\text{-}55}$ and were incubated at 30 °C for 30 min. Reactions were stopped by the addition of SDS loading buffer and were subjected to SDS-PAGE. Proteins were electrotransferred to PVDF membranes and blotted with a phospho-specific anti-IκBα (Ser32-36) antibody. To evaluate the extent of tyrosine phosphorylation of GST-IκBα by immunoprecipitated c-Src, HeLa cells were lysed for 10 min on ice in whole cell extraction buffer (10 mM HEPES–KOH, 0.1 mM EDTA, 10 mM KCl, 2 mM $MgCl_2$, 0.5% Igepal, 1 mM DTT, 0.5 mM PMSF, 20 mM NaF, 1 mM Na_3VO_4, 25 mM β-glycerophosphate, 10 mM NPP and proteases inhibitors (complete, Roche)). c-Src was precipitated as described in immunoprecipitation assays. Immunoprecipitates were then resuspended in 30 μL of kinase buffer (50 mM Tris–HCl pH 8, 100 mM NaCl, 2 mM $MgCl_2$, 1 mM DTT, 20 mM NaF, 1 mM Na_3VO_4, 25 mM β-glycerophosphate, 10 mM NPP and proteases inhibitors (complete, Roche)) supplemented with ATP (1 mM) in the presence of wild-type glutathione S-transferase (GST)-IκBα$_{1\text{-}55}$ and were incubated at 30 °C for 30 min. Reactions were stopped by the addition of SDS loading buffer and were subjected to SDS-PAGE. Proteins were electrotransferred to PVDF membranes and blotted with an anti-phosphotyrosine or c-Src antibodies.

2.6. Quantitative real-time reverse transcription-PCR

Total RNA samples were extracted with Tripur isolation reagent (Roche, Mannheim, Germany). 1 μg of RNA was submitted to reverse transcription with the M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA, USA). For quantitative real-time RT-PCR, the obtained cDNA was analyzed, in duplicate, with the SYBR Green Master Mix (Applied Biosystems, Foster city, CA, USA) in the ABI Sequence Detection System. The results were normalized with the 18S transcript. The primers used to analyze the different transcripts were designed with the software Primer Express™ (Applied Biosystems): *iκbα*, FW 5′-CCAACCAGCCAGAAATTGCT-3′ and RV 5′-TCTCGGAGCTCAGGATCACA-3′; 18S, FW 5′-AACTTTC-GATGGTAGTCGCCG-3′ and RV 5′-CCTTGGATGTGGTAGCCG-TTT-3′ (Eurogentec).

2.7. Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) assays were carried out with solutions prepared in our laboratory following the Upstate Cell Signaling protocol. After cross-linking with formaldehyde, treated cells were lysed and sheared by sonication for 25 min to obtain DNA fragments between 200 and 1000 basepairs in length. To reduce non-specific background, protein A agarose (Pierce Biotechnology Inc., Rockford, IL, USA), used for immunoprecipitation, was presaturated with herring-sperm DNA (Sigma). Immunoprecipitation were performed with 2 μg of anti-p65 antibody (Santa Cruz Biotechnology, CA, USA). To test aspecific binding to the beads, an irrelevant antibody was used as control for immunoprecipitation (anti-flag antibody, Sigma). The next day, precipitation was carried out with saturated protein A-agarose beads. Cross-link was reversed at 65 °C for 4 h and precipitated DNA was purified using a phenol/chloroform extraction. Quantitative real-time PCR (using the SYBR Green Master Mix in the ABI Sequence Detection System) was performed on the immunoprecipitated DNA by normalizing to input DNA for each sample. The following primers, amplifying specific κB sites of the *iκbα* gene, were used: FW 5′-CGCTCA-TCAAAAAGTTCCCTG-3′ and RV 5′-GGAATTTCCAAGCCAGT-CAGAC-3′.

2.8. Detection of apoptosis by FACS analysis

GA and PV-induced apoptosis was measured using an apoptosis detection kit (Annexin V-fluorescein and propidium iodide, Roche, Manheim, Germany), according to the manufacturer's instructions. Cells were analyzed on a FACSCanto® II (Benton Dickinson, Erembodegem, Belgium). A total of 30,000

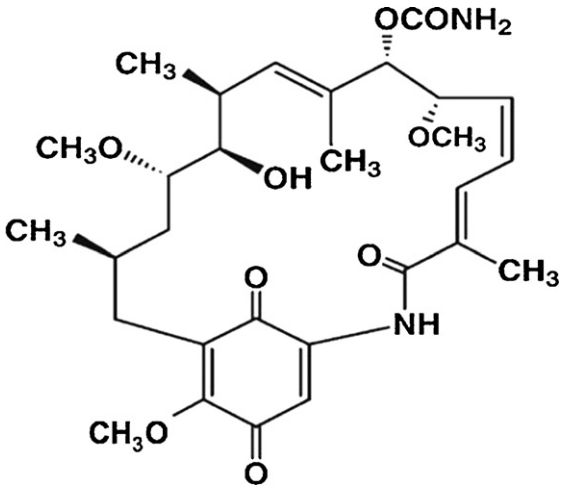

Fig. 1 – Structure of geldanamycin.

events per sample were collected in a dot plot displaying the FSC and SSC properties of the cell. The FITC signal of Annexin V was detected at 488 nm and iodide fluorescence was detected at 680 nm.

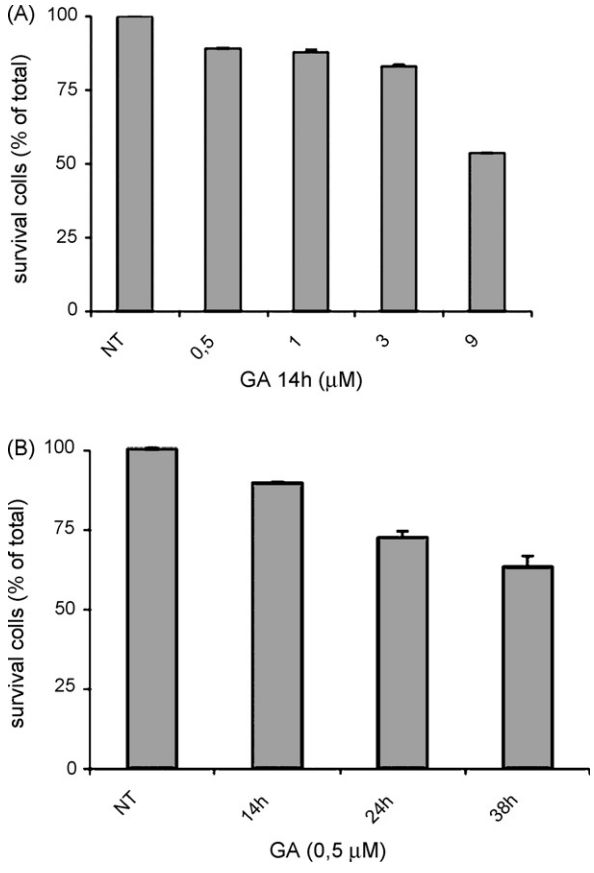

Fig. 2 – Effects of various doses and times of GA treatment on Jurkat cell viability. Jurkat cells were treated with various concentrations of GA for 14 h (A) or with 0.5 μM of GA during 14 h, 24 h and 38 h (B). Then cells were stained with Annexin V/propidium iodide and subjected to FACS analysis.

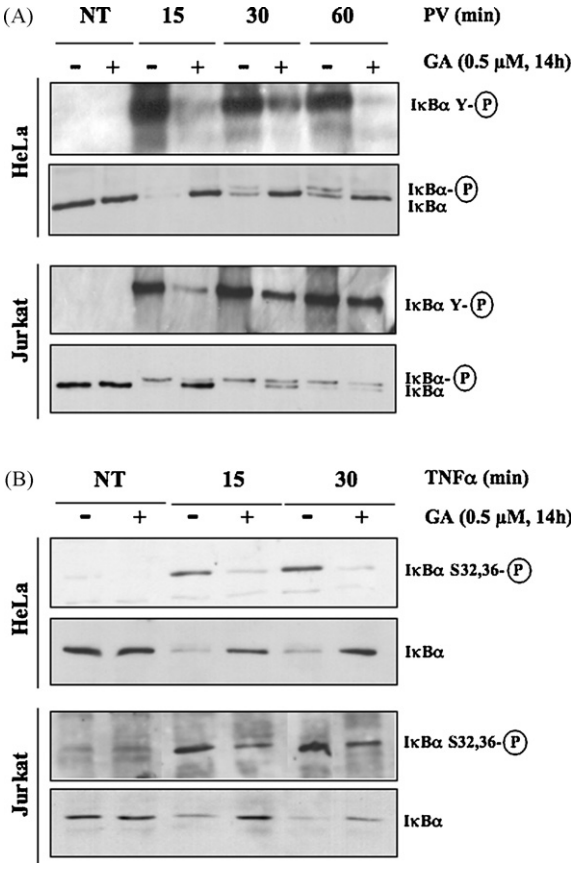

Fig. 3 – Geldanamycin inhibits tyrosine phosphorylation of IκBα. (A) HeLa and Jurkat cells were pre-treated or not with GA (0.5 μM) for 14 h and stimulated with PV (200 μM) for the indicated times. Whole cell extracts were prepared and IκBα was immunoprecipitated. The immunocomplex was then analyzed by Western blotting using phosphotyrosine and IκBα monoclonal antibodies. (B) HeLa and Jurkat cells were pre-treated or not with GA (0.5 μM) for 14 h and then stimulated with TNF-α (200 U/mL) for the indicated times. After treatment, cytoplasmic extracts were prepared and analyzed by Western blotting using a phospho-specific S32-36 anti-IκBα antibody and a specific anti-IκBα monoclonal antibody.

3. Results

3.1. *Geldanamycin inhibits tyrosine phosphorylation of IκBα*

To study the effects of geldanamycin (GA, Fig. 1) on IκBα tyrosine phosphorylation, HeLa and Jurkat cells were pre-treated or not with GA and then stimulated with sodium pervanadate (PV). Because this dose and time of treatment induced very few apoptosis in Jurkat cells (Fig. 2A), we pre-treated cells with GA at a concentration of 0.5 μM during 14 h. Nevertheless, we observed that higher concentrations of GA or longer time of 0.5 μM treatment induced apoptosis (Fig. 2A and B). IκBα was immunoprecipitated and its phosphotyrosine content was detected by Western blotting using a specific antibody. As shown in Fig. 3A, IκBα tyrosine phosphorylation

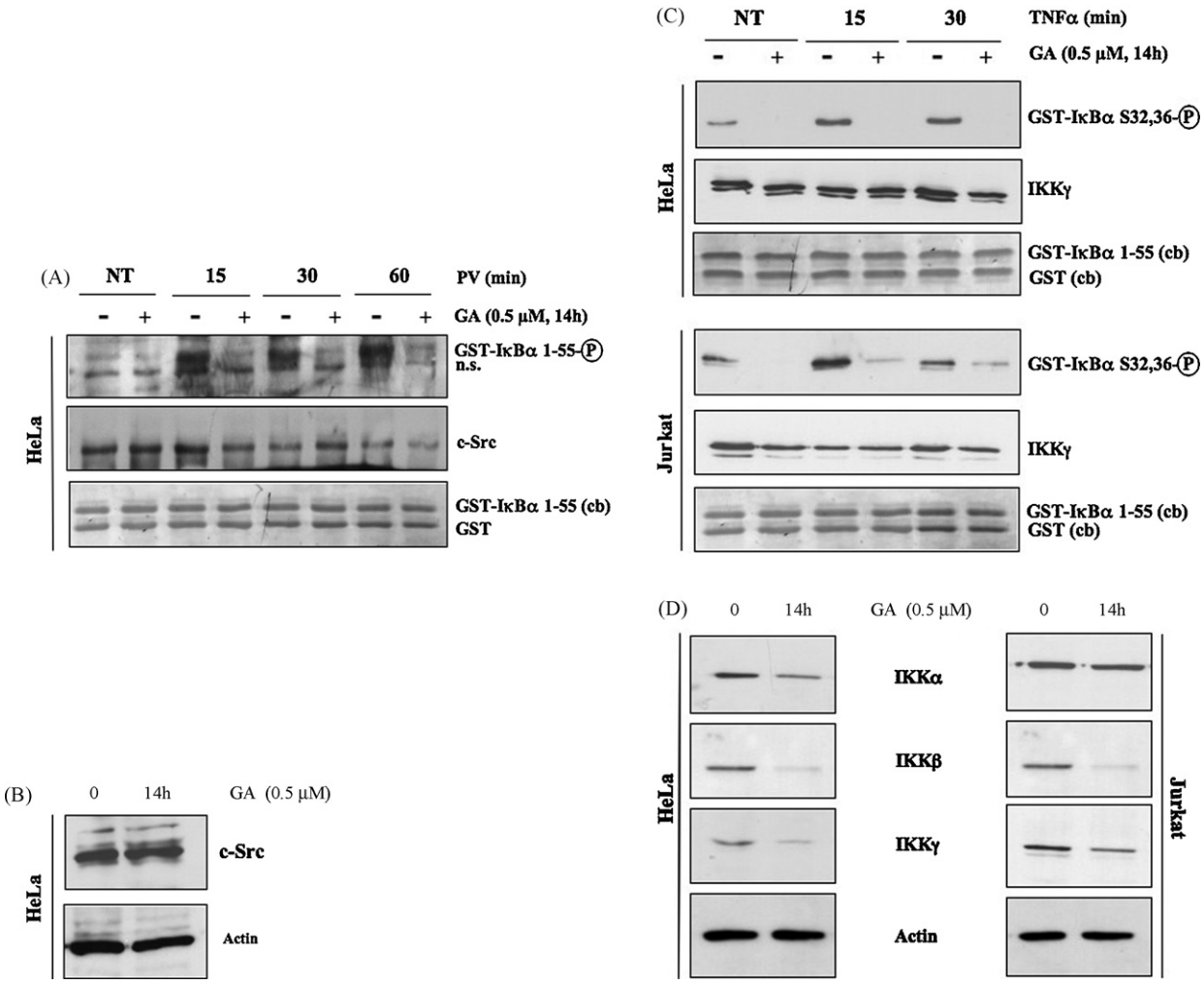

Fig. 4 – c-Src-mediated IκBα tyrosine phosphorylation is inhibited by geldanamycin. (A) HeLa cells were pre-treated or not with GA (0.5 μM) for 14 h and stimulated with PV (200 μM) for the indicated times. After immunoprecipitation of c-Src, an *in vitro* kinase assay using GST-IκBα$_{1-55}$ was carried out as described in Section 2. n.s. = non-specific. Western blotting was then performed using phosphotyrosine and c-Src antibodies (upper panels). Coomassie blue staining of the substrate (cb) is shown as a loading control (lower panel). (B) HeLa cells were pre-treated or not with GA (0.5 μM) for 14 h. After treatment, total extracts were prepared and a Western blotting was performed using an anti-c-Src antibody. (C) HeLa and Jurkat cells were pre-treated or not with GA (0.5 μM) for 14 h and then stimulated with TNF-α (200 U/mL) for the indicated times. After immunoprecipitation of the IKK complex with an anti-IKKγ antibody, an *in vitro* IKK kinase assay was carried out by incubation of the immunoprecipitated proteins with a purified GST-IκBα$_{1-55}$ fusion protein as substrate. A Western blotting was then performed using an antibody specific for Ser32-36 phosphorylated IκBα and IKKγ (upper panels). Coomassie blue staining of the substrate (cb) is shown as a loading control (lower panels). (D) HeLa and Jurkat cells were pre-treated or not with GA (0.5 μM) for 14 h. The expression levels of IKKα, β and γ were analyzed by Western blotting.

is completely inhibited by GA pre-treatment in Hela cells, whereas this inhibition is more transient in Jurkat. Inhibition of IκBα tyrosine phosphorylation and degradation was also detected on IκBα in Western blot, where the apparition of a slower migrating species upon PV stimulation was clearly reduced by GA pre-treatment (Fig. 3A). As control, we also followed IκBα Ser-32, -36 phosphorylation upon TNF-α stimulation with a specific antibody. As expected, GA clearly inhibited TNF-α-induced Ser-32, -36 IκBα phosphorylation in both cell lines (Fig. 3B). Altogether, these data suggest that IκBα phosphorylation on serines and tyrosine relies on Hsp90 activity.

3.2. *c-Src-mediated IκBα tyrosine phosphorylation is inhibited by geldanamycin*

A recent paper reported that c-Src directly phosphorylates IκBα on Tyr42 in HeLa cells upon PV stimulation [7]. To further explore the effect of GA on c-Src activity, we carried out an *in vitro* kinase assay using GST-IκBα$_{1-55}$ as substrate. Immunoprecipitated c-Src from PV-treated HeLa cells was able to directly phosphorylate GST-IκBα on tyrosine (Fig. 4A). The phosphorylated residue is very likely Y42, since there is no other tyrosine residues in the 55 first amino acids of IκBα [6]. Interestingly, pre-treatment with GA completely inhibited c-

Src-mediated IκBα tyrosine phosphorylation (Fig. 4A). Given that c-Src activity is inhibited in the presence of GA, we analyzed cellular c-Src expression by Western blotting in HeLa cells treated or not with GA. As presented in Fig. 4B, GA treatment has no effect on cellular c-Src expression. We also measured IKK activity in HeLa and Jurkat cells stimulated with TNF-α in the presence of GA by immunoprecipitating IKKγ and carrying out an *in vitro* kinase assay using the same substrate. GA completely blocked TNF-α-induced IKK activation in HeLa cells, and nearly completely in Jurkat cells (Fig. 4C). But on the contrary of c-Src and in agreement with others [29], GA induced an important reduction of the expression of IKKβ and IKKγ, and a less pronounced reduction of the level of IKKα in HeLa and Jurkat cells (Fig. 4D).

3.3. *Geldanamycin inhibits NF-κB DNA-binding on an exogenous κB site and p65 recruitment on the iκbα promoter after PV induction*

Given that GA inhibited PV-induced IκBα degradation, we next wanted to study the effects of GA on the nuclear phase of the NF-κB pathway, i.e., on NF-κB DNA-binding activity. HeLa and Jurkat cells were pre-treated or not with GA then stimulated with PV. NF-κB activation induced by TNF-α, which has already been reported to be sensitive to Hsp90 inhibition, was used as positive control [29,41]. Electrophoretic mobility shift assays (EMSA) performed on nuclear extracts revealed that GA dramatically inhibited NF-κB DNA-binding activity induced by PV and TNF-α in both cell lines (Fig. 5A). To extend these results, we used p65 ChIP assays to determine whether inhibition of NF-κB DNA-binding observed by EMSA was also confirmed on an endogenous promoter. After chromatin immunoprecipitation with p65 antibody, real-time PCR was used to amplify κB consensus sequences of the *iκbα* promoter. p65 recruitment was observed at 30 min of treatment with PV, and this recruitment is clearly inhibited upon GA pre-treatment (Fig. 5B). The same results were obtained when cells were treated with GA and TNF-α.

3.4. *Geldanamycin reduces PV-induced IκBα mRNA synthesis*

A quantitative reverse transcription-PCR (qRT-PCR) was used to determine whether IκBα mRNA synthesis was influenced by GA treatment upon PV stimulation. HeLa cells were treated with PV with or without GA and total RNA were isolated. IκBα specific primers were used to examine the relative RNA expression. The results were normalized with the 18S transcript. When cells

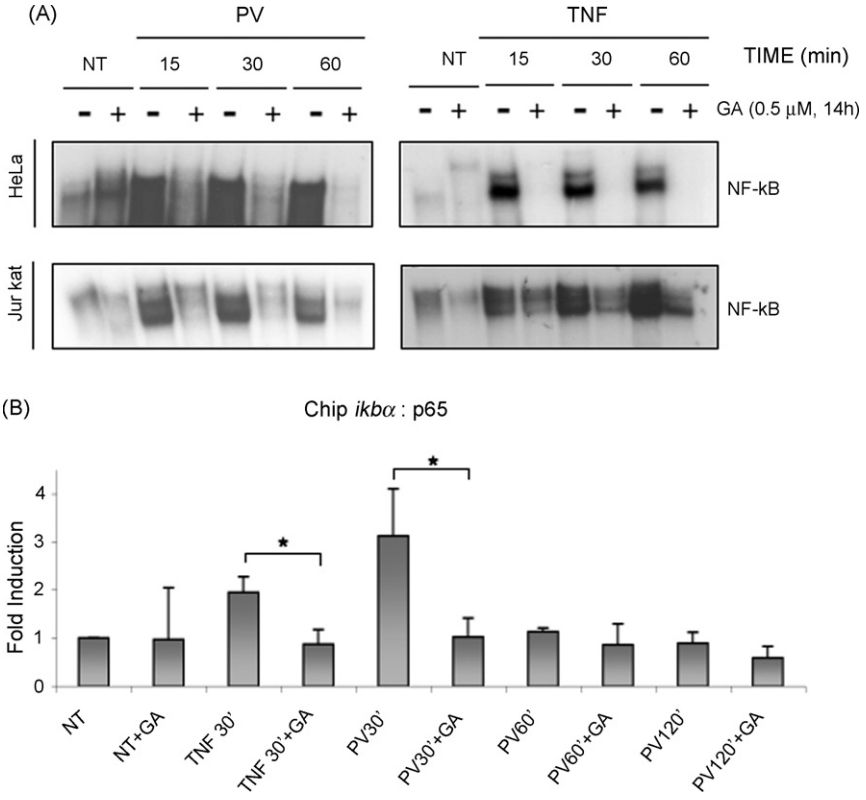

Fig. 5 – Geldanamycin inhibits NF-κB DNA-binding on an exogenous κB site and p65 recruitment on the *iκbα* promoter after PV induction. (A) HeLa and Jurkat cells were pre-treated or not with GA (0.5 μM) for 14 h and then stimulated with PV (200 μM) or TNF-α (200 U/mL) for the indicated times. After treatment, nuclear extracts were prepared. NF-κB DNA-binding activity was measured by electrophoretic mobility shift assay (EMSA) with a probe corresponding to the HIV-1 LTR-κB site (upper panels). (B) ChIP assays were performed on HeLa cells pre-treated or not with GA and treated with PV or TNF-α using p65 antibody for the immunoprecipitation. The immunoprecipitated chromatin was submitted to a quantitative real-time PCR analysis using primers amplifying the promoter region of *iκbα*. The fold induction is relative to untreated controls (NT). *Significantly different (p value < 0.05).

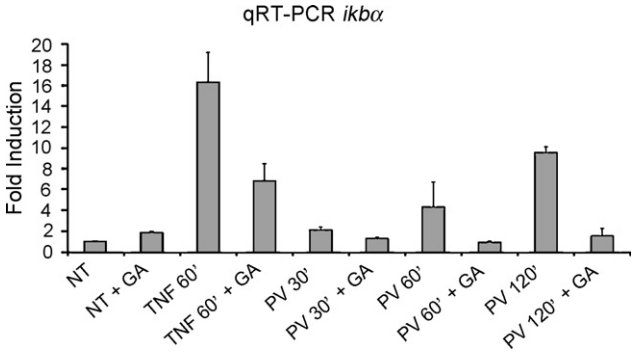

Fig. 6 – Geldanamycin inhibits PV-induced *iκbα* mRNA synthesis. Total RNAs were isolated from HeLa cells at various times of treatment with either PV (200 μM) or PV + GA (0.5 μM) and TNFα (200 U/mL) or TNFα + GA. IκBα mRNA expression level was analyzed by quantitative real-time RT-PCR with specific primers. The fold induction is relative to untreated controls (NT). Values are means from three independent experiments.

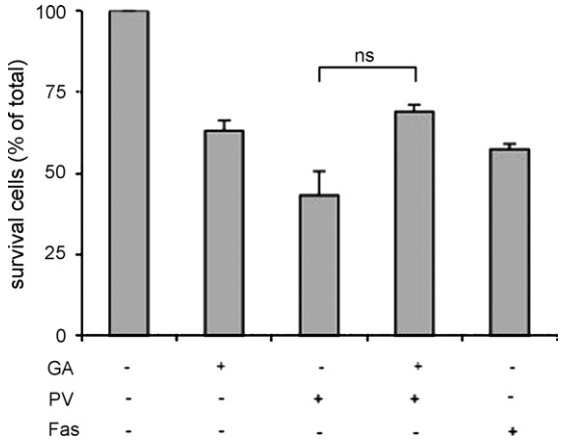

Fig. 7 – Combination of GA and PV treatments has not synergistic effect on Jurkat apoptosis. Jurkat cells were treated with GA (0.5 μM) for 38 h or PV (200 μM) for 24 h or pre-treated with GA for 14 h followed by a stimulation with PV for 24 h. Fas-L (50 ng/mL, 24 h) was used as positive control. Then cells were stained with Annexin V/propidium iodide and subjected to FACS analysis. ns: non-significantly different (p value > 0.05).

were treated for 30 min with PV alone, a two-fold stimulation of the IκBα mRNA synthesis was observed, and is gradually increased at 60 and 120 min of treatment (Fig. 6). Upon GA addition, IκBα mRNA synthesis was strongly reduced at 60 and 120 min of treatment, bringing it to levels quite comparable to the non-treated signal (Fig. 6). The same data were observed when cells were treated with TNF-α plus GA (Fig. 6).

3.5. *Combination of GA and PV treatments has no synergistic effect on apoptosis*

PV was reported to induce apoptosis in Jurkat through the intrinsic mitochondrial pathway [42]. To explore whether GA potentiates PV-induced apoptosis, we pre-treated Jurkat cells with GA for 14 h and stimulated them with PV for 24 h. Apoptosis was then analyzed by FACS using Annexin V/PI staining. Fas-ligand was used as positive control. As shown in Fig. 7, PV treatment induced more than 50% of cell death. Interestingly, the combination of GA and PV treatments did not increase apoptosis significantly.

4. Discussion

Geldanamycin (GA), an anti-tumor drug, is the main inhibitor of Hsp90, a protein chaperone involved in cytokine-induced NF-κB signalling pathways [16,29]. Pervanadate (PV) induces an atypical NF-κB activation pathway, leading to IκBα phosphorylation on Tyr42 dependent of the tyrosine kinase c-Src [5-7]. Several studies reported an interaction between Hsp90 and c-Src, but the exact role of Hsp90 on c-Src activity and expression is poorly understood [34–36,39,43]. In this study, we have investigated the effect of GA on tyrosine phosphorylation-dependent NF-κB activation, and report for the first time that Hsp90 is an essential component for the c-Src-dependent NF-κB pathway. First, we have shown that GA inhibits tyrosine phosphorylation and degradation of IκBα in HeLa and Jurkat cells. As a result, NF-κB nuclear translocation and DNA-binding on both an exogenous κB element and on the endogenous *iκbα* promoter were blocked, thereby inhibiting *iκbα* mRNA synthesis. Using an *in vitro* kinase assay, we also demonstrated for the first time that the activity of c-Src as an IκBα kinase is completely inhibited by GA in HeLa cells, suggesting that Hsp90 is indeed required for c-Src activity. The same results were obtained in yeast by Xu et al. [34]. Moreover, on the contrary to IKKα, β and γ, we observed that the cellular c-Src expression was not affected by GA treatment. This is in agreement with other works demonstrating that total levels of c-Src are not affected by GA [35,37], and suggests that the mature protein is stable in the absence of Hsp90 activity. In the same way, the expression of the Src family kinase Lck is unaffected by GA treatment, unless the protein is activated by cellular stimulation [44]. We were unable to detect any c-Src degradation in cells pre-treated by GA and stimulated by PV, even after a long-time activation (data not shown), ruling out the involvement of a general mechanism in the Hsp90-mediated regulation of Src-related tyrosine kinases. In the same way, despite several works reported that some members of Src family kinases, *i.e.*, fyn and v-Src, interact directly with Hsp90 [38,45], we were unable to reproducibly demonstrate any interaction between Hsp90 and c-Src, mainly due to aspecific binding of Hsp90 to the beads used for immunoprecipitation experiments (data not shown). This observation, in agreement with a recent work [38], reflects the need of more powerful biochemical tools to analyze the interactions between Hsp90 and its partners, which would be highly desirable to avoid artifactual results.

Since GA was already demonstrated to inhibit the classical NF-κB pathway, we thus propose that Hsp90 functions as a key chaperone protein in all NF-κB signalling pathways, induced by both serine or tyrosine kinases [29,46]. On the other hand,

since Cdc37 and ELKS have been described to be associated with the IKK complex, we can suggest that these proteins may be associated with c-Src [28,32,47].

Interestingly, we also demonstrated in this work that, despite NF-κB inhibition, combination of GA and PV treatments has no significant synergistic effect on Jurkat cells apoptosis. PV-induced apoptosis was reported to rely on the intrinsic mitochondrial pathway and can be blocked by herbimycin A, a tyrosine kinase inhibitor, suggesting that tyrosine kinase activity is crucial in PV-induced cell death [42]. Since GA treatment of activated T cells was reported to deplete important tyrosine kinases, such as Lck [48], it is highly possible that, like herbimycin A, GA reverses PV-induced apoptosis. This result also indicates that NF-κB has no or little role in the protection against cell death induced by PV. It was recently reported that combination of GA and LPS induces macrophage apoptosis [45]. This led us to conclude that GA may have opposite effects on cellular apoptosis depending on the nature of the signalling pathway.

In conclusion, the work presented here suggests that tyrosine phosphorylation-dependent NF-κB activation is sensitive to pharmacological inhibition of Hsp90 by GA, and also clearly position c-Src as a GA-sensitive tyrosine kinase. These results emphasize the key role of Hsp90 in the signalling pathway leading to NF-κB activation upon oxidative stress, which may have interesting therapeutic implications to fight inflammatory diseases caused by the accumulation of reactive oxygen species.

Acknowledgements

This work was supported by the ''Action de Recherche Concertée'' (ARC, University of Liège) and IAP6/18 programs, the FNRS (Brussels, Belgium) and the '' Centre Anticancéreux près l'Université de Liège''. We thank S. Ormenese from the Imaging and flow cytometry GIGA-Research technological platform for FACS analysis. JC was supported by an ARC fellowship. GG, M-PM and JP are, respectively, Postdoctoral Researcher, Senior Research Associate and Research Director from the FNRS.

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