Reactive Oxygen Species Mediate TNF-α Cytotoxic Effects in the Multidrug-Resistant Breast Cancer Cell Line MCF-7/MX

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Introduction

Reactive oxygen species (ROS) are generated primarily in the mitochondria as by-products of the cellular metabolism. Growth factors and cytokines also induce ROS generation by specialized plasma membrane oxidases in normal physiological signaling. Since free radical species are highly unstable and reactive, they acquire electrons from lipids, nucleic acids, proteins, carbohydrates, or any nearby molecule; thus, when the cellular generation of ROS overwhelms their antioxidant capacity, a cascade of damage and disease may ensue [1, 2]. ROS are not only harmful by-products of the cellular metabolism, but they are also essential components in cell signaling, contributing to various physiologic features including cytokine and growth factor signaling [3]. ROS play their role in cell signaling mainly through the modification of specific cysteine residues found within signaling-related proteins. The activity of the target proteins is modulated by oxidation of these specific and reactive cysteine residues [3].

ROS can be generated from different sources. Reduced nicotinamide adenine dinucleotide phosphate (NADPH)-dependent plasma membrane oxidases and mitochondria are mostly responsible for the generation of ROS within cells. Some of the intracellular enzymes that produce oxidants as part of their normal enzymatic function, including cyclooxygenases, cytochrome P450 enzymes, xanthine oxidase, and lipoygenases, are other sources of intracellular ROS. The maintenance of intracellular redox homeostasis depends on the activity of several antioxidant enzymes, including superoxide dismutase (SOD); reduces O$_2^-$ to H$_2$O$_2$, catalase, and glutathione peroxidase (GP; reduces H$_2$O$_2$ to H$_2$O). These enzymes protect cells against the potential damaging effects of ROS [3]. It should be noted that the regulation of the antioxidant levels in cells is related to the levels of intracellular ROS. Transcription factors including Nrf2 and FoxO regulate the expression of antioxidant and detoxifying genes. The activity of these transcription factors depends on the intracellular redox state [4, 5].

 Materials and Methods

ROS levels were evaluated following TNF-α exposure using 2',7'-dichlorodihydrofluorescein diacetate (H$_2$DCF-DA) as fluorescent probe, and the TNF-α cytotoxic effects were examined using the dimethylthiazolyl-2,5-diphenyl tetrazolium bromide (MTT) assay. Results: TNF-α led to ROS accumulation only in MCF-7/MX and not in MCF-7 cells. The role of ROS in the cytotoxic effects of TNF-α was further evaluated by inhibition of ROS accumulation in MCF-7/MX cells and by induction of ROS generation in MCF-7 cells along with TNF-α treatment. ROS accumulation sensitized the MCF-7 cells to the cytotoxic effects of TNF-α while inhibition of ROS accumulation attenuated the cytotoxic effects of TNF-α in MCF-7/MX cells. Following TNF-α treatment, the activities of antioxidant enzymes (superoxide dismutase, glutathione peroxidase, glutathione reductase, and catalase) were evaluated in both cell lines. The results of the enzyme assays revealed that superoxide dismutase activity was enhanced in MCF-7 but not in MCF-7/MX cells. Conclusions: ROS accumulation in MCF-7/MX cells may be involved in the higher cytotoxic effects of TNF-α in the MCF-7/MX cell line.
The pro-inflammatory cytokine tumor necrosis factor-α (TNF-α) plays a role in different physiological and pathological processes, including acute and chronic inflammation, autoimmune diseases, and cancer-related inflammation [6]. It can activate different downstream mediators, including caspases, nuclear factor kappaB (NF-κB), c-Jun N-terminal kinase (JNK), ROS, and phosphatidylinositol 3-kinase (PI3K)/Akt by binding to its specific receptors, TNF receptor 1 (TNFR1) and TNFR2. TNF-α exerts its different effects, ranging from inflammation and cell survival to cell death, on different cells and tissues as well as on the same cell or tissue in different states [7, 8]. Generation of ROS after TNF-α exposure has been reported in different cell lines, suggesting a role for ROS as a signaling mediator for TNF-α [9–12]. Following TNF-α exposure, the mitochondria have been reported to be the main source of ROS generation, while NADPH oxidase-dependent ROS generation has also been reported [13]. In TNF-α signaling, ROS can contribute to transcription factor activation (e.g. NF-κB) [14] and cell proliferation [15] as well as cell death [16]. Induction of sustained JNK activation plays a role in ROS-mediated TNF-α toxicity [17, 18]. NF-κB signaling has been reported to be activated or repressed by ROS. Dissimilar effects of ROS on NF-κB described in the literature may be due to the use of different methodologies and/or cell-specific behaviors. NF-κB regulates transcription of the genes coding for manganese-containing SOD (Mn-SOD), glutathione S-transferase, GP-1, and catalase, the enzymes influencing the ROS levels [14].

Multidrug resistance (MDR) is a major obstacle to successful therapy of breast cancer, the most common cancer in women worldwide [19]. In MDR, tumors do not respond to the cytotoxic effects of various chemically and pharmacologically unrelated drugs. Tumors usually contain both drug-resistant and drug-sensitive cells. After the first round of chemotherapeutical therapy, tumors can be repopulated by drug-resistant cells and may resist further treatments. Numerous mechanisms, including increased drug efflux, decreased drug uptake, activation of deoxyribonucleic acid (DNA) repair mechanisms, activation of detoxifying systems, and evasion of drug-induced apoptosis, may be involved in the MDR [20]. Interestingly, TNF-α treatment exerted higher cytotoxic effects in some drug-resistant cell lines than in their parental drug-sensitive cell lines [21–24]. In our previous study, we showed that MCF-7/MX, the mitoxantrone (MX)-resistant derivative of the human breast carcinoma cell line MCF-7, was more sensitive to the cytotoxic effects of TNF-α than its parental cells [25]. Based on the above-mentioned investigation, we suggested a role for ROS in this phenomenon. Here, we investigated the role of ROS in the cytotoxicity of TNF-α in MCF-7 and MCF-7/MX cells in more detail.

### Materials and Methods

**Cell Culture**

MCF-7 and MCF-7/MX cells (both gifts from Dr. Erasmus Schneider, Wadsworth Center, New York State Department of Health, University at Albany, State University of New York, Albany, NY, USA) were cultured in RPMI 1640 medium containing 10% fetal bovine serum and penicillin (100 U/ml)/streptomycin (100 μg/ml) (all from Gibco, Grand Island, NY, USA). Cells were incubated at 37 °C in the presence of 5% CO₂, 250 mM MX (Sigma-Aldrich, Taufkirchen, Germany) was added to the culture medium of the MCF-7/MX cells to maintain the MDR phenotype, but was removed 1 week before the experiments.

**Determination of Intracellular ROS Levels**

2’,7’-Dichlorodihydrofluorescein diacetate (H₂DCF-DA) (Sigma-Aldrich, St. Louis, MO, USA) was used to assess the intracellular levels of ROS. Following penetration into the cells, the non-fluorescent ester is hydrolyzed to 2’,7’-dichlorodihydrofluorescein (H₂DCF) by the cellular esterases. Then, H₂DCF is oxidized to the highly fluorescent compound 2’,7’-dichlorofluorescein (DCF) by intracellular ROS. After treatment with TNF-α for 5, 24, and 72 h, the cells were further incubated in a medium containing 50 μM H₂DCF-DA for 30 min in the dark and at 37 °C. Stained cells were washed in fresh medium and fluorescence was measured with a microplate spectrophorometer (Synergy 4; BioTek, VT, USA) where the excitation and emission wavelengths were adjusted to 485 and 530 nm, respectively. ROS generation was reported as the percentage of the fluorescence of control wells treated with vehicle.

**Cell Viability Assays**

After seeding at a density of 6,000 cells/well and overnight incubation, MCF-7 and MCF-MX cells were treated with different concentrations of TNF-α (Millipore, Billerica, MA, USA) alone or in combination with 100 μM Trolox as ROS-scavenging agent (Sigma-Aldrich, St. Louis, MO, USA) or a ROS-generating system. After 5, 24, and 72 h, the cells were incubated with 0.5 mg/ml dimethylmethoxyazobenzene-2,5-diphenyl tetrazolium bromide (MTT) for 3 h; then, 100 μl dimethyl sulfoxide was added to each well to lyse the cells. Absorption of each well at 570 nm was determined using a microplate reader (Synergy 4; BioTek, VT, USA). Cell viability was calculated as a percentage of controls treated with vehicle.

To investigate the role of ROS in TNF-α toxicity, ROS generation was inhibited or activated along with the TNF-α treatment. A combination of H₂O₂ (20 μM), paraquat (20 μM), CuSO₄ (1 μM), 1,10-phenanthroline (1 μM), and ascorbic acid (400 μM) (all from Sigma-Aldrich, St. Louis, MO, USA) was employed to generate ROS in the cells [26]. Dose-response experiments were performed to find those concentrations causing a cell survival rate of at least 80% of the untreated cultures after 72 h. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), a water-soluble analog of vitamin E, was used to suppress ROS production in the desired cell line.

### Analysis of the Activities of Antioxidant Enzymes

Since the activity of antioxidant enzymes plays an important role in the maintenance of intracellular redox homeostasis, the activities of enzymes including SOD, GP, glutathione reductase (GR), and catalase were measured using activity assay kits from Abcam (Cambridge, MA, USA) according to the manufacturer’s instructions. Briefly, lysates of treated cells were prepared using the lysis buffers provided. The samples then underwent centrifugation at 14,000 × g at 4 °C for 15 min. A standard Bio-Rad Bradford protein assay with bovine serum albumin (internal standard) (Bio-Rad, Hemel Hempstead, UK) was employed to determine protein concentrations. Enzyme activities were assessed using 10 μg of the total protein extracts. Absorbance values were measured using a microplate spectrophotometer (Synergy 4; BioTek, VT, USA).

### Results

**Determination of Intracellular ROS Levels**

MCF-7 and MCF-7/MX cells were treated with 1, 10, 25, and 50 ng/ml TNF-α for 5, 24, and 72 h. Then intracellular ROS levels were calculated by measuring the oxidation of the non-fluorescent H₂DCF to its highly fluorescent derivative DCF. As shown in fig-
Fig. 1. Analyzing ROS accumulation after TNF-α treatment. ROS production was analyzed by measuring the fluorescence intensity of DCF in a fluorescent plate reader. (A) MCF-7 and (B) MCF-7/MX cells were treated with TNF-α at various concentrations for 5, 24, and 72 h; then, ROS generation was analyzed. tert-Butyl hydroperoxide (tBHP) was used as positive control. *Significantly different from control, p < 0.05.

Fig. 2. Role of ROS in the TNF-α cytotoxicity. To investigate the role of ROS in TNF-α toxicity, cells were treated with TNF-α and a ROS-generating system (RGS) or a ROS-scavenging agent. (A) MCF-7/MX cells were treated with TNF-α and/or Trolox as ROS-scavenging agent. As demonstrated, Trolox diminished ROS generation after TNF-α treatment. (B) MCF-7 cells were treated with TNF-α and/or a standard ROS-generating system. The ROS-generating system induced ROS production after TNF-α treatment. *Significantly different from control, p < 0.05.

ure 1B, TNF-α stimulated ROS generation in MCF-7/MX cells in a concentration-dependent manner (p < 0.05) while in MCF-7 cells the ROS levels did not change significantly (fig. 1A).

Cell Viability Assays

We have already reported that TNF-α treatment exerted higher cytotoxic effects in MCF-7/MX than in MCF-7 cells [25]. As TNF-α increased the production of ROS in the MCF-7/MX cells, we further investigated the role of ROS production in TNF-α cytotoxicity by inhibiting ROS production along with the TNF-α treatment, using Trolox in MCF-7/MX cells. Trolox diminished the ROS production in response to TNF-α in MCF-7/MX cells (fig. 2A). Trolox also attenuated the TNF-α toxicity in MCF-7/MX cells, demonstrating the role of ROS production in the cytotoxic effects of TNF-α in MCF-7/MX cells (fig. 3A). Induction of ROS production using the above-mentioned ROS-generating system along with TNF-α treatment enhanced the cytotoxic effects of TNF-α (fig. 2B and fig. 3B).

Analysis of the Activities of Antioxidant Enzymes

The activities of antioxidant enzymes, including SOD, catalase, GR, and GP, were analyzed using commercially available kits. MCF-7 and MCF-7/MX cells were treated with 50 ng/ml TNF-α for 5, 24, and 72 h. Then, the enzyme activities were measured according to the manufacturer’s instructions. All tested enzymes showed higher activities in MCF-7 cells compared to MCF-7/MX cells (data not shown). TNF-α treatment led to an enhancement of the SOD and GP activities in MCF-7 cells while in MCF-7/MX cells the SOD and GP activities did not change following TNF-α treatment (fig. 4). A non-significant increase in catalase activity was observed in MCF-7 cells (p < 0.05) while no changes were observed in the catalase activity of MCF-7/MX cells after TNF-α treatment. TNF-α treatment did not lead to any significant changes in GR activity in both MCF-7 and MCF-7/MX cells (data not shown).
Discussion

Exposure to TNF-α may lead to cell survival or death, and ROS play an important role in TNF-α signaling [16]. Therefore, we examined the role of ROS in the cytotoxic effects of TNF-α in MCF-7 and MCF-7/MX cells. TNF-α exerted higher cytotoxic effects on MCF-7/MX cells than on MCF-7 cells. We found that TNF-α exposure increased the ROS levels only in MCF-7/MX cells in a dose-dependent manner. For a better understanding of the role of ROS in TNF-α toxicity, ROS accumulation was inhibited in MCF-7/MX cells by co-treatment of the cells with TNF-α and the ROS-scavenging agent Trolox. The TNF-α cytotoxicity in MCF-7/MX cells was attenuated by inhibiting the generation of ROS. Moreover, using a ROS-generating system along with TNF-α treatment, we could sensitize MCF-7 cells to the cytotoxic effects of TNF-α. These results demonstrate that ROS was responsible, at least in part, for the TNF-α cytotoxicity in MCF-7/MX cells, while MCF-7 cells resist TNF-α toxicity through the prevention of ROS generation following TNF-α exposure. Since the activity of antioxidant enzymes is involved in the maintenance of intracellular redox homeostasis, the activities of antioxidant enzymes, including SOD, GR, GP, and catalase, were also analyzed. TNF-α treatment increased the SOD and GP activities in MCF-7 cells while it did not lead to any significant changes in the activities of these enzymes in the MCF-7/MX cells. Moreover, all tested enzymes showed higher activities in MCF-7 compared to MCF-7/MX cells.

The balance between cell death (apoptotic and non-apoptotic) and cell survival signaling pathways determines the cellular response (e.g., proliferation vs. cell death) to TNF-α [8, 27]. ROS accumulation after TNF-α exposure has frequently been reported in several cell lines including murine L-M tumorigenic fibroblast cells [28], L929 murine fibrosarcoma cells [29], and human endothelial cells [11]. Moreover, membrane TNF-α could induce ROS and necrotic cell death in RAW 264.7 monocytic and L929 fibroblast cells [30]. ROS are important mediators of signaling pathways and can regulate cell death signaling as well as cell survival signaling pathways following TNF-α exposure. NF-κB activation is mainly responsible for the pro-survival effects of TNF-α and ROS, while ac-

Fig. 3. Role of ROS in the TNF-α cytotoxicity. (A) MCF-7/MX cells were treated with TNF-α and/or Trolox as ROS-scavenging agent and the viability of the cells was analyzed using the MTT assay. Trolox attenuated the cytotoxic effects of TNF-α. (B) MCF-7 cells were treated with TNF-α and/or a standard ROS-generating system. The ROS-generating system sensitized the MCF-7 cells to the cytotoxic effects of TNF-α. *Significantly different from control, p < 0.05. #Significantly different from TNF-α-treated cells, p < 0.05.

Fig. 4. Analysis of GP and SOD activities. The GP and SOD activities in MCF-7 cells increased following treatment with TNF-α. The (A) GP and (B) SOD activities were assessed in the MCF-7 and MCF-MX cell lines following TNF-α treatment for 5, 24, and 72 h. Data are presented as mean ± standard deviation (SD) of 3 independent experiments. *Significantly different from the corresponding control, p < 0.05.
tivation of pro-death proteins, including JNK, and inhibition of NF-kB might promote TNF-α-induced cell death [27]. Proteins such as IkappaB kinase (IKK) involved in NF-kB activation and thioredoxin-1 (Trx-1) involved in JNK activation (through ASK-1 binding) contain critical redox-sensitive cysteins that can be modulated by ROS [31, 32]. Overall, ROS in moderate amounts can induce cell proliferation while excessive rises of ROS trigger cell death [33]. Our preliminary results demonstrated the important role of ROS in the cytotoxicity of TNF-α on MCF-7/MX cells, while MCF-7 cells resist TNF-α cytotoxicity through the inhibition of ROS accumulation.

Intracellular ROS are converted to non-harmful products by cellular antioxidant enzymes. Therefore, the activity of antioxidant enzymes could be involved in the maintenance of intracellular redox homeostasis. GR plays an important role in the conversion of oxidized glutathione (GSSG) to reduced glutathione (GSH). GP biological activity detoxifies organic hydroperoxides. Highly reactive O2− is converted to the less toxic H2O2 by SOD, and catalase reduces H2O2 to H2O. Overexpression of Mn-SOD could block the TNF-α cytotoxicity in the MCF-7 and ME-180 human cervical carcinoma cell lines [34, 35]. TNF-α exposure increased the mRNA level and activity of Mn-SOD in some cell lines, including endometrial stromal cells (ESC) and MCF-7 cells [36–38]. Similarly, in this study, TNF-α exposure increased the SOD activity in MCF-7 cells while its activity remained unchanged after TNF-α treatment in MCF-7/MX cells. There could be a relation among the ROS, NF-kB and SOD levels in cells. The intracellular ROS level is affected by the SOD activity and the expression of different types of SOD enzymes such as Mn-SOD is up-regulated by increased NF-kB levels. Finally, the ROS level affects NF-kB activation. As observed in this study, accumulation of ROS facilitated TNF-α cytotoxicity. Therefore, the lack of SOD activation (which could facilitate ROS accumulation) might be one of the mechanisms of TNF-α cytotoxicity in MCF-7/MX cells. Moreover, ROS inhibits NF-kB activation, a transcription factor that is able to activate SOD expression. Consequently, it seems that NF-kB also plays a role in the effect of TNF-α in MCF-7 and MCF-7/MX cells.

Overall, in our previous study, MCF-7/MX cells were found to be more sensitive than MCF-7 cells to the cytotoxic effects of TNF-α. Here, we demonstrated that, following TNF-α treatment, ROS accumulation in MCF-7/MX cells is involved at least in part in the sensitivity of MCF-7/MX cells to the cytotoxic effects of TNF-α. Some other MDR cell lines have also demonstrated higher sensitivity to TNF-α cytotoxicity than their drug-sensitive parental cell lines [22, 23]. Therefore, investigating the role of events underlying the sensitivity of drug-resistant cancer cells to TNF-α may help to clarify the mechanisms for overcoming the MDR. Moreover, such studies could help to shed light on the mechanisms underlying the pleiotropic nature of TNF-α.

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Disclosure Statement

The authors declare that they have no conflicts of interest.

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