NF-κB is involved in SHetA2 circumvention of TNF-α resistance, but not induction of intrinsic apoptosis

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Treatment of cancer with tumor necrosis factor-α (TNF-α) is hindered by resistance and toxicity. The flexible heteroarotinoid, SHetA2, sensitizes resistant ovarian cancer cells to TNF-α-induced extrinsic apoptosis, and also induces intrinsic apoptosis as a single agent. This study tested the hypothesis that nuclear factor-κB (NF-κB) is involved in SHetA2-regulated intrinsic and extrinsic apoptosis. SHetA2 inhibited basal and TNF-α-induced or hydrogen peroxide-induced NF-κB activity through counter-regulation of upstream kinase (IκB kinase) activity, inhibitor protein (IκB-α) phosphorylation, and p-65 NF-κB subunit nuclear translocation, but independently of reactive oxygen species generation. Ectopic over-expression of p-65, or treatment with TNF-α receptor 1 (TNFR1) small interfering RNA or a caspase-8 inhibitor, each attenuated synergistic apoptosis by SHetA2 and TNF-α, but did not affect intrinsic apoptosis caused by SHetA2. In conclusion, NF-κB repression is involved in SHetA2 circumvention of resistance to TNF-α-induced extrinsic apoptosis, but not in SHetA2 induction of intrinsic apoptosis. ***Anti-Cancer Drugs*** **21:297–305 © 2010 Wolters Kluwer Health | Lippincott Williams & Wilkins.**

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Introduction

Development of resistance to platinum-based chemotherapy is the major cause of ovarian cancer deaths. New treatment approaches include administration of death receptor ligands, such as tumor necrosis factor-α (TNF-α), to induce the extrinsic apoptosis pathway. TNF-α was the first death receptor ligand to be considered as an anti-tumor drug; however, the clinical trials failed because of TNF-α resistance and unacceptable toxicity [1,2]. Currently, multiple National Cancer Institute-sponsored clinical trials are evaluating various ways of tumor-selective delivery of TNF-α to reduce the toxicity (Protocols NCT00483509, NCT00181025, and NCT00496535, *www.cancer.gov*). SHetA2 is a flexible heteroarotinoid compound that has been shown to sensitize ovarian cancer cells to TNF-α induction of the extrinsic apoptosis pathway, but the mechanism has yet to be elucidated [3].

Induction of apoptosis by TNF-α binding to the TNF receptor 1 is hindered by the simultaneous activation of the nuclear factor κB (NF-κB) survival pathway [4,5]. NF-κB is an inducible dimeric transcription factor complex of subunits [p65 (RelA), RelB, Rel (c-Rel), p50/p105, and p52/p100] that are upregulated in many cancers [6–8]. The activity of NF-κB is regulated by inhibitors of NF-κB (IκB) proteins, which bind NF-κB dimers and sequester them in the cytoplasm by masking the nuclear localization sequences [9]. The IκB proteins are, in turn, regulated by the IκB kinase (IKK) complex composed of two catalytic subunits: IKK-α and IKK-β, and a regulatory subunit IKKγ/NEMO [10]. TNF-α and other cytokines induce IκB-α phosphorylation by IKK, thus targeting IκB-α for ubiquitin-dependent proteasomal degradation. The released NF-κB subunits translocate into the nucleus where the dimers bind to consensus DNA sequences (κB sites) in several hundred genes involved in immune response, growth, tumorigenesis, inflammation, carcinogenesis, and apoptosis [8,11,12].

We hypothesized that SHetA2 sensitizes cancer cells to TNF-α by interfering with the NF-κB survival pathway. In support of this hypothesis, SHetA2 has been shown to counter-regulate the expression of NF-κB-controlled genes [13–15] and induce reactive oxygen species (ROS) generation that has the potential to regulate redox-sensitive transcription factors, such as NF-κB [16,17]. Furthermore, a systems biology analysis of microarray data implicated the TNF-α pathway as a major player in the SHetA2 mechanism of action [18]. Earlier studies showed that SHetA2 induces apoptosis in cancer cells, with very little effect on normal cells, by directly targeting mitochondria and the Bcl-2 family of proteins leading to the induction of the caspase 9-dependent intrinsic apoptosis pathway [16,17,19]. TNF-α, in contrast, initiates the caspase 8-dependent extrinsic apoptosis pathway [20]. Although the intrinsic and extrinsic pathways are initiated through separate mechanisms, they converge through the activation of the Bid protein [21]. The objective of this study was to

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determine whether SHetA2 treatment alters NF-κB signaling and potential downstream effects on intrinsic apoptosis and sensitization to TNF-α-driven extrinsic apoptosis in ovarian cancer cells.

Materials and methods

Reagents and cell lines

SHetA2 was synthesized by Dr K. Darrel Berlin [22], dissolved in dimethyl sulfoxide (DMSO) and stored at −20°C. Human recombinant TNF-α (R&D Systems, Minneapolis, Minnesota, USA), Caspase 8/FLICE Inhibitor (Z-IETD-FMK) (Medical & Biological Laboratories Ltd., Japan) and MG132 (Calbiochem, San Diego, California, USA) were stored according to the manufacturer's instructions. A2780 ovarian cancer cells (Michael Birrer, National Cancer Institute, Bethesda, Maryland, USA) and SK-OV-3 ovarian cancer cells (ATCC, Manassas, Virginia, USA) were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 1 mmol/l HEPES buffer, 1 mmol/l sodium pyruvate, and 1X antimycotic/antibiotic.

Plasmids, transfections, and luciferase reporter assays

Cultures were co-transfected with a 10 : 1 ratio of NF-κB-specific reporter plasmid and pNF-κB-luc (Dr Wei Qun Ding, University of Oklahoma Health Sciences Center) to evaluate NF-κB transcriptional activity and pRenilla luciferase (pRL)-thymidine kinase (TK) (Dr A.L. Olson, University of Oklahoma Health Sciences Center) as a transfection control using Metafectene Pro (Biontex, Germany). For p65 overexpression, 3 μg pCytomegalovirus (pCMV)4 or pCMV4-p65 (Dr W.C. Greene, University of San Francisco, California, USA) were co-transfected with pNF-κB-luc and pRL-TK to assess NF-κB activity or transfected alone to determine the effects on apoptosis or cell viability. Twenty-four hours after transfection, cells were trypsinized and re-plated onto 12-well plates. After a further 24-h incubation, cultures were treated with 10 μmol/l SHetA2 for various times. In some instances, 20 ng/ml TNF-α was added for the last 30 min of treatment or varying concentrations of hydrogen peroxide (H_2O_2) were added for the last 240 min. Cells were prepared and evaluated for luciferase activity as described [13]. Firefly luciferase activity was normalized to Renilla luciferase activity and expressed as fold of luciferase activity in untreated cells. For additional controls the pTATA box-binding protein (pTBP)-luc or cFos-luc (both from Dr D.L. Johnson, University of Southern California) was co-transfected with pRL-TK plasmid. For antioxidants studies, A2780 cultures were pretreated with butylated hydroxyanisol; 50 μmol/l), MnTBAP (100 μmol/l), Trolox (0.5 mmol/l), or NAC (5 mmol/l) for 24 h before SHetA2 or DMSO treatments. All experimental results are presented as the average and standard error of three independent experiments performed in triplicate.

Western blotting

Whole cell extracts prepared from cultures using M-PER (Pierce, Rockford, Illinois, USA) were evaluated by western blots using the following primary antibodies: IKK-α, GAPDH, PARP-1, β-actin, (Santa Cruz Biotechnology, Santa Cruz, California, USA); IκB-α ser 32/36, total IκB-α, IκK ser 176/180, Bid, caspase 8, caspase 9, and caspase 3 (Cell Signaling Technology, Danvers, Massachusetts, USA) as described earlier [15]. Results presented are representative blots of three independent experiments producing similar results.

Immunocytochemistry

Cells were seeded at 20 000 cells per chamber in an 8-chamber slide, grown overnight and treated with 10 μmol/l SHetA2 for 16 h. TNF-α (20 ng/ml) was added for the last 20 min of treatment. After treatment, immunocytochemistry was performed using p65 primary antibody (Santa Cruz Biotechnology) and Alexa-488 conjugated secondary antibody (Invitrogen, Carlsbad, California, USA) with propidium iodide nuclear staining. Results presented are representative photomicrographs of three independent experiments producing similar results.

Preparation of IκB kinase complex and immunocomplex kinase assay

IKK activity was evaluated using the protocol described by Lou and Kaplowitz [23]. Briefly, whole cell extracts (250 μg) were immunoprecipitated with the IKK-α antibody and protein G-agarose (Roche, Indianapolis, Indiana, USA). IKK activity of the precipitates was measured using 1 μg of GST-fused IκB-α (Millipore, Billerica, Massachusetts, USA) as a substrate in the presence of 20 μl kinase buffer supplemented with 0.3 mmol/l ATP in a 30-min incubation at 30°C. IκB-α phosphorylation by IKK was visualized by western blotting of kinase reaction components using ser 32/36 phospho-specific antibody (Cell Signaling Technology). Results presented are representative blots of three independent experiments producing similar results.

Small interfering RNA experiments

Cultures were transfected with small interfering RNA (siRNA) from the NF-κB signaling pathway, SiRNA Array (SABiosciences, Frederick, Maryland, USA), using the manufacturer's reverse transfection protocol. Sixteen hours after transfection, the medium was changed and cells were treated with 10 μmol/l SHetA2 alone or in combination with 20 ng/ml TNF-α for 24 h. Control cultures were treated with DMSO solvent only. Apoptosis was measured using the DNA fragmentation cell death ELISA kit (Roche). Results that are presented are representative of two independent repeats of the experiment producing similar results.

Cell viability and apoptosis assays

Twenty-four hours after transfection with pCMV4-p65 plasmids or mock transfection, cultures were treated with

a range of SHetA2 concentrations alone or in combination with 20 ng/ml TNF-α for 24 h. Cell viability was measured with the MTS reagent (Promega, Madison, Wisconsin, USA) and expressed as fold of the untreated control. Apoptosis was detected using Annexin V FITC/propidium iodide from the Vybrant Apoptosis assay kit #3 (Invitrogen) and evaluated by flow cytometry using a Becton Dickinson FACS Caliber automated bench-top flow cytometer at an excitation wavelength of 488 and observation wavelengths of 530 and 575 nm. All results are presented as the average and standard error of two to three independent experiments performed in duplicate.

Data analysis and statistics

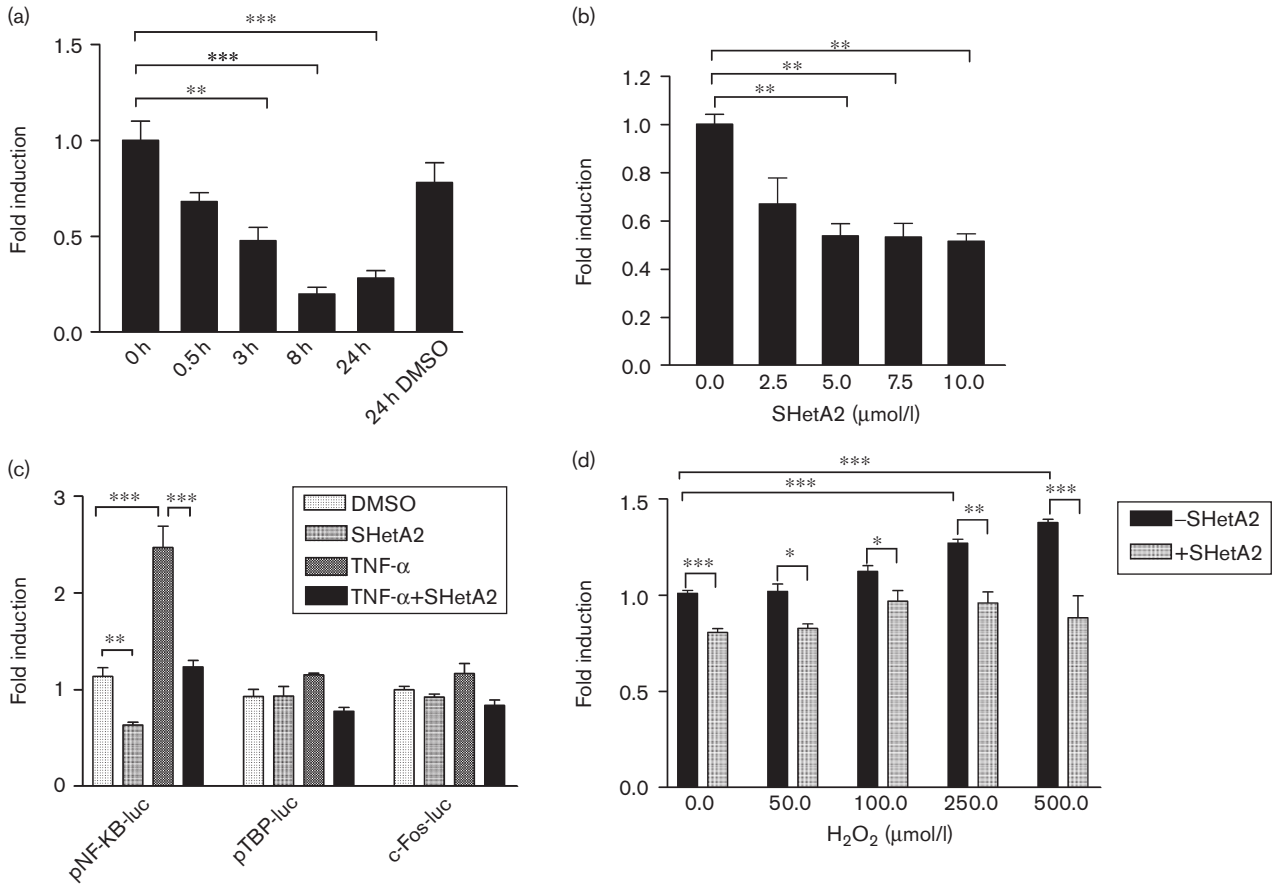
Graphpad Prism software and Microsoft Excel were used to plot the graphs and test for statistical significance using two-tailed paired *t*-test and significance was established at a 95% confidence interval ($P < 0.05$). For comparing more than three groups at once, one-way analysis of variance with a Bonferroni post test was used to compare all pairs of groups with significance established at *P* value of less than 0.05.

Results

SHetA2 inhibits basal, TNF-α- and H_2O_2-induced NF-κB activity

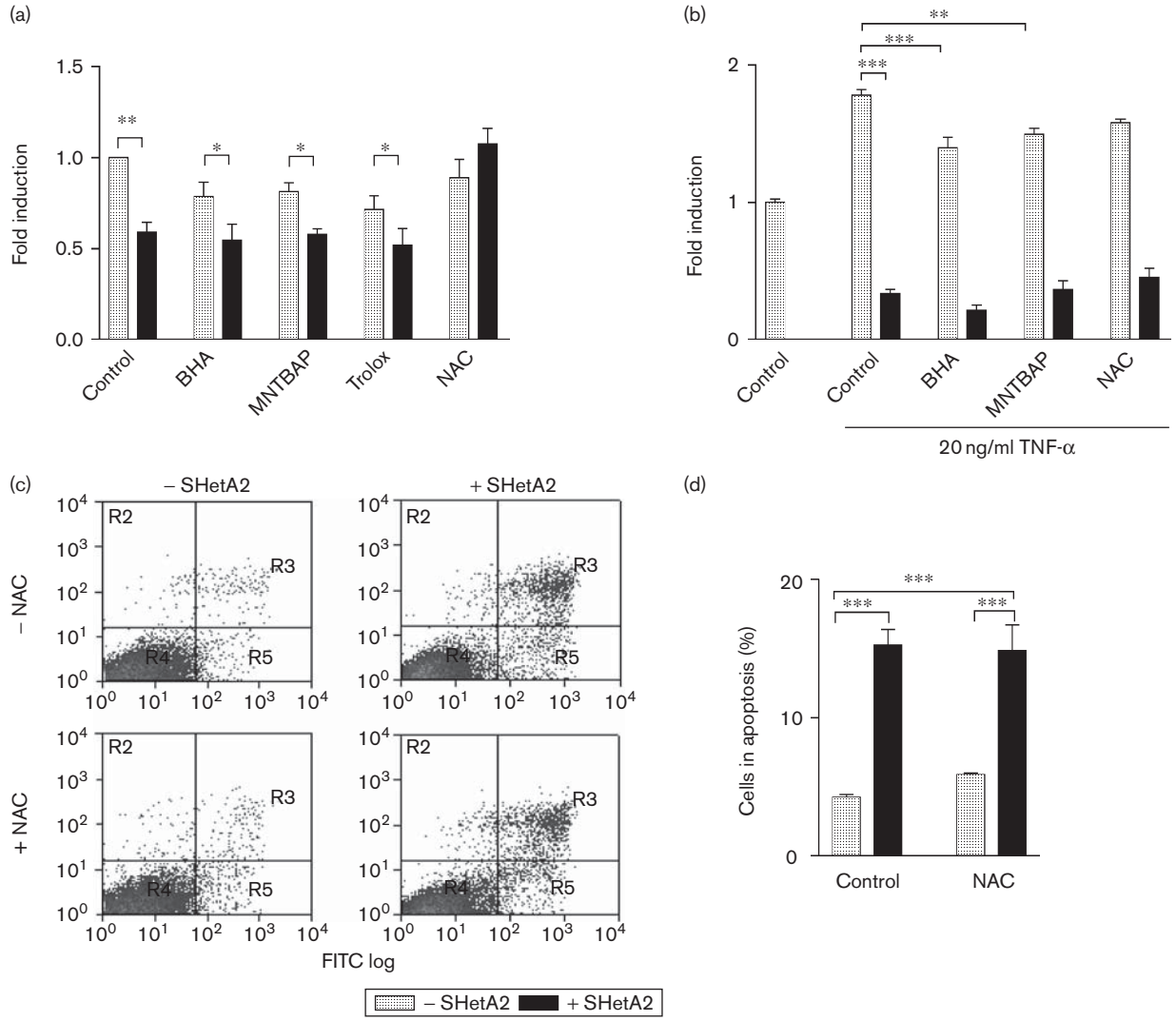
Regulation of NF-κB activity was evaluated in A2780 ovarian cancer cells transfected with NF-κB reporter and transfection control plasmids. SHetA2 suppressed basal NF-κB activity in a dose-dependent and time-dependent manner within the earliest time point of 30 min and lowest dose of 2.5 μmol/l evaluated (Fig. 1a and b, respectively). Induction of NF-κB activity through two different mechanisms, TNF-α (Fig. 1c) and H_2O_2 (Fig. 1d), was also repressed by SHetA2. Lack of SHetA2 repression of different promoter sequences in the c-Fos and TATA

Fig. 1



Effects of SHetA2 on basal and inducible NF-κB activity. (a and b) Cultures co-transfected with the NF-κB inducible Firefly luciferase reporter plasmid (pNF-κB–luc) and the transfection control Renilla luciferase reporter plasmid (pRL-TK) were treated with 10 μmol/l SHetA2 for the indicated times (a) or treated with the indicated doses of SHetA2 for 12 h (b) and tested for luciferase activity. (c) Cultures co-transfected with NF-κB, TBP-driven or c-Fos-driven luciferase reporter plasmid and the pRL-TK transfection control plasmids were treated for 4 h with 10 μmol/l SHetA2. TNF-α (10 ng/ml) was added for the last 30 min of incubation before evaluation of luciferase activity. (d) Cells co-transfected with pNF-κB-luc reporter and pRL-TK were treated with different doses of H_2O_2, with or without 10 μmol/l SHetA2 for 4 h before evaluation of luciferase activity. Firefly luciferase activity was normalized to Renilla luciferase activity and expressed as fold of luciferase activity in untreated cells. *$P<0.05$, **$P<0.01$, ***$P<0.001$. DMSO, dimethyl sulfoxide; H_2O_2, hydrogen peroxide; TNF-α, tumor necrosis factor-α.

Fig. 2



Evaluation of ROS in NF-κB induction and apoptosis in A2780 cells. (a) Cells co-transfected with pNF-κB-luc reporter and pRL-TK were treated with different antioxidants in the presence and absence of 10 μmol/l SHetA2 for 24 h before evaluation of luciferase activity. (b) Same as a, except that TNFα was added for the last 30 min of incubation before evaluation of luciferase activity. (c) Cells were treated with 10 μmol/l SHetA2 or dimethyl sulfoxide solvent only (Control) for 24 h with or without 5 mmol/l NAC and evaluated for apoptosis using annexin FITC/propidium iodide staining and flow cytometry. (d) Quantification of two independent experiments performed in duplicate as described in d are presented as average and standard error and expressed as fold activity of the untreated control. *$P<0.05$, **$P<0.01$, ***$P<0.001$. TNF-α, tumor necrosis factor-α.

box-binding protein-driven reporter plasmids showed that this repression was specific for NF-κB sites and not because of global inhibition of transcription (Fig. 1c). Similar results were observed for the SK-OV-3 ovarian cancer cell line (data not shown).

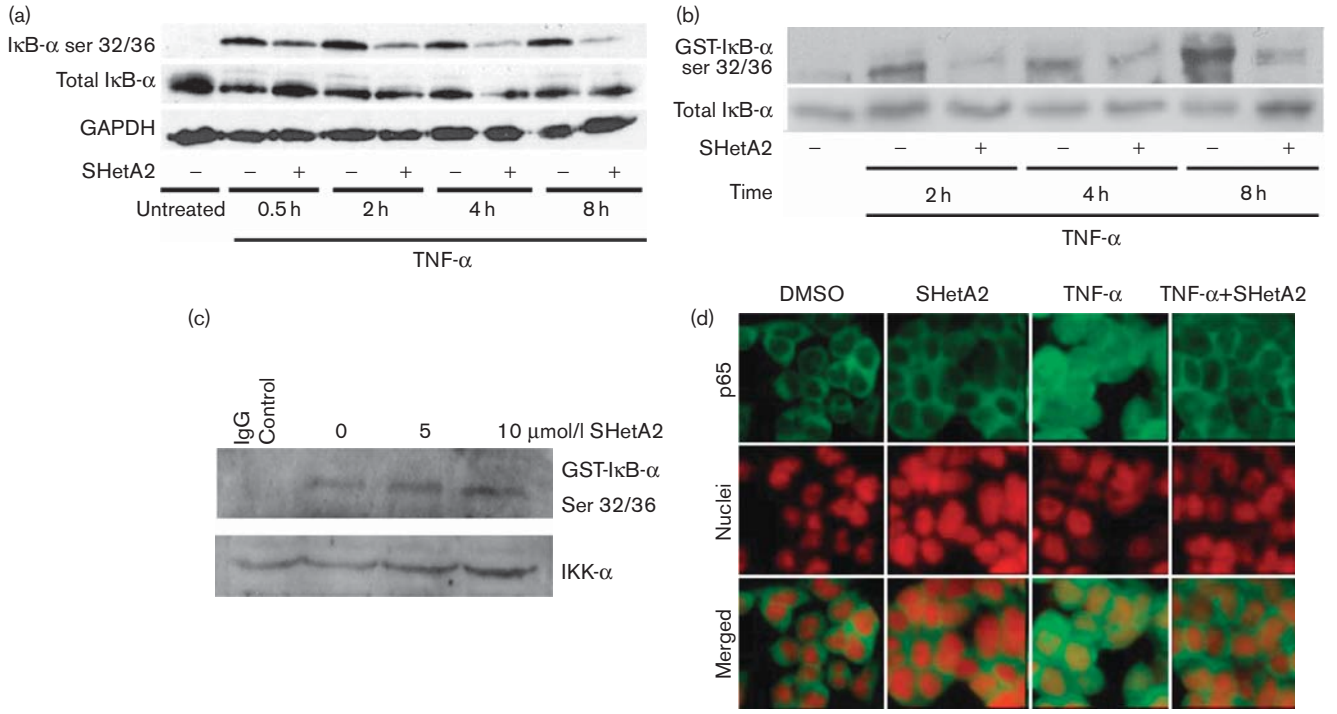
Reactive oxygen species are not involved in SHetA2 suppression of NF-κB activity

As NF-κB activity can be regulated by the redox state of the cell, and SHetA2 can induce ROS generation [16,22], we hypothesized that SHetA2 repression of NF-κB is a result of increased intercellular ROS. To test this, NF-κB reporter assays were performed in the presence and absence of various antioxidants. Several ROS scavengers did not prevent SHetA2 inhibition of basal (Fig. 2a) or TNF-α-induced (Fig. 2b) NF-κB activity. Although NAC, a precursor of the natural cellular tripeptide antioxidant, glutathione, prevented SHetA2 repression of basal NF-κB activity (Fig. 2a), it had no effect on the repression of TNF-α-induced NF-κB activity (Fig. 2b) or the induction of apoptosis (Fig. 2c and d).

Upstream events leading to SHetA2 inhibition of NF-κB

Phosphorylation of IκB-α on serine 32 and serine 36 triggers the degradation of IκB-α and release of NF-κB

Fig. 3



SHetA2 repression of IκB-α phosphorylation and degradation, IKK activity and p65 nuclear translocation. (a–c) Whole cell extracts were prepared from A2780 cells treated with 10 μmol/l SHetA2 or DMSO solvent control (Untreated) for the indicated time points. (a) 100 μg of extracts were analyzed for IκB-α total and ser 32/36 IκB-α by western blotting. (b) Whole cell extracts were immunoprecipitated with IKK-α antibody or IgG isotype control antibody and subjected to the immune complex kinase assay. Phosphorylation of IκB-α was detected by western blot analysis of immune complex assay products using ser 32/36 IκB-α antibody were compared to IKK-α levels in the cell extract, which were evaluated by western blot analysis using the same volume of whole cell extracts used in the assay. (c) To test direct effects of SHetA2 on IKK activity, untreated whole cell extracts were used to immunoprecipitate IKK enzyme. Kinase buffer containing the indicated concentrations of SHetA2 was used in immune complex kinase assay as described in b. (d) A2780 cells treated with 10 μmol/l SHetA2 for 24 h with or without exposure to 10 ng/ml TNF-α for the last 30 min were fixed, and subjected to immunocytochemistry of p65. DMSO, dimethyl sulfoxide; IKK, IκB kinase; TNF-α, tumor necrosis factor-α.

repression. Western blot analysis of cytoplasmic extracts showed that TNF-α treatment of A2780 ovarian cancer cells resulted in the phosphorylation of IκB-α and reduction in total IκB-α levels, whereas SHetA2 inhibited phosphorylation at all the time points that were evaluated (Fig. 3a). This inhibition was followed by the preservation of IκBα total levels only at the 30-min time point suggesting an IκB-α-independent mechanism in the inhibition of NF-κB activity upon prolonged exposure with SHetA2.

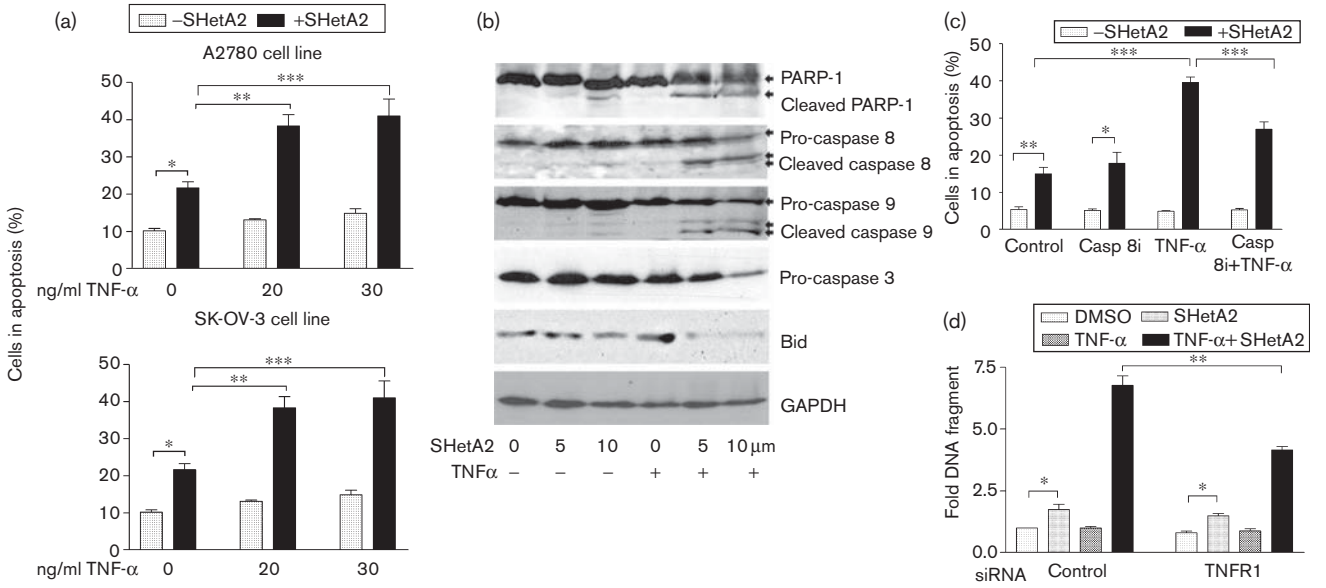
Inhibition of IKK activity was hypothesized to be responsible for SHetA2 inhibition of basal and induced IκB-α phosphorylation because activation of this kinase is required for both TNF-α- and H_2O_2-induced phosphorylation of IκB-α and p65. An immune complex assay showed that SHetA2 treatment of intact cells suppressed TNF-α-induced IKK activity at each time point that was evaluated (Fig. 3b). Basal IKK activity in the absence of TNF-α was also repressed by SHetA2 (data not shown). The inhibition appears to be indirect, however, as SHetA2 did not alter IKK activity when added directly to the kinase assay *in vitro* (Fig. 3c).

As IκB-α phosphorylation and subsequent degradation are known to release the cytoplasmic retention of NF-κB subunits, it was anticipated that SHetA2 repression of this phosphorylation would prevent nuclear accumulation of NF-κB subunits. Immunocytochemistry revealed that the p65 protein was predominantly localized in the cytoplasm in both DMSO-treated and SHetA2-treated cells, and that TNF-α induced p65 nuclear translocation, whereas SHetA2 inhibited this translocation (Fig. 3d).

Evaluation of intrinsic and extrinsic apoptosis pathway components and cross-talk in SHetA2-regulated apoptosis

The activation and cross-talk of the intrinsic and extrinsic apoptosis pathways in the apoptosis mechanisms induced by SHetA2, as a single agent, and when used as a TNF-α sensitizer, were evaluated. TNF-α, as a single agent, did not induce apoptosis in either the A2780 or SK-OV-3 ovarian cancer cell lines, as shown by the absence of Annexin V staining to detect apoptosis (Fig. 4a). Lack of cleavage (indicating activation) of PARP-1, caspase 8, caspase 9, and caspase 3 in cultures treated with TNF-α

Fig. 4



Evaluation of apoptosis pathway components and cross-talk in SHetA2-induced and TNF-α-induced apoptosis. (a) A2780 (top graph) and SK-OV-3 (bottom graph) cells were treated with 10 μmol/l SHetA2 with or without the indicated concentrations of TNF-α for 24 h and evaluated for apoptosis as described in Fig. 2c and d. (b) A2780 cells were treated with indicated concentrations of SHetA2 with or without 20 ng/ml TNF-α for 24 h. Protein extracts (100 μg) were prepared and subjected to western blot analysis with antibodies against indicated proteins. (c) A2780 cells were treated with 10 μmol/l SHetA2 with or without 20 ng/ml TNF-α or caspase 8 inhibitor (casp 8i) for 24 h and evaluated for apoptosis as described in Fig. 2c and d. (d) A2780 cells transfected with validated siRNA against TNFR1 or scrambled siRNA were treated with DMSO solvent only or 10 μmol/l SHetA2 with or without 20 ng/ml TNF-α 24 h after transfection for 24 h and evaluated for apoptosis. DMSO, dimethyl sulfoxide; siRNA, small interfering RNA; TNF-α, tumor necrosis factor-α; TNFR1, TNF-α receptor 1.

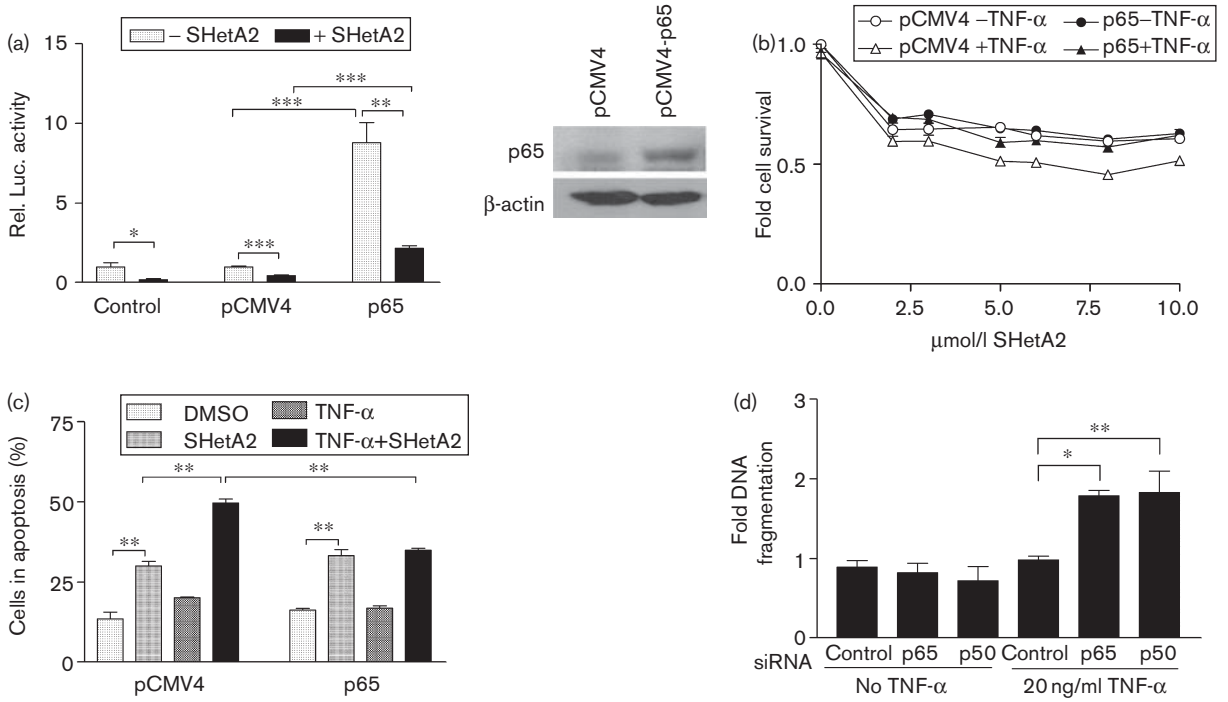
only confirmed the resistance of the A2780 (Fig. 4b) and SK-OV-3 cell lines (data not shown). Simultaneous treatment of cells with TNF-α and SHetA2 resulted in potentiation of SHetA2-induced apoptosis in both cell lines (Fig. 4a) and induction of PARP-1, caspase 3, and caspase 8 cleavage in the A2780 (Fig. 4b) and SK-OV-3 cell lines (data not shown). The weak cleavage of caspase 9 observed in A2780 cultures treated with SHetA2 only was enhanced by the addition of TNF-α, most likely due to the cleavage of Bid (Fig. 4b), which is known to connect the extrinsic to the intrinsic apoptosis pathway [21].

Consistent with earlier studies showing that SHetA2 acts through the intrinsic apoptosis pathway [16], a caspase-8 inhibitor did not prevent SHetA2-induced apoptosis (Fig. 4c). The ability of the caspase-8 inhibitor to partially attenuate apoptosis induced by simultaneous SHetA2 and TNF-α treatment confirmed the participation of the extrinsic pathway in the synergistic induction of apoptosis (Fig. 4c). The residual potentiation of apoptosis is most likely because of the contribution from caspase 10, another initiator caspase for the extrinsic apoptotic pathway. To complement this data knockdown of TNFR1, the cognate receptor for TNF-α by a validated siRNA reduced the potentiation of apoptosis (Fig. 4d). The partial effect may be because of a low 10% knockdown of TNFR1.

Role of NF-κB repression in TNF-α sensitization, but not intrinsic apoptosis

If SHetA2 induction of apoptosis is mediated by the inhibition of the NF-κB cell survival pathway as our study suggests, then overexpression of p65 to elevate NF-κB activity should modulate apoptosis induction by SHetA2 alone and when combined with TNF-α. To test this hypothesis, ovarian cancer cells were transfected with a constitutive p65 cDNA expression vector. Overexpression of p65 in ovarian cancer cells resulted in increased p65 protein expression and enhanced NF-κB transcriptional activity (Fig. 5a). SHetA2 treatment inhibited NF-κB transcriptional activity in both mock and p65 overexpressing cells (Fig. 5a). The level of NF-κB activity in p65 transfected cells was significantly higher than mock-transfected cells even when treated with SHetA2, thus providing a model to test our hypothesis (Fig. 5a). Under these conditions ectopic overexpression of p65 did not repress loss of cell viability and apoptosis induced by SHetA2 as a single agent (Fig. 5c), which is consistent with the finding that NAC reversal of the SHetA2 NF-κB repression did not attenuate SHetA2-induced apoptosis (Fig. 2d). When SHetA2 was combined with TNF-α treatment, however, the p65-induced elevated NF-κB activity prevented the enhanced decrease in cell viability caused by the combination of the two agents (Fig. 5b), indicating a vital role of NF-κB repression in the

Fig. 5



Effects of p65 on SHetA2-induced and TNFα-induced apoptosis. (a) Cells co-transfected with pNF-κB-Luc and pRL-TK either alone (Control) or with empty vector (pCMV4) or p65 expression vector (pCMV4-p65) were treated with DMSO solvent only (–SHetA2) or 10 µmol/l SHetA2 (+SHetA2) for 16 h and evaluated for luciferase activity. Firefly luciferase activity was normalized to Renilla luciferase activity. Inset shows western blot confirming p65 expression in pCMV4-p65 transfected cells. (b) Cells were transfected with pCMV4-p65 or pCMV4 and treated with indicated concentrations of SHetA2 with or without 20 ng/ml TNF-α for 24 h and evaluated with an MTS assay in triplicate. Fold survival was derived by dividing the average optical density of each treatment by the optical density of the control treated with solvent only. (c) Parallel cultures treated as in panel b were analyzed for apoptosis using Annexin FITC/propidium iodide staining as described in 2c and d. (d) A2780 cells transfected with validated siRNA against p50, p65, and control scrambled siRNA were treated with 20 ng/ml TNF-α for 24 h, and then evaluated for apoptosis with a DNA fragmentation assay. DMSO, dimethyl sulfoxide; pCMV4, pCytomegalovirus 4; siRNA, small interfering RNA; TNF-α, tumor necrosis factor-α.

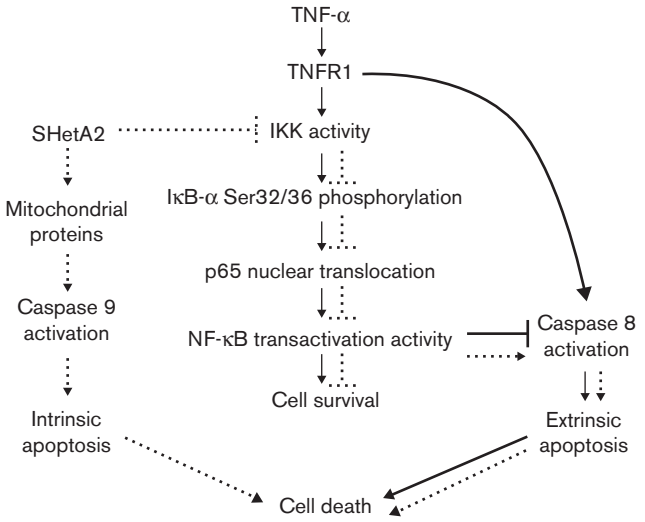
mechanism of SHetA2 sensitization of ovarian cancer cells to TNF-α. These results were confirmed at the level of apoptosis (Fig. 5c) and in the SK-OV-3 ovarian cancer cell line (data not shown). To further support the role of NF-κB inhibition in the sensitization of ovarian cancer cells to TNF-α, validated siRNA from the NF-κB signaling pathway array against p65 and p50 NF-κB subunits significantly sensitized A2780 cells to TNF-α-induced cell death (Fig. 5d).

Discussion

The results of this study show that SHetA2 suppresses NF-κB transactivation activity in ovarian cancer cell lines. In support of our hypothesis that this NF-κB repression is involved in the mechanism of SHetA2-regulated extrinsic apoptosis, prevention of the reduced levels of NF-κB activity by the overexpression of the p65 NF-κB protein subunit also suppressed the synergistic induction of apoptosis when SHetA2 was used in combination with TNF-α. In opposition to the hypothesis that NF-κB repression is involved in SHetA2 regulation of intrinsic apoptosis, the elevated NF-κB activity did not attenuate apoptosis caused by SHetA2 as a single agent. These different effects are consistent with the different pathways induced by SHetA2 and TNF-α, which ultimately lead to the apoptotic death of the cell.

Induction of the intrinsic apoptosis pathway by SHetA2 has been well characterized and validated in multiple cancer cell types [13,16,17,24,25], whereas binding TNF-α to its cell surface death receptors is known to induce the extrinsic apoptosis pathway [20]. The ability of a caspase-8 inhibitor to prevent the synergistic level of apoptosis, but not the level of apoptosis induced by SHetA2 as a single agent, confirms that the enhanced apoptosis induced by the SHetA2 and TNF-α combination is because of the induction of the extrinsic apoptosis pathway, which adds to the level of apoptosis caused by SHetA2 induction of the intrinsic apoptosis pathway. These results are consistent with the findings of others that various NF-κB inhibitors can sensitize other types of cancer cell lines to death receptor ligands [26–28], and that the induction of intrinsic apoptosis by curcumin does not involve NF-κB repression [29]. Although constitutive NF-κB activation has been reported to prevent the

Fig. 6



Working model. Solid lines: TNF-α activities. Dashed lines: SHetA2 activities. In ovarian cancer cells, binding of TNF-α to its cell surface receptor; TNFR1 induces NF-κB activity. SHetA2 inhibits NF-κB activation through inhibition of IKK phosphorylation of IκB-α allowing TNFα-induced apoptosis mediated by caspase 8 to occur in cell death receptor ligands-resistant cells. Activation of TNF-α-induced extrinsic apoptosis and SHetA2-induced intrinsic apoptosis results in synergistic induction of apoptosis. IKK, IκB kinase; NF-κB, nuclear factor-κB; TNF-α, tumor necrosis factor-α; TNFR1, TNF-α receptor 1.

induction of intrinsic apoptosis in lymphoma cell lines [30], our results indicate that the level of basal NF-κB activity in ovarian cancer cell lines does not cause resistance to intrinsic apoptosis.

The mechanism of SHetA2 NF-κB repression appears to involve upstream signaling components, as this drug caused the repression of IKK phosphorylation of IκB-α leading to the inhibition of translocation of the p65 NF-κB subunit to the nucleus in which it can regulate gene expression. Expression of several NF-κB-controlled genes, including E-cadherin, VEGF and cyclin D1, has been shown to be counter-regulated by SHetA2 [13–15]. The mechanism of SHetA2 IKK inhibition appears to be indirect in that SHetA2 only caused repression when administered to intact cells and not when tested *in vitro*, suggesting that a metabolic product of SHetA2 may be responsible for the repression, or that SHetA2 may inhibit a kinase upstream of IKK. The NF-κB-inducing kinase is a likely candidate for the next upstream level of control, as SHetA2 inhibits induction of NF-κB by both TNF-α and H_2O_2, which activate NF-κB through different mechanisms that converge at the activation of NF-κB-inducing kinase, which then activates the IKK complex [31,32]. Alterations in IKK phosphorylation may not be entirely responsible for mediating SHetA2 regulation of IKK activity, however, because another mechanism of IKK activation has been observed. IKK phosphorylation has been shown to be insufficient for the induction of IKK activity by G protein-coupled receptors, which can increase IKK activity by stimulating the physical association of the IKK complex with CARMA3 (caspase recruitment domain-associated and membrane-associated guanylate kinase domain-containing protein 3) [33,34].

Although NF-κB activity can be regulated by the redox state of the cell, it is unlikely that the known generation of intracellular ROS by SHetA2 is involved in NFκB repression or regulation of apoptosis. Carefully controlled antioxidant studies showed that the induction of intrinsic apoptosis by SHetA2 can proceed despite quenching of the mitochondrial and cytoplasmic ROS generated [16]. ROS generation therefore appears to be a consequence, and not a cause, of the mitochondrial swelling and apoptosis induced by SHetA2 in cancer cells. Although metabolism studies have shown that SHetA2 binds to the natural cellular tripeptide antioxidant, glutathione [35], this activity is unlikely to be responsible for the ROS generated because glutathione is present in mmol/l concentrations, whereas only μmol/l SHetA2 concentrations are required for the induction of mitochondrial swelling, ROS generation and intrinsic apoptosis [13,16,19,36]. The ability of the glutathione precursor, NAC, to prevent SHetA2-repression of basal, but not induced, NF-κB activity levels could be explained by the ability of NAC to prevent oxidation of cys 62 on the p50 protein and thereby prevent the decreased DNA binding caused by this oxidation [37]. Although this suggests that SHetA2 causes p50 oxidation, the inability of general ROS scavengers to prevent SHetA2 repression of basal NF-κB activity discounts this possibility.

The results from these studies support a model in which repression of NF-κB signaling sensitizes cells to TNF-α-induced extrinsic apoptosis, whereas SHetA2 induction of intrinsic apoptosis can occur regardless of NF-κB status (Fig. 6). Upon binding to its cognate receptor, TNFR1, TNF-α induces a survival pathway governed by NF-κB transcription factors, which blocks extrinsic apoptosis. SHetA2 inhibits TNF-α induction of the NF-κB pathway by inhibiting IKK-induced phosphorylation and subsequent proteasomal degradation of IκBα thereby retaining the p65 NF-κB subunit in the cytoplasm. The reduced NF-κB transcription factor activity removes the repression of extrinsic caspase-8-dependent apoptosis by TNF-α.

In summary, these results show that SHetA2 repression of NF-κB activity circumvents resistance to TNF-α-induced extrinsic apoptosis, thereby enhancing the level of apoptosis caused by a NF-κB-independent intrinsic pathway induced by SHetA2 as a single agent. SHetA2 has not shown any general toxicity, liver toxicity, skin irritancy, mutagenicity or teratogenicity in several animal models [13,36,38], and therefore could be used to reduce the effective dose of TNF-α thereby reducing inflammation and organ injury observed at high concentrations

of TNF-α. These results also suggest that SHetA2 may sensitize ovarian cancer cells to other death receptor ligands currently in clinical trials, such as TRAIL and agonistic antibodies to TRAIL.

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