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Hyperthermia Engages the Intrinsic Apoptotic Pathway by Enhancing Upstream Caspase Activation to Overcome Apoptotic Resistance in MCF-7 Breast Adenocarcinoma Cells

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Abstract Febrile hyperthermia enhanced TNF-stimulated apoptosis of MCF-7 cells and overcame resistance in a TNF-resistant, MCF-7 variant (3E9), increasing their TNF-sensitivity by 10- and 100-fold, respectively. In either cell line, the hyperthermic potentiation was attributable to increased apoptosis that was totally quenched by caspase inhibition. In MCF-7 cells, hyperthermic potentiation of apoptosis was associated with sustained activation of upstream caspases in response to TNF and more prominent engagement of the intrinsic apoptotic pathway. Apoptotic enhancement by hyperthermia was primarily mediated by caspase-8 activation, as the specific inhibitor, Z-IETD, blocked cell death, whereas direct engagement of the intrinsic apoptotic pathway (with doxorubicin) was not affected. In 3E9 cells, hyperthermia alone induced activation of caspase-8, and was further enhanced by TNF. In 3E9 cells, hyperthermia caused TNF-dependent loss of mitochondrial membrane potential and activation of capspase-9 that was initiated and dependent on upstream caspases. MCF-7 and 3E9 cells were equally sensitive to exogenous C_6-ceramide, but mass spectroscopic analysis of ceramide species indicated that total ceramide content was not enhanced by TNF and/or hyperthermia treatment, and that the combination of TNF and hyperthermia caused only modest elevation of one species (dihydro-palmitoyl ceramide). We conclude that febrile hyperthermia potentiates apoptosis of MCF-7 cells and overcomes TNF-resistance by sustained activation of caspase-8 and engagement of the intrinsic pathway that is independent of ceramide flux. This report provides the first evidence for regulation of caspase-dependent apoptosis by febrile hyperthermia. J. Cell. Biochem. 98: 356–369, 2006.

Key words: Caspase-8; intrinsic pathway; hyperthermia; MCF-7; PARP; ceramide; TNF

Abbreviations used: BHA, butylated hydroxy-anisole; BHT, butylated hydroxy-toluene; CD95, cluster designate 95-member of TNF receptor family; cIAP, cellular inhibitor of apoptosis; Crm-A, cytokine response modifier A; DISC, death-inducing signaling complex; Dox, doxorubicin; FADD, Fas-associated death domain; Fas/APO1, alternative designation for CD95; FLIP, FLICE inhibitory protein; IAP, inhibitor of apoptosis protein; IC_{50}, 50% inhibitory concentration; ICE, interleukin-1b converting enzyme; i-FLICE, inhibitor of FLICE (FADD-like ICE); LC/MS, liquid chromatography/mass spectroscopy; MMP, mitochondrial membrane potential; MnSOD, manganous superoxide dismutase; NF-κB, nuclear factor, kappa, first described on B-cell lineage; PARP, Poly(ADP)-ribose polymerase; ROI, Reactive oxygen intermediate; SAPK/JNK, stress-activated protein kinase/Jun–N-terminal kinase; SP600125, selective reversible inhibitor of JNK1, 2, and 3; TNF, tumor necrosis factor; TMRE, tetramethylrhodamine ethyl ester; TRADD, TNF receptor-associated death domain; TRAF2, TNF receptor associated factor-2; Z-IETD-fmk, Ile-Glu-Thr-Asp-*o*-methyl-fluoromethyketone; Z-VAD-fmk, benzyloxy-valine-alanine-aspartate-*o*-methyl-fluoromethylketone.

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TNF is capable of inducing apoptotic as well as necrotic forms of cell death in tumor cells [Goossens et al., 1995; Wallach et al., 1997]. A number of docking proteins proximal to the p55 TNF receptor, such as TRADD [Hsu et al., 1995], FADD [Chinnaiyan et al., 1995, 1996; Boldin et al., 1996] and TRAF2 [Liu et al., 1996], are members of complexes that activate upstream caspases, specifically, caspase-8 [Boldin et al., 1996; Muzio et al., 1996] and initiate the apoptotic cascade [Martin et al., 1998]. Alternatively, a role for TNF in upregulation of reactive oxygen intermediate (ROI) species has been described in certain models of TNF killing, including the human MCF-7 breast adenocarcinoma [Li and Oberley, 1997] and the murine L929 fibrosarcoma [Vercammen et al., 1998; Leroux et al., 2001]. More recently, TNF induction of ceramide synthesis via sphingomyelinase-mediated degradation of membrane sphingomyelin has been proposed to be a key component of the apoptotic pathway in MCF-7 cells [Liud et al., 1998; Luberto et al., 2002].

The MCF-7 cell line has been reported from various laboratories as displaying either resistance or sensitivity to TNF [Tang et al., 1994; Li and Oberley, 1997; Ameyar et al., 1998], presumably reflecting the influence of selective pressures of in vitro culture on this phenotype. We have previously described a TNF-sensitive parental MCF-7 cell line from which a TNF-resistant clonal variant (3E9) was derived by selection in this cytokine [Doman et al., 1999]. In this model, TNF-resistance was associated with reduced apoptotic potential and downregulation of stress pathways, including SAPK/JNK and p53; however, the NF-κB pathway was functionally equivalent between the parental and resistant lines.

Hyperthermia has been shown to potentiate TNF killing in vitro in certain TNF-sensitive models, such as L-M [Niitsu et al., 1988; Tsuji et al., 1992; Yamauchi et al., 1992; Watanabe et al., 1998], L929 [Klostergaard et al., 1989, 1996; Tomasovic et al., 1989a,b; Leroux et al., 2001], and MCF-7 cells [Li and Oberley, 1997]. However, there is minimal experimental analysis of whether the combination of hyperthermia and TNF potentiates apoptotic or necrotic forms of cell death, or both, in any tumor model. In the MCF-7 model, acute hyperthermia (43°C, 1 h) only slightly increased the cytotoxic action of TNF, and manganous superoxide dismutase (MnSOD)-overexpressing transfectants were far more resistant than parental cells to TNF, acute hyperthermia or their combination. The latter result strongly supported a major role for reactive oxygen intermediates (ROI), specifically superoxide anion, in TNF cytotoxicity [Li and Oberley, 1997]. However, the involvement of caspases in the hyperthermic response was not examined.

CD95 (Fas/APO-1), another member of the TNF receptor superfamily, is known to activate caspases and induce mitochondrial cytochrome-*c* release following receptor ligation [Schulze-Osthoff et al., 1998; Krammer, 1999]. CD95 ligation-induced apoptosis in human malignant glioma cells has been shown to be enhanced by hyperthermia (41°C); hyperthermic augmentation was quenched by ectopic expression of Bcl-2 or crm-A, and appeared to occur upstream of caspase-3 activation [Hermisson et al., 2000]. The mobilization of cytochrome-*c* and inhibitory effects of Bcl-2 suggest a significant role for the intrinsic pathway in hyperthermic potentiation of CD95-dependent apoptosis in this cell model.

Since hyperthermia can affect stress pathways [SAPK/JNK [Doman et al., 1999; Ducret et al., 1999; Li et al., 1999]], we examined the mechanism of hyperthermic potentiation of TNF-mediated cytotoxicity in MCF-7 and 3E9 cells. We observed that moderate hyperthermia (febrile; 40.0–40.5°C and long-duration; 24 h) augmented TNF sensitivity of MCF-7 cells and overcame TNF-resistance in 3E9 cells. The molecular basis for hyperthermic potentiation of TNF-mediated apoptosis was linked to enhanced and sustained activation of caspase-8, leading to recruitment of caspase-9 into the pathway, most notably in 3E9 cells. Importantly, hyperthermia did not enhance chemotherapy- or ceramide-induced cell death, suggesting a prominent role for upstream caspases in hyperthermic potentiation of apoptosis in MCF-7 cells.

MATERIALS AND METHODS

Cell Lines, TNF, Reagents, and Antibodies

The MCF-7 breast carcinoma cell line [Soule et al., 1973] was originally provided to us by Dr. Kapil Dhingra, Department of Medical Breast Oncology, University of Texas M.D., Anderson Cancer Center. Similar results were observed with the line available from ATCC. These cells were grown in Minimal Essential Medium containing 5% fetal bovine serum (FBS;

Hyclone, Logan UT). The TNF-resistant MCF-7/3E9 cell variant was selected by culturing MCF-7 cells in the presence of increasing concentrations of TNF (starting at 0.05 nM and escalating to 1.6 nM TNF as previously reported; Doman et al., 1999).

Antibody against PARP and its cleavage fragment was from PharMingen (San Diego, CA). Antibodies against caspase-8 and caspase-9 were from Cell Signaling, (Beverly, MA). The broad spectrum caspase inhibitor, Z-VAD-fmk, and the caspase-8-specific inhibitor, Z-IETD-fmk, were from Calbiochem (La Jolla, CA), and were made up as stock solutions in DMSO. Recombinant human TNF was kindly supplied by Genentech (South San Francisco, CA). BHA was from Sigma (St. Louis, MO) and was made up as an ethanolic stock. Doxorubicin was from Ben Venue Laboratories (New Bedford, OH). SP600125, a selective reversible inhibitor of JNK1, 2, and 3, was from Biomol (Plymouth Meeting, PA).

Measurements of Cell Survival

Treatment effects on survival were determined by MTT or neutral red staining of cells following TNF and/or hyperthermia exposure in 96-well plates as previously described [Tang et al., 1994; Vercammen et al., 1998]. 1×10^4 MCF-7 parental or 3E9 cells were cultured overnight in 100 μl of media per well prior to treatment. TNF was added over a dose-range from ~6 pM to ~4.4 nM (0.1–75 ng/ml) of the monomer, depending on the experiment. In selected experiments, Z-VAD-fmk (final concentration: 5 μM) or BHA (final concentration: 50 μM) was added to wells 30 min before introduction of TNF or doxorubicin. Cultures were treated in CO_2 incubators set at 37°C (for 24–48 h), 40.0–40.5°C (for 24 h), or 41.5–41.8°C (for up to 2 h). Optical density values were normalized to a 100% survival value for untreated control cells at the appropriate temperature or, where appropriate, to the optical density from cells treated with SP600125. Febrile hyperthermia alone caused a 10–20% reduction in survival after 24 h. Percent survival was calculated as the ratio of the optical densities from experimental vs. control cultures. Survival was reported as mean ± S.E.M.; results from two or more experiments of each type are shown. In experiments designed to assess the possible role of the JNK pathway, SP600125 was included at a final concentration of 20 μM.

Cell Extraction and Immunoblot Analysis

Total cell lysates were prepared as previously described [Doman et al., 1999]. Equal protein aliquots were resolved by SDS–PAGE, transferred to nitrocellulose membranes, and immunoblotted with antibody against PARP, caspase-8 or caspase-9. Antigen was detected with anti-mouse antibodies coupled to horseradish peroxidase and visualized with ECL reagent as described by the supplier (Amersham, Arlington Heights, IL).

Analysis of the Role of Caspases-8 and -9

Caspase-8 activity. Cultures of parental MCF-7 or 3E9 cells from treatment with TNF and/or febrile hyperthermia were prepared as described above. Lysates were prepared from these cultures harvested at various times after initiation of treatment, using lysis buffer without protease inhibitors. After determining protein concentrations, equal protein aliquots (representing 50–100 μg of protein) were incubated with the caspase-8-specific colorimetric substrate, IETD-*p*-nitro-anilide for various intervals at 37°C (R&D Systems, Minneapolis, MN). Resultant optical densities after 1–3 h (parental cells) or after up to 24 h (3E9 cells) of incubation were assessed in a plate reader set at 405 nm. Background corrected experimental values were calculated and shown directly and were compared to various control lysates.

Caspase-8 inhibition. 1×10^4 MCF-7 parental cells were cultured overnight in microwells prior to treatment with the caspase-8 inhibitor, Z-IETD-fmk, at up to 20 μM for 40–75 min before introduction of TNF. Cultures were treated at 37°C for 48 h or at 40.0–40.5°C for 24 h before neutral red staining. Percent inhibition by Z-IETD-fmk was based on reduction in TNF killing of control cells.

Time-Lapse Fluorescence Microscopy

Digital time-lapse acquisition of phase contrast and fluorescence images from MCF-7 and 3E9 cells was performed using the Cell Observer function of a Zeiss Axiovert 200 M motorized inverted microscope. Tumor cells were plated in a 12-well tissue culture plate (1×10^5 cells/well); after 1–2 days of incubation, TMRE (25 nM) was added 1 h before TNF (1 nM).

The plate was placed in a chamber on a temperature-controlled stage and maintained in a heated, humidified, 5% CO_2 atmosphere. The Axiovision Software was used to program the motorized stage to move sequentially from well to well and acquire phase contrast and fluorescent color images once per hour. Phase images and fluorescent images, using appropriate excitation and emission settings for TMRE (546 nm and 580 nm, respectively) were acquired.

Liquid Chromatographic/Mass Spectroscopic (LC/MS) Analysis of Ceramides

Initial experiments were designed to identify the most predominant ceramide species in parental MCF-7 cells. Standards of known selected ceramides (Sigma, Biomol), with acyl chain lengths ranging from C2 to C24, either with or without the double bond in the sphingoid backbone, and with either full saturation or with a *cis*-double bond in the acyl chain, were first characterized for their retention time via HPLC. Parameters of HPLC were generally as follows: Column: YMC ODS-Aqua 3 m 2×100 mm (Waters Corp.); Guard column: Eclipse XDB C8 5 m 2×12 mm (Mac-Mod Analytical, Chadds Ford, PA); Mobile phase: A: 0.05% formic acid (aqueous) pH 3.0, B: methanol; Column temperature: 40°C; Sample injection volume: 25 μl; Flow rate: 200 μl per min; Gradient: 70% B to 95% B in 5 min, hold at 95% for an additional 5 min. The fragmentation patterns of the predominant peak of each standard were established by mass spectroscopy (see below).

Parental MCF-7 cells were plated in DMEM/F12 and 5% serum at 3×10^6 cells per 60-mm dishes and placed in a 37°C incubator overnight. Cells were subsequently harvested, washed 3× with PBS, and resuspended in 1 ml methanol with 0.1 mg/ml BHT in 10 ml round bottom glass screw cap tubes. One to two milliliter chloroform were added, and the tubes flushed with nitrogen to remove oxygen. The mixture was vortexed and placed on ice in the dark for 1 h. One milliliter of 0.15 M sodium chloride was added, followed by vortexing, and then centrifugation at 4,000 rpm for 10 min to separate phases. The upper aqueous layer was removed with a transfer pipette, and the lower organic layer was evaporated to dryness with nitrogen. Reconstitution was with 150 μl methanol, and 50 μl 12 mM ammonium acetate, pH 8, with vortexing prior to injection into the HPLC guard column as described above.

Peaks with characteristic retention times corresponding to the ceramide standards were confirmed by mass spectroscopy. Parameters of MS were generally as follows: Ionization mode: electrospray negative (M-H); Detection mode: Multiple reaction monitoring (MRM); Capillary voltage: 2.75 kV; Desolvation temperature: 400°C at 900 L/h; Source temperature: 125°C; Cone gas: 100 L/h; Collision gas: Argon a $2.5 \times$ 10e-3 torr.

To evaluate ceramide fluxes in response to TNF and/or hyperthermia treatment, cells were plated as described above. TNF (68 ng/ml) was added and control and treated cells were further incubated at 37°C or 40.5°C; cells were harvested at selected intervals and lysates subjected to ceramide analysis by LC/MS as described above. Detector units were normalized to values for known masses of control ceramides. Final values were expressed in nanogram of each ceramide per million cells harvested.

Statistical Analyses

Selected statistical analyses of the data were done by analysis of variance (ANOVA, Statview 4.5 Abacus Concepts, Inc., Berkeley, CA) and post-hoc testing by Fisher's protected least significant difference (Fisher's PLSD, Statview 4.01) at $P = 0.05$. The reported data was based on triplicate determinations for each of two to five independent experiments.

RESULTS

Febrile Hyperthermia Enhances MCF-7 Response to TNF, Overcomes Resistance to TNF in 3E9 Cells, but has Minimal Effects on Sensitivity to Doxorubicin in Either Cell Line

As previously reported [Doman et al., 1999; Donato and Klostergaard, 2004], 3E9 cells were ~30-fold less sensitive to TNF than parental MCF-7 cells as 75 ng/ml of TNF failed to reduce survival of 3E9 cells to 50% in 48 h. In order to determine the possible effects of febrile hyperthermia on the TNF response of these cells, cells were incubated with a dose-range of TNF and following 24 h of TNF-treatment at either 37°C or 40–40.5°C, were stained with neutral red. The percent survival of TNF-treated versus control cells incubated at the corresponding temperature is shown in Figure 1A. Parental

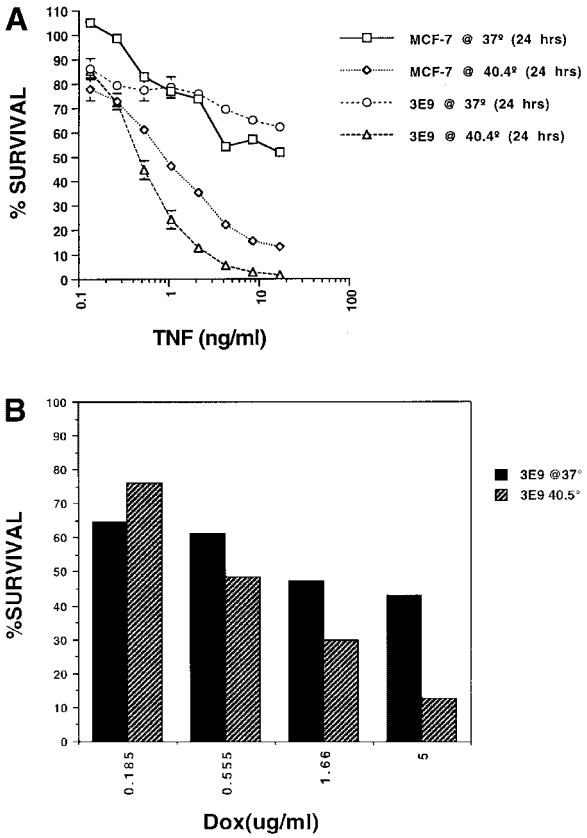

Fig. 1. **Panel A**: Survival of parental MCF-7 and clone 3E9 cells in response to treatment with TNF with and without febrile hyperthermia. Cells were established in microwells and after overnight incubation, treated with a dose range of TNF immediately prior to 24 h of incubation at 37°C or at 40.0–40.5°C. Survival is expressed compared to normothermic or hyperthermic controls, as appropriate. Hyperthermia enhanced TNF killing of parental cells, and even more so of 3E9 cells. **Panel B**: Effects of febrile hyperthermia on doxorubicin-mediated killing of 3E9 cells. Cells were established as above, prior to addition of a dose range of doxorubicin and 24 h culture at 37°C or at 40.0–40.5°C. Survival is expressed compared to normothermic or hyperthermic controls, as appropriate. Hyperthermia only nominally enhanced doxorubicin-mediated killing of 3E9 cells.

MCF-7 cells treated with hyperthermia demonstrated ~10-fold increased TNF sensitivity, with an IC_{50} of ~60 pM (~1 ng/ml). Remarkably, TNF-resistant 3E9 cells became even more sensitive than parental cells when treated with TNF at 40.0–40.5°C, with an IC_{50} of value ~30 pM (~0.5 ng/ml). In contrast to the effects of prolonged febrile hyperthermia, acute hyperthermia (41.5–41.8°C for up to 2 h) had minimal effects on the responses of either cell line to TNF (data not shown), consistent with the minimal augmentation of TNF cytotoxicity against MCF-7 cells reported with 1 h of heating at 43°C [Li and Oberley, 1997].

In order to determine whether febrile hyperthermia caused more global alterations in the responses of these cell lines to apoptotic stimuli, MCF-7 and 3E9 cells were treated with a dose range of doxorubicin (Dox) and were then incubated at 37°C or 40.0–40.5°C for 24 h prior to analysis of cell survival. Dox caused a modest dose-dependent loss of survival by 24 h compared to control cells, and drug-sensitivity was essentially equivalent between these lines (data not shown). Hyperthermia had only modest effects on the Dox response in either cell line (shown in Fig. 1B for 3E9 cells). These results suggest mechanistic distinctions between the effect(s) of hyperthermia on TNF- (extrinsic) and Dox- (intrinsic) mediated death-signaling pathways in these cells.

Caspase Inhibition, but not Anti-Oxidants, Blocks TNF Cytotoxicity, With and Without Hyperthermia, in MCF-7 and 3E9 Cells

Parental MCF-7 and clone 3E9 cells were pretreated with BHA (50 μM), previously shown to quench reactive oxygen intermediates (ROI) in L929 cells [Vercammen et al., 1998; Leroux et al., 2001] or with Z-VAD (5 μM) for 30 min at 37°C prior to incubation with a dose-range of TNF. After 24 h, cell survival was quantified, and is compared to control cells in Figures 2A,B for MCF-7 and 3E9 cells, respectively, incubated at 37°C or 40.0–40.5°C. As noted above, parental cells incubated at 37°C demonstrated only modest sensitivity to TNF at this timepoint, which was strongly quenched by co-incubation with the broad-spectrum caspase inhibitor, Z-VAD (Fig. 2A). The combination of TNF and hyperthermia, which led to increased sensitivity to TNF, was unaffected by the presence of 50 μM BHA, a concentration that effectively quenches TNF killing of L929 cells (data not shown and ref. 12) but was totally quenched by Z-VAD (Fig. 2A). A similar pattern was evident with 3E9 cells: the normally TNF-resistant phenotype was modified by hyperthermia such that TNF-sensitivity increased ~100-fold with hyperthermia (Fig. 2B). Again, this response was unaffected by BHA, but was totally ablated by Z-VAD. In contrast, Z-VAD only slightly protected against Dox-mediated killing (data not shown), further distinguishing the drug response from the TNF-response in these cells. Thus, the normothermic and hyperthermic responses of parental MCF-7 cells, as well as the hyperthermic response

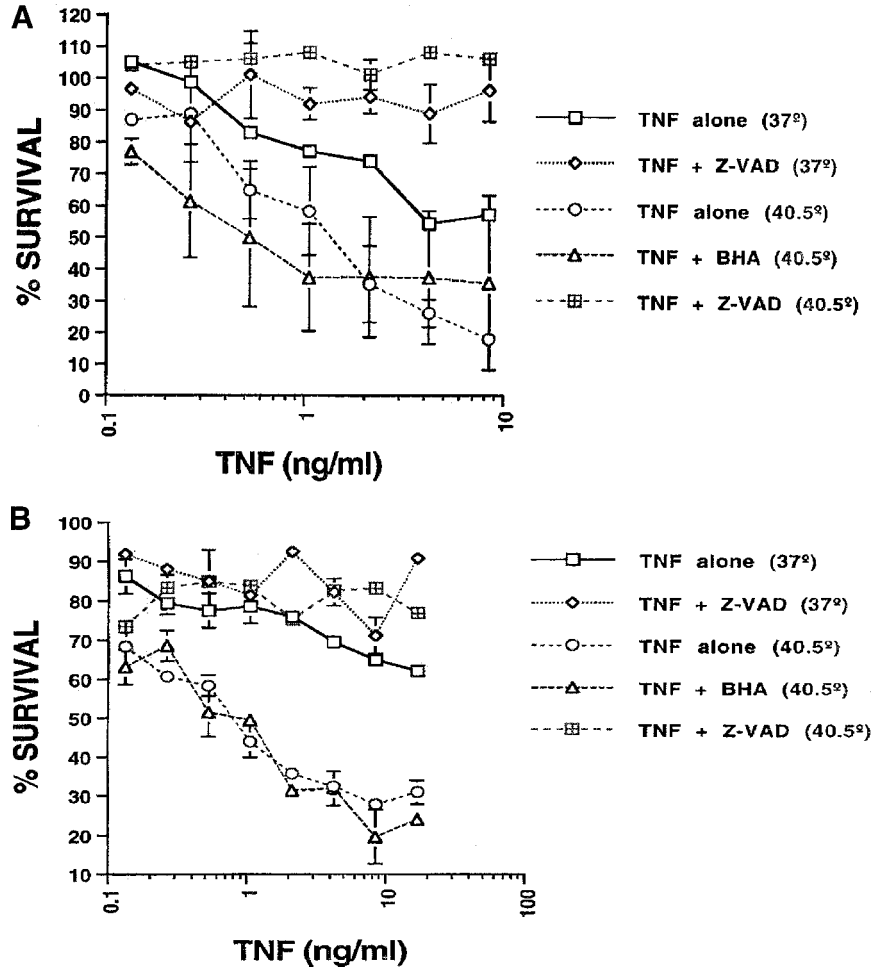

Fig. 2. **Panel A**: Survival of parental MCF-7 cells in response to treatment with TNF, febrile hyperthermia, BHA, and/or Z-VAD. Cells were established as in Figure 1 and selected wells were pretreated for 30 min with Z-VAD (5 μM) or BHA (50 μM). Then cells were treated with TNF immediately prior to 24 h of incubation at 37°C or at 40.0–40.5°C. Survival is expressed compared to normothermic or hyperthermic controls, with or without Z-VAD or BHA, as appropriate. Z-VAD, but not BHA, strongly inhibited TNF killing. **Panel B**: Survival of 3E9 cells in response to treatment with TNF, febrile hyperthermia, BHA, and/or Z-VAD. These experiments were conducted identically to those in Panel A conducted with parental MCF-7 cells. Z-VAD, but not BHA, strongly inhibited TNF killing.

of 3E9 cells to TNF, were caspase-mediated. This response was consistent with apoptosis, and based upon the BHA experiments, was apparently independent of production of ROI, contrasting with the results of Li and Oberley [Li and Oberley, 1997].

Febrile Hyperthermia Accelerates and Increases the Extent of TNF-Induced Caspase Cascade Activation in MCF-7 Cells

Parental MCF-7 cells were seeded in replicate 60-mm dishes and treated with 1 nM TNF followed by incubation at either 37°C or 40.0–40.5°C. At selected times up to 24 h thereafter, cell lysates were prepared and equivalent protein aliquots were subjected to PARP immunoblotting. As shown in Figure 3, by 6 h the 85 kDa PARP cleavage product was initially detectable in the TNF-treated cells cultured at 40.0–40.5°C, but not in the normothermic or non-TNF-treated controls. PARP cleavage in the normothermic, TNF-treated MCF-7 cells was evident by 18 h (and beyond); however, a far more prominent signal was apparent when the cells were incubated under hyperthermic conditions. Of note, non-TNF-treated hyperthermic cells also demonstrated intrinsic PARP cleavage by 24 h, consistent with reduced MTT formazan production in heated compared to normothermic controls (data not shown). These

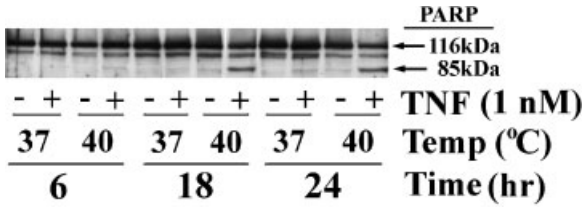

Fig. 3. Effect of TNF with and without febrile hyperthermia on PARP cleavage in parental MCF-7 cells. Parental MCF-7 cells were established in 60-mm dishes, and after overnight incubation, were treated with 1 nM (17 ng/ml) TNF immediately prior to further incubation at 37°C or at 40.0–40.5°C. Lysates were prepared from cells harvested at 6, 18, or 24 h and subjected to SDS–PAGE and PARP immunoblotting. The uncleaved PARP (116 kDa) and cleaved (89 kDa) PARP product are depicted by arrows. Hyperthermia accelerated and enhanced the extent of TNF-induced PARP cleavage.

results strongly suggest that hyperthermia potentiates TNF-induced apoptosis by increased activation of caspase pathways.

Febrile Hyperthermia Increases the Extent and Accelerates the Onset of TNF-Induced Caspase-8 Activation In MCF-7 Cells

Parental MCF-7 cells were treated with 1 nM TNF followed by incubation at either 37°C or 40.0–40.5°C. At selected times thereafter, replicate cell lysates were prepared and equivalent protein aliquots were assayed for caspase-8 activity. As shown in Figure 4, hyperthermia alone caused elevation of basal caspase-8 activity, ~50% greater than the normothermic control, consistent with some PARP cleavage (Fig. 3) and loss of survival in these cultures (see above). TNF treatment resulted in rapid elevation (~two-fold) of caspase-8 activity, in either normothermic or hyperthermic cultures. By 3 h, however, the activity in the normothermic culture declined significantly, whereas in the hyperthermic culture, activity was sustained or further increased. Thus, hyperthermia may facilitate the engagement of upstream caspases and their ligand-induced activation or retard their subsequent downregulation.

TNF/Hyperthermia Killing of MCF-7 Cells is Blocked by Inhibition of Caspase-8

Parental MCF-7 cells were pretreated with up to 20 μM Z-IETD-fmk, a caspase-8 inhibitor, and then treated with up to 1 nM TNF followed by incubation at either 37°C or 40.0–40.5°C. Effects on survival are shown in Table I for 1 nM TNF. Z-IETD-fmk effectively blocked killing induced by TNF alone at 48 h (under

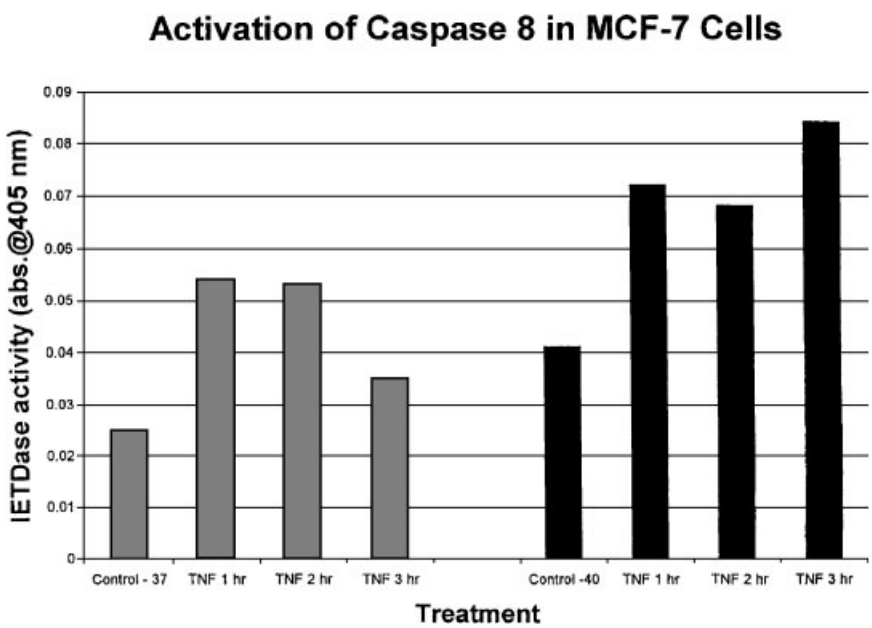

Fig. 4. Effect of TNF with and without febrile hyperthermia on caspase-8 activation in parental MCF-7 cells. Cultures were prepared as described in Figure 3 and cells harvested at 1, 2, and 3 h. Caspase-8 activity in cell lysates was evaluated by degradation of the colorimetric substrate, IETD-p-nitro-anilide at 37°C, and is expressed as increased absorbance at 405 nm. Hyperthermia caused greater and more durable caspase-8 activation by TNF in parental MCF-7 cells compared to normothermia.

TABLE I. Inhibition of TNF- and TNF/Hyperthermia-Induced Apoptosis of Parental MCF-7 Cells by the Caspase-8 Inhibitor Z-IETD

Conditions	Z-IETD concentration (μM)	Percent inhibiton	
37°C, 48 h, 1 nM TNF	5	59.8 ± 0.16	
	10	85.3 ± 0.05	
	20	78.2 ± 0.65	
		1st Experiment	2nd Experiment
40.5°, 24 h, 1 nM TNF	20	78.1 ± 0.80	85.3 ± 0.19

normorthermic conditions), as well as the killing induced by the combination of TNF and febrile hyperthermia at 24 h. This indicates that hyperthermic augmentation of TNF-induced apoptosis was strongly dependent on caspase-8 activation.

Febrile Hyperthermia Increases the Extent and Accelerates the Onset of TNF-Induced Caspase-8 Activation In 3E9 Cells

3E9 cells were treated with 1 nM TNF followed by incubation at either 37°C or 40.0–40.5°C. At various times up to 24 h thereafter, cell lysates were prepared and equivalent protein aliquots were assayed for caspase-8 activity. As shown in the two experiments in Figure 5, TNF treatment of heated 3E9 cells resulted in a time-dependent, ~two-fold increase in caspase-8 activity compared to 3E9 cells under normothermia (upper panel; Figure 5); further, slightly more marked elevation (2–2.5-fold) of caspase-8 activity was observed than with parental MCF-7 cells (lower panel; Figs. 5 and 4), but with slower onset and decline from peak activation. Loss of caspases-8 activity at later time points is commonly associated with an advanced state of downstream caspases activation. We conclude that febrile hyperthermia accelerates and increases the extent of TNF- induced PARP, pro-caspase-8 cleavage in 3E9 cells.

Although the studies of parental MCF-7 cells pointed to an essential role for the extrinsic pathway in TNF-induced apoptosis, and particularly to caspase-8 as a key target, they did not define a role for engagement of the intrinsic caspase pathway in this response. We next investigated this possibility and extended previous observations with parental MCF-7 cells to 3E9 cells. 3E9 cells were seeded in replicate 60-mm dishes and treated with 1 nM TNF followed by incubation at either 37°C or 40.0–40.5°C. At 6, 12, and 24 h thereafter, cell lysates were prepared and equivalent protein aliquots were subjected to immunoblotting for PARP, caspase-8 and caspase-9. As shown in Figure 6, already at the earliest time point examined (6 h) the 89 kDa PARP cleavage product was detectable in TNF-treated 3E9 cells cultured at 40.0–40.5°C (8th lane), but not in the normothermic (2nd lane) or non-TNF-treated controls (1st and 7th lanes). The PARP cleavage product became more prominent in TNF-treated hyperthermic cells and marked degradation of PARP was evident at 24 h (12th lane). Loss of full length PARP reflects a more advanced stage of apop-

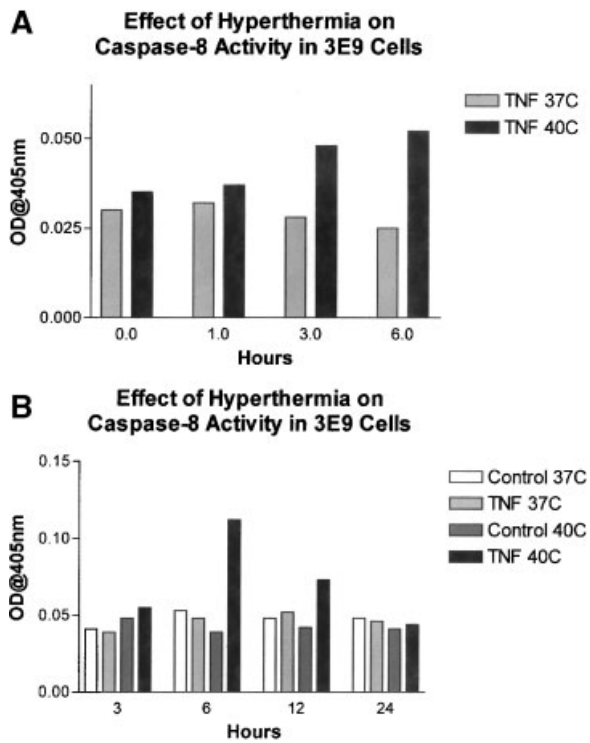

Fig. 5. Effect of TNF with and without febrile hyperthermia on caspase-8 activation in 3E9 cells. Cultures were prepared as described in Figure 4 and cells harvested at several times up to 24 h. Caspase-8 activity in cell lysates was evaluated by degradation of the colorimetric substrate, IETD-p-nitro-anilide at 37°C, and is expressed as increased absorbance at 405 nm. Hyperthermia caused greater and more durable caspase-8 activation by TNF in 3E9 cells compared to normothermia.

tosis. PARP cleavage in the normothermic, TNF-treated 3E9 cells was evident only after 24 h (6th lane), and was not observed without TNF treatment (e.g., 5th lane). Degradation of the pro-caspase-8 band became evident by 12 h in hyperthermic 3E9 cells (9th lane), was enhanced by TNF (10th lane), and was similar to the caspases-8 activation profile (Fig. 5). The pattern for caspase-9 processing was similar, but more pronounced (Fig. 6); degradation of pro-caspase-9 required hyperthermia and was markedly enhanced by hyperthermia alone by 12 h (9th lane), but was more extensive when combined with TNF (10th lane). These results suggest that hyperthermia potentiates TNF-induced apoptosis by increased activation of the extrinsic apoptotic pathway and by recruitment of intrinsic caspases.

Time-lapse fluorescence microscopy. Parental MCF-7 or 3E9 cells were treated with the mitochondrial membrane potential (MMP)-sensitive dye, TMRE, prior to triggering with TNF and initiation of image acquisition on a temperature-controlled stage. Due to a progressive decline in TMRE fluorescence over time even in controls only images acquired through 8 h of incubation were evaluated.

Neither cell line incubated at 37°C demonstrated a distinct TNF-dependent decline in TMRE fluorescence over 8 h of incubation, indicating a lack of perturbation of MMP under these conditions (data not shown). However, at 40–40.5°C, TMRE fluorescence from TNF-treated 3E9 cells was markedly reduced by ~8 h, compared to the fluorescence from non-TNF-treated control cells (Fig. 7). This reduction was not evident at earlier time-points, nor was it evident in parental MCF-7 cells, indicating that effects on MMP were not induced under these conditions. Taken together, the data in Figures 4–7 are consistent with early TNF-dependent, hyperthermia-augmented activation of caspase-8 in parental MCF-7 cells, whereas in 3E9 cells, activation of caspase-8 is more protracted and closely coincident with MMP decline, activation of caspase-9 and engagement of the intrinsic pathway.

Pharmacological Blockade of JNK Pathway Does not Inhibit TNF/Hyperthermia-Mediated Killing of Parental MCF-7 Cells

Parental MCF-7 cells were pretreated for 30 min with SP600125, a selective reversible inhibitor of JNK1, 2, and 3, previously shown to inhibit TNF-induction of c-jun phosphorylation in these cells [Donato and Klostergaard, 2004]. Cells were incubated with a concentration range-range of TNF up to 4 nM. After 24 h of further incubation at 40.0–40.5°C, cell survival was quantified, and is compared to control cells

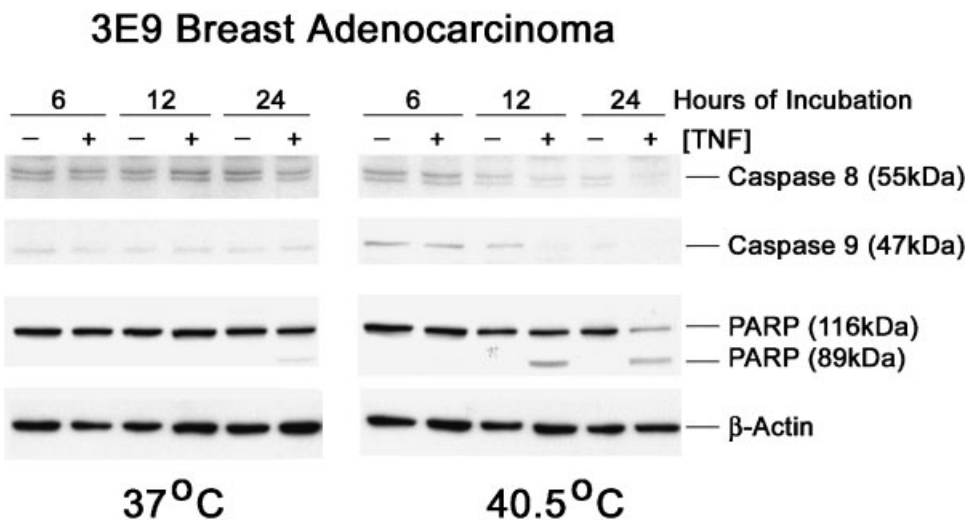

Fig. 6. Effect of TNF with and without febrile hyperthermia on PARP, pro-caspase-8 and pro-caspase-9 cleavage in 3E9 cells. Cells were plated, and after overnight incubation, were treated with 1 nM (17 ng/ml) TNF immediately prior to further incubation at 37°C or at 40.0–40.5°C. Lysates were prepared from cells harvested at 6, 12, and 24 h and subjected to SDS–PAGE and immunoblotting for PARP, caspase-8 and caspase-9. The uncleaved PARP (116 kDa) and cleaved (89 kDa) PARP product, as well as pro-caspase-8 (55 kDa) and pro-caspase-9 (47 kDa) are depicted by arrows. Hyperthermia accelerated and enhanced the extent of TNF-induced PARP cleavage.

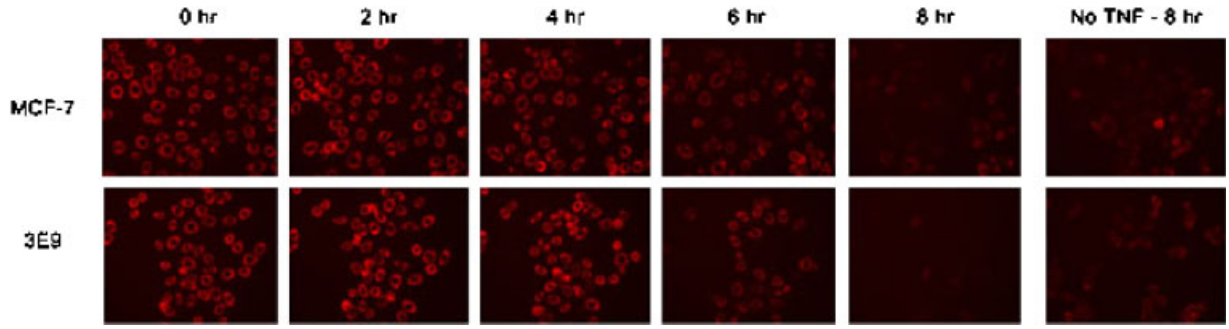

Fig. 7. Effect of TNF and hyperthermia on MMP in parental MCF-7 and 3E9 cells. MCF-7 and 3E9 cells were established in 12-well plates prior to staining with TMRE for 1 h, preceding TNF treatment under hyperthermic conditions and commencement of fluorescence image acquisition at time 0 h. Images collected through 8 h are shown, and are compared to the non-TNF control at 8 h (right-most panels). There was no significant decline in TMRE fluorescence for either cell line through 6 h, but by 8 h, TNF treatment of 3E9 cells caused a marked decrease in fluorescence compared to the non-TNF control. Thus, there was no evidence for early, TNF-dependent mitochondrial perturbation. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

in Figure 8. Direct pharmacological inhibition of the SAPK/JNK pathway clearly did not abrogate TNF-mediated killing of MCF-7 cells in this hyperthermia model.

Ceramide Fluxes in TNF- and/or Hyperthermia-Treated Parental MCF-7 Cells

LC/MS studies of the distribution of ceramide species in parental log-phase control MCF-7 cells incubated at 37°C revealed that the principal ceramide species were nervonyl (C24:1, cis-15), lignoceroyl (C24:0), stearoyl (C18:0), dihydro-palmitoyl (C16), and oleoyl (C18:1, cis-9), in order of decreasing abundance [Donato and Klostergaard, 2004; Table II]. Expressed in ng per 1×10^6 cells, the sum of nervonyl (50.3 ± 3.0), lignoceroyl (37.1 ± 1.5), stearoyl (17.3 ± 1.0), dihydro-palmitoyl (10.8 ± 0.9), and oleoyl (0.21 ± 0.04) ceramides for control cells harvested at 0 h was 115.7. For control cells incubated at 37°C and harvested at 8 h, total ceramide mass declined slightly to 94.6 ng/1×10^6 cells, mostly due to decreased signals for nervonyl (40.0 ± 3.3 ng), lignoceroyl (32.2 ± 7.9 ng), and stearoyl (12.9 ± 0.5 ng) ceramides. For TNF-treated cells incubated at 37°C and harvested at 8 h, the time at which the first signs of cytotoxic effects were evident microscopically, total ceramide mass declined further to 85.4 ng/1×10^6 cells, due to additional decrements in nervonyl (37.0 ± 5.6 ng) and particularly in lignoceroyl (23.7 ± 3.0 ng) ceramides. Only the signal for dihydro-palmitoyl ceramide (10.5 ± 1.2 ng) increased slightly compared to 8 h controls (9.4 ± 0.6 ng), but was not above the 0 h control levels (10.8 ± 0.9 ng).

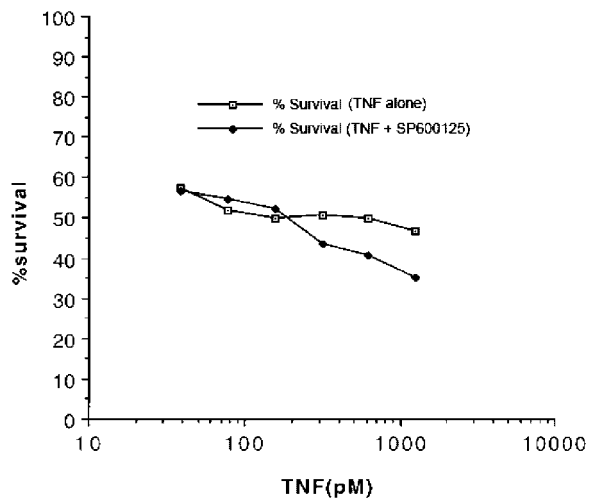

Fig. 8. Effect of pharmacological inhibition of the JNK pathway on TNF-mediated, hyperthermia-enhanced killing of parental MCF-7 cells. Parental MCF-7 cells were treated with the specific JNK inhibitor, SP600125 (20 μM), before addition of TNF at up to 4 nM and then incubated at 40.0–40.5°C.; cell survival (at 24 h) was determined by neutral red assay. The results represent the mean of sextuplicate determinations; the error bars are obscured by the symbols for the mean. The addition of SP600125, which alone caused some growth inhibition (data not shown) tended to increase, not decrease sensitivity to TNF.

Hyperthermia (incubation at 40.0–40.5°C) alone for 8 h caused a marked reduction in total ceramides, to 68.3 ng/1×10^6 cells, again mostly due to decreased nervonyl (30.0 ± 9.2 ng) and stearoyl (10.0 ± 5.3 ng) ceramides. Hyperthermia combined with TNF treatment resulted in slightly higher total ceramide (95.9 ng/1×10^6 cells) than TNF treatment under normothermic conditions (94.6 ng/1×10^6 cells);

TABLE II. Effects of TNF/Hyperthermia Treatment on Ceramide Content in Parental MCF-7 Cells

	Ceramide				
Treatment	Lignoceroyl	Nervonyl	Stearoyl	Oleoyl	Dihydro-palmitoyl
0 h, No TNF	37.1 ± 1.5[a]	50.3 ± 3.0	17.3 ± 1.0	0.21 ± 0.04	10.8 ± 0.9
37°C					
8 h, No TNF	32.2 ± 7.9	40.0 ± 3.3	12.9 ± 0.5	0.13 ± 0.00	9.4 ± 0.6
8 h, TNF (+)[b]	23.7 ± 3.0	37.0 ± 5.6	14.0 ± 0.7	0.21 ± 0.04	10.5 ± 1.2
40.0–40.5°C					
8 h, No TNF	19.4 ± 4.5	30.0 ± 9.2	10.0 ± 5.3	0.21 ± 0.08	8.7 ± 4.1
8 h, TNF (+)[b]	23.2 ± 2.4	41.0 ± 5.7	16.5 ± 2.8	0.25 ± 0.02	14.9 ± 1.4

[a]Nanogram per 1×10^6 cells; mean ± SEM from quadruplicates.
[b]68 ng/ml (4 nM).

however, this was still lower than initial control levels (115.7 ng/1×10^6 cells).

Thus, TNF treatment, with or without hyperthermia, induced a decrease in total ceramide, contrary to the increase anticipated; the only component that showed a tendency for even slight increase was dihydro-palmitoyl ceramide: this species increased ~38% with the combination of TNF and hyperthermia (14.9 ± 1.4 ng compared to 10.8 ± 0.9 ng for controls). However, this species lacks the *trans*-4,5 double bond and should therefore be inert in apoptosis induction.

DISCUSSION

In the current study, several key observations have been made regarding the effects of hyperthermia on breast tumor cell apoptosis. This is the first report that the strong TNF-resistance of the human MCF-7 breast adenocarcinoma can be markedly reversed by febrile (40–40.5°C), long-duration hyperthermia. In contrast, a previous study using TNF-sensitive MCF-7 cells [Li and Oberley, 1997] demonstrated only minimal enhancement of TNF killing by acute hyperthermia (43°C, 1 h). This advantage with febrile hyperthermia is of particular note, given the increasing interest in the systemic application of febrile hyperthermia in clinical oncology [Buell et al., 1997; Robins et al., 1997; Hafstrom and Naredi, 1998; Olieman et al., 1998; Lindner et al., 1999; Rossi et al., 1999].

We have strongly linked the molecular basis for hyperthermic potentiation of TNF-mediated apoptosis in this model to caspase activation: first, by using the broad-spectrum caspase inhibitor, Z-VAD-fmk to block killing (Fig. 2A,B); then, more specifically, we have defined a key for role for activation of caspase-8, a component of the DISC associated with TNF receptor. The role for caspase-8 was established both by an activity assay (Figs. 4 and 5) and by a more selective inhibitor of caspase-8, Z-IETD-fmk (Table I). Thus, hyperthermia acted at, or upstream of caspase-8, potentiated the apoptotic signal or lowered the apoptotic threshold. Of note, hyperthermic potentiation of TNF-induced PARP cleavage occurred in these cells (Fig. 3), reported to be caspase-3 deficient [Janicke et al., 1998]. Nevertheless, the MCF-7 model has been extensively studied with respect to mechanisms of apoptosis.

Our results differ in some respects from those revealed in human malignant glioma cells following CD95 ligation in conjunction with hyperthermia [Hermisson et al., 2000]. These investigators observed strong apoptotic potentiation with this combination, which was inhibited by the viral caspase inhibitor, crm-A, implicating convergence on caspase-8. Nevertheless, levels of the active cleavage product of caspase-8, p18, were actually reduced, and not increased, in CD95-activated cells subject to hyperthermia. However, hyperthermia markedly increased CD95-mediated cleavage of Ac-DEVD-amc, which was interpreted as evidence for activation of caspase activity upstream from caspase-3. Curiously, levels of cleaved caspase-3 were not enhanced by hyperthermia, nor was cleavage of PARP, the latter contrasting with our results (Fig. 3). These investigators suggest that a feedback loop linking caspases-3, -8, and -9 [Slee et al., 1999] might be operant. In that light and with regard to possible crosstalk between the extrinsic and intrinsic apoptotic pathways and a role for caspase-9 in our model, we have shown that hyperthermia, but not normothermia, leads to activation of caspase-9, and that this was enhanced by combination

with TNF (Fig. 6). We were not able to find evidence supporting the existence of a retrograde pathway for activation of caspase-8, as neither loss of MMP nor degradation of procaspase-9 preceded caspase-8 activation. These responses were, in fact, closely linked temporally. Of note, our observation of caspase-9 activation with febrile hyperthermia appears unique, as numerous other studies have used acute levels of hyperthermia [Enomoto et al., 2001; Little and Mirkes, 2002; Choi et al., 2003; Mauz-Korholz et al., 2003; Zhang et al., 2003; Souslova and Averill-Bates, 2004; Yasumoto et al., 2004].

In contrast to the role we have defined for caspase activation in this MCF-7 model, the anti-oxidant BHA was without effect on TNF killing, with or without hyperthermia (Fig. 2A,B), differing significantly from the results of Li and Oberley [Li and Oberley, 1997]. These investigators did not report studies in their model regarding a possible role for caspase activation in TNF or TNF/hyperthermia killing. Further, in our studies, hyperthermia sustained activation of caspase-8 following TNF incubation. Finally, the distinctions between TNF- and Dox-induced cell-death were evident both in their interactions with hyperthermia (Fig. 1A, B) and in their susceptibility to inhibition by the broad-spectrum caspase inhibitor, Z-VAD. Hyperthermia had a much greater effect on TNF-induced apoptosis and Dox-mediated killing did not appear to be solely dependent on caspases (data not shown).

Increased intracellular ceramide, derived by sphingomyelinase-mediated cleavage of membrane sphingomyelin, has been proposed to be an essential downstream mediator of TNF-induced killing, including MCF-7 cells [Liud et al., 1998; Luberto et al., 2002]. Our results indicate that the combined levels of the most abundant ceramide species in MCF-7 cells actually decline slightly in response to TNF treatment, rather than increase (Table II); the combination of TNF and hyperthermia, which markedly increases apoptosis (Figs. 1–3), still did not result in increased total ceramide (Table II). The only species that increased modestly in response to TNF and hyperthermia, dihydro-palmitoyl (Table II), lacks the *trans*-4, 5 double bond, essential for apoptosis induction by ceramides, although not for sphingosines [Bielawska et al., 1993]. Therefore, we are lead to conclude that increased ceramide levels are not essential for induction of apoptosis in MCF-7 cells in response to TNF and/or hyperthermia. However, it remains possible that hyperthermia affects other aspects of the metabolism of ceramides, such that although ceramide levels themselves remain at a somewhat steady state, the flux of ceramide into sphingosines, via the action of ceramidase, is enhanced.

The apoptotic mechanism underlying the TNF death response in parental MCF-7 and in heated 3E9 cells contrasts markedly with that observed in murine L929 cells [Vercammen et al., 1998; Leroux et al., 2001]. In L929 cells, TNF induces a necrotic response dependent on ROI, which is quenched by BHA and enhanced strongly, rather than inhibited, by the caspase inhibitor, Z-VAD [Vercammen et al., 1998]. We have observed that febrile hyperthermia markedly increases TNF-induced ROI production and necrotic death in the L929 model, which is again quenchable by BHA, but enhanced by Z-VAD [Leroux et al., 2001]. In L929 cells, a likely molecular target of hyperthermia is the mitochondrial mechanism of ROI production.

As mentioned, in our MCF-7 model a possible target of hyperthermia is upstream of caspase-8 activation, such as enhancement of assembly of TNF-receptor/DISC (death-inducing signaling complex) complexes. Other downstream targets or targets on other converging pathways aside from caspase-9, ultimately inhibitable by Z-VAD, are also possible candidates. These targets would include the inhibitor of apoptosis protein (IAP), which is initiated at TRAF2 [Rothe et al., 1995; Liston et al., 1996; Orth and Dixit, 1997]. However, the rapid kinetics of hyperthermic potentiation of TNF killing, as measured by activation of caspases-8, suggests that a rather direct mode of action is operant, not one involving downstream loops. It is also unlikely that hyperthermia affects signaling downstream of caspase-8 since Z-IETD provided effective protection from apoptosis induced by TNF combined with hyperthermia (Table I). The inability of pharmacological blockade of the JNK pathway to even slightly block killing of parental MCF-7 cells by TNF/hyperthermia (Fig. 8) suggests that this pathway is of minimal mechanistic significance in this response, as it is in the normothermic response [Donato and Klostergaard, 2004]. Further, since alterations in TNF-induced NF-κB activation were not evident in the 3E9 cells compared to parental cells and did not correlate

with TNF resistance [Doman et al., 1999], we see little indication for its contribution to the hyperthermic potentiation of TNF apoptotic activity.

Several contributors may underlie the effects of hyperthermia on caspase-8 activation, recruitment of the intrinsic pathway, and increased apoptosis of both parental and TNF-resistant MCF-7 cells. Assembly of the TNF receptor complex, including accessory or docking proteins such as cIAP, FLIP, or TRAF2, may be regulated by hyperthermia. It is also possible that recruitment of post-mitochondrial regulators of apoptosis and involvement of downstream caspases that facilitate caspase-8 activation also play a role in the hyperthermic effects on MCF-7 cells. These possibilities are currently being examined. The sustained activation of caspase-8 with TNF and hyperthermia, compared to TNF and normothermia, may point to blocking of proteosome-dependent degradation or i-FLICE/FLIP downregulation.

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