Free Radical Research, Volume 38 Number 10 (October 2004), pp. 1119–1132

Taylor & Francis
healthsciences

Overexpression of Manganese Superoxide Dismutase Promotes the Survival of Prostate Cancer Cells Exposed to Hyperthermia

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Accepted by Professor B. Kalyanaraman

(Received 12 February 2004; In revised form 3 August 2004)

It has been hypothesized that exposure of cells to hyperthermia results in an increased flux of reactive oxygen species (ROS), primarily superoxide anion radicals, and that increasing antioxidant enzyme levels will result in protection of cells from the toxicity of these ROS. In this study, the prostate cancer cell line, PC-3, and its manganese superoxide dismutase (MnSOD)-overexpressing clones were subjected to hyperthermia (43°C, 1 h). Increased expression of MnSOD increased the mitochondrial membrane potential (MMP). Hyperthermic exposure of PC-3 cells resulted in increased ROS production, as determined by aconitase inactivation, lipid peroxidation, and H_2O_2 formation with a reduction in cell survival. In contrast, PC-3 cells overexpressing MnSOD had less ROS production, less lipid peroxidation, and greater cell survival compared to PC-3 Wt cells. Since MnSOD removes superoxide, these results suggest that superoxide free radical or its reaction products are responsible for part of the cytotoxicity associated with hyperthermia and that MnSOD can reduce cellular injury and thereby enhance heat tolerance.

Keywords: Superoxide dismutase; Hyperthermia; Aconitase; Lipid peroxidation; Heat shock protein; Electron paramagnetic resonance

Abbreviations: 3-CP, 3-carboxy-proxyl; CuZnSOD, copper–zinc superoxide dismutase; DMPO, 5,5-dimethyl-pyrroline-1-oxide; DTPA, diethylenetriaminepentaacetic acid; ETC, electron transport chain; FAMES, fatty acid methyl esters; GPx, glutathione peroxidase; HRP, horseradish peroxidase; HSP, heat shock protein; HT, hyperthermia; MMP, mitochondrial membrane potential; MnSOD, manganese superoxide dismutase; PI, propidium iodide; PUFA, polyunsaturated fatty acids; ROS, reactive oxygen species

INTRODUCTION

Therapeutic hyperthermia is being investigated as an adjuvant for many cancer therapies, including radiation and photodynamic therapy. During hyperthermia, tissue is heated to 41–43°C. These elevated temperatures could induce oxidative stress[1–4] and may enhance cytotoxicity in cells/tissues.[5] Based on research observations, we hypothesize an oxidative stress theory of heat shock with the following components:

(a) heat shock can promote the formation of free radicals and related reactive oxygen species (ROS) from metabolic pathways;
(b) these ROS can cause part of the cellular injury produced by heat; and
(c) this cellular injury can be blunted if protective proteins are induced.

Heat shock induces numerous protective proteins, referred to as heat shock proteins (HSP)[6,7] that protect cells by maintaining structure and function of proteins. Antioxidant enzymes can eliminate or repair heat-induced, ROS-mediated cell injury and are a subset of these protective proteins.[8–11] In cancer cells antioxidant enzymes are often down regulated.[12,13] Changes

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ISSN 1071-5762 print/ISSN 1029-2470 online © 2004 Taylor & Francis Ltd
DOI: 10.1080/10715760400010470

in antioxidant enzyme levels can have profound effects on the biology of tumor cells, e.g. change in growth rate,[14–16] phenotype[17–19] and altered resistance to antitumor therapies.[20–23] Thus, cancer therapies that have a mechanism of action involving oxidation/reduction reactions could be influenced by altered levels of antioxidants.

It was reported that more superoxide radical is generated in hyperthermia-treated cells than in cells at physiological temperatures, which suggests that heat increases oxidative stress in cells and tissues.[24] This study investigates the oxidative stress theory of heat shock. Several lines of evidence have shown that the toxic cellular injury caused by hyperthermia is due to oxidative stress with subsequent lipid peroxidation.[4,25,26] Therefore, we propose that heat toxicity is partially due to oxidative stress from superoxide generation with resultant lipid peroxidation and that antioxidant enzymes, like SOD, can eliminate or blunt heat-induced, ROS-mediated cell injury.

In normal metabolism, a very small percent of the oxygen consumed is converted to $O_2^{\cdot-}$ by the mitochondrial electron transport chain.[27] In isolated muscle cell mitochondria it was found that an increase in temperature causes mitochondrial uncoupling leading to an increased rate of $O_2^{\cdot-}$ production.[28] Manganese superoxide dismutase (MnSOD) is an antioxidant enzyme located in the mitochondria where it protects cells from the detrimental effects of superoxide by converting $O_2^{\cdot-}$ to H_2O_2. Thus, we hypothesize that up-regulation of MnSOD could protect cells from the toxicity associated with hyperthermia.

The overall objectives of this study were:

(1) to investigate the mechanism of cytotoxic effect of hyperthermia on prostate cancer cells, specifically to determine if ROS such as $O_2^{\cdot-}$ have a role in the heat-induced toxicity in PC-3 cells;
(2) to determine the role of MnSOD, a mitochondrial antioxidant enzyme, on heat-induced toxicity; and
(3) to examine the downstream consequence of ROS production in cells after HT. We measured free radical-, H_2O_2- and lipid hydroperoxide-production, changes in mitochondrial membrane potential (MMP), and cell survival after heating prostate cancer cells.

METHODS

Cell Culture

The human prostate carcinoma cell line, PC-3 (Wt), was purchased from the American Type Culture Collection (ATCC, Rockville, MD). The PC-3 Wt cells were cultured in modified Ham's F12 medium with 2 mM L-glutamine, 1.5 g/l sodium bicarbonate, 10% fetal bovine serum (Hyclone), and 1% penicillin/streptomycin.

To overexpress MnSOD, PC-3 cells were stably transfected with appropriate pcDNA3 plasmids; the MnSOD clones have been characterized with respect to their antioxidant profile (see "Results" section). They were grown in modified Ham's F12 medium with 2 mM L-glutamine, 1.5 g/l sodium bicarbonate, 10% fetal bovine serum (Hyclone) and 350 μg/ml G418. For all experiments, cells were harvested when they were 70–80% confluent. For heat exposure, cells were seeded in tissue culture flasks, allowed to grow for 24 h, and then exposed to heat. Antibiotics were removed one passage before conducting experiments.

Heat Treatment and Experimental Strategy

Cells were grown in standard T-25 or T-75 cell culture flasks. Cells were provided with fresh medium immediately before heating. Hyperthermia was applied at 43°C for 1 h in a gently circulating water bath maintained at 43 ± 0.1°C for 1 h. Controls were maintained at 37 ± 0.1°C. After heat treatment, the medium was replaced with fresh medium and cells were returned to a 37°C incubator for recovery. At desired times (0, 1, 3, 6, or 24 h) after heat-exposure, cells were harvested either by scraping or trypsinizing depending on the experiments. The 0 h-time point represents cells that were harvested immediately after heat-exposure (< 5 min).

Assays

Cells were washed 3 times with cold PBS and then harvested by scraping. Cell pellets (centrifuged at 200*g* for 5 min at 4°C) were resuspended in potassium phosphate buffer (pH 7.8, 50 mM) and sonicated with three bursts of 20 s each using a Vibra Cell sonicator with a cup horn at full power (Sonics and Materials Inc., Danbury, CT). Cell protein content was determined by the method of Bradford.[29] The concentration of H_2O_2 was measured in the cell culture medium before and after heat treatment spectroflurometrically as described by Panus *et al.*[30] and the results are presented as pmol $cell^{-1}\,h^{-1}$. The cells were cultured in phenol red-free HBSS during the collection period. To determine relative levels of ROS, aconitase activity was measured in the whole cell lysate by the oxidation of citrate to α-ketoglutarate coupled to the reduction of $NADP^+$ as described by Gardner *et al.*[31] Aconitase activity was expressed as ΔAbsorbance/50 μg soluble protein.

Western Blot Analysis

The protein expression of MnSOD and CuZnSOD were visualized by western blot analysis. Cells

were washed thrice with PBS and cell pellets were resuspended in 50 mM potassium phosphate buffer (pH 7.8) and sonicated. Western blotting was performed as described previously[32] using primary antibodies against either MnSOD (1:1000) or CuZn-SOD (1:250) (both made in Dr L.W. Oberley's laboratory).[33] Anti-rabbit IgG-horseradish peroxidase conjugate was used as the secondary antibody and detection was via the chemiluminescence ECL kit. After transfer, the gels were stained with Coomassie blue to check the protein loading.

Native Gel Assay

SOD activity was visualized by native PAGE according to the method of Beauchamp and Fridovich.[34] Briefly, cell extracts were prepared as described under western blotting and were loaded onto a 12% polyacrylamide gel with a 5% stacking gel for electrophoresis. Achromatic bands corresponding to MnSOD and CuZnSOD appeared against a blue background after the gel was stained by incubating it in 2.43 mM nitroblue tetrazolium (NBT) and 28 μM riboflavin/28 mM N,N,N′,N′-tetramethylethylenediamine (TEMED) for 30 min in the dark, followed by washing, and illumination under bright fluorescent light.

Clonogenic Assay

To determine clonogenic cell survival, cells (9×10^5) were plated in a tissue culture flask 24 h before experiments. To determine the effect of heating for different lengths of time (Fig. 1), cells were heated at 43°C for 1, 3, or 5 h. After heating, cells were returned to a 37°C incubator (5% CO_2) for 12 h to recover and then trypsinized and seeded into 60 mm tissue culture dishes after appropriate dilutions. To perform the clonogenic survival assay, cells were heated at 43°C for 1 h. Cells were then moved back to the incubator to recover and at different time points cells were trypsinized, counted, and seeded into 60 mm dishes.

After 14 days of incubation at 37°C, individual colonies (>50 cells/colony) were fixed with 70% ethanol and stained with Coomassie blue. The colonies were counted with the FluorChem 8800 Imaging System (Alpha Innotech Corporation, San Leandro, CA) using a visible light source.

EPR Detection of Radical Formation during HT

PC-3 (Wt and Mn32) cells (1.5×10^6) were grown to 70% confluence and were subjected to HT at 43°C for 1 h. After heat treatment, the flasks were returned to 37°C in a tissue culture incubator and allowed to recover for 0, 1, 6, or 24 h. After recovery, the media was removed and the cells were rinsed with PBS buffer (pH 7.4). To the cell monolayer, 1 ml of chelated (using the chelating resin, iminodiacetic acid, sodium form, dry mesh 50–100, from Sigma, St Louis, MO) PBS buffer (pH 7.4) containing DTPA (110 μM) and 100 mM of the spin trap DMPO was added. Then $FeSO_4$ (100 μM) was added to the cells to initiate radical reactions and the cells were scraped and transferred immediately into an extraction flask. The lipid-derived radical adducts of DMPO when separated by Folch extraction[35] are stabilized.[36] Briefly, the lipids were extracted with ice-cold chloroform:methanol with 0.9% saline (2:1:0.5 vol/vol). Phase separation was accomplished by incubating at room temperature for 12 h. The bottom organic layer was removed, dried under nitrogen, resuspended in 500 μl of degassed ethyl acetate, and transferred to a flat cell for EPR measurements. The extraction was also carried out in the absence of cells and without the addition of Fe^{2+} as controls.

As control experiments, linolenic acid and DHA were exposed to air-oxidation and subjected to the above described extraction procedure and EPR spectra were recorded. EPR spectra were recorded using a Bruker EMX-300 series spectrometer with a magnetic field modulation frequency of 100 kHz, microwave power of 40mw modulation amplitude 1.0 G, and receiver gain 10^4–10^6.

The spectral analyses were done by simulation of the spectra using the NIEHS simulation program.[37] To assist in the assignment, DMPO spin adduct splitting constants and *g*-values were compared with published values given in the Spin Trapping Database.[38] The spectra were simulated using hyperfine values reported for individual components that were generated independently.[39–42] The EPR spectra derived from linolenic acid and DHA were also simulated and the parameters were compared with that of cell samples.

Estimates on the concentration of radical adducts were obtained by measuring the normalized area under the radical adduct peak, using 3-carboxy proxyl (3-CP) (Aldrich Chem Co., Milwaukee, WI) as standard. The 3-CP solution was standardized spectrophotometrically.[43]

PUFA Analysis

Fatty acid analysis was performed by gas chromatography after the conversion of fatty acids into fatty acid methyl esters (FAMEs). PC-3 Wt and Mn32 cells were subjected to HT. After heating, the cells were incubated at 37°C for 24 h. Cells were then scraped and centrifuged for 5 min at 400*g*. The supernatant was removed, the cell pellet resuspended in PBS (pH 7.4), and centrifuged at 400*g*. Lipid analysis was done as described by Wagner *et al.*[44] The FAMEs were identified and quantitated by comparison with authentic FAME standards (Supelco).

Lipid Hydroperoxide Assay

Total cellular lipid hydroperoxides were determined using the LPO assay kit (Cayman Chemical Co., Ann Arbor, MI) according to the manufacturer's instructions. Briefly, in this assay, hydroperoxides oxidize ferrous iron to ferric iron. Ferric iron forms a complex with thiocyanate that can be measured spectrophotometrically at 500 nm.

Cells (3×10^6) were subjected to hyperthermia and then harvested by scraping in PBS at specific time points along with the control cells. Cells were then subjected to chloroform/methanol (2:1 v/v) extraction. The lipids were separated into the chloroform layer and the hydroperoxides were measured.

Determination of Mitochondrial Membrane Potential

It has been shown that uptake of rhodamine123 into mitochondria depends on MMP.[45] After heat treatment, at different recovery times cells were trypsinized, washed, pelleted, and resuspended in F-12 media containing 25 μM rhodamine123 (Rh123, at 37°C) for 30 min. Rh123 fluorescence was determined using FACScan (Becton Dickinson, San Jose, CA.). To allow elimination of the dead cells, propidium iodide (2 μg/ml) was added just before the FACS analysis.

Statistical Analysis

A single factor ANOVA, followed by a *post hoc* Bonferroni test, or Student's *t*-test was used to compare statistical differences between means. To compare different groups over time for aconitase activity, the linear mixed model analysis was used[46] and for other time course experiments, two-way ANOVA was used. Statistical analyses were done with SAS software (version 8.2) for Windows at a significance level of 0.05.

RESULTS

To study the relationships between the generation of ROS, SOD activity, and lipid peroxidation, PC-3 cells and their MnSOD-transfected clones were subjected to HT. PC-3 cells were transfected with MnSOD cDNA and three clones overexpressing MnSOD have been characterized.[47] The transfected clones (Mn5, Mn32, Mn98) had a 7- to 8-fold increase in MnSOD activity compared to parental (Wt) or vector alone control (Neo).

The CuZnSOD-activity was below detection limit in the MnSOD-transfected cells. The vector control (Neo) had a 5-fold increase in CuZnSOD activity compared to parent PC-3 Wt cells. The H_2O_2-removing enzyme GPx had slightly decreased activity in all three clones. Another H_2O_2-removing enzyme, catalase, did not change significantly from that of parental PC-3 Wt cells, except for clone Mn98 (~4-fold increase).

Hyperthermia: Optimal Conditions and Change in SOD Levels

Cells that were heated at 44 and 45°C had very low survival compared to those exposed to 43°C (data not shown). Therefore, 43°C was chosen for this study.

The clonogenic cell survivals of PC-3 Wt cells and MnSOD clones after hyperthermia at 43°C for 1, 3, or 5 h are shown in Fig. 1. The transfected clones had a 7- to 8-fold increase in MnSOD activity. Those clones that overexpressed MnSOD had greater survival at all time points compared to Wt. The Mn32 clone had the greatest survival for all heating times; Mn98 had intermediate survival and the survival of Mn5 was only a little greater than the controls. After 1 h of heating, Mn32 cells had 72% survival compared to only 45% for the Wt ($P < 0.05$). There was a marked decrease in cell survival with increasing heating time at 43°C (3 and 5 h); the survival of the Wt was < 20% at 3 h and <10% at 5 h in comparison with the unheated control cells. From these results, we chose 1 h at 43°C as the conditions for the hyperthermia experiments in this study. These results demonstrate that MnSOD protects PC-3 cells from heat-induced toxicity.

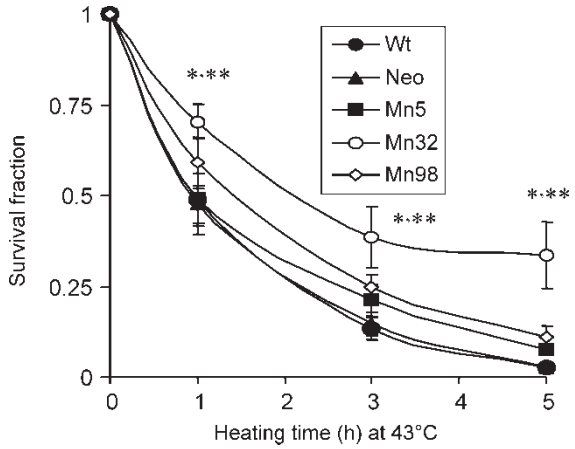

FIGURE 1 Clonogenic survival of PC-3 cells exposed to hyperthermia for different heating times. PC-3 cells were subjected to heat at 43°C for 1, 3, and 5 h and the effect of heat on cell survival was determined. After heat treatment, cells were incubated for 12 h at 37°C, 5% CO_2 and then plated to form colonies. Survival curves were plotted as the log of the surviving fraction of cells vs. heating time. Values are mean ± SE. According to two-way ANOVA, there is not a statistically significant interaction between group and time ($P = 0.72$). According to Bonferroni's multiple comparison procedure, the following groups are significantly different ($P < 0.05$): Wt vs. Mn32 and Mn98, Neo vs. Mn32 and Mn98, Mn5 vs. Mn32. Statistical differences at each time point 1, 3, and 5, are: *$P < 0.0001$ for Wt vs. Mn32, **$P < 0.0001$ Neo vs. Mn32.

When PC-3 Wt cells were heated for 1 or 4 h, an increase in MnSOD protein [Fig. 2(a)] and a corresponding increase in activity [Fig. 2(b)] were observed. However, when PC-3 cells that overexpress MnSOD were subjected to the same heat treatment, no detectable change in MnSOD protein levels or activity was noted. In contrast, the CuZnSOD protein levels [Fig. 2(a)] and activity [Fig. 2(c)] did not change with heat in Wt cells. However, in the MnSOD-overexpressing clones, there was an increase in both CuZnSOD protein and activity levels after heat exposure. Thus, SOD activity is affected by heat. ROS production, particularly superoxide radical, has been shown to induce SOD; these results suggest but do not prove that ROS production is increased during hyperthermia.

We have found that in general vector-only transfected clones are not a good control.[16] Other groups have simply picked the Neo clone that most resembles wild type, which biases the results. For this reason, we decided to use the parental cell, PC-3 Wt, as control in our experiments. The three MnSOD-overexpressing clones have almost the same level of MnSOD activity. However, the survival of Mn5 was not statistically increased compared to Wt, moreover, Mn98 had markedly elevated catalase activity. For these reasons, we chose Mn32 for most of the experiments in this study.

Overexpression of MnSOD Preserves MMP before and after HT

Figure 3 illustrates the effect of MnSOD and HT on MMP. As a cationic dye, Rh123 accumulation in mitochondria is driven by a membrane potential, $\Delta\psi_m$. Changes in membrane potential are reflected by changes in total fluorescence intensity.[48] Transfection of MnSOD, a mitochondrial antioxidant enzyme, increased dye uptake significantly, by a factor of 2.5 compared to parent cells ($P < 0.0001$). The mean fluorescence intensity of Wt and Mn32 were 84 ± 3 and 201 ± 18 A.U. ($n = 3$), respectively.

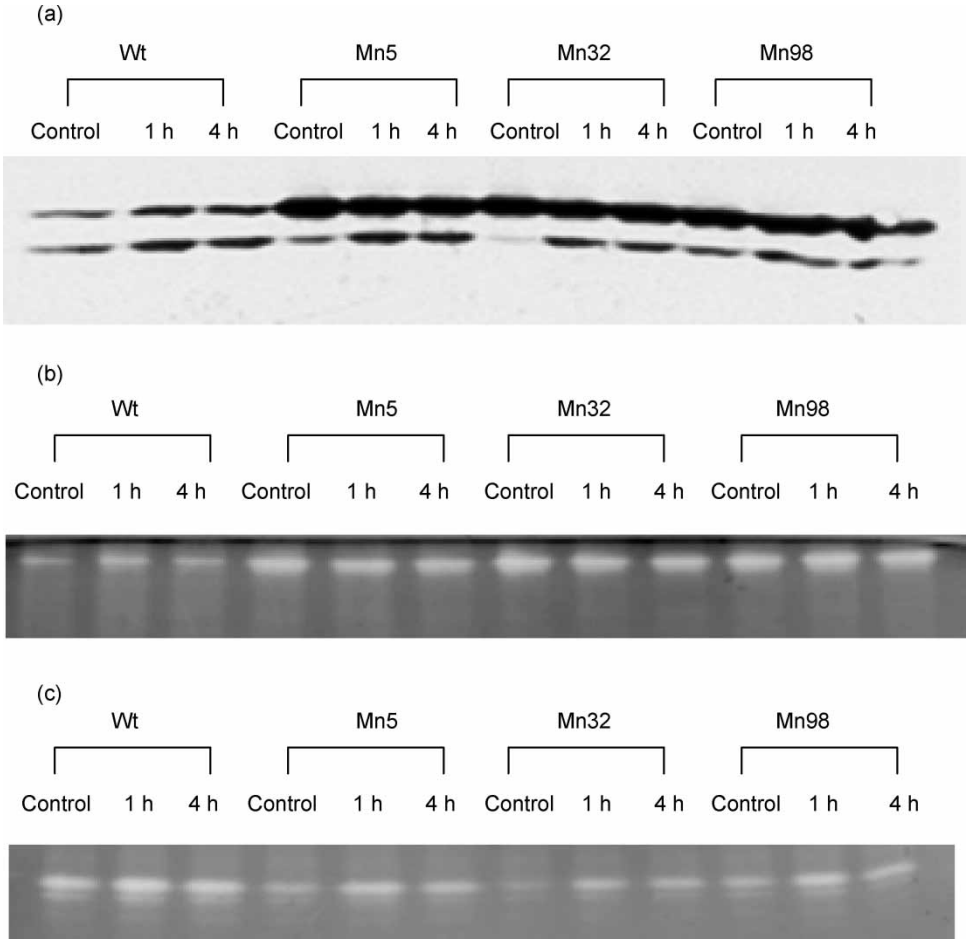

FIGURE 2 SOD protein and activity levels in PC-3 cells before and after heat treatment. (a) Western blot analysis showing the MnSOD (*top band*) and CuZnSOD (*bottom band*) protein measured in PC-3 cells after heat treatment at 43°C. Protein levels were measured prior to heat, and immediately after 1 and 4 h of heat exposure. The gels were stained with Coomassie blue and found to be equally loaded with protein. (b) Activity gel analysis for MnSOD activity. (c) CuZnSOD activity before and after heat treatment (43°C, 1 and 4 h). The activity was measured as the inhibition of the reduction of nitroblue tetrazolium by MnSOD and CuZnSOD in a native gel. The gels were illuminated under fluorescent light and the achromatic band corresponding to MnSOD and CuZnSOD activity appeared on a blue background. The experiment has been done at least twice.

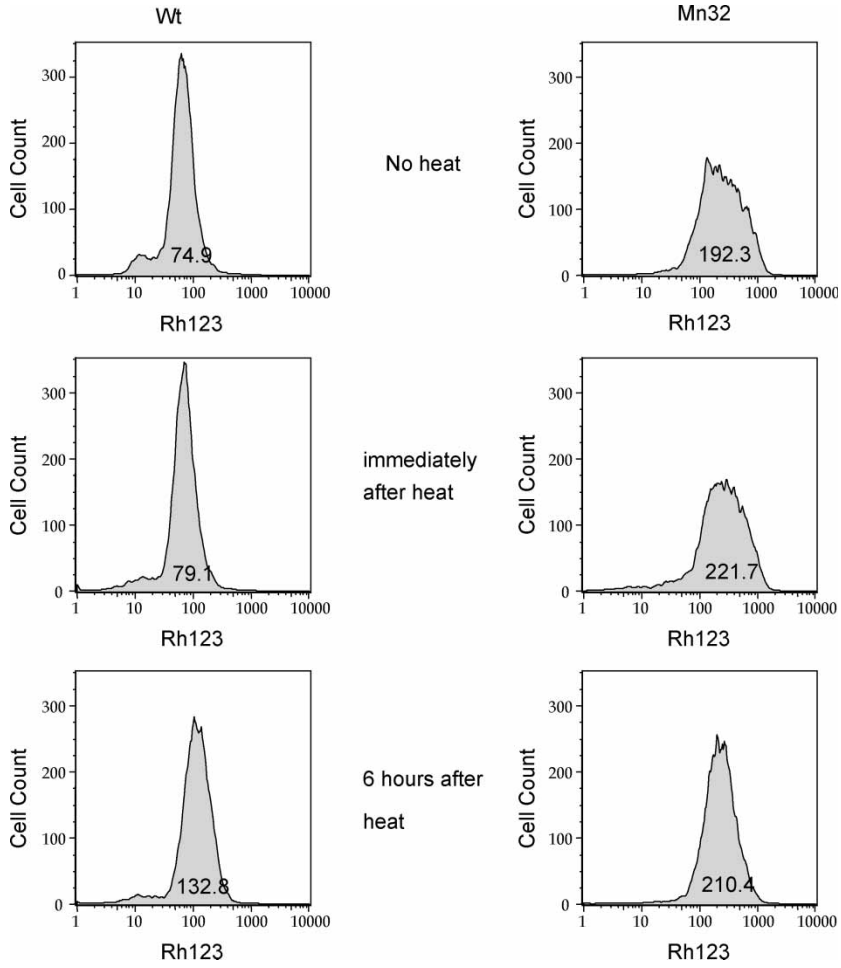

FIGURE 3 Rhodamine 123 fluorescence of PC-3 cells before and after HT. A representative Rh 123 histograms is shown for PC-3 Wt and Mn32 cells that were subjected to HT (43°C for 1 h). Fluorescent intensity values are given for cells, before HT, immediately after HT, and 6 h after HT. There was a significant difference between the mean fluorescent intensities of Wt and Mn32 values: no heat $P < 0.0001$; immediately after heat $P < 0.0001$; 6 h after heat $P = 0.0003$. Results are expressed in arbitrary fluorescence units as a mean of three samples. The mean fluorescence intensities ($n = 3$) for Wt and Mn32 at different time points are given in the results section.

Immediately after heating, the MMP increased only slightly in Wt and Mn32 (Wt = 81.6 ± 1.5 and Mn32 = 235 ± 8 A.U.). However, 6 h after heat treatment dye uptake was 1.8 times greater (Wt = 145 ± 12 and Mn32 = 237 ± 22 A.U.) in Wt compared to unheated samples ($P < 0.0035$), while the membrane potential for Mn32 did not change significantly ($P = 0.125$). The observation that overexpression of MnSOD significantly increased the membrane potential suggests improved mitochondrial membrane integrity. On the other hand, Wt cells with lower MMP may be more vulnerable to heat-induced toxicity. After heat treatment, the increased MMP in Wt cells is suggestive of increased ROS formation during HT.

Generation of ROS after HT as Measured by Aconitase Activity

Increased ROS production in PC-3 Wt cells was demonstrated by measuring the activity of aconitase after heat treatment. It has been previously reported that aconitase is sensitive to superoxide[31] and hydrogen peroxide[49] and that the reaction of superoxide with aconitase releases iron, which can enhance the oxidative damage in mitochondria.[6,50–52] Thus, a decrease in aconitase activity can be regarded as an increase in ROS production.

As seen from the slopes, aconitase activity in Wt and Mn32 cells was nearly identical in the absence of heat treatment [Fig. 4(a)]. When measured immediately after HT, both Wt and Mn32 cells showed a decrease in aconitase activity [Fig. 4(b)]. However, the loss in activity in Wt cells was significantly more (47% loss) than the loss in Mn32 cells (15%) comparing each to their non-heated controls ($P < 0.05$). When the cells were incubated for 1 h after HT, aconitase activity returned in both cell lines [Fig. 4(c)], but was still somewhat diminished compared to that of the respective control, Wt (18% loss) and Mn32 (9%).

The decrease in aconitase activity after hyperthermia in PC-3 Wt cells suggests that there was an increased ROS formation and the fact that this decrease in aconitase was inhibited by MnSOD

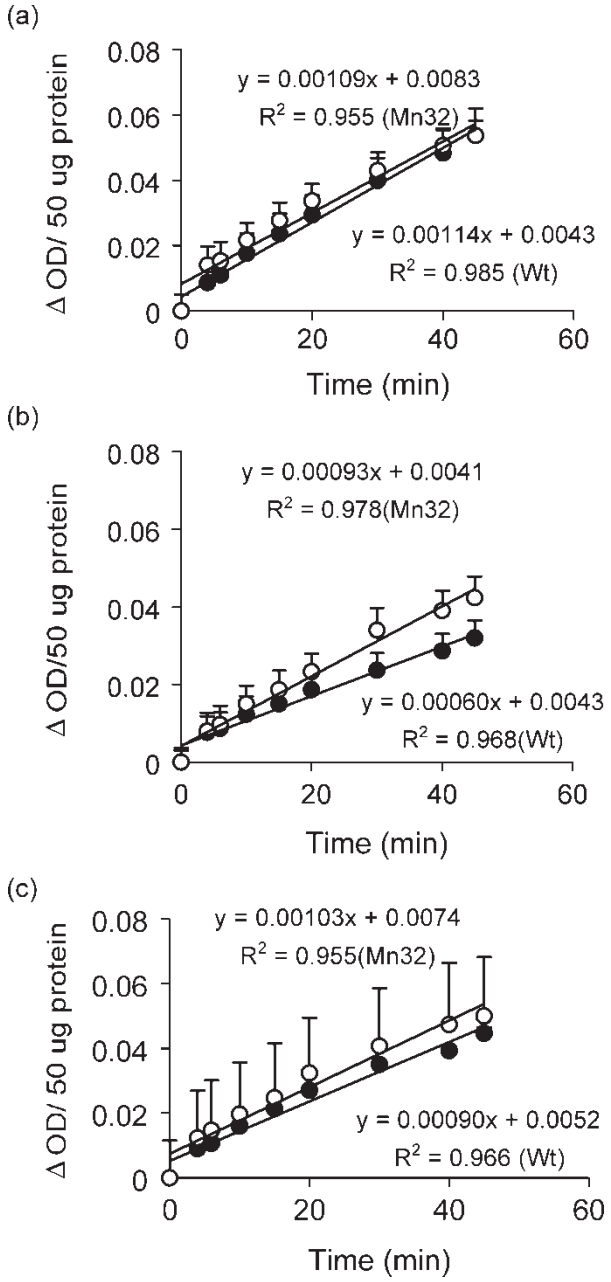

FIGURE 4 Time course of change in aconitase activity in PC-3 cells before and after heat treatment (43°C for 1 h). Cellular aconitase activity was determined for whole cell lysates at the indicated time points: (a) aconitase activity in PC-3 Wt (O) and Mn32 (•) cells prior to heating; (b) immediately after heating; (c) 1 h after heating. Aconitase activity is expressed in ΔOD/50 μg protein. Data from the average of three representative experiments are shown. The error bars represents SE of the mean; error bars that are not visible are within the data symbol. There is a significant difference in the pair wise comparison of slopes: $P < 0.001$ for Mn32 control vs. Mn32 at 0 h, $P = 0.047$ for Wt control vs. Wt 0 h, $P = 0.031$ for Wt vs. Mn32 at 0 h.

overexpression indicates the involvement of superoxide.

Hyperthermia Increases H_2O_2 Production

Before heat treatment, the ambient rate of accumulation of extracellular H_2O_2 measured in PC-3 Wt and Mn32 cells were 1.92 ± 0.09 and 3.33 ± 0.08 pmol cell^{-1} h^{-1}, respectively. After HT, the rate of accumulation of H_2O_2 from Wt and Mn32 cells was 3.78 ± 0.26 and 5.37 ± 0.42 pmol cell^{-1} h^{-1}, respectively; the values were significantly different ($P = 0.0008$). Therefore, after HT an ≈60% increase in the rate of production of H_2O_2 was seen in Mn32 cells and an ≈95% increase in the rate of production of H_2O_2 was seen in Wt cells. But most importantly, the absolute increase in the rate of H_2O_2 production after HT was the same in both cell lines.

SOD catalyzes the dismutation of superoxide to hydrogen peroxide. Thus, in the presence of MnSOD, if superoxide is formed then an increase in the rate of formation of superoxide will result in an increase in the rate of production of hydrogen peroxide. H_2O_2 diffuses through cell membranes and in our setting it can be used as an indicator of mitochondrial superoxide production.[53]

HT-induced LOOH Formation is Decreased by MnSOD Overexpression

Consistent with previous studies,[4,26] an increase in LOOH formation was observed in PC-3 cells subjected to hyperthermia. In PC-3 Wt cells, LOOH reached a maximum (9-fold increase) at the end of the heat treatment (0 h) (Fig. 5). After a 1 h incubation at 37°C, the LOOH level decreased but was still twice that of unheated controls. In contrast, Mn32 cells had only a slight increase (<1.5-fold) in LOOH at the end of the heat treatment (0 h). After a 1 h incubation at 37°C, LOOH dropped below baseline levels in Mn32 cells. Interestingly, 24 h after heat treatment PC-3 Wt cells had a somewhat higher LOOH level (~1.5) than unheated controls ($P < 0.0001$). In summary, HT increases the LOOH content of cells suggesting increased cellular lipid peroxidation. The change in LOOH correlated with the increase in ROS production as reflected in aconitase activity in Wt cells and by the protection afforded by MnSOD in Mn32 cells.

PUFA Profile before and after HT

Because changes in the lipid profile of cells can result in changes in the rate of lipid oxidation,[44] we determined whether stable transfection of MnSOD changed the lipid content of PC-3 cells, before and after HT. In Table I the saponifiable fatty acid content of PC-3 Wt and PC-3 Mn32 cells as determined by GC are summarized as well as additional selected global indices. The fatty acid profile of both PC-3 Wt and Mn32 were quite similar. However, there were some minor differences between the cell lines, such as:

(1) the higher content of 16:0 in PC-3 Wt (15%) compared to Mn32 cells (6%);

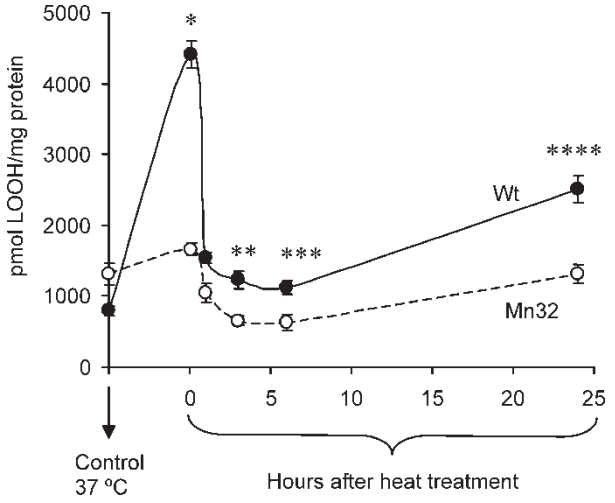

FIGURE 5 LOOH-formation after HT is time-dependent. Cellular lipids were extracted with chloroform: methanol (2:1, v/v). The amount of lipid hydroperoxide that partitioned into the chloroform layer was measured with the Cayman Lipid Hydroperoxide Assay Kit. The LOOH levels were maximally elevated immediately after heat exposure with $^{*}P = 0.019$ for Wt vs. Mn32. According to two-way ANOVA, there is a significant interaction between group and time ($P = 0.0001$). The *P*-values for the groups at individual time points were each significant: Wt vs. Mn32 at time 3 h, $^{**}P = 0.008$; 6 h, $^{***}P = 0.016$; and 24 h, $^{****}P < 0.0001$. Values are mean ± SE, $n = 3$. The pair-wise comparison between no heat and heat treatment that are statistically significant are: $P < 0.0001$, Wt control vs. 0 h; $P < 0.0007$, Wt control vs. 1 h; $P < 0.0001$, Wt control vs. 24 h; $P < 0.0013$, Mn32 control vs. 6 h.

(2) 16:1 was 3.5% vs. 0.6% for Wt and Mn32 cells, respectively; and
(3) Mn32 cells have a higher 22:4 content (6.7%) than the Wt (3.2%). These differences result in minor variations in some of the global parameters. Twenty-four hours after HT, PC-3 Wt cells showed no significant changes in lipid profile compared to the unheated control. However, even though Mn32 cells had a slight increase in PUFA content, they actually underwent less lipid peroxidation upon being subjected to HT. Thus, as expected these minor variations should have little effect on the overall consequences of HT.

Increased Radical Formation after HT as Measured by EPR

In the presence of redox-active metals, hydroperoxides undergo a one-electron reduction reaction to yield free radicals. An increase in the LOOH content of cells after HT will likely result in an increase in lipid-derived radical formation in the presence of ferrous iron. In free radical-mediated lipid peroxidation many different kinds of lipid-derived radicals can be produced.[54,55] They are carbon-centered radicals (lipid-derived alkyl $L_d^{\cdot}$, epoxy-allylic $OL_d^{\cdot}$) and oxygen-centered radicals (alkoxyl $L_dO^{\cdot}$, peroxyl $L_dOO^{\cdot}$).

In cells grown at 37°C or immediately after HT, higher levels of DMPO spin adducts were observed in PC-3 Wt cells compared to Mn32 cells [Fig. 6(A)]. One hour after heat treatment, the total amount of carbon-centered and oxygen-centered radicals detected was higher than that seen immediately after heat. Throughout the recovery period, greater spin adduct formation was seen in Wt cells compared to Mn32. Even 24 h after heat treatment, the radical concentration was higher in Wt cells compared to Mn32 cells. As a control experiment, the extraction was done with no cells and the spectra show the presence of only DMPO/$^{\cdot}$OH adduct. Also, parallel experiments with air-oxidized linolenic acid and DHA produced similar type of spectra suggesting that these radicals are lipid-derived carbon- and oxygen-centered adducts (data not shown). In the absence of Fe^{2+} only a small amount of DMPO/$^{\cdot}$OH adduct was seen (data not shown).

The reduction in radical formation in MnSOD-overexpressing cells suggests that superoxide may be involved in the lipid-derived carbon- and oxygen-centered radical formation. These results demonstrate that MnSOD protects against HT-induced lipid peroxidation.

The spectra obtained from the cell experiments [Fig. 6(B)/(I)] were simulated to determine the types of free radicals formed [Fig. 6(B)/(II–IV)]. At least three different radical spin adducts were produced in both Wt and Mn32 cells with HT. The major components were spin adducts resulting from the trapping of two isomeric alkoxyl radicals, denoted as $L_1O^{\cdot}$ and $L_2O^{\cdot}$. Dikalov *et al.* have shown that $L_2O^{\cdot}$ results from the conversion of a peroxyl adduct to an alkoxyl adduct.[39] A small contribution (10%) from a carbon-centered spin adduct of DMPO ($L_d^{\cdot}$) was also seen.

The increase in lipid hydroperoxides and the corresponding increase in the carbon- and oxygen-centered radical adduct formation after heat suggest that these radicals are lipid-derived and that hyperthermia increased radical formation. Although the exact identity of the lipid-derived radicals is not known, the expected general types of lipid-derived adducts were observed.

HT Decreases Clonogenic Survival of PC-3 Cells

As shown in Fig. 7, the time course for clonogenic survival after HT (43°C) followed similar trends for both Wt and Mn32 cells; however, the decrease in survival was greater in Wt cells compared to that of Mn32 cells. Immediately after heat treatment, there was a 70% decrease in survival of Wt cells compared to only 28% decrease in Mn32 cells. This difference was maintained even after a 24 h recovery time. That the difference is maintained between the two cell lines indicates that heat-induced cell-damage was not fully reversible. The mode of cell death was examined by gel electrophoresis and Annexin V flow cytometric analysis. Following HT, no evidence of

TABLE I PUFA Content (mole%) in Wt and Mn32 before and after heat treatment*

Fatty acids†	PC-3 Wt‡,¶	PC-3 Wt, HT‡,¶	Mn32‡,¶	Mn32, HT‡,¶
16:0	15.2 (4.2)	14.1 (3.0)	5.8 (1.2)	8.0 (1.7)
16:1	3.5 (1.4)	2.9 (0.8)	0.6 (0.2)	1.1 (0.4)
18:0	15.4 (1.2)	16.5 (1.0)	17.9 (0.7)	16.7 (0.7)
18:1	25.5 (0.7)	25.8 (0.7)	26.7 (1.6)	25.6 (1.3)
18:2n6	2.6 (0.1)	3.0 (0.1)	2.3 (0.1)	2.3 (0.1)
18:3n6	2.4 (2.1)	0.7 (0.1)	0.4 (0.2)	1.3 (0.5)
18:3n3	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
18:4n3	0.0 (0.0)	0.0 (0.0)	0.1 (0.1)	0.0 (0.0)
20:0	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
20:1n9	1.7 (0.3)	0.3 (0.3)	0.9 (0.7)	2.4 (0.3)
20:2n6	1.4 (0.7)	1.8 (0.2)	2.7 (0.8)	3.2 (0.4)
20:3n6	3.0 (0.2)	3.1 (0.3)	3.4 (0.1)	3.1 (0.3)
20:4n6	14.9 (1.6)	15.6 (1.5)	17.9 (0.6)	16.0 (0.8)
20:5n3	0.8 (0.0)	1.2 (0.3)	0.9 (0.2)	1.0 (0.4)
22:0	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
22:1n9	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
22:4n6	3.2 (0.4)	3.7 (0.8)	6.7 (0.5)	6.1 (0.9)
22:5n3	2.9 (0.5)	3.1 (0.4)	3.9 (0.5)	3.4 (0.4)
22:6n3	3.7 (0.6)	2.8 (0.5)	5.4 (0.6)	4.6 (0.5)
24:0	1.3 (0.2)	2.1 (0.7)	1.7 (0.9)	2.4 (0.5)
24:1n9	0.9 (0.1)	1.3 (0.3)	1.7 (0.2)	1.8 (0.3)
Fatty acid parameters	PC-3 Wt‡,¶	PC-3 Wt, HT‡,¶	Mn32‡,¶	Mn32, HT‡,¶
Saturates	32.5 (3.4)	32.8 (1.8)	25.6 (0.2)	27.7 (1.9)
Monounsaturates	32.5 (1.3)	32.3 (1.6)	30.8 (2.7)	31.5 (1.3)
Polyunsaturates	34.9 (4.6)	34.9 (3.1)	43.5 (2.8)	40.8 (3.2)
n3's	7.4 (1.0)	7.0 (0.4)	10.1 (1.2)	9.0 (1.1)
n6's	27.5 (3.7)	27.9 (2.8)	33.4 (1.7)	31.8 (2.1)
Average chain length	18.5 (0.2)	18.6 (0.2)	19.2 (0.2)	19.1 (0.2)
Double bond index§	1.7 (0.2)	1.7 (0.1)	2.1 (0.1)	1.9 (0.1)
Methylene bridge Index‖	1.0 (0.1)	1.0 (0.1)	1.3 (0.1)	1.2 (0.1)

* PC-3 cells were subjected to HT and PUFA analysis was done after 24 h of incubation. Cells were washed and extracted with $CHCL_3/CH_3OH$, 2:1 (v/v). After alkaline hydrolysis, fatty acids in the saponifiable fraction were methylated and the methyl esters separated by gas-liquid chromatography. † Fatty acids are designated as number of carbon atoms:number of double bonds. ‡ Mole percents of various fatty acids were calculated on the basis of weight percents from integrated peak areas on chromatograms and respective molecular weights of fatty acid methyl esters. ¶ Values are the mean (± SE) of three independent determinations of three different samples. § Double bond index is the mean number of double bonds per fatty acid in the cells; ‖ Methylene bridge index is the mean number of *bis*-allylic methylene positions per fatty acid in the cells.

apoptotic DNA fragmentation was observed and also no necrotic or apoptotic cells were found by flow cytometric analysis (data not shown). Cells overexpressing MnSOD displayed resistance to heat-induced cytotoxicity, consistent with a mechanism that involves superoxide.

DISCUSSION

Hyperthermia is now considered as an established modality for cancer treatment either alone or combined with chemo- or radiotherapy.[56] The heat sensitivities of different cancer cells may vary widely, due to the differences in development of tolerance and intrinsic protective enzymes.[57] An important consideration is that cancer cells have varying levels of antioxidant enzymes.[58,59] Because the antitumor effect of HT depends in part on the increased formation of free radicals, changes in the levels of antioxidant enzymes could modulate the heat sensitivity of cancer cells. The present study demonstrates that overexpression of MnSOD in human prostate cancer PC-3 cells confers resistance to cytotoxicity due to hyperthermia, consistent with an increased rate of formation of superoxide with HT.

In this study it was observed that MnSOD levels in PC-3 Wt cells increased after hyperthermia, whereas no change was found in the overexpressing clones. This is consistent with earlier reports on the induction of MnSOD during hyperthermia or ionizing radiation mediated by ROS.[10,60,61] The CuZnSOD levels in PC-3 Wt cells remained the same. Interestingly, there was an increase in CuZnSOD protein and activity in the MnSOD-overexpressing cells. The induction of SOD is most likely a response to the increased flux of ROS during hyperthermia. The lack of induction of exogenous MnSOD upon exposure to HT in the MnSOD-overexpressing clones could be anticipated because the upstream promoter regions are missing in the MnSOD vector we used. Thus, it will not be responsive to HT-induced transcription factors.

Superoxide and other ROS are known to increase leakage of electrons from the ETC, thereby altering MMP and permeability.[62] The PC-3 Wt cells had a much lower MMP than the MnSOD-overexpressing

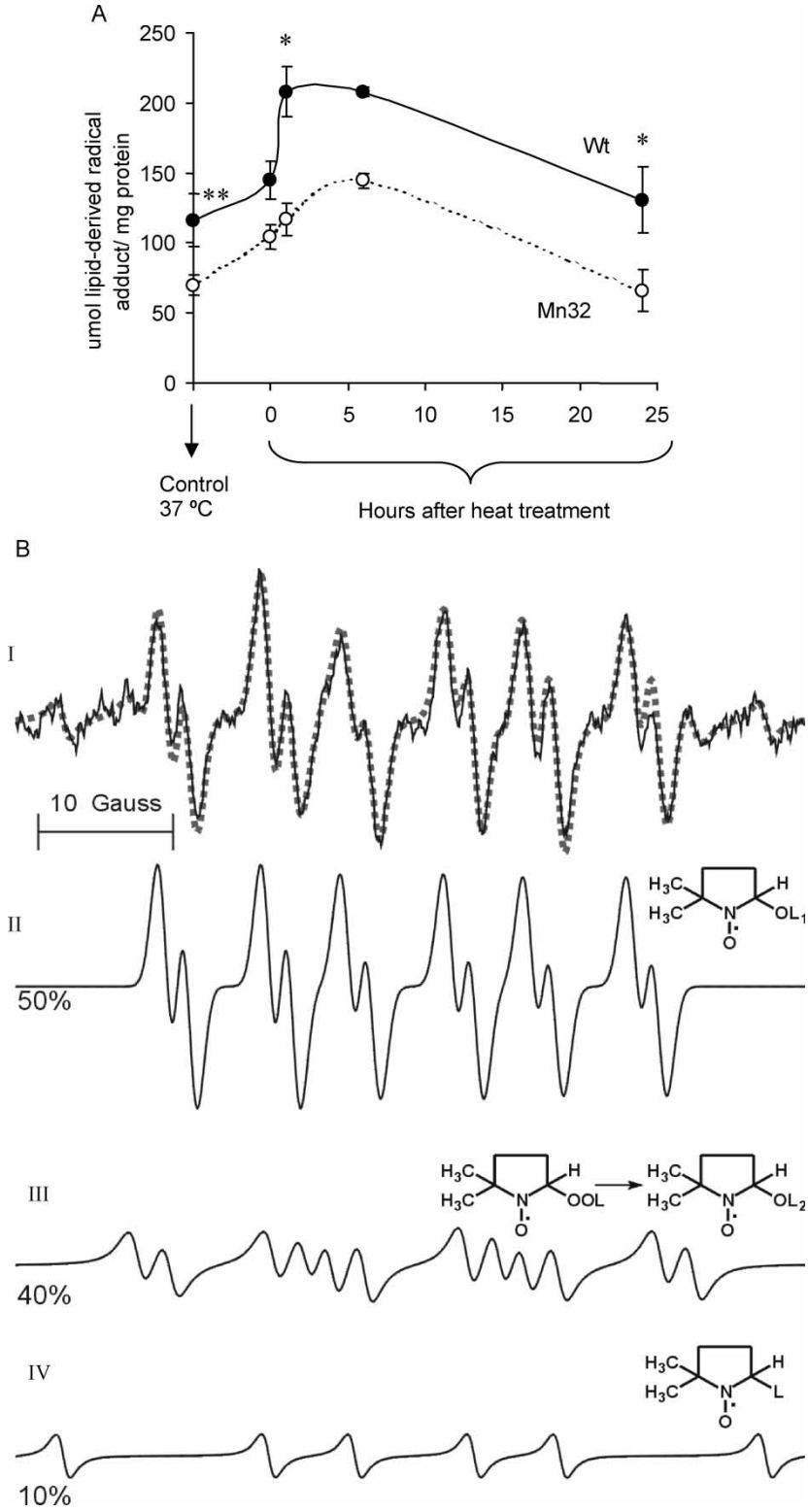

FIGURE 6 Detection of radicals by EPR. (A) Cells were heated at 43°C for 1 h. At the indicated time points, the EPR spectra of radicals were obtained using EPR spin trapping and Folch extraction as described in "Methods" section. To quantitate the lipid-derived radical adducts formed, the areas under the EPR spectra were measured and calibrated using 3-CP as standard. The contribution of any non-lipid radicals was removed from the quantitation by simulation. Data are the mean of three different samples. The *P*-values for the group comparisons at the individual time points were each significant: Wt and Mn32 cells $^{*}P < 0.0001$ after 1, 6, and 24 h after heat exposure and $^{**}P = 0.0003$ before heat. The pair-wise comparison between no heat and heat treatment that are statistically significant are: $P < 0.0001$, Wt control vs. 1 h; $P < 0.0001$, Wt control vs. 6 h; $P < 0.0011$, Mn32 control vs. 24 h. (B). EPR Spectral Analysis (I) Experimental EPR spectrum of DMPO/lipid-derived radical adducts formed in PC-3 cells before and after heat exposure. Dotted lines represent the computer-simulated spectrum that results from a composite of DMPO/$L_1O^{\cdot}$, DMPO/$L_1O^{\cdot}$, and DMPO/$L^{\cdot}$; see (II), (III) and (IV). The percentage contributions of each species to the spectra obtained are given. With no heat-treatment, the spectral intensity was substantially reduced as shown in 6A. (II) Computer simulation of DMPO/$L_1O^{\cdot}$ radical adduct component having $a^{N} = 13.13$ G, $a_{\beta}^{H} = 10.59$ G, $a_{\gamma}^{H} = 1.34$ G(50%).

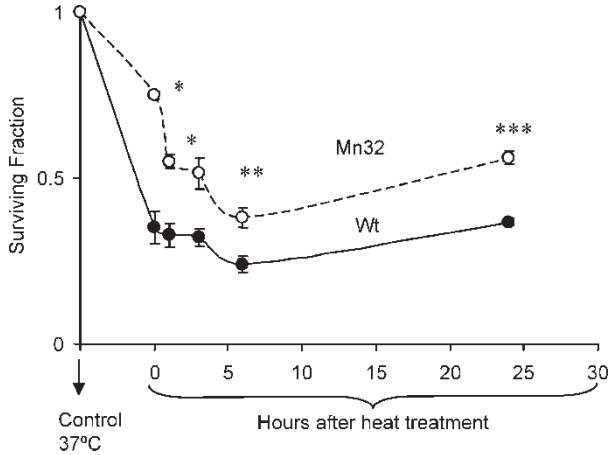

FIGURE 7 Increase in MnSOD activity increases clonogenic survival of PC-3 cells exposed to hyperthermia. Cells were heated at 43°C for 1 h. Cells at 37°C served as a control. Cells were trypsinized and plated in 60 mm dishes at different time points after HT. Colonies containing more than 50 cells were counted after 14 days. Survival curves were plotted as the log of the survival fraction of cells vs. the hours of incubation after HT. Experimental points are plotted as the mean ± SE, $n = 3$, three different cell cultures. The *P*-values for Wt vs. Mn32 at individual time points were each significant: $^{*}P < 0.0001$ at 0, 1, and 3 h; $^{**}P = 0.0009$ at 6 h; and $^{***}P < 0.0001$ at 24 h.

cell line, Mn32. A higher MMP implies a more intact, better functioning mitochondrial membrane. The importance of the mitochondria in this process is clear from the observation that HT brings about a change in MMP in WT cells, while it is preserved in the MnSOD-overexpressing clone.

Inhibition of aconitase was used to access the production of ROS by HT. Aconitase, a Kreb's cycle enzyme, is inactivated by $O_2^{\cdot-}$ and H_2O_2. Immediately after heat treatment, there was a 47% inhibition in aconitase activity in Wt cells compared to the unheated control, consistent with increased production of ROS. In Mn32 cells there was only 15% loss of aconitase activity. The observation that overexpression of MnSOD preserved aconitase activity suggests that during hyperthermia there is an increase in the production of superoxide in cells and that the $O_2^{\cdot-}$ is removed by the mitochondrial enzyme, MnSOD, resulting in the formation of H_2O_2.

From the results obtained with the fluorescent measurement of $(pHPA)_2$ in the presence of HRP, there was an increase in extracellular H_2O_2 after HT. The higher levels of H_2O_2 found outside the cell are consistent with an increase in the flux of $O_2^{\cdot-}$ and its conversion to H_2O_2 by SOD. Since aconitase can also be inactivated by H_2O_2 and since the MnSOD-overexpressing cell line, Mn32, had higher steady-state levels of H_2O_2, it would be expected to show more aconitase inactivation. As Mn32 cells had less inactivation, it can be assumed that the aconitase inactivation is mainly due to the formation of $O_2^{\cdot-}$ and that MnSOD by removing $O_2^{\cdot-}$ inhibits inactivation of aconitase.

Oxygen radicals are highly toxic and they can induce cytotoxicity through lipid peroxidation and DNA damage; increase in the levels of antioxidant enzymes have been shown to decrease their toxicity.[63,64] Previous studies have reported an increase in lipid peroxidation in mouse liver, lung, and heart after hyperthermia treatment.[65,66] Our data show that immediately after HT in Wt cells, there was a large increase in the level of LOOH that was reduced in cells that overexpressed MnSOD. As might be expected, the content of LOOH in cells decreased as they recovered from heat treatment. Based on the experimental evidence presented here and in previous studies, increased levels of lipid hydroperoxides are a consequence of HT.

The differences in the levels of LOOH between Wt and Mn32 after heat treatment could be due to differences in PUFA content. However, there was no significant difference in the overall PUFA content between Wt and Mn32 cells before or after heat treatment. Thus, the difference in lipid peroxidation would appear to be due to the differences in the levels of MnSOD.

The inactivation of aconitase by ROS leads to the oxidation of [4Fe–4S] clusters followed by loss of Fe (II).[67,68] This "free" iron could react with alkyl hydroperoxides forming alkoxyl radicals, which can initiate oxidation of PUFA by free radical chain reactions.[69] In EPR spin trapping experiments with hyperthermia-treated PC-3 cells, an increase in three types of spin adducts was observed: lipid-derived carbon- and oxygen-centered radicals ($LO^{\cdot}$, $L_d^{\cdot}$, $LOO^{\cdot}$). Consistent with the LOOH-measurement, the total amount of radical adduct formed was less in MnSOD-overexpressing cells compared to the Wt PC-3 cells. DHA and linolenic acid gave EPR spectral features similar to those from PC-3 cells; controls without cells showed none of these spectral features, suggesting that the radicals observed were lipid-derived carbon- and oxygen-centered radicals. However, detailed identification of specific lipid radicals is yet to be done. These results are consistent with the role of formation of superoxide as the initial event in the oxidation processes associated with HT.

When examined by a clonogenic survival assay to assess a biological consequence of HT, a decrease in

(III) Computer simulated spectrum of DMPO/$L_2O^{\cdot}$ radical adduct component using the parameter $a^{N} = 13.28\,G$, $a_{\beta}^{H} = 6.88\,G$, $a_{\gamma}^{H} = 2.0\,G$ (40%). The DMPO/$L_2O^{\cdot}$ adduct results from the conversion of the first formed product DMPO/$LOO^{\cdot}$. $L_1O^{\cdot}$ and $L_2O^{\cdot}$ are the two diasteromers of the DMPO/lipid-derived alkoxyl radical adducts.[40] (IV) Computer simulation of DMPO/$L_d^{\cdot}$ a carbon-centered radical adduct component with $a^{N} = 15.10\,G$, $a_{\beta}^{H} = 22.36\,G$ (10%). All spectra are the result of 40 signal-averaged scans. Spectra were simulated using the NIEHS simulation program.[37]

survival of PC-3 Wt and Mn32 cells was observed. However, survival of Mn32 cells was significantly greater than Wt cells, as would be predicted from the observations of decreased ROS, decreased lipid-derived radicals, and increased mitochondrial potential. These results are consistent with increased oxidative stress in cells subjected to hyperthermia. Studies have shown that hyperthermia leads to apoptotic or necrotic cell death.[70,71] When PC-3 cells were subjected to hyperthermia, no apoptotic or necrotic cell death was seen (data not shown). In summary, PC-3 cells overexpressing MnSOD show increased thermoresistance.

The HSPs are involved in the protection of cells against heat treatment.[60,72] We examined the modulation in HSP's 70 and 27 in both cell lines; there was a slight increase in HSP's after HT. However, there was no difference in the induction of HSP's between Wt and Mn32 cells (data not shown). Thus, these HSPs did not appear to play a role in the increased heat resistance of MnSOD-overexpressing cells.

It is widely recognized that MnSOD-overexpression has an antitumor effect.[14,73] Some authors suggest that this is due to the removal of superoxide,[74] while others believe it is caused by an increase in hydrogen peroxide production.[75] On the other hand, it has been reported that the concentration of SOD in cells can regulate the steady-state levels of superoxide or H_2O_2 and their subsequent toxicity.[76,77] If not removed by CAT/GPx, SOD-driven accumulation of H_2O_2 can be toxic to cells as H_2O_2 can form hydroxyl radical in the presence of Fe^{2+}. This hydroxyl radical will bring about lipid peroxidation. In the hyperthermia scenario it appears that superoxide production from the electron transport chain is the initial ROS produced because:

(a) increased MnSOD suppresses the cytotoxicity of HT, while at the same time increasing the level of H_2O_2 formed;
(b) the clone Mn98, although having ~4 times more catalase activity than Mn32, has no significant change in clonogenic survival after HT (Fig. 1);
(c) MnSOD is a mitochondrial enzyme that removes superoxide implicating the ETC as its source;
(d) MnSOD protects against the inactivation of aconitase; and
(e) increased MnSOD in cells decreases lipid peroxidation markers. This is consistent with superoxide releasing "free" iron from aconitase, which can then enhance oxidant formation and the initiation of lipid peroxidation.

These results suggest that superoxide free radical or its reaction products are responsible for part of the cytotoxicity associated with hyperthermia and that increased levels of MnSOD can reduce cellular injury, thereby enhancing heat tolerance.

Acknowledgements

We thank Drs Douglas Spitz and Fredrick Domann for helpful discussions, Ms Elisabeth Buettner for her help with the hydrogen peroxide assay and Dr Yunxia O'Malley for her help with aconitase assay. Thanks are due to Justin Fishbaugh in the Flow Cytometry Core Facility for his help with flow techniques and to Ms Kellie Bodeker for her editorial assistance.

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