

Inhibition of Akt phosphorylation attenuates resistance to TNF- α cytotoxic effects in MCF-7 cells, but not in their doxorubicin resistant derivatives

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ABSTRACT

Objective(s): Acquisition of TNF- α resistance plays role in the onset and growth of malignant tumors. Previous studies have demonstrated that MCF-7 cell line and its doxorubicin resistant variant MCF-7/Adr are resistant against the cytotoxic effects of TNF- α . In this study, we investigated the role of Akt activation in resistance of MCF-7 and MCF-7/Adr against TNF- α cytotoxicity.

Materials and Methods: The role of Akt activation in TNF- α cytotoxicity was investigated by MTT cell viability assay following treatment of the cells with the chemical inhibitor of Akt activation with or without TNF- α treatment. Phosphorylation of Akt at Ser473 before and after 72 hr TNF- α treatment was also determined by western blot.

Results: TNF- α treatment led to enhancement of Akt Ser473 phosphorylation. Treatment of MCF-7 cells with TNF- α along with Akt-inhibitor agent, triciribine, attenuated Akt Ser473 phosphorylation and sensitized these cells to the cytotoxic effects of TNF- α in a dose and time dependent manner while triciribine treatment did not cause any significant cytotoxicity in MCF-7/Adr cells alone or in combination with TNF- α .

Conclusion: These results demonstrate that Akt phosphorylation plays pivotal role in the resistance of MCF-7 cells against TNF- α -induced cytotoxicity while it might play no significant role in the resistance of MCF-7/Adr cells against TNF- α .

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Introduction

Pro-inflammatory cytokine tumor necrosis factor- α (TNF- α) plays various roles in the different physiological and pathological processes. TNF- α is involved in autoimmune disease, chronic inflammation, acute inflammation, and cancer-related inflammation (1). Binding of TNF- α to its specific receptors TNFR1 and TNFR2, activation of different downstream mediators including NF- κ B, c-jun N-terminal kinase (JNK), caspase cascade, phosphatidylinositol 3-kinase (PI3K)/Akt and reactive oxygen species (ROS) lead cells to inflammation and cell survival as well as cell death (2, 3). There are conflicting reports regarding the effects of TNF- α on MDR. Although various *in vitro* and *in vivo* investigations demonstrated MDR modulatory effects for TNF- α and numerous studies have been designed to evaluate its potential as chemosensitizers of resistant tumor cells (4-6), there are also investigations indicating TNF- α leads

to overexpression of MDR proteins and enhancement of the resistance of cancer cells (7-9).

Chemotherapy as therapeutic strategy against various cancers including breast cancer is failed by multidrug resistance (MDR). In MDR resistance against cytotoxic effects of anti-cancer drugs with different structure and mechanism can be intrinsic or acquired. In the acquired MDR, although chemotherapy leads to initial responses but tumors would be repopulated by drug resistant tumor cells and become resistant to retreatment (10). Overexpression of ATP-binding cassette (ABC) transporters, alteration in signaling pathways causing cellular death, overexpression or activity enhancement of drug detoxifying enzymes and improvement in DNA repair are mechanisms causing cells to show MDR (11-16).

It has been reported that TNF- α mediates some of its effects through PI3K/Akt signaling pathway (17).

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Cellular events including transcription, translation, cell proliferation and survival are affected by the PI3K/Akt pathway in normal as well as neoplastic tissues (18). Seventy percent of breast cancers have shown aberrations in this pathway. Moreover PI3K/Akt signaling plays a key role in resistance of tumors to the cancer chemotherapy (19-21). Activation of this pathway leads to phosphorylation of Akt kinase at Ser473 which is directly related to Akt activation.

For analysis of the biological role of the PI3k/Akt signaling pathway in MDR cancer cells, we employed the breast adenocarcinoma cell line MCF-7 and its MDR subline MCF-7/Adr which have been shown to be resistant against TNF- α cytotoxic effects (22, 23).

Materials and Methods

Cell culture

Human breast carcinoma cell lines MCF-7 and MCF-7/Adr were cultured in RPMI 1640 containing 10% fetal bovine serum (GIBCO, Grand Island, NY, USA) and penicillin (100 units/ml)/streptomycin (100 μ g/ml) (GIBCO, Grand Island, NY, USA). Cells were incubated at 37 °C in the presence of 5% CO₂. MCF-7/Adr cells were cultured in the presence of doxorubicin (Sigma-Aldrich, Taufkirchen, Germany) (250 nM) to maintain the MDR phenotype but doxorubicin was removed one week before the experiments.

Inhibition of Akt phosphorylation

To investigate the exact role of Akt phosphorylation in TNF- α toxicity, Akt activation was inhibited along with TNF- α treatment. Inhibition of Akt phosphorylation was done by triciribine (TCN, Sigma-Aldrich, St. Louis, MO, USA) which is a potent small-molecule inhibitor of activation and phosphorylation of all three isoforms of Akt *in vitro* (24). It is highly selective for Akt and does not inhibit PI3K, PDK1, PKC, SGK, PKA, Stat3, Erk-1/2 or JNK (25).

Cell viability assays

MCF-7 and MCF-7/Adr cells were seeded at a density of 6000 cell/well in 96-well cell culture plates and incubated overnight. Then cells were treated with 50 ng/ml TNF- α alone or in combination with 1, 10 and 30 μ M of TCN. After indicated times cells were incubated with 0.5 mg/ml dimethylthiazolyl-2,5-diphenyl tetrazolium bromide (MTT) for 3 hr, then cells were lysed using 100 μ l dimethyl sulfoxide. A microplate reader (Synergy 4, BioTek, VT, USA) was used to determine absorption at 570 nm of each well. Cell viability in treated cells was calculated as a percentage of controls treated with vehicle.

Western blot analysis

Akt Ser473 phosphorylation level was evaluated using Western blot analysis as described previously (26). In brief, cells were treated with 50 ng/ml TNF- α alone or in combination with 10 or 30 μ M of Akt inhibitor, Triciribine (TCN, Sigma-Aldrich, St. Louis, MO, USA). After 72 hr whole-cell lysates were prepared

using a lysis buffer containing 0.2% w/v sodium deoxycholate, 2 mM EDTA, 2 mM EGTA, 50 mM Tris-HCl (pH 7.4), 10 mM NaF, 1 mM sodium orthovanadate, 10 mM β glycerophosphate, 1 mM phenylmethylsulfonyl fluoride (PMSF) and complete protease inhibitor cocktail as instructed by the manufacturer (Roche, Welwyn, UK) on ice for 15 min. Insoluble materials in samples were removed using centrifugation at 10,000 \times g for 15 min at 4 °C. Protein concentration was determined using standard Bio-Rad Bradford protein assay with bovine albumin as standard (Bio-Rad, Hemel Hempstead, UK). Protein samples were size fractionated using sodium dodecyl sulfate-polyacrylamide gel electro-phoresis (SDS-PAGE) and then electro transferred to a poly (vinylidene difluoride) (PVDF) membranes (Bio-Rad, Hemel Hempstead, UK). Membranes incubated with specific antibodies recognizing Akt phosphorylated at the Ser473, Akt and β -actin all purchased from Cell signaling technology (Beverly, MA, USA). Horseradish peroxidase-linked anti-mouse or anti-rabbit conjugates antibodies (Cell Signaling Technology Beverly, MA, USA) were used to detect primary antibodies and the blots were developed using enhanced chemiluminescences (ECL) reagent (Pierce, Rockford, IL, USA) and Alliance 4.7 Geldoc (UK). UVtec software (UK) was used to analyze the protein bands. The protein levels were normalized against their corresponding β -actin band intensities.

Statistical analysis

Results were expressed as mean \pm SD. Tukey-Kramer *post hoc* test following one-way ANOVA was employed to compare the differences between means. Differences were considered statistically significant when $P < 0.05$.

Results

Effect of TNF- α treatment on Akt phosphorylation at Ser473

Phosphorylation of Akt at Ser473 leads to Akt activation (27). TNF- α treatment significantly enhanced Akt phosphorylation in both MCF-7 and MCF-7/Adr cells after 72 hr. Moreover, in these cells TCN treatment alone or in combination with TNF- α decreased Akt phosphorylation level (Figure 1 and 2).

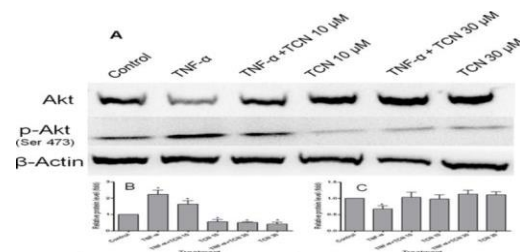


Figure 1. Western blot analysis of MCF-7 cells. A, MCF-7 cells were treated with TNF- α (50 ng/ml) and/or TCN (10, 30 μ M), then phospho- and total Akt in whole cell lysates were immunoblotted. β -Actin was used as the loading control. B, Bar graph is derived from densitometric scanning of the phospho-Akt blots. C, Bar graph is derived from densitometric scanning of the Akt blots. Results are mean \pm SD from two independent experiments. * Significantly different from control, $P < 0.05$

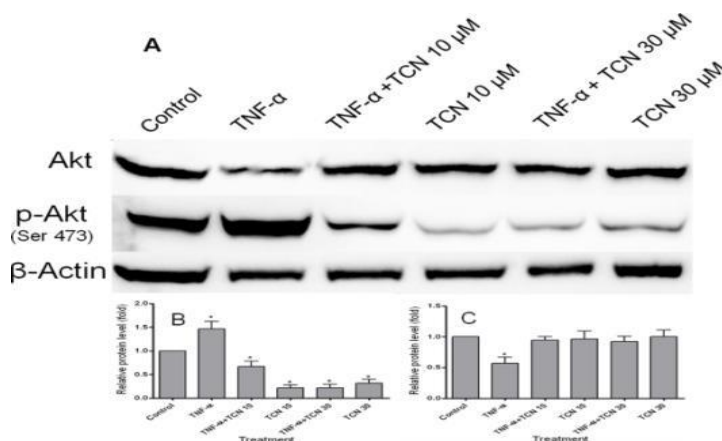


Figure 2. Western blot analysis of MCF-7/Adr cells. A, MCF-7/Adr cells were treated with TNF- α (50 ng/ml) and/or TCN (10, 30 μ M), then phospho and total Akt in whole cell lysates were immunoblotted. B-Actin was used as the loading control. B Bar graph is derived from densitometric scanning of the phospho-Akt blots. C Bar graph is derived from densitometric scanning of the Akt blots. Results are mean+SD from two independent experiments. *Significantly different from control, $P < 0.05$

Effect of TNF- α treatment on cell viability

To investigate the role of Akt Ser473 phosphorylation in resistance of MCF-7 and MCF-7/Adr cells against TNF- α toxicity, TNF- α treatment was used along with inhibition of Akt phosphorylation using TCN in MCF-7 and MCF-7/Adr cells. After 24 and 72 hr treatment, TNF- α (50 ng/ml) alone did not exert significant cytotoxic effects in both cell lines.

In the MCF-7 cells, after 24 hr TCN treatment alone or in combination with TNF- α did not lead to significant ($P < 0.05$) cytotoxic effect whereas after 72 hr, while TCN treatment did not cause significant cytotoxicity co-treatment with TCN and TNF- α led to significant cytotoxic effects in MCF-7 cells in comparison with control and only TCN treated samples ($P < 0.05$) (Figure 3). In MCF-7/Adr cells, TCN at none of the tested concentrations alone or in combination with TNF- α caused significant changes in the cells viability ($P < 0.05$) (Figure 4).

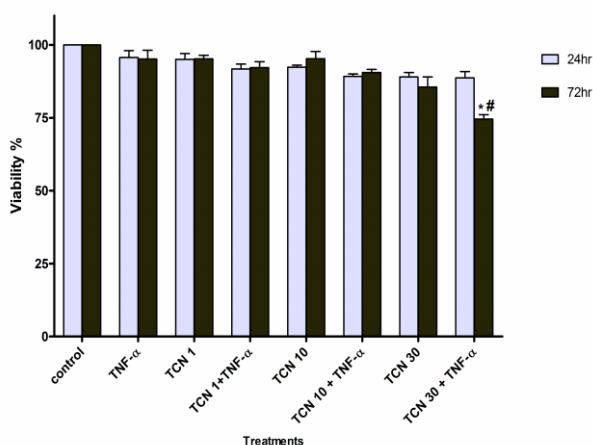


Figure 3. The cytotoxic effects of TNF- α and TCN on MCF-7 cells. The viability of MCF-7 cells were measured by MTT assay after 24 hr and 72 hr treatment with 50 ng/ml TNF- α and/or 1, 10, 30 μ M of TCN. Results are mean+SD from three independent experiments. *Significantly different from control, $P < 0.05$. #Significantly different from TCN 30 treated samples

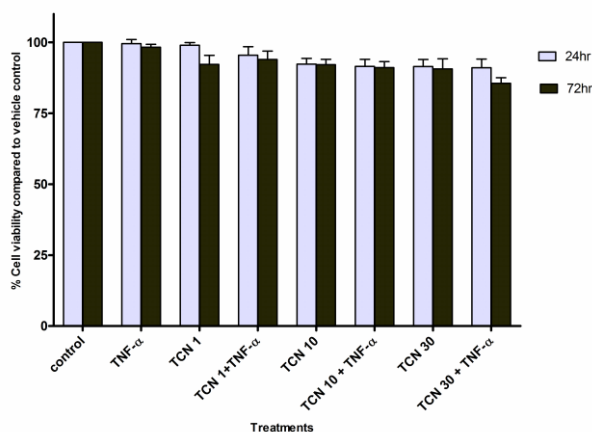


Figure 4. The cytotoxic effects of TNF- α and TCN on MCF-7/Adr cells. The viability of MCF-7/Adr cells were measured by MTT assay after 24 hr and 72 hr treatment with 50 ng/ml TNF- α and/or 1, 10, 30 of TCN. Results are mean+SD from three independent experiments

Discussion

Here, we investigated the role of Akt kinase phosphorylation/activation in resistance of MCF-7 and MCF-7/Adr cells against TNF- α cytotoxicity. TNF- α treatment increased Akt Ser473 phosphorylation in both cell lines indicating Akt activation after TNF- α treatment. Co-treatment of the cells with TCN (a chemical inhibitor of Akt phosphorylation) and TNF- α led to attenuation of Akt Ser473 phosphorylation in MCF-7 and MCF-7/Adr cells. Inhibition of Akt phosphorylation sensitized MCF-7 cells to the cytotoxic effects of TNF- α while in MCF-7/Adr cells TCN did not sensitized cells to the cytotoxic effects of TNF- α .

TNF- α is a pleiotropic cytokine representing dual function. Binding of TNF- α to its specific receptors TNFR1 and TNFR2 is followed by activation of different downstream pathways and signaling mediators including NF- κ B, caspases, ROS, JNK, and PI3K/Akt. Activation of these pathways leads cells to inflammation and survival as well as cell death (2, 3). Acquisition of TNF- α resistance

plays role in the onset and growth of malignant tumors (23). Antiapoptotic proteins contract the ability of apoptotic stimuli including TNF- α to induce cell death. In our study, it was shown that Akt phosphorylation was involved in resistance of MCF-7 cells to TNF- α toxicity. Serine/threonine-specific protein kinase, Akt, is mostly activated in PI3k/Akt pathway which plays a key role in many cellular events including transcription, translation, cell proliferation and survival in normal as well as neoplastic tissues (18). Growth and progression of a variety of human tumors, including glioblastomas (GBM), breast, colon and endometrial cancers are promoted by deregulation of the PI3K and its downstream effector, Akt. Chemotherapy resistance has also been shown to be affected by Akt aberrant activation (28-32).

Akt was activated after TNF- α exposure in the HaCaT (pre-malignant keratinocyte), 1321N1 (glioblastoma) and PC-3 (human prostate cancer) cells (33-35). According to these investigations we hypothesized a role for Akt Ser473 phosphorylation, which is directly related to its activation, in resistance to TNF- α cytotoxicity in MCF-7 and MCF-7/Adr cell lines. As expected, Akt Ser473 phosphorylation in MCF-7 cells was increased following TNF- α treatment. To address the role of Akt Ser473 phosphorylation after TNF- α treatment on the resistance of MCF-7 cells against TNF- α cytotoxicity, Akt phosphorylation was inhibited using a chemical specific Akt inhibitor, TCN. The cytotoxic effect of TNF- α was significantly increased by inhibition of Akt Ser473 phosphorylation along with TNF- α treatment in MCF-7 cells. Since co-treatment of MCF-7 cells (TCN along with TNF- α) demonstrated significant higher cytotoxicity than treatment with TCN alone, it can be concluded that Akt phosphorylation plays a key role in MCF-7 resistance against TNF- α cytotoxicity. TNF- α treatment enhanced Akt Ser473 phosphorylation in MCF-7/Adr cells too. Further investigations using TCN suggested that in MDR cell line the role of Akt phosphorylation in resistance against TNF- α is doubtful. Treatment of MCF-7/Adr cells by TCN (30 μ M) alone or in combination with TNF- α inhibited Akt Ser 473 phosphorylation however, TCN (30 μ M) alone and co-treatment with TCN (30 μ M) and TNF- α did not exert any significant decrease in viability of MCF-7/Adr cells after 24 hr and 72 hr treatment.

Conclusion

Overall, it seems that resistance of MCF-7/Adr cells against TNF- α is orchestrated by multiple mechanisms in which Akt phosphorylation does not play an important role. More investigations on other prosurvival mediators of TNF- α signaling are needed to specify mechanisms underlying TNF- α resistance in MCF-7/Adr cells. To do so, chemical inhibitors and siRNA technology can be employed to inhibit the TNF- α prosurvival mediators followed by investigation of TNF- α effects. MDR is a multiple mechanism event with contribution of several gene/proteins and signaling pathways. Investigating events underlying resistance of MCF-7/Adr cells to TNF- α may help clarify

the mechanisms contributing to MDR as well as development of new therapeutic strategies against it.

Conflict of Interest

The authors declare no financial or commercial conflict of interest.

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