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Catalase Overexpression Impairs TNF-α Induced NF-κB Activation and Sensitizes MCF-7 Cells Against TNF-α

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Abstract The pleiotropic cytokine tumor necrosis factor alpha (TNF-α) can induce apoptosis but also supports cell survival pathways. Among the possible anti-apoptotic mechanisms of TNF-α is the activation of the transcription factor NF-κB. Since reactive oxygen species (ROS) are assumed to contribute to TNF-α mediated cytotoxicity but can also facilitate NF-κB activation this study investigates the relationship between TNF-α treatment, NF-κB activation and the expression of the anti-oxidative enzyme catalase. TNF-α treatment caused downregulation of catalase expression in MCF-7, Caco-2 and Hct-116 cancer cell lines. Overexpression of catalase in MCF-7 cells, resulting in lower intracellular ROS levels upon challenge with H_2O_2, caused a transient nuclear p65 translocation upon TNF-α treatment as compared to the sustained NF-κB activation in wild type cells. This was due to a lack of sufficient H_2O_2 to co-stimulate NF-κB activation as demonstrated by the observation that addition of exogenous H_2O_2 led to a second increase of NF-κB activity. The rapid decline of nuclear translocation of NF-κB in the catalase overexpressing cells resulted in a slower increase of NF-κB mediated reporter gene expression. These results indicate that TNF-α mediated downregulation of catalase expression and accordingly sufficient H_2O_2 is required for appropriate function of the NF-κB dependent survival pathway.
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Key words: apoptosis; catalase; MnSOD; NF-κB; TNF-α

An anti-oxidant enzyme system consisting of $O_2^{\cdot -}$ degrading superoxide dismutase(s) and H_2O_2 degrading catalase(s)/peroxidase(s) is present in bacteria, plants and animals. These enzymes have long been understood mainly as a barrier against the attack by supraphysiological amounts of reactive oxygen species (ROS) on cellular integrity while in recent years their role is increasingly interpreted in terms of fine tuning of the physiological concentration of ROS required in the redox regulation of the cell cycle and of programmed cell death [Adler et al., 1999; Bauer, 2002; Herrlich and Bohmer, 2000; Kahl et al., 2004].

Mammalian catalase belongs to the enzymes studied most extensively with respect to their catalytic properties. This enzyme does not mediate direct actions other than peroxide degradation. Since H_2O_2 is degraded in the presence of transition metals to yield the highly reactive hydroxyl radical it is generally assumed that catalase fulfils a cytoprotective role against oxidative stress. Numerous papers show that the administration of exogenous catalase, the up-regulation of catalase expression or catalase gene transfer can protect cells against cytotoxicity and apoptosis induced by a wide variety of stimuli, and this protection has most often been assigned to the removal of H_2O_2 as reviewed in Kahl et al. [2004]. However, there are also some findings which indicate that cell protection by catalase cannot be achieved against all types of stimuli [Brown et al., 1999; Bai and Cederbaum, 2001, 2003; Bernard et al., 2001; Zanetti et al., 2002].

Tumor necrosis factor-α (TNF-α) is a pleiotropic cytokine with different and even antagonistic

Abbreviations used: ANOVA, analysis of variance; BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; CAT, catalase; CMV, cytomegalovirus; GAPDH, glycelraldehyde-3-phosphate dehydrogenase; LSD, least significant difference; MnSOD, manganous superoxide dismutase; ROS, reactive oxygen species; SEAP, secreted alkaline phosphatase; TBS-T, tris buffered saline with Tween 20; TNF-α, tumor necrosis factor alpha.

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effects on cell function eliciting the formation of proinflammatory molecules, exerting mitogenic actions and supporting both proapoptotic and anti-apoptotic signaling pathways [Luster et al., 1999]. TNF-α initiates apoptosis via the death receptor pathway. On the other hand, the intrinsic pathway leading to the release of mitochondrial cytochrome *c* and subsequent caspase-9 activation can additionally be activated by TNF-α via mitochondrial ROS production [Bradham et al., 1998] and is also involved in the cytotoxicity of TNF-α [Schulze-Osthoff et al., 1992; Goossens et al., 1999; Schmelz et al., 2004].

Among the anti-apoptotic mechanisms of TNF-α is the induction of protective proteins, which counteract the proapoptotic action of TNF-α. Induction of the mitochondrial antioxidant enzyme manganous superoxide dismutase (MnSOD) by TNF-α has been reported in a wide variety of cell types and has been assumed to function as a protective mechanism against TNF-α induced apoptosis [Wong and Goeddel, 1988; Wong et al., 1989]. NF-κB activation is required for the induction of MnSOD and other anti-apoptotic proteins playing a central role in survival pathways of the cell [Wong and Goeddel, 1988; Wong et al., 1989]. NF-κB can also be activated by H_2O_2 albeit by a different mechanism as compared to TNF-α [Baeuerle and Sies, 2000]. H_2O_2 has been described to act as a co-stimulator of NF-κB together with stimuli such as TNF-α [Janssen-Heininger et al., 1999; Baeuerle and Sies, 2000] although the inverse effect, inhibition of NF-κB activation by H_2O_2, has also been observed [Korn et al., 2001]. It is therefore obvious that a link between catalase activity and NF-κB activation should exist; because of the H_2O_2 degrading capacity of catalase. However, data on this issue is rare. It was the aim of the present study to investigate the relationship between TNF-α treatment, NF-κB activation and catalase expression.

MATERIALS AND METHODS

Cell Lines and Culture Conditions

The human breast adenocarcinoma cells (MCF-7) were grown in RPMI 1640 medium containing 2 mM L-glutamine, 1% non-essential amino acids, 1 mM sodium pyruvate, 10 μg/ml insulin and 10% heat-inactivated fetal calf serum at 37°C in a humidified atmosphere of 5% CO_2. Hct-116, Caco-2 (human colon carcinoma cell lines), Huh-7 (human hepatoma cell line) and H4IIE cells (rat hepatoma cell line) were grown in Dulbecco's modified Eagle's medium (4.5 g/l glucose, 2 mmol/l L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin, 10% fetal calf serum) at 37°C in a humidified atmosphere of 5% CO_2.

Plasmid Construction

Total RNA was extracted from MCF-7 cells using Trizol® reagent (Sigma). Full-length human catalase cDNA were amplified by RT-PCR using following primer pairs: hCat-forward 5′-ATGGCTGACAGCCGGGAT-3′ and hCat-reverse 5′-TCA CAG ATT TGC CTT CTC CCT-3′. PCR products were then isolated from agarose gels with the QIAquick gel extraction kit (Qiagen) and subcloned in the pGem®-T Easy cloning vector (Promega) before cloning into the *Not*I site of pcDNA™-Zeo(−) plasmid (Invitrogene) downstream of a CMV promoter to generate the mammalian expression construct pZeo-hCat. Recombinant plasmids with the required correct insert orientation were identified by restriction enzyme digestion and were further confirmed by DNA sequencing.

Catalase Activity

Catalase activity was determined by measuring the initial rate of decay of H_2O_2 absorbance at 240 nm, using an extinction coefficient of 43.6 $M^{-1} cm^{-1}$ as described previously [Chovolou et al., 2003]. Each assay mixture consisted of 80 μl cell extracts (300 μg protein/ml) and 920 μl H_2O_2 at an initial concentration of 19 mM H_2O_2 in 50 mM phosphate buffer (pH 7.0). The specific catalase activity was expressed as U/mg protein.

RNA Isolation RT-PCR

Total RNA was isolated from MCF-7 cells treated with TNF-α for the indicated time points using Trizol® reagent (Sigma). Total RNA (1 μg) was reverse transcribed using 2.0 μM oligo-d(T)16 primers, 0.2 mM of each dNTP, 25 units RNase inhibitor (Fermentas), and 200 units M-MLV reverse transcriptase (Promega, Mannheim, Germany) in a final volume of 25 μl. Subsequent PCR was performed using 0.2 mM of each dNTP, 1 unit Taq DNA polymerase (Promega) and 0.4 μM of each PCR primer. PCR was then carried out for 1 min 94°C, 1 min 60°C and 1 min 72°C. The following primer pairs

were used for amplification: human catalase (sense, 5′-GGT GAG ATC GAA TGG AT-3′; anti-sense, 5′-GGC GAT GGC ATT GAA A-3′), human MnSOD (sense, 5′-CCT GAA CGT CAC CCG AGG AGA A-3′; anti-sense, 5′-CTG CAG TAC TCT ATA CCA CTA CA-3′), human GAPDH (sense, 5′-ACC ACA GTC CAT GCC ATC AC-3′; anti-sense, 5′-TCC ACC ACC CTG TTG CTG TA-3′). Amplified products were analyzed by agarose gel electrophoresis at cycles within the linear range of cDNA amplification using for detection and densitometric analysis the Quantity One system from Bio-Rad (München, Germany). Real-time quantitative PCR analysis were performed using SYBR Green JumpStart Taq Ready Mix (Sigma) and Qiagen QuantiTect Primer assays for catalase and GAPDH as recommended by the manufacturers instructions. Statistical analysis of relative expression results was carried out using Relative Expression Software Tool (REST©) [Pfaffl, 2001; Pfaffl et al., 2002, 2004].

Neutral Red Assay

The cytotoxicity was assayed by the neutral red assay according to Borenfreund and Babich [1987]. Briefly, MCF-7 cells (4×10^4 cells/well of 96-well plate) were incubated with TNF-α in a final volume of 0.1 ml for various times at 37°C. For the neutral red assay, culture medium was replaced by RPMI 1640 medium containing neutral red (50 μg/ml) and 10 mM Hepes pH 7.4. Cells were incubated for 3 h at 37°C to allow the viable cells to take up and retain neutral red by lysosomes. Thereafter, cells were fixed with solution F (1% formaldehyde, 1% $CaCl_2$) and washed once with phosphate-buffered saline pH 7.4. The incorporated dye was extracted from the cells by solution E (50% ethanol and 1% acetic acid in dH_2O) and quantified using a Wallac Victor^2 multilabel counter at 540 nm. The cells incubated with control medium were considered 100% viable.

Transient Transfection

The jetPEI™ transfection reagent (Polyplus Transfection) was used for transient transfection according to the batch protocol recommended by the manufacturer. In brief, complex forming was carried out using a ratio of 2 μl of jetPEI™ reagent per μg plasmid DNA. After 30 min incubation at RT up to 5×10^6 cells were mixed with the DNA-jetPEI™ complexes and then plated in suitable dishes for 24–48 h. Thereafter cells were incubated with TNF-α for indicated time points. Efficiency of gene transfer was assessed by applying an aliquot of transfected cells to Western blot analysis with a polyclonal anti-catalase antibody.

Reporter Gene Assay

A pNFκB-SEAP vector, which contains five NF-κB sites (5′-AGGGGACTTTCCGAGAGG-3′, NF-κB consensus is underlined) upstream of a minimal SV40 promoter and the coding sequence for the secreted alkaline phosphatase (SEAP) was used for reporter gene assay. Cells were transfected using jetPEI™ transfection reagent (Polyplus Transfection). Forty-eight hours after transfection cells were treated with TNF-α and SEAP activities were quantified using the Great EscAPE SEAP detection kit according to manufacturers recommendations (Clontech Laboratories, Heidelberg, Germany).

Protein Isolation

To determine the levels of p65, nuclear extracts were prepared from MCF-7 cells. Cells were washed with PBS pH 7.4 and solubilized with buffer A (10 mM Hepes pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM DTT, 1 mM PMSF, 10 mM NaF, 0.6% NP40) and then centrifuged at 10,000*g* for 1 min and 4°C. The supernatants were used as cytosolic protein fraction. The pellets were resuspended in buffer B (20 mM Hepes pH 7.9, 400 mM KCl, 1 mM EDTA, 1 mM EGTA, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM DTT, 1 mM PMSF, 10 mM NaF, 0.6% NP40), incubated for 25 min at 4°C and then centrifuged at 15,000*g* for 5 min and 4°C. The supernatants were used as nuclear protein fraction. To determine catalase expression whole cell lysates were prepared using RIPA buffer (50 mM Tris pH 8.0, 1% NP40, 0.5% sodium deoxycholate, 150 mM NaCl, and 5 mM EDTA, 0.1% SDS) supplemented with protease inhibitors 10 μg/ml leupeptin, 1 mM DTT, 1 mM PMSF. The protein concentration of each sample was measured using DC protein assay (Bio-Rad).

Antibodies and Western Blot Analysis

SDS–PAGE was performed with equal amounts of protein (7.5 μg) separated on mini gels (Peqlab) and electrotransferred onto PVDF membranes (Amersham). Antibodies were used

at the following dilutions: 1:2,000 for anti-catalase (Calbiochem), 1:1,250 for anti-MnSOD (Upstate) and 1:500 for anti-p65 (sc-109, Santa Cruz). Antibody incubations were done at 4°C overnight (p65, MnSOD antibodies) or at RT for 1h (catalase antibody) in TBS-T (+5% milk powder) buffer. HRP conjugated goat anti-rabbit (Southern Biotechnology) was used at a dilution of 1:5,000–1:7,500. Bands were detected by enhanced chemiluminescence using the BM chemiluminescence Western blotting kit (Roche Diagnostics).

Immunofluorescence Microscopy

Cells were grown on glass cover slips. After 24 h cells were rinsed twice with phosphate buffer saline and fixed for 15 min in 4% paraformaldehyde. Cells were washed twice with phosphate buffer saline and permeabilized for 20 min with 0.2% Triton-X100 at room temperature, washed three times, and incubated for 30 min with 10% fetal goat serum. Cells were incubated for 60 min with a 1:50 dilution of a rabbit antihuman catalase antibody (Calbiochem), followed by three washing steps for 10 min with phosphate buffer saline containing 2% fetal goat serum. Cells were then incubated with a 1:200 dilution of a rhodamine conjugated goat anti-rabbit antibody (Calbiochem). The cover slips were washed as above, and stained with 12.5 nM Hoechst 33342. Cells were mounted on slides, and examined using a Zeiss Axiolab fluorescence microscope. For negative control, primary antibody was omitted, and no fluorescence could be observed.

Intracellular H_2O_2 Measurement

The effect of catalase overexpression on H_2O_2 production in MCF-7 cells was determined by a fluorometric assay using 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA) from Molecular Probes. H_2DCF-DA readily diffuses through the cell membrane and is enzymatically hydrolyzed by intracellular esterases to the non-fluorescent H_2DCF, which can then be rapidly oxidized to highly fluorescent DCF in the presence of ROS. Cells were incubated with 50 μM H_2DCF-DA in DMEM for 30 min at 37°C, washed once with PBS pH 7.4 and treated with 1 mM H_2O_2 in DMEM. Cellular fluorescence was measured in a Wallac Victor^2 multilabel counter at 485 nm for excitation and 525 nm for emission.

Caspase Assay

Determination of caspase activity was carried out in 96-well plates using 75 μg protein. Colorimetric substrates for caspase-3/7 (Ac-DEVD-pNA), caspase-9 (Ac-LEHD-pNA) were used following the protocol of the manufacturer (Calbiochem). After treatment with TNF-α, attached and floating cells were collected, washed and lysed with ice-cold lysis buffer (50 mM Hepes, 100 mM NaCl, 100 μM EDTA pH 7.4, 0.1% CHAPS and 1mM DTT). Activity of caspase-3/7 and caspase-9 was detected by measuring proteolytic cleavage of 200 μM caspase substrates in assay buffer (55 mM Hepes, 110 mM NaCl, 110 μM EDTA, 0.1% CHAPS, 10 mM DTT, 10% glycerol, pH 7.4) using the absorbance of released *p*-nitroaniline at 405 nm.

Statistics

All data are expressed as mean ± SEM of at least three independent experiments and were analyzed using one-way ANOVA, followed by LSD post hoc analysis to determine the statistical significance. P values <0.05 were considered statistically significant.

RESULTS

Characterization of MCF-7 Cells Overexpressing Catalase

MCF-7 cells were transiently transfected with human catalase cDNA and the capacity of our construct to enhance catalase expression and catalase activity was examined. Catalase mRNA expression was 6.7-fold increased in MCF-7 cells transfected with catalase cDNA (MCF-7-cat) compared to control cells (Fig. 1A). Catalase expression at protein level was 5-fold higher (Fig. 1B), and catalase activity was 10.5-fold higher in MCF-7cat cells than in MCF-7 wild type cells (Fig. 1C). To determine the subcellular distribution of exogenous catalase cell fractionation experiments and immunohistochemical analysis were performed in MCF-7 cells. We found in catalase overexpressing cells catalase predominantly localized in the cytosolic fraction but also a small portion in the mitochondrial fraction (data not shown). A punctuate staining pattern was observed in wild type cells, matching to a mainly cytosolic distribution of catalase. This staining was increased in catalase overexpressing cells (Fig. 1D).

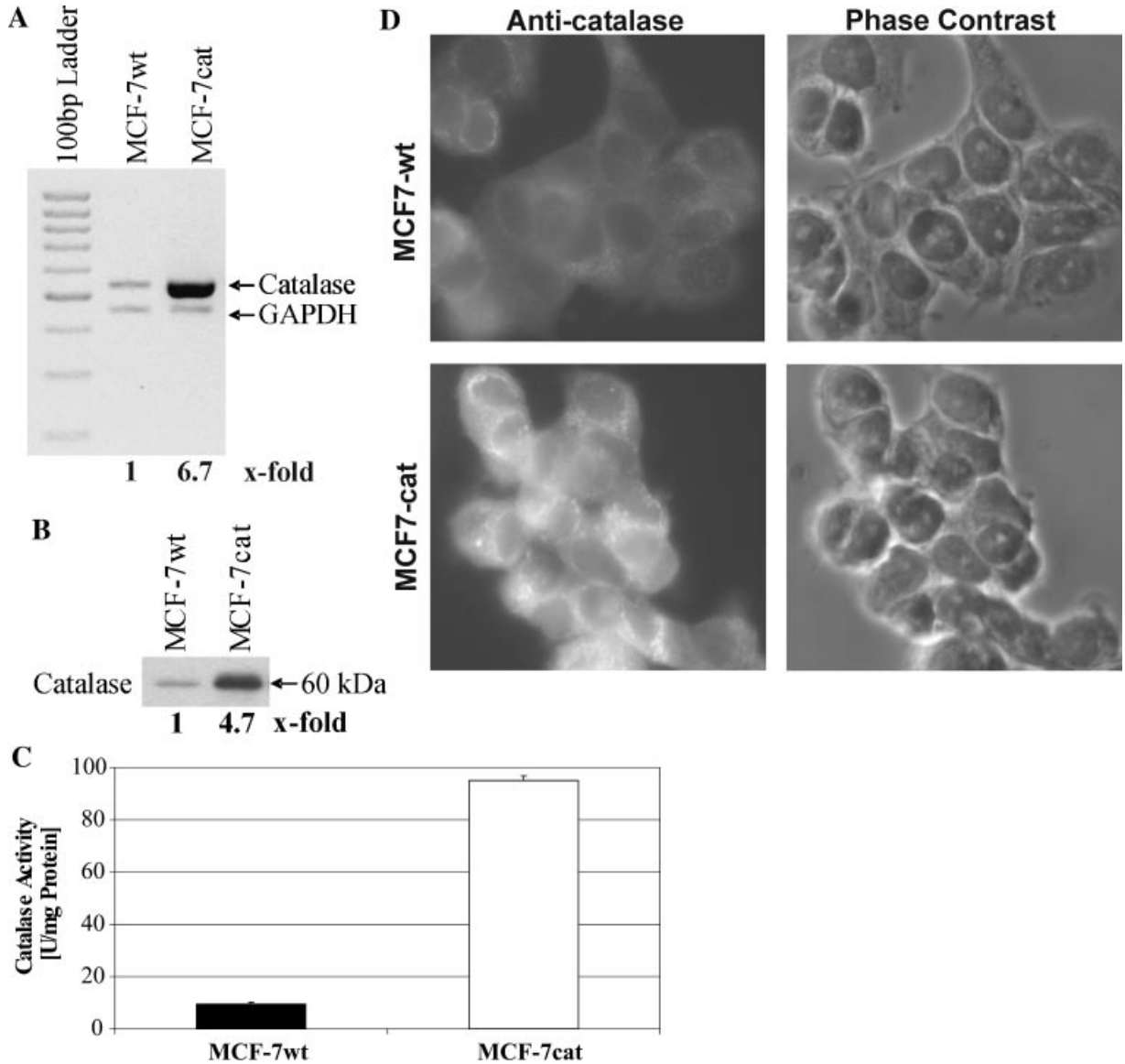

Fig. 1. Characterization of MCF-7 cells overexpressing catalase. Wild type MCF-7 cells (MCF-7wt) and MCF-7 cells overexpressing catalase (MCF-7cat) were analyzed for catalase expression by RT-PCR (**A**) and Western blot (**B**). Catalase activity assay was performed with cell homogenates from MCF-7wt and MCF-7cat cells as described under Experimental Procedures (**C**). Data are means ± SEM (n=3). Immunofluorescence images of MCF-7 wild type and MCF-7 cells overexpressing catalase showing the cytosolic distribution of immunoreactive catalase. Nuclei were counterstained with Hoechst 33342. Representative images are shown (**D**). Magnification, 400×.

We next addressed the question of whether there are differences in intracellular levels of ROS between MCF-7 cells overexpressing catalase and wild type MCF-7 cells. Using the oxidant-sensitive dye DCFH-DA we observed that a bolus of H_2O_2 results in lower intracellular concentrations of ROS over time in the catalase overexpressing MCF-7cat cells than in the wild type MCF-7wt cells (Fig. 2). These findings confirm that catalase overexpression results in improved elimination of intracellular ROS.

Then we examined the effect of catalase overexpression on H_2O_2-mediated cytotoxicity. Cells were treated with increasing concentrations of H_2O_2 and cell viability was assessed by neutral red assay. The catalase-overexpressing MCF-7cat cells showed a significant protection against H_2O_2-mediated cytotoxicity compared to MCF-7 wild type cells (Fig. 3).

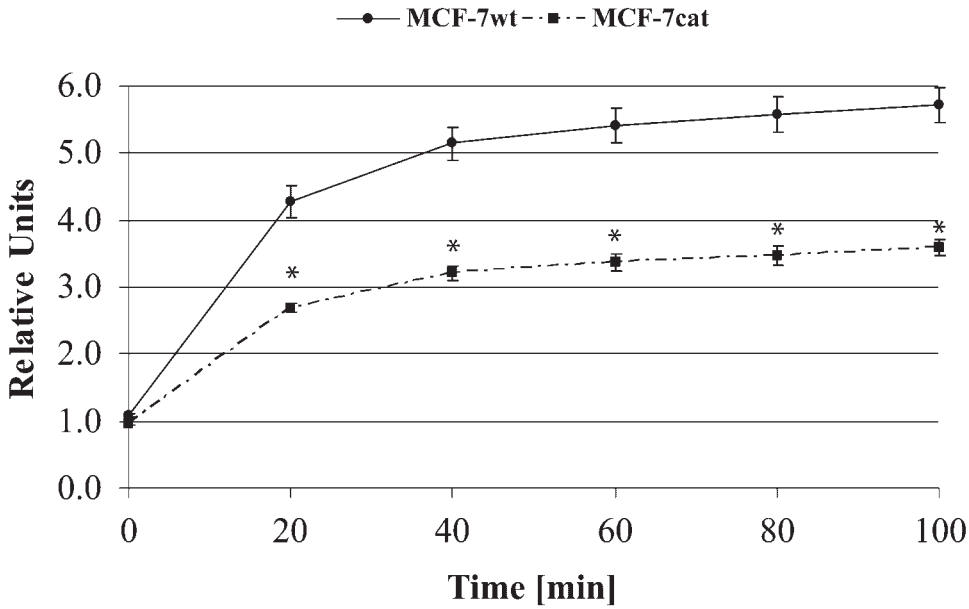

Fig. 2. Effect of catalase overexpression on intracellular H_2O_2 accumulation. MCF-7wt or MCF-7 cells overexpressing catalase (MCF-7cat) were pre-incubated with 50 μM H_2DCF-DA for 30 min, cells were then treated with 1 mM H_2O_2 for 100 min. Fluoerescence was measured in Wallac Victor^2 multilabel counter. The results were expressed as relative units with the intensity of control cells assigned a value of 1. Data are means ± SEM (n > 3); *$P < 0.05$ significant different from corresponding MCF-7wt value.

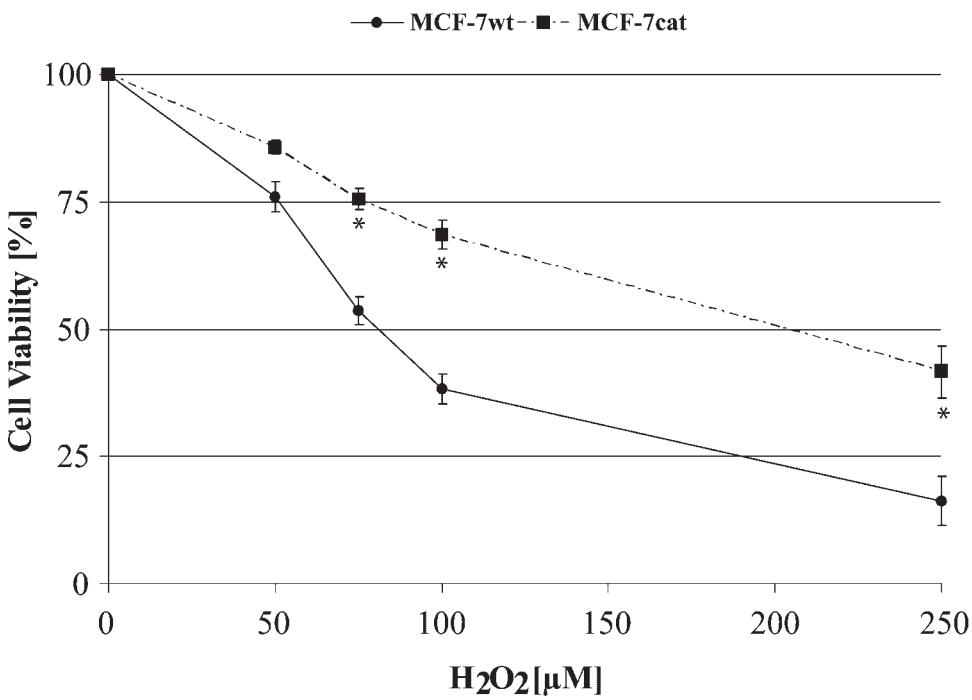

Fig. 3. Overexpression of catalase decreases sensitivity to H_2O_2-induced cytotoxicity. Cells were treated with various concentrations of H_2O_2 for 24 h and cell viability was determined by neutral red assay. Viability was expressed as percentage of control value ± SEM (n = 5); *$P < 0.05$ significant different from corresponding MCF-7wt value.

Taken together, these results suggest that transfection with human catalase cDNA clearly increased the amount of functional catalase protein and protected cells from H_2O_2-mediated cytotoxicity.

Catalase Overexpression is Associated With Increased Sensitivity to TNF-α

Since oxidative stress has been linked to TNF-α toxicity the increase in H_2O_2 degrading capacity might be expected to alleviate the cytotoxic action of TNF-α. To evaluate the effects of exogenous TNF-α on cell viability, MCF-7 wild type and MCF-7cat cells were incubated in the presence of increasing concentrations of TNF-α for 48 h. In contrast to our expectations, catalase overexpressing MCF-7cat cells are more sensitive to TNF-α induced cytotoxicity than the wild type MCF-7 cells as confirmed by neutral red assay (Fig. 4). To test whether the increased sensitivity of the MCF-7cat cells against TNF-α mediated cytotoxicity was associated with increased apoptosis, caspase-3/7 and caspase-9 activity were determined by measuring the proteolytic cleavage of caspase substrates. A significant activation of caspase-3/7 (Fig. 5A) and caspase-9 (Fig. 5B) upon treatment with TNF-α were found in

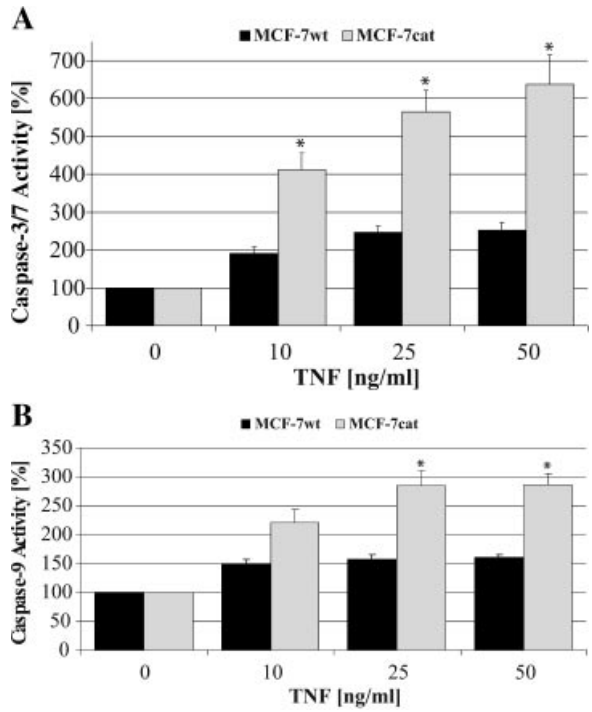

Fig. 5. Overexpression of catalase increases sensitivity to TNF-α induced apoptosis. Wild type MCF-7 and MCF-7cat cells were treated with medium or with medium containing 10, 25, and 50 ng/ml TNF-α for 48 h. Activation of caspase-3/7 (Ac-DEVD-pNA) (**A**) and caspase-9 (Ac-LEDH-pNA) (**B**) were measured colorimetrically. Activity was expressed as percentage of control value ± SEM (n = 4–5); $*P < 0.05$ significant different from corresponding MCF-7wt value.

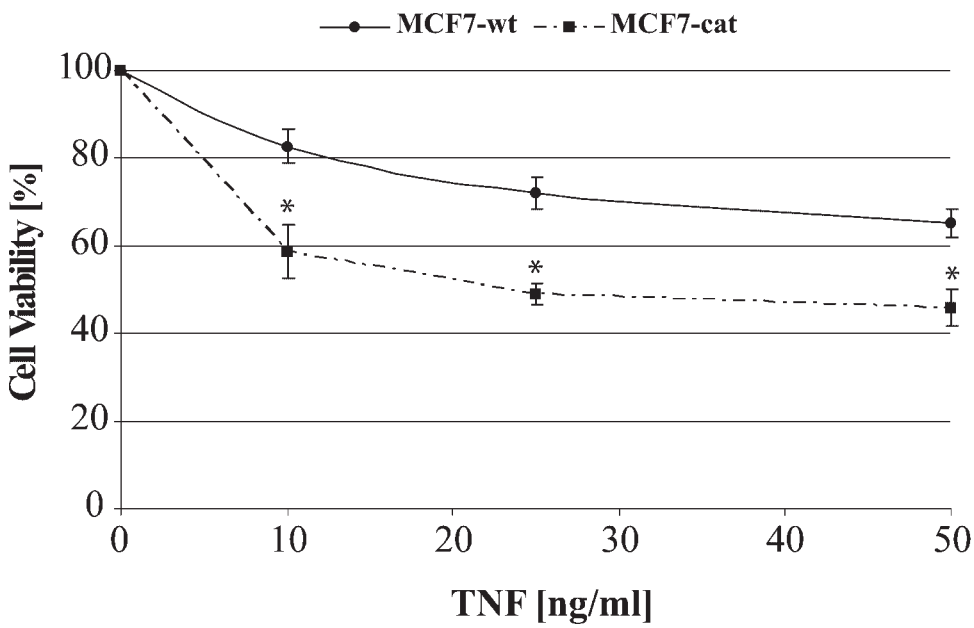

Fig. 4. MCF-7 cells are sensitized to TNF-α cytotoxicity by catalase overexpression. Cells were treated with various concentrations of TNF-α for 48 h and cell viability was determined by neutral red assay. Viability was expressed as percentage of control value ± SEM (n = 5); $*P < 0.05$ significant different from corresponding MCF-7wt value.

catalase overexpressing MCF-7cat cells compared to wild-type MCF-7 cells.

We have previously reported that low catalase expression is associated with TNF-α resistance in H4IIE rat hepatoma cells [Chovolou et al., 2003]. We assessed therefore the catalase expression after TNF-α treatment in wild type MCF-7 cells. A significant dose-dependent down-regulation of catalase was observed by RT-PCR (Fig. 6A), real-time quantitative PCR (Fig. 6B), Western blot (Fig. 6C) and activity measurement (Fig. 6D) in the human breast cancer cell line MCF-7. The down-regulation of catalase expression upon incubation with TNF-α was not restricted to MCF-7 cells but was also observed in the human intestinal cancer cell lines Caco-2 and Hct-116 and in the rat hepatoma cell line H4IIE (Fig. 6E). No decrease was observed in the human hepatoma cell line Huh-7.

While catalase downregulation by TNF-α has only rarely been described in the literature [Yasmineh et al., 1991; Beier et al., 1997] it has often been reported that the mitochondrial enzyme, manganese superoxide dismutase (MnSOD) can be induced by TNF-α and is correlated with resistance against TNF-α-mediated cytotoxicity [Schmelz et al., 2004]. MnSOD expression assessed on mRNA (Fig. 7A) and protein level (Fig. 7B) was increased in both cell lines after TNF-α treatment and was not significant different. Figure 7 demonstrates the dose dependency of MnSOD induction by TNF-α in MCF-7cat and MCF-7 wild type cells.

These results suggest that the quotient of MnSOD expression—known to produce H_2O_2—and catalase expression—known to degrade H_2O_2—increases in TNF-α treated MCF-7wt cells, resulting in more H_2O_2. Overexpression of catalase decreases the MnSOD/catalase quotient which may be one of the mechanisms leading to the observed altered TNF-α sensitivity. Overall, these results imply that a decrease of intracellular H_2O_2 concentration was associated with increased TNF-α cytotoxicity in MCF-7 cells overexpressing catalase.

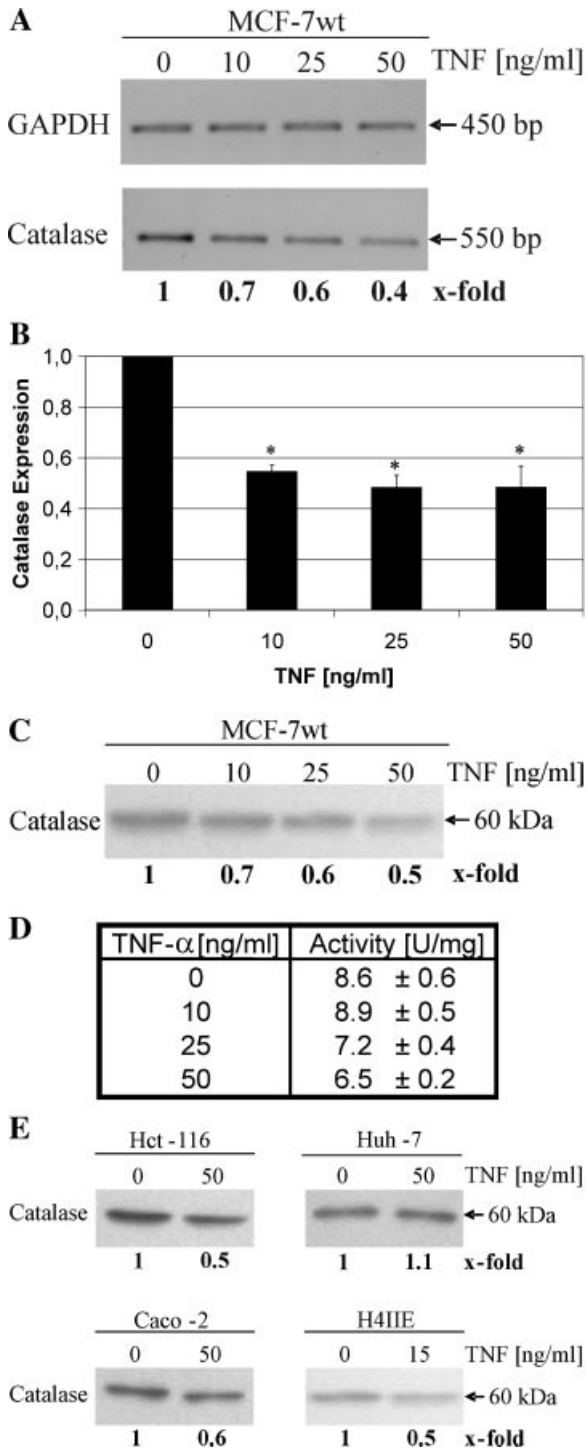

Fig. 6. Dose dependent down-regulation of catalase after TNF-α treatment. Wild type MCF-7 cells were incubated with medium or with medium containing various concentrations of TNF-α for 48 h and the expression of catalase was examined by RT-PCR (**A**), Real-time quantitative analysis (**B**), Western blot analysis (**C**) and photometric activity measurement (**D**). Hct-116, Caco-2, Huh-7, and H4IIE cells were incubated with indicated concentrations of TNF-α for 24 h and the expression of catalase was examined by Western blot analysis (**E**). Each blot is a representative of at least three independent experiments. Blots were scanned by densitometry and the data presented as x-fold expression over control cells. Data are means ± SEM (n = 3).

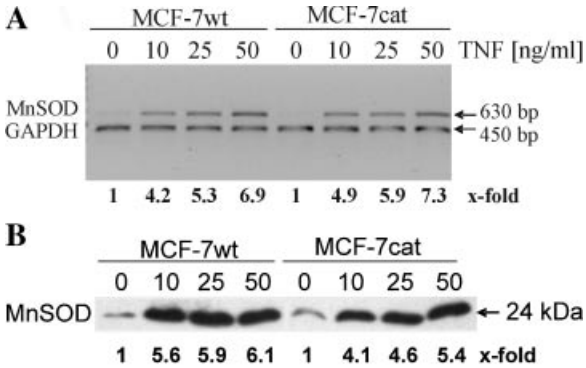

Fig. 7. Increased MnSOD expression after TNF-α treatment. MCF-7 wild type cells were incubated with medium or with medium containing various concentrations of TNF-α for 48 h and the expression of MnSOD was examined by RT-PCR. Data are means ± SEM (n = 3–4) (**A**) and Western blot analysis (**B**).

Catalase Overexpression is Associated With an Altered Nuclear Translocation of p65 Upon TNF-α Treatment

The sensitizing effect of catalase overexpression towards TNF-α induced apoptosis indicates that a signaling pathway requiring H_2O_2 might be affected in these cells. Since it is known that NF-κB activation can be stimulated by H_2O_2 and is part of a survival pathway, we hypothesized that NF-κB activation by TNF-α might be impaired in catalase overexpressing cells. Figure 8 shows that in the MCF-7 wild type cells a sustained nuclear p65 translocation lasting at least 9 h is elicited by TNF-α treatment while in the MCF-7cat cells NF-κB activation is only transient with markedly elevated NF-κB activity at early time points that decreased gradually following the activation. The assumption that this is due to a lack of sufficient H_2O_2 to co-stimulate NF-κB activation is supported by the observation that addition of exogenous H_2O_2 at 1.5 h after the start of TNF-α treatment leads to a second increase of NF-κB activity and shifts the time course of decay to later time points compared to TNF-α treatment (Fig. 9A,B). Nuclear translocation of NF-κB by a bolus of H_2O_2 in the absence of TNF-α was barely detectable (Fig. 9c)

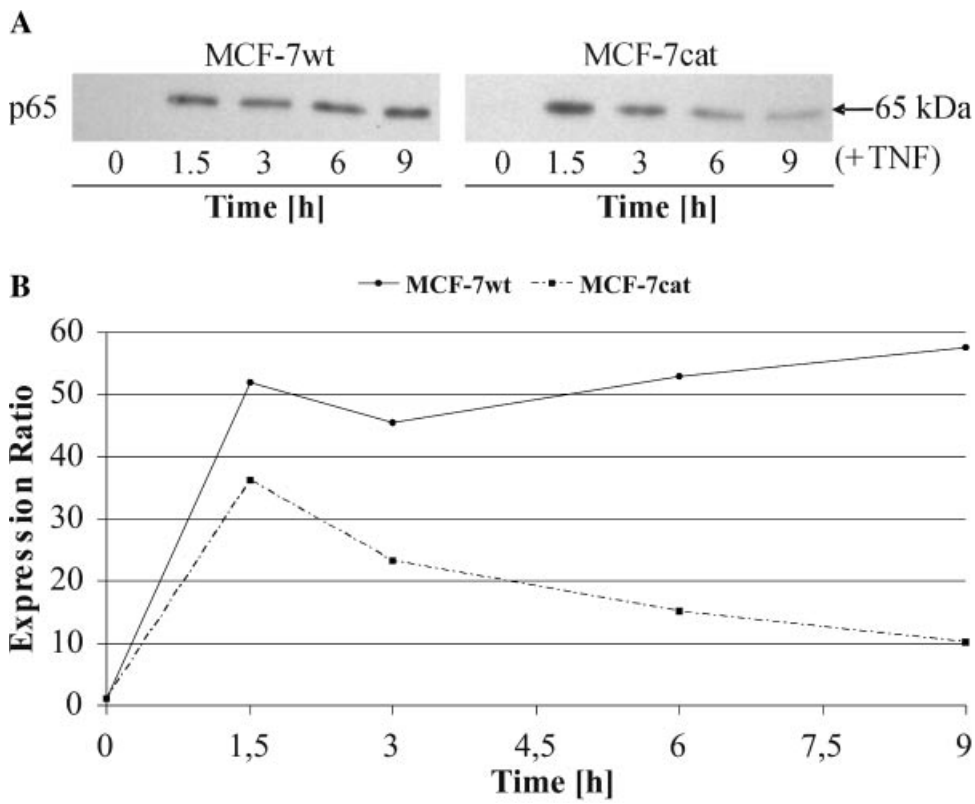

Fig. 8. Effects of catalase overexpression on TNF-α induced NF-κB activation. Time course of p65 translocation after treatment of MCF-7wt and MCF-7cat cells with 50 ng/ml TNF-α for indicated time points. Translocation of p65 was examined by Western blotting analysis using a specific antibody against p65 (**A**). Blots are representative of at least three independent experiments. Blots were scanned by densitometry and the data presented as expression ratio with the intensity of control cells assigned a value of 1 (**B**).

indicating that the increase of NF-κB activity is not just a synergistic effect due to an H_2O_2 action occurring independently from the TNF-α action. To investigate the functional outcome of the impairment of nuclear translocation of NF-κB obtained by catalase overexpression, NF-κB mediated gene expression in both cell types was analyzed using a NF-κB reporter gene construct. Figure 10 demonstrates the time course of gene expression up to 10.5 h after the onset of TNF-α treatment. The rise in gene expression is markedly reduced in the MCF-7cat cells as compared to the MCF-7 wild type cells indicating that the inability of the catalase overexpressing cells to maintain a constant level of nuclear translocation of NF-κB results in lower levels of NF-κB mediated gene expression.

DISCUSSION

Our finding that catalase overexpression sensitizes MCF-7 breast cancer cells against TNF-α adds to the scarce data showing a pro-apoptotic role of catalase. Rather, the published literature provides impressive evidence for the contrary, protection against apoptosis induced by a wide variety of stimuli following overexpression or upregulation of catalase. Thus, overexpression of catalase prevented apoptosis induced by H_2O_2 itself [Chen et al., 2005], menadione, antimycin A [Bai et al., 1999], cytostatic drugs [Bai and Cederbaum, 2003]. Protection against H_2O_2 was even increased when a mitochondrial leader sequence was linked to the catalase gene thus localizing it to a main compartment of intracellular ROS formation [Bai et al., 1999; Arita et al., 2006]. Recently, it was reported that overexpression of human catalase in mitochondria of murine tissues prolongs median and maximal life span of these mice without apparent deleterious side effects [Schriner et al., 2005]. The protective role of catalase against apoptosis could either be due to a requirement for H_2O_2 in one or more steps in the apoptotic program or to an inhibitory action of H_2O_2 in a survival pathway.

Inversely, as demonstrated in the present paper, the overexpression of catalase can also facilitate apoptosis. Adenoviral transfer of the catalase gene into vascular smooth muscle cells or human aortic endothelial cells inhibited DNA synthesis and proliferation while spontaneous apoptosis was increased [Brown et al., 1999; Zanetti et al., 2002]. Bai and Cederbaum [2003] reported that catalase overexpressing cells were protected against apoptosis induced by cytostatic drugs but were sensitized against TNF-α induced apoptosis [Bai and Cederbaum, 2000]. We have previously shown that cells made resistant towards TNF-α were lower in catalase expression than their wild type counterparts and that pharmacological inhibition of catalase with 3-aminotriazole rendered the wild type cells more resistant to TNF-α [Chovolou et al., 2003]. A pro-apoptotic action of catalase could be due to the requirement of H_2O_2 for a survival pathway or an inhibitory action of H_2O_2 on a step within the apoptotic program. In this situation an upregulation of catalase would increase the danger of cell death and one might rather expect a downregulation of catalase expression in order to strengthen the survival pathway or to support the inhibition of apoptosis. The observation that tumor cells in general show low catalase levels and chronically elevated ROS—which are claimed to contribute to growth advantage and malignant phenotype [Oberley and Oberley, 1997]—also points to a pro-apoptotic role of catalase. The results obtained in our cell model are in accordance with this view. In the MCF-7wt cells TNF-α caused little apoptosis and decreased catalase expression while the catalase overexpressing cells were more sensitive to TNF-α induced apoptosis. Catalase downregulation by TNF-α was not restricted to MCF-7 cells but was also observed in two human colon cancer cells lines, Caco-2 and Hct-116. Moreover, we detected catalase downregulation also in TNF-α treated rat hepatoma H4IIE cells as previously reported [Chovolou et al., 2003]. We know of only a few reports on impaired catalase activity or

Fig. 9. Stimulatory effects of H_2O_2 on TNF-α induced NF-κB activation. MCF-7wt and MCF-7 cells overexpressing catalase (MCF-7cat) were treated with 50 ng/ml TNF-α alone or with 1 mM H_2O_2 following pretreatment with 50 ng/ml TNF-α for 1.5 h. At the indicated time p65 translocation to the nucleus were analyzed by Western blotting (**A**). Blots are representative of two independent experiments, were scanned by densitometry and the data presented as expression ratio with the intensity of control cells assigned a value of 1 (**B**). MCF-7wt and MCF-7cat cells were treated with 1 mM H_2O_2 and at the indicated time p65 translocation to the nucleus were analyzed by Western blotting. p65 translocation by a bolus of H_2O_2 in the absence of TNF-α was barely detectable (**C**).

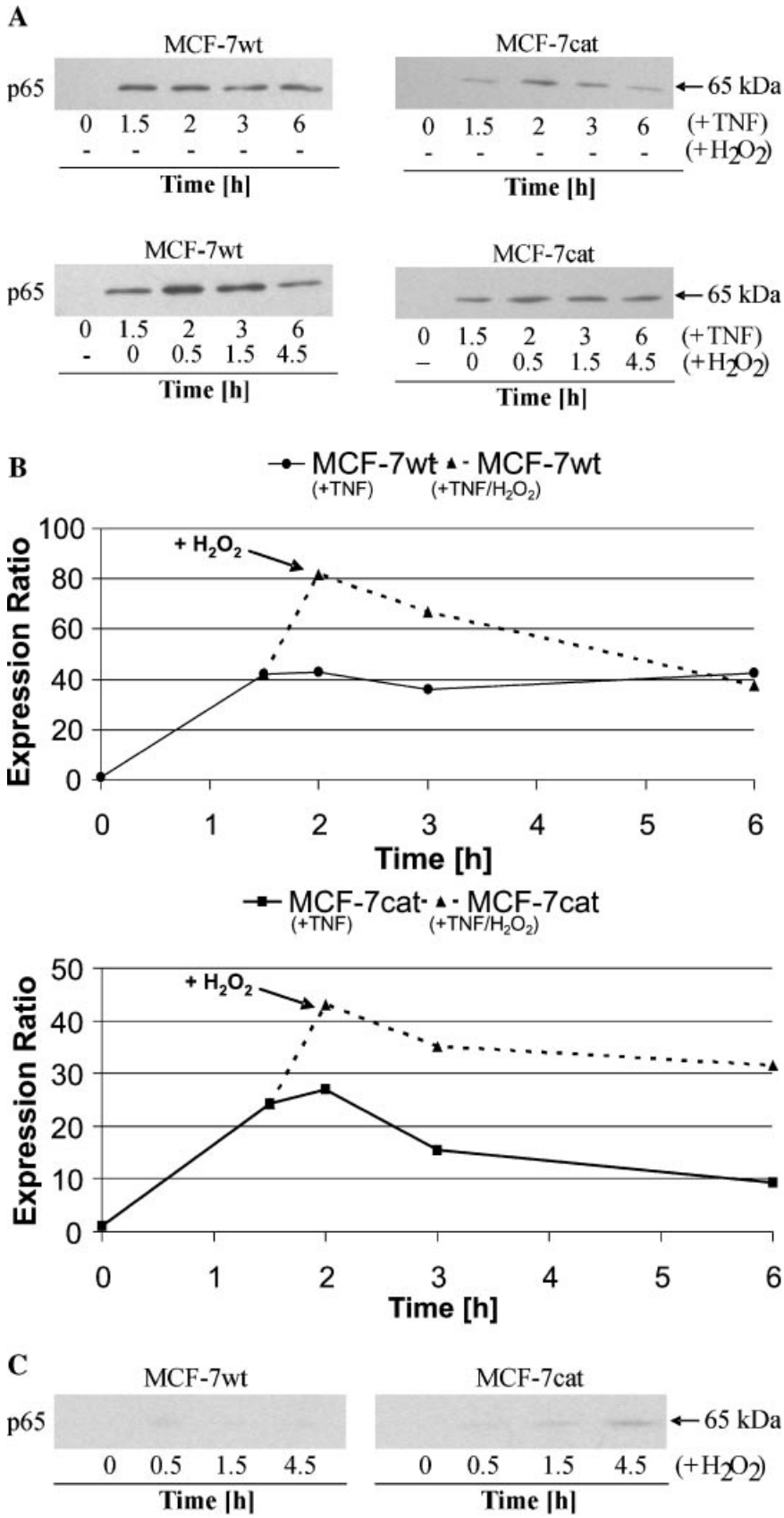
A
MCF-7wt
MCF-7cat
p65
65 kDa
0 1.5 2 3 6
(+TNF)
(+H2O2)
Time [h]
0 1.5 2 3 6
- - - - -
0 1.5 2 3 6
- 0 0.5 1.5 4.5
B
MCF-7wt (+TNF)
MCF-7wt (+TNF/H2O2)
+ H2O2
Expression Ratio
Time [h]
MCF-7cat (+TNF)
MCF-7cat (+TNF/H2O2)
C

Fig. 9.

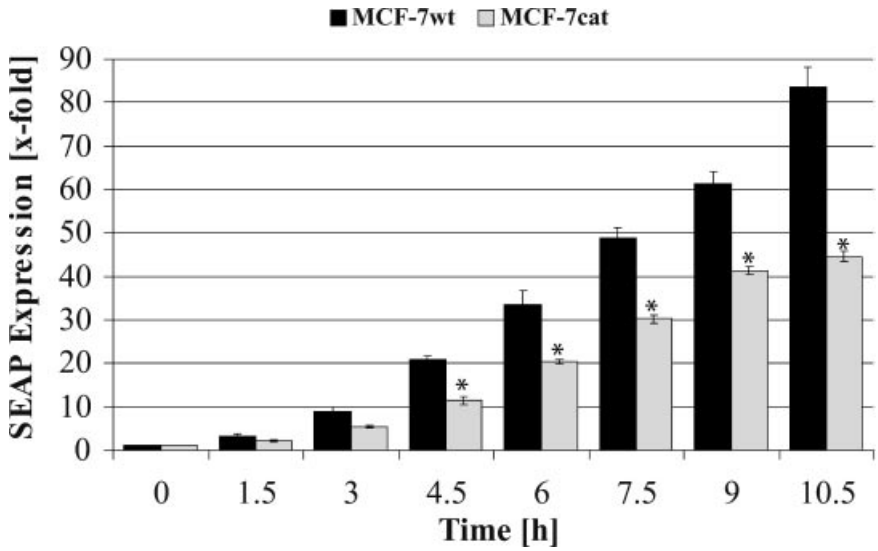

Fig. 10. NF-κB mediated reporter gene expression after treatment with TNF-α. MCF-7wt and MCF-7cat cells were transfected with NF-κB SEAP reporter plasmid. Forty-eight hours after transfection cells were treated with 50 ng/ml TNF-α for indicated time points and SEAP activity was measured. The results were expressed as relative SEAP activity with the activity of control cells assigned a value of 1. Data are means ± SEM (n = 3); $^*P < 0.05$ significant different from corresponding MCF-7wt value.

expression due to TNF-α. Yasmineh et al. [1991] described a decrease in catalase activity in rat liver after TNF-α treatment in vivo. Beier et al. [1997] demonstrated that catalase mRNA in rat liver was reduced by TNF-α treatment in vivo. In contrast, Wong and Goeddel in their seminal 1988 paper on MnSOD induction [Wong and Goeddel, 1988] as well as others [Shaffer et al., 1990; Siemankowski et al., 1999] did not find a change in catalase expression by TNF-α treatment.

It is well known that TNF-α, besides its pro-apoptotic effects, also causes the transcription of anti-apoptotic genes including the MnSOD gene and that NF-κB activation is involved in MnSOD induction [Wong and Goeddel, 1988; Wong et al., 1989] and provides a survival pathway in TNF-α treated cells [Bernard et al., 2002]. The role of reactive oxygen species in NF-κB activation is highly controversial [Janssen-Heininger et al., 1999; Li and Karin, 1999; Baeuerle and Sies, 2000; Bowie and O'Neill, 2000; Korn et al., 2001] and obviously dependent on cell type (e.g., differing activities of signaling pathways) and/or experimental conditions (e.g., medium requirements, time frame of measurements) but the reasons for the opposite outcome have not finally been elucidated. With respect to NF-κB activation by TNF-α, both facilitating [Janssen-Heininger et al., 1999; Baeuerle and Sies, 2000] and inhibitory effects [Korn et al., 2001; Strassheim et al., 2004; Panopoulos et al., 2005] of reactive oxygen species have been found, sometimes even in similar cells and under similar experimental conditions [Korn et al., 2001].

Given the two possibilities of either a stimulatory or an inhibitory effect of H_2O_2 on TNF-α induced NF-κB activation, catalase overexpression could either impair or support NF-κB activation via removal of H_2O_2. In the MCF-7 cells investigated in this paper, the former turned out to be the case: TNF-α merely elicits a transient NF-κB translocation and a diminished NF-κB mediated reporter gene expression in the catalase overexpressing cells while in the wild type cells a sustained NF-κB translocation lasting for several hours and a more rapid increase in NF-κB mediated reporter gene expression is obtained. The failure of catalase overexpressing cells to maintain NF-κB translocation appears to be due to low intracellular H_2O_2 concentration since bolus administration of H_2O_2 allows for prolongation of NF-κB translocation.

To our best knowledge, it has not been reported before, that the impairment of NF-κB activation by TNF-α in cells overexpressing catalase affects control of cell death responses. No influence of catalase overexpression on NF-κB activation, either basal [Azevedo-Martins et al., 2003; Lin et al., 2004] or induced by TNF-α

[Suzuki et al., 1995] or a cytokine mix [Azevedo-Martins et al., 2003] was found by others. A parallel increase of catalase expression and NF-κB activation by TNF-α treatment of glycolysis depleted cells was even observed [Boada et al., 2002]. Only Schmidt et al. [1995] showed that cells stably overexpressing catalase were deficient in activating NF-κB in response to TNF-α. However measurement of NFκB activation was limited to the first hour of TNF treatment without any consideration of subsequent cellular survival responses.

Temporal control of NF-κB activation by TNF-α has been studied in detail. In most but not all cells, nuclear translocation of NF-κB is biphasic with an early phase due to IKK activation and IκBα degradation and a second phase when IKK activity declines and IκBα is re-synthesized [Kemler and Fontana, 1999; Hoffmann et al., 2002]. This second phase lasts for 4 to >24 h [Kemler and Fontana, 1999; Ahmed-Choudhury et al., 2003]. The time course of NF-κB activation can influence the pattern of gene expression and the balance between pro-apoptotic and anti-apoptotic pathways. NF-κB responsive genes can be divided in those requiring persistent NF-κB activation and those for which the burst of NF-κB activation in the first phase is sufficient to initiate gene expression [Hoffmann et al., 2002]. It has been shown that transient activation of NF-κB by CD40 ligand binding triggers apoptosis in hepatocytes in the presence of sustained AP-1 activation while prolonged activation of NF-κB by CD40 ligand binding in the absence of AP-1 activation in intrahepatic endothelial cells results in proliferation [Ahmed-Choudhury et al., 2003]. In our experimental model, a similar outcome as in the experiments of Ahmed-Choudhury et al. [2003] was obtained: In the MCF-7cat cells only a transient NF-κB translocation with an early sharp decline accompanied by lower levels of NF-κB mediated gene expression was achieved due to a lack of the co-stimulator H_2O_2. These cells were sensitive to TNF-α induced apoptosis. In the wild type cells expressing lower catalase levels, a constant level of NF-κB activation and a rapid increase of NF-κB mediated gene expression was obtained, and these cells turned out to be less sensitive to TNF-α induced apoptosis.

In conclusion, we have shown that the ability of TNF-α to drive the MCF-7 cell into apoptosis is modulated by the expression level of catalase. In catalase overexpressing cells, there is not enough H_2O_2 to maintain a sustained NF-κB activation during the first hours of TNF-α treatment. This results in lower levels of NF-κB mediated reporter gene expression and in higher sensitivity towards TNF-α as compared to wild type cells. One might speculate that the downregulation of catalase expression by TNF-α described in this paper occurs as a negative feedback in order to avoid insufficient NF-κB activation. Our results add to the view that antioxidant enzymes play a role in fine-tuning of the physiological concentrations of ROS required in the regulation of redox sensitive cellular processes.

REFERENCES

Adler V, Yin Z, Tew KD, Ronai Z. 1999. Role of redox potential and reactive oxygen species in stress signaling. Oncogene 18:6104–6111.

Ahmed-Choudhury J, Russell CL, Randhawa S, Young LS, Adams DH, Afford SC. 2003. Differential induction of nuclear factor-kappaB and activator protein-1 activity after CD40 ligation is associated with primary human hepatocyte apoptosis or intrahepatic endothelial cell proliferation. Mol Biol Cell 14:1334–1345.

Arita Y, Harkness SH, Kazzaz JA, Koo HC, Joseph A, Melendez JA, Davis JM, Chander A, Li Y. 2006. Mitochondrial localization of catalase provides optimal protection from H2O2-induced cell death in lung epithelial cells. Am J Physiol Lung Cell Mol Physiol 290:L978–L986.

Azevedo-Martins AK, Lortz S, Lenzen S, Curi R, Eizirik DL, Tiedge M. 2003. Improvement of the mitochondrial antioxidant defense status prevents cytokine-induced nuclear factor-kappaB activation in insulin-producing cells. Diabetes 52:93–101.

Baeuerle PA, Sies H. 2000. Antioxidant and redox regulation of genes. New York: Academic Press.

Bai J, Cederbaum AI. 2000. Overexpression of catalase in the mitochondrial or cytosolic compartment increases sensitivity of HepG2 cells to tumor necrosis factor-alpha-induced apoptosis. J Biol Chem 275:19241–19249.

Bai J, Cederbaum AI. 2001. Mitochondrial catalase and oxidative injury. Biol Signals Recept 10:189–199.

Bai J, Cederbaum AI. 2003. Catalase protects HepG2 cells from apoptosis induced by DNA-damaging agents by accelerating the degradation of p53. J Biol Chem 278: 4660–4667.

Bai J, Rodriguez AM, Melendez JA, Cederbaum AI. 1999. Overexpression of catalase in cytosolic or mitochondrial compartment protects HepG2 cells against oxidative injury. J Biol Chem 274:26217–26224.

Bauer G. 2002. Signaling and proapoptotic functions of transformed cell-derived reactive oxygen species. Prostaglandins Leukot Essent Fatty Acids 66:41–56.

Beier K, Volkl A, Fahimi HD. 1997. TNF-alpha down-regulates the peroxisome proliferator activated receptor-alpha and the mRNAs encoding peroxisomal proteins in rat liver. FEBS Lett 412:385–387.

Bernard D, Quatannens B, Begue A, Vandenbunder B, Abbadie C. 2001. Antiproliferative and antiapoptotic effects of crel may occur within the same cells via the up-regulation of manganese superoxide dismutase. Cancer Res 61:2656–2664.

Bernard D, Monte D, Vandenbunder B, Abbadie C. 2002. The c-Rel transcription factor can both induce and inhibit apoptosis in the same cells via the upregulation of MnSOD. Oncogene 21:4392–4402.

Boada J, Cuesta E, Roig T, Gamez A, Carbonell T, Ventura F, Bermudez J. 2002. Enhanced antioxidant defenses and resistance to TNF-alpha in a glycolysis-depleted lung epithelial cell line. Free Radic Biol Med 33:1409–1418.

Borenfreund E, Babich H. 1987. In vitro cytotoxicity of heavy metals, acrylamide, and organotin salts to neural cells and fibroblasts. Cell Biol Toxicol 3:63–73.

Bowie A, O'Neill LA. 2000. Oxidative stress and nuclear factor-kappaB activation: A reassessment of the evidence in the light of recent discoveries. Biochem Pharmacol 59:13–23.

Bradham CA, Qian T, Streetz K, Trautwein C, Brenner DA, Lemasters JJ. 1998. The mitochondrial permeability transition is required for tumor necrosis factor alpha-mediated apoptosis and cytochrome c release. Mol Cell Biol 18:6353–6364.

Brown MR, Miller FJ, Li WG, Ellingson AN, Mozena JD, Chatterjee P, Engelhardt JF, Zwacka RM, Oberley LW, Fang X, Spector AA, Weintraub NL. 1999. Overexpression of human catalase inhibits proliferation and promotes apoptosis in vascular smooth muscle cells. Circ Res 85:524–533.

Chen H, Li X, Epstein PN. 2005. MnSOD and catalase transgenes demonstrate that protection of islets from oxidative stress does not alter cytokine toxicity. Diabetes 54:1437–1446.

Chovolou Y, Watjen W, Kampkotter A, Kahl R. 2003. Resistance to tumor necrosis factor-alpha (TNF-alpha)-induced apoptosis in rat hepatoma cells expressing TNF-alpha is linked to low antioxidant enzyme expression. J Biol Chem 278:29626–29632.

Goossens V, Stange G, Moens K, Pipeleers D, Grooten J. 1999. Regulation of tumor necrosis factor-induced, mitochondria- and reactive oxygen species-dependent cell death by the electron flux through the electron transport chain complex I. Antioxid Redox Signal 1:285–295.

Herrlich P, Bohmer FD. 2000. Redox regulation of signal transduction in mammalian cells. Biochem Pharmacol 59:35–41.

Hoffmann A, Levchenko A, Scott ML, Baltimore D. 2002. The IkappaB-NF-kappaB signaling module: Temporal control and selective gene activation. Science 298:1241–1245.

Janssen-Heininger YM, Macara I, Mossman BT. 1999. Cooperativity between oxidants and tumor necrosis factor in the activation of nuclear factor (NF)-kappaB: Requirement of Ras/mitogen-activated protein kinases in the activation of NF-kappaB by oxidants. Am J Respir Cell Mol Biol 20:942–952.

Kahl R, Kampkotter A, Watjen W, Chovolou Y. 2004. Antioxidant enzymes and apoptosis. Drug Metab Rev 36: 747–762.

Kemler I, Fontana A. 1999. Role of IkappaBalpha and IkappaBbeta in the biphasic nuclear translocation of NF-kappaB in TNFalpha-stimulated astrocytes and in neuroblastoma cells. Glia 26:212–220.

Korn SH, Wouters EF, Vos N, Janssen-Heininger YM. 2001. Cytokine-induced activation of nuclear factor-kappa B is inhibited by hydrogen peroxide through oxidative inactivation of IkappaB kinase. J Biol Chem 276:35693–35700.

Li N, Karin M. 1999. Is NF-kappaB the sensor of oxidative stress? FASEB J 13:1137–1143.

Lin SJ, Shyue SK, Liu PL, Chen YH, Ku HH, Chen JW, Tam KB, Chen YL. 2004. Adenovirus-mediated over-expression of catalase attenuates oxLDL-induced apoptosis in human aortic endothelial cells via AP-1 and C-Jun N-terminal kinase/extracellular signal-regulated kinase mitogen-activated protein kinase pathways. J Mol Cell Cardiol 36:129–139.

Luster MI, Simeonova PP, Gallucci R, Matheson J. 1999. Tumor necrosis factor alpha and toxicology. Crit Rev Toxicol 29:491–511.

Oberley TD, Oberley LW. 1997. Antioxidant enzyme level in cancer. Histol Histopathol 12:525–535.

Panopoulos A, Harraz M, Engelhardt JF, Zandi E. 2005. Iron-mediated H2O2 production as a mechanism for cell type-specific inhibition of tumor necrosis factor alpha-induced but not interleukin-1beta-induced IkappaB kinase complex/nuclear factor-kappaB activation. J Biol Chem 280:2912–2923.

Pfaffl MW. 2001. A new mathematical model for relative quantification in real-time RT–PCR. Nucleic Acids Res 29:45.

Pfaffl MW, Horgan GW, Dempfle L. 2002. Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucleic Acids Res 30:36.

Pfaffl MW, Tichopad A, Prgomet C, Neuvians TP. 2004. Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper—Excel-based tool using pair-wise correlations. Biotechnol Lett 26:509–515.

Schmelz K, Wieder T, Tamm I, Muller A, Essmann F, Geilen CC, Schulze-Osthoff K, Dorken B, Daniel PT. 2004. Tumor necrosis factor alpha sensitizes malignant cells to chemotherapeutic drugs via the mitochondrial apoptosis pathway independently of caspase-8 and NF-kappaB. Oncogene 23:6743–6759.

Schmidt KN, Amstad P, Cerutti P, Baeuerle PA. 1995. The roles of hydrogen peroxide and superoxide as messengers in the activation of transcription factor NF-kappa B. Chem Biol 2:13–22.

Schriner SE, Linford NJ, Martin GM, Treuting P, Ogburn CE, Emond M, Coskun PE, Ladiges W, Wolf N, Van Remmen H, Wallace DC, Rabinovitch PS. 2005. Extension of murine life span by overexpression of catalase targeted to mitochondria. Science 308:1909–1911.

Schulze-Osthoff K, Bakker AC, Vanhaesebroeck B, Beyaert R, Jacob WA, Fiers W. 1992. Cytotoxic activity of tumor necrosis factor is mediated by early damage of mitochondrial functions. Evidence for the involvement of mitochondrial radical generation. J Biol Chem 267:5317–5323.

Shaffer JB, Treanor CP, Del Vecchio PJ. 1990. Expression of bovine and mouse endothelial cell antioxidant enzymes following TNF-alpha exposure. Free Radic Biol Med 8: 497–502.

Siemankowski LM, Morreale J, Briehl MM. 1999. Antioxidant defenses in the TNF-treated MCF-7 cells: Selective increase in MnSOD. Free Radic Biol Med 26: 919–924.

Strassheim D, Asehnoune K, Park JS, Kim JY, He Q, Richter D, Mitra S, Arcaroli J, Kuhn K, Abraham E. 2004. Modulation of bone marrow-derived neutrophil signaling by H2 O2:disparate effects on kinases, NF-kappaB, and cytokine expression. Am J Physiol Cell Physiol 286:C683–C692.

Suzuki YJ, Mizuno M, Packer L. 1995. Transient overexpression of catalase does not inhibit TNF- or PMA-induced NF-kappa B activation. Biochem Biophys Res Commun 210:537–541.

Wong GH, Goeddel DV. 1988. Induction of manganous superoxide dismutase by tumor necrosis factor: Possible protective mechanism. Science 242:941–944.

Wong GH, Elwell JH, Oberley LW, Goeddel DV. 1989. Manganous superoxide dismutase is essential for cellular resistance to cytotoxicity of tumor necrosis factor. Cell 58:923–931.

Yasmineh WG, Parkin JL, Caspers JI, Theologides A. 1991. Tumor necrosis factor/cachectin decreases catalase activity of rat liver. Cancer Res 51:3990–3995.

Zanetti M, Katusic ZS, O'Brien T. 2002. Adenoviral-mediated overexpression of catalase inhibits endothelial cell proliferation. Am J Physiol Heart Circ Physiol 283: H2620–H2626.