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# Manganese Superoxide Dismutase (*SOD2*)-Mediated Delayed Radioprotection Induced by the Free Thiol Form of Amifostine and Tumor Necrosis Factor α

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**Murley, J. S., Kataoka, Y., Baker, K. L., Diamond, A. M., Morgan, W. F. and Grdina, D. J. Manganese Superoxide Dismutase (*SOD2*)-Mediated Delayed Radioprotection Induced by the Free Thiol Form of Amifostine and Tumor Necrosis Factor α. *Radiat. Res.* 167, 465–474 (2007).**

**RKO36 cells, a subclone of RKO colorectal carcinoma cells that have been stably transfected with the pCMV-EGFP2Xho vector, were grown to confluence and then exposed to either the radioprotector WR-1065, i.e. the active thiol form of amifostine, for 30 min at doses of 40 μ*M* and 4 m*M* or the cytokine tumor necrosis factor α (TNFα, TNFA) for 30 min at a concentration of 10 ng/ml and then washed. Total protein was isolated as a function of time up to 32 h after these treatments. Both doses of WR-1065 as well as the concentration of TNFα used were effective in elevating intracellular levels of the antioxidant protein SOD2 (also known as MnSOD) at least 15-fold over background levels as determined by Western blot analysis, while measured SOD2 activity was elevated between 5.5- and 6.9-fold. SOD2 reached a maximal level 24 h and 20 h after WR-1065 and TNFα treatments, respectively. The antioxidant proteins catalase and glutathione peroxidase (GPX) were also monitored over the 32-h period. In contrast to the robust changes observed in intracellular levels of SOD2 as a function of time after exposure of cells to WR-1065, catalase levels were elevated only 2.6-fold over background as determined by Western blot analysis, while GPX activity was unaffected by WR-1065 exposure. GPX protein levels were extremely low in cells, and analysis of GPX activity using a spectrophotometric method based on the consumption of reduced NADPH also revealed no measurable change as a function of WR-1065 or TNFα exposure. RKO36 cells either were irradiated with X rays in the presence of either 40 μ*M* or 4 m*M* WR-1065 or 10 ng/ml TNFα or were irradiated 24 or 20 h later, respectively, when SOD2 protein levels were most elevated. The concentrations and exposure conditions used for WR-1065 and TNFα were not cytotoxic and had no effect on plating efficiencies or cell survival compared to untreated controls. No protection or sensitization was observed for cells irradiated in the presence of 40 μ*M* WR-1065 or TNFα. Survival was elevated 1.90-fold for cells irradiated in the presence of 4 m*M* WR-1065. When RKO36 cells were irradiated with 2 Gy 24 h after 40 μ*M* or 4 m*M* WR-1065 and 20 h after TNFα treatments when SOD2 levels were the most increased, survival was elevated 1.42-, 1.48- and 1.36-fold, respectively. This increased survival represents a SOD2-mediated delayed radioprotective effect. *SOD2* appears to be an important antioxidant gene whose inducible expression is an important element in adaptive cellular responses in general, and the delayed radioprotective effect in particular. It can be induced by a range of agents including cytoprotective nonprotein thiols such as WR-1065 and pleiotropic cytokines such as TNFα.** **© 2007 by Radiation Research Society**

## INTRODUCTION

*SOD2* encodes manganese superoxide dismutase, a protein that is an inducible antioxidant enzyme localized in the mitochondria of cells that protects against oxidative damage caused by reactive oxygen species (ROS) generated during normal aerobic metabolism and by oxidative stress-inducing agents including ionizing radiation (*1*). Superoxide dismutases catalyze the dismutation of superoxide radicals to oxygen and hydrogen peroxide. The antioxidant enzymes catalase and glutathione peroxidase (GPX) in turn detoxify the resultant hydrogen peroxide into water and oxygen. Of these endogenous antioxidant enzymes, SOD2 has been identified as the most effective in protecting against radiation-induced toxicity to cells (*2–6*).

*SOD2* contains multiple transcription factor binding motifs, but only the nuclear transcription factor κB (NFκB, NFKB) has been linked to its enhanced transcription after exposure to stress-inducing agents. In particular, an intronic NFκB binding element has been identified as being essential for the induction of *SOD2* transcription after exposure of cells to stress-inducing agents such as tumor necrosis factor α (TNFα, TNFA) and interleukin 1β (*7*). NFκB is a redox-sensitive transcription factor. Not only can it be activated by oxidative stress-inducing deleterious agents such

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as inflammatory cytokines and ionizing radiation (*8, 9*), it can also be induced by nonprotein thiols (NPT) having a free sulfhydryl group. NPT that include *N*-acetylcysteine, oltipraz, mesna, captopril, dithiothreitol and WR-1065, the free thiol active form of amifostine, all have been found to be effective in activating NFκB and inducing *SOD2* transcription in exposed cells, resulting in elevated intracellular levels of SOD2 protein (*10–13*). While the underlying mechanism of action is unclear at present, data indicate that NPT can reduce disulfide bonds associated with cysteine residues on the p50 and p65 subunits of NFκB, resulting in its activation and enhanced binding to DNA (*14*). Exposure of cells to inhibitors of NFκB activation such as BAY 11-7082 and Helenalin prior to exposure to NPT abrogates their ability to elevate *SOD2* transcription (*15, 16*). Likewise, *SOD2* gene expression is also unaffected by NPT exposure in cells stably transfected with a mutant IκBα gene that prevents the subsequent ligand-induced degradation that results in an inhibition of NFκB activation (*15*).

The activation of NFκB and subsequent elevation of *SOD2* gene expression leading to increased intracellular levels of active protein as a function of time after NPT exposure suggested that this process could also induce a corresponding elevated level of cellular resistance to reactive oxygen species (ROS) induced by ionizing radiation exposure. Such a delayed radioprotective effect capable of being induced by a number of different NPT was identified and characterized in mouse SANH tumor cells (*15, 17*) and in human microvascular endothelial cells (HMEC) (*16*). In both systems, intracellular levels of SOD2 increased with time after NPT exposure, with the maximum elevation in active protein levels occurring between 22 and 26 h later. If challenged with a 2- or 4-Gy dose of radiation at that time, cell survival levels were elevated between 30 and 40% compared to non-NPT-treated controls irradiated in a similar manner. This effect was referred to as an NPT-induced but SOD2-mediated delayed radioprotective effect (*15–17*). In several ways this NPT-induced effect is similar to the phenomenon identified as the adaptive response in which exposure of certain types of cells to a relatively low dose of ionizing radiation induces an enhanced downstream enhanced resistance to a second but much larger dose of radiation (*18*). Mothersill and Seymour (*19*) have generalized this effect as including any response engendered in cells that can transduce signals from an initial ''inducing'' event into a downstream more robust response. Key factors identified in such adaptive responses include both the generation of ROS and effects on mitochondria (*19*).

In this study, we further characterize the ability of NPT to induce a delayed radioprotective effect in human colorectal carcinoma cells (RKO36). This cell line has a wild-type functional p53 gene (*TP53*) and a near diploid karyotype (*20*). Specifically, we have investigated the ability of the free thiol form of amifostine, WR-1065 [2-(aminopropylamino)ethane thiol], to affect intracellular levels of SOD2, and we also assessed its effects on the protein levels of the antioxidants catalase and glutathione peroxidase. The ability of WR-1065 to affect SOD2 and induce the delayed radioprotective effect was contrasted to that of a non-NPT agent, the cytokine tumor necrosis factor α (TNFα), which is a known inducer of *SOD2* gene expression (*7, 11, 21*).

## METHODS AND MATERIALS

### *Cells and Culture Conditions*

RKO36 cells are a subclone of RKO colorectal carcinoma cells that have been transfected with the pCMV-EGFP2Xho vector that allows analysis of delayed hyper-recombination and/or deletion/mutation events in the progeny of surviving cells (*20*). They were grown in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (HyClone, Logan, UT) at 37°C in 5% $CO_2$ and 95% air as described in detail elsewhere (*20*). Cells were grown to confluence for use in all delayed radioprotection studies. When they reached confluence, cells were refed with fresh medium and maintained for an additional 3 days. Cultures were again refed with fresh medium 1 day prior to each experiment.

### *WR-1065 and TNFα*

WR-1065 was supplied by the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD. Immediately before use it was dissolved in PBS at a concentration of 1 *M* and filter sterilized. Cells were exposed to WR-1065 at concentrations of 40 μ*M* or 4 m*M* for 30 min.

TNFα (1 mg/ml in PBS) was purchased from Sigma-Aldrich (St. Louis, MO). Cells were exposed to a final concentration of 10 ng/ml of TNFα for 30 min in complete medium. Medium was removed, cells were washed with PBS, and fresh medium was added.

### *Western Blotting*

Protein isolation and Western blot analysis were performed following methods described previously (*17*). Briefly, cell lysates were prepared from RKO36 cells that were grown to confluence in 100-mm tissue culture dishes. After isolation and determination of protein concentrations using the Bradford method (*22*), SOD2, catalase and glutathione peroxidase (GPX) protein levels were assessed using the WesternBreeze Chemiluminescent Western Blotting Immunodetection System (Invitrogen). Total proteins were electrophoresed at a concentration of 5 μg on a 12% SDS polyacrylamide gel according to the method of Laemmli (*23*). Proteins were transferred onto polyvinylidene difluoride (PVDF) membranes, blocked in Blocking solution (Invitrogen) at room temperature for 30 min, and then incubated with primary antibody (1:1000 dilution of rabbit anti-SOD2, Upstate, Charlottesville, VA; 1:1000 dilution of rabbit anti-catalase, EMD Biosciences, San Diego, CA; 1:1000 dilution of mouse-anti-β-actin, Sigma-Aldrich) for 1 h at room temperature. The antibody for GPX Western blotting was affinity purified from the eggs of hens immunized with a KLH-conjugated peptide representing amino acid residues 83–100 of the human *GPX1* gene, CZHQENAKNEEIL NSLKYVR (Aves Labs, Inc., Tigard, OR). Anti-human GPX2 antibody was purchased from Abcam (Cambridge, MA). Blots were washed four times with Antibody Wash (Invitrogen) and were then incubated with goat anti-rabbit IgG conjugated with alkaline phosphatase (Invitrogen) for 30 min. The blots were again washed four times with Antibody Wash, and protein bands were visualized using Chemiluminescent Substrate (Invitrogen) as per instructions. Membranes were exposed to BioMax XAR film (Kodak, Rochester, NY) and scanned using an HP ScanJet 8200 (Houston, TX). NIH ImageJ software was used to analyze band intensities.

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*SOD2 Enzyme Activity Assay*

SOD2 activity was measured using the Superoxide Dismutase Assay Kit from Trevigen (Gaithersburg, MD) following the manufacturer's instructions. Briefly, RKO36 cells grown to confluence were trypzinized and counted. Five million cells were lysed with 500 μl of lysis solution. The resulting suspension was centrifuged at 14,000*g* for 5 min at 4°C and then transferred to a clean 1.5-ml tube. Activity was determined at room temperature using a colorimetric assay based on the ability of SOD to form $H_2O_2$ from superoxide radicals generated by an exogenous reaction involving xanthine and xanthine oxidase which converts nitroblue tetrazolium (NBT) to NBT-diformazan. NBT-diformazan absorbs light at 550 nm. The extent of reduction in the appearance of NBT-diformazan is a measure of SOD activity. Activity was measured using 400 μl of cell suspension in the presence of 5 m*M* sodium cyanide (NaCN) (Sigma-Aldrich) to inhibit copper/zinc SOD (SOD1) activity. Absorbance changes were recorded for 5 min, and the percentage inhibition was calculated for each experimental sample. SOD activity was calculated using a SOD standard curve generated with known concentrations of purified SOD supplied with the kit.

*GPX Enzyme Activity Assay*

Total GPX activity in protein extracts was measured using a standard coupled spectrophotometric method based on the consumption of reduced NADPH measured spectrophotometrically by absorbance at 339 nm in a reaction containing reduced glutathione, glutathione reductase, cell lysate and hydrogen peroxide as described in detail elsewhere (*24*). Briefly, RKO36 cells are washed with PBS, harvested by scraping, and resuspended in cold sodium phosphate buffer (0.1 *M*, pH 7.0) and lysed by sonication. GPX activity is reported as nanomoles of NADPH oxidized per minute per milligram of protein.

*SOD2 siRNA Transfection*

RKO36 cells grown to confluence in 100-mm dishes were transfected with 100 n*M* SOD2 siRNA (5′ GCU GCU CUA UUG UAG CAU UTT 3′; graciously supplied by Dr. Jian Jian Li, Purdue University) using Lipofectamine 2000 transfection reagent (Invitrogen) following the manufacturer's instructions. Briefly, siRNA oligomer (100 n*M* final concentration) was diluted in 1.5 ml serum-free DMEM. Thirty microliters of Lipofectamine 2000 reagent was diluted in 1.5 ml of serum-free DMEM, mixed gently, and then incubated for 5 min at room temperature. The siRNA oligomer was then combined with the diluted Lipofectamine 2000, gently mixed, and incubated for 20 min at room temperature to allow complex formation. During this incubation the growth medium was aspirated from the dishes and the cells were washed with PBS at 37°C to remove traces of serum. The siRNA-Lipofectamine 2000 complexes were added to the dishes and gently swirled to ensure uniform distribution. The cells were incubated for 24 h with the transfection complexes under their normal growth conditions of 37°C and 5% $CO_2$. The cells were then washed with PBS at 37°C and fresh growth medium was added. Transfected cells were then treated with WR-1065 and irradiated as described below.

*Irradiation Conditions and Survival Assay*

Confluent RKO36 cells, normal or transfected with SOD2 siRNA, were irradiated at room temperature using a Philips X-ray generator operating at 250 kVp and 15 mA at a dose rate of 1.65 Gy per minute. Clonogenic cell survival was assayed after a dose of 2 Gy. To assess the immediate radioprotective effect of WR-1065, cells were exposed for 30 min to either 40 μ*M* or 4 m*M* of the NPT and then irradiated. RKO36 cells transfected with SOD2 siRNA were evaluated for their response to irradiation with 2 Gy either alone or 24 h after exposure to 4 m*M* of WR-1065 for 30 min. RKO36 cell survival was also evaluated after their exposure to 10 ng/ml of TNFα for 30 min and then irradiated to assess the immediate effects of TNFα on cellular response to radiation exposure. Its effects on viability of unirradiated cells were also determined under these conditions. Immediately after exposure to radiation, cells were washed free of WR-1065 or TNFα, trypsinized, counted and plated into fresh growth medium at appropriate numbers to give rise to about 100 colonies per dish. Five dishes per experimental point were used and experiments were repeated two or three times. Cells were incubated under standard conditions for 10 to 14 days and resultant colonies were stained with 20% crystal violet. Colonies containing 50 or more cells were scored as surviving cells. To assess the SOD2-mediated delayed radioprotective effect, RKO36 cells were first monitored to determine the kinetics of changes in SOD2 as a function of thiol or cytokine exposure. SOD2 levels were at their highest approximately 20 and 24 h after TNFα and WR-1065 treatment, respectively. Cells were washed free of TNFα and WR-1065 after 30 min of exposure and were then incubated in fresh growth medium for 20 and 24 h, respectively, before being exposed to 2 Gy. To further characterize the role of elevated SOD2 on the delayed radioprotective effect, RKO36 cells transfected with SOD2 siRNA were also exposed to 4 m*M* of WR-1065 for 30 min and then irradiated 24 h later. Pairwise comparisons of surviving fractions at 2 Gy between each of the experimental conditions were performed using a Student's two-tailed *t* test.

## RESULTS

### *Kinetics of Induced Antioxidant Proteins*

The kinetics of changes in SOD2, catalase and GPX as a function of WR-1065 treatment was monitored by Western blotting. Consistent with previous reports of SOD2 induction by WR-1065 in both SANH mouse tumor cells and human microvascular endothelial cells (HMEC) (*13, 15–17*), intracellular levels of SOD2 increased as a function of time after exposure of RKO36 cells to WR-1065 regardless of the dose used, with maximal levels being achieved at 24 h postexposure. Plots of the kinetics of SOD2 induction as a function of time after exposure to either 40 μ*M* or 4 m*M* WR-1065 derived from their corresponding Western blot data corrected for β-actin loading controls is presented in Figs. 1 and 2, respectively, for comparison. Also included are measurements of the fold increase in SOD2 activity determined at 24 h when SOD2 levels, as determined by Western blot analysis, were maximally elevated over background levels. SOD2 levels fell to pretreatment background values by 32 h after exposure to either dose of WR-1065.

SOD2 was also significantly induced as a function of time after a 30-min exposure of RKO36 cells to 10 ng/ml of the cytokine TNFα. A plot of SOD2 levels corrected for corresponding β-actin loading controls obtained from Western blot data are presented in Fig. 3 for comparison. Also included is the measured increase in SOD2 activity at 20 h, the time at which intracellular SOD2 protein levels were maximally elevated as determined by Western blot analysis. Based on these data, all subsequent experiments to assess effects on radiation response were performed 20 h after TNFα treatment and 24 h after exposure of cells to WR-1065.

Neither WR-1065 nor TNFα exposure affected intracellular protein levels of catalase or GPX as robustly as observed for SOD2 in RKO36 cells. A plot of the kinetics of changes in intracellular catalase levels as determined by

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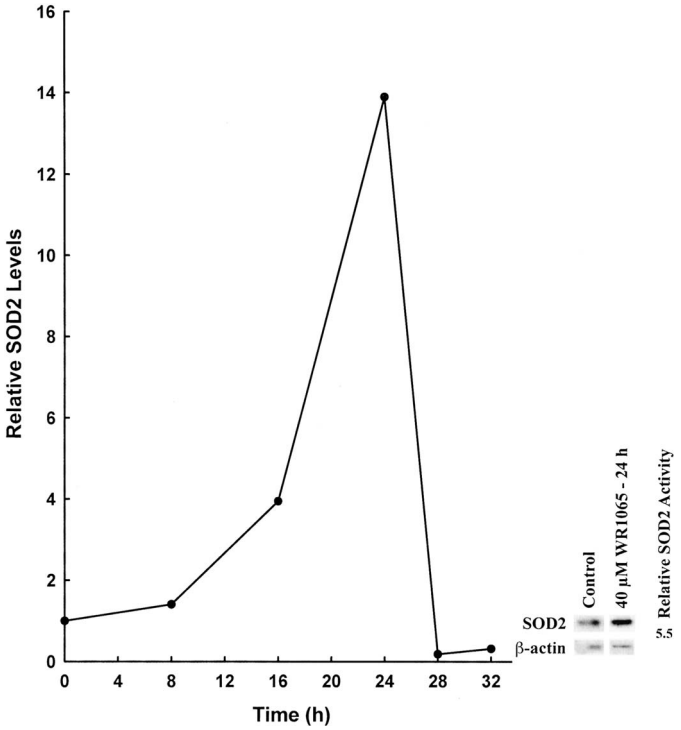


**FIG. 1.** Time course showing changes in intracellular SOD2 protein levels after a 30-min exposure of RKO36 cells to 40 μ*M* of the free thiol form of amifostine, WR-1065. SOD2 protein levels, as determined by Western blotting (see inset), were maximally elevated 24 h after exposure of cells to WR-1065. Also presented is the measured SOD2 activity relative to background determined at 24 h. This Western blot is representative of three separate experiments.

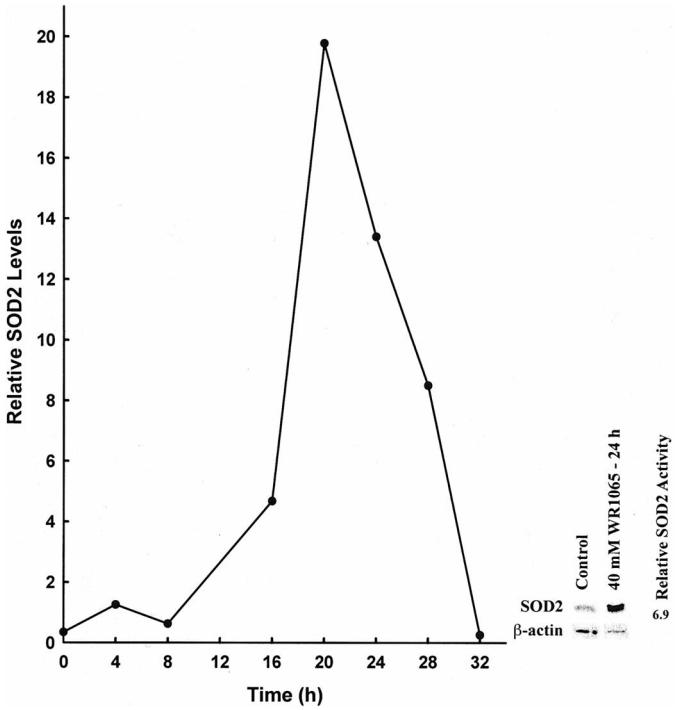


**FIG. 2.** Time course showing changes in intracellular SOD2 protein levels after a 30-min exposure of RKO36 cells to 4 m*M* of the free thiol form of amifostine, WR-1065. SOD2 protein levels, as determined by Western blotting (see inset), were maximally elevated 24 h after exposure of cells to WR-1065. Also presented is the measured SOD2 activity relative to background determined at 24 h. This Western blot is representative of three separate experiments.

Western blot analysis describing the effects of 4 m*M* WR-1065 exposure for 30 min is presented in Fig. 4. Catalase levels remained relatively unchanged throughout the 32-h post-treatment time monitored, with only a modest 2.6-fold increase observed at 24 h. GPX levels were too low to effectively be compared by Western blotting (data not shown), requiring a more sensitive method of direct measurement of GPX activity. GPX activity, expressed as nanomoles of NADPH oxidized per milligram of protein per minute, as a function of time after WR-1065 exposure is plotted in Fig. 5. GPX activity was unaffected by WR-1065 exposure and remained relatively constant over the post-exposure time studied.

*Radiation Response*

WR-1065, the active thiol form of amifostine, has been studied extensively as a radiation protector. Its ability to protect cells against radiation-induced cytotoxicity under *in vitro* culture conditions is known to be a function of its concentration, with 4 m*M* being the most nontoxic cytoprotective concentration for a 30-min exposure, e.g., the classical immediate radioprotective effect (*25*). WR-1065 at a concentration of 40 μ*M* is not cytoprotective but is sufficient to protect against radiation-induced mutagenesis at the hypoxanthine phosphoribosyl transferase (*HPRT*) locus in mammalian cells (*25*). This relationship between WR-1065 concentration and the ability to protect against radiation-induced cytotoxicity was confirmed for RKO36 cells (see Fig. 6). A concentration of 40 μ*M* (in contrast to 4 m*M*) WR-1065 was not cytoprotective, e.g. survival protection ratios of 0.98 and 1.90, respectively. As presented earlier in Figs. 1 and 2, both the 40 μ*M* and 4 m*M* concentrations of WR-1065 were similar in their ability to induce a 15- to 20-fold increase in intracellular SOD2, with the maximum induction occurring 24 h after treatment. RKO36 cells irradiated with 2 Gy 24 h after WR-1065 exposure at either concentration exhibited a similar and significant increase in radiation resistance, e.g. enhanced survival protection ratios of 1.42 and 1.48, respectively. This ability to induce an enhanced resistance to a 2-Gy challenge dose of radiation 24 h later that correlates with the increase of WR-1065-induced SOD2 intracellular levels is referred to as the delayed radioprotective effect (see Fig. 7). No delayed radioprotection was observed for RKO36 cells transfected with SOD2 siRNA when they were irradiated 24 h after treatment with 4 m*M* WR-1065, e.g. radiation protection ratio of 1.02 (see Fig. 8).

Exposure of RKO36 cells to the cytokine TNFα at a concentration of 10 ng/ml for 30 min was not cytotoxic under these experimental conditions, but it was effective in inducing over a 20-fold elevation in intracellular SOD2 20

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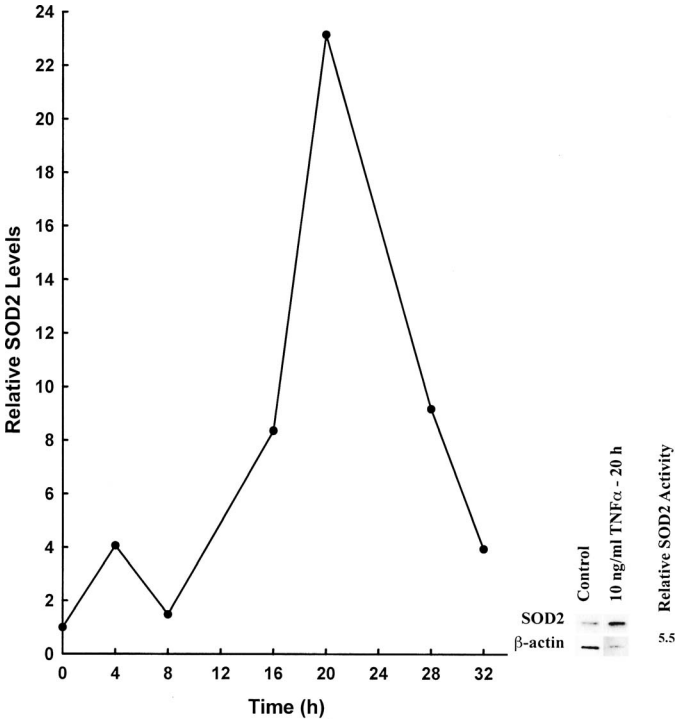


**FIG. 3.** Time course showing changes in intracellular SOD2 protein levels after a 30-min exposure of RKO36 cells to 10 ng/ml of the cytokine TNFα. SOD2 protein levels, as determined by Western blotting (see inset), were maximally elevated 20 h after exposure of cells to TNFα. Also presented is the SOD2 activity relative to background, determined for the 20 h. This Western blot is representative of three separate experiments.

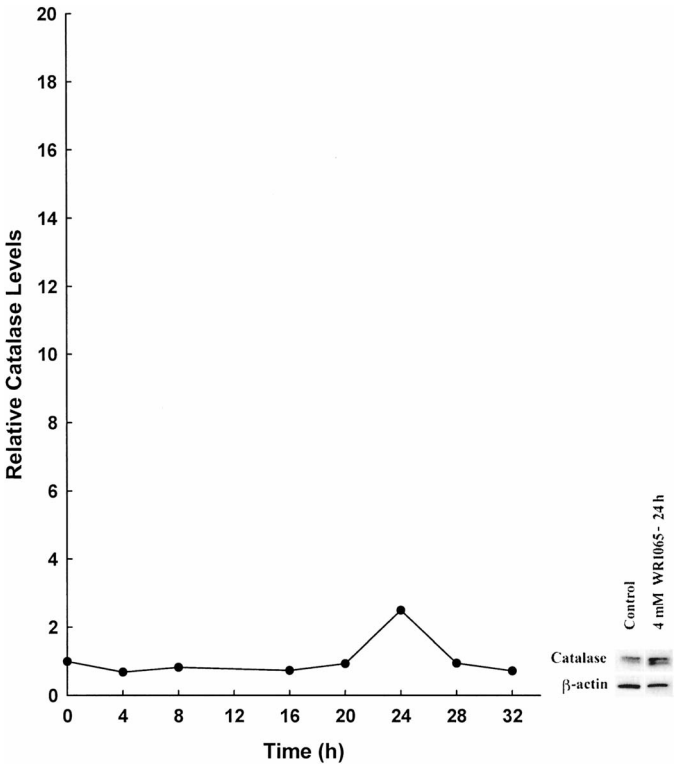


**FIG. 4.** Time course showing changes in intracellular catalase levels after a 30-min exposure of RKO36 cells to 4 m*M* WR-1065 as determined by Western blotting (see inset). This Western blot is representative of three separate experiments.

h later. TNFα administered to cells 30 min prior to irradiation with 2 Gy had no effect on the radiation response of the cells, e.g., the immediate radioprotective effect routinely associated with classical radioprotective agents such as WR-1065 (see Fig. 9). In contrast, when RKO36 cells were exposed to TNFα for 30 min and then irradiated with 2 Gy 20 h later when SOD2 intracellular levels were maximal, cell survival was increased significantly by 36% (see Fig. 10).

## DISCUSSION

Thiol-containing reducing agents in general, and WR-1065, the active thiol form of amifostine, in particular, have been demonstrated in a number of neoplastic and non-tumor cell systems to be effective in activating the redox-sensitive transcription factor NFκB, leading to the subsequent expression and translation of the antioxidant gene *SOD2* and the elevation of intracellular levels of active SOD2 protein (*10, 12, 13, 15–17*). SOD2 is an antioxidant enzyme localized within the mitochondria that facilitates the dismutation of the highly reactive superoxide radical into hydrogen peroxide, an oxidative species that is further converted into water and oxygen through the action of catalase and glutathione peroxidase (*1*). The ability to induce elevated levels of SOD2 in cells is not limited to thiol-containing drugs but is also shared by the pleiotropic cytokine TNFα (*17*). Elevation of intracellular SOD2 should presumably lead to an increased cellular resistance to ROS producing oxidative damaging agents such as ionizing radiation (*2–6, 15–17*). While this appears to be a general phenomenon of thiols, the role of TNFα as an inducer of this process is somewhat controversial, with conflicting reports in the literature regarding its ability to be a radiosensitizing (*26–28*) and a radioprotective (*29–32*) agent. For these reasons we investigated and contrasted the abilities of WR-1065 and TNFα not only with regard to their ability to affect intracellular SOD2 levels but also to their ability to confer an enhanced radioresistance to cells, referred to as the delayed radioprotective effect (*15–17*).

Consistent with our earlier studies using mouse SANH tumor (*15, 17*) and human microvascular endothelial cells (HMEC) (*16*), WR-1065 was effective in elevating SOD2 levels 15- to 20-fold in human colorectal RKO36 cells 24 h after exposure, as determined by Western blotting, and increased SOD2 activity by 5.5- to 6.9-fold. This effect could be induced using the low anti-mutagenic dose of 40 μ*M* as well as the much greater cytoprotective dose of 4 m*M* (see Figs. 1 and 2). Likewise, and as reported by others (*6, 7, 11, 17*), TNFα was also found to be a potent inducer of SOD2, with the peak level of enzyme concentration occurring at 20 h after a 30-min exposure (see Fig. 3).

In contrast to its robust effect on SOD2, WR-1065 ex-

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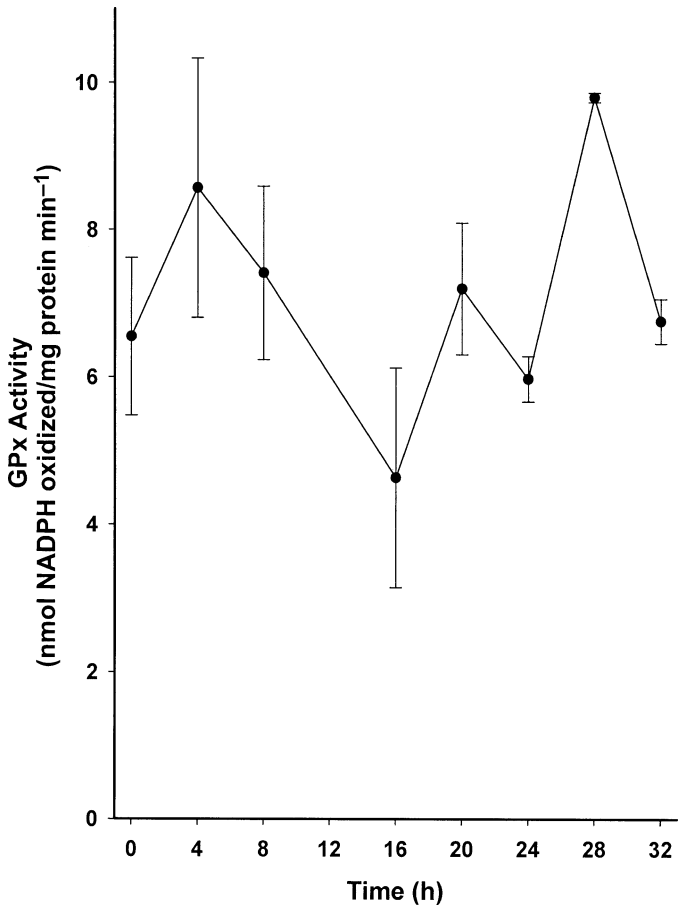


**FIG. 5.** Time course of glutathione peroxidase (GPX) activity after exposure of cells to 4 m*M* WR-1065 for 30 min. Error bars represent the means ± SEM of three separate treatment samples per experimental point.

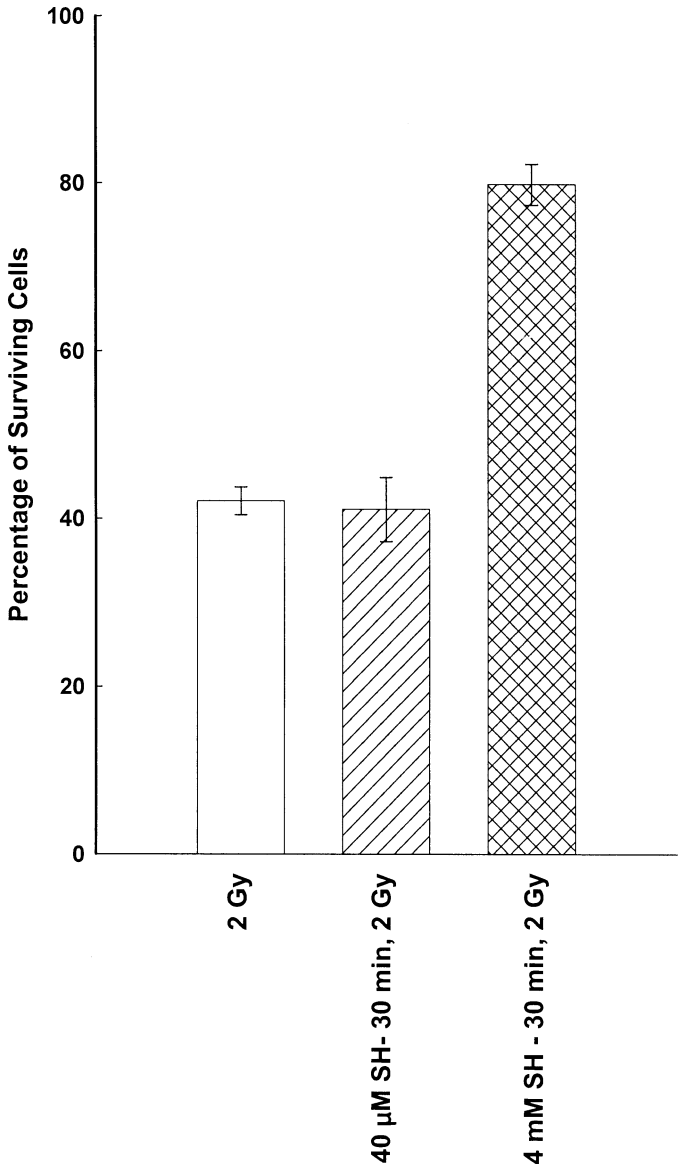


**FIG. 6.** Survival of RKO36 cells exposed to 2 Gy of 250 kVp X rays immediately after a 30-min treatment with either 40 μ*M* or 4 m*M* of WR-1065 (SH) and of cells irradiated in the absence of thiol treatment. Each bar represents the mean ± SEM of three separate experiments. Compared to the radiation only group, protection ratios of 0.98, $P = 0.97$, and 1.90, $P < 0.001$, were calculated for the 40 μM and 4 m*M* WR-1065 treatment conditions, respectively. *P* values were calculated using a two-tailed *t* test.

posure did not significantly affect catalase or GPX levels (see Figs. 4 and 5). These results are similar to those reported elsewhere for TNFα in which exposure of cells resulted in no effect on mRNA levels of both catalase and GPX (*4*).

To test whether elevated levels of SOD2 corresponded to an enhanced cellular resistance to the cytotoxic effects of ionizing radiation, RKO36 cells were challenged with a 2-Gy dose of radiation at 0 and 24 h after WR-1065 exposure. Consistent with numerous reports, WR-1065 significantly protected cells against radiation toxicity, e.g. the immediate radioprotective effect, when it was administered to cells 30 min prior to irradiation at a concentration of 4 m*M* (see Fig. 6) (*25*). A dose of 40 μ*M* was not cytoprotective under these conditions. However, when cells were irradiated with 2 Gy 24 h after a 30-min exposure to either dose of WR-1065, during which time SOD2 levels were elevated at least 15-fold over background values, cellular resistance to the cytotoxic effects of radiation was enhanced over 40% compared to non-thiol-exposed cells (see Fig. 7). RKO36 cells transfected with SOD2 siRNA did not exhibit an enhanced radiation resistance when irradiated under these conditions, indicating that elevation of SOD2 is an essential element of the delayed radioprotective effect (see Fig. 8). As demonstrated earlier with SANH (*15, 17*) and HMEC (*16*), WR-1065 is also effective in inducing a SOD2-mediated delayed radioprotective effect in human colorectal RKO36 cells, suggesting that this is a general phenomenon reflective of a novel paradigm for use in radiation protection.

While the delayed radioprotective effect reported in this paper has been defined in terms of a thiol-induced SOD2-mediated process, it is clear that elevation of SOD2 by any agent should also result in an elevated resistance to ROS-

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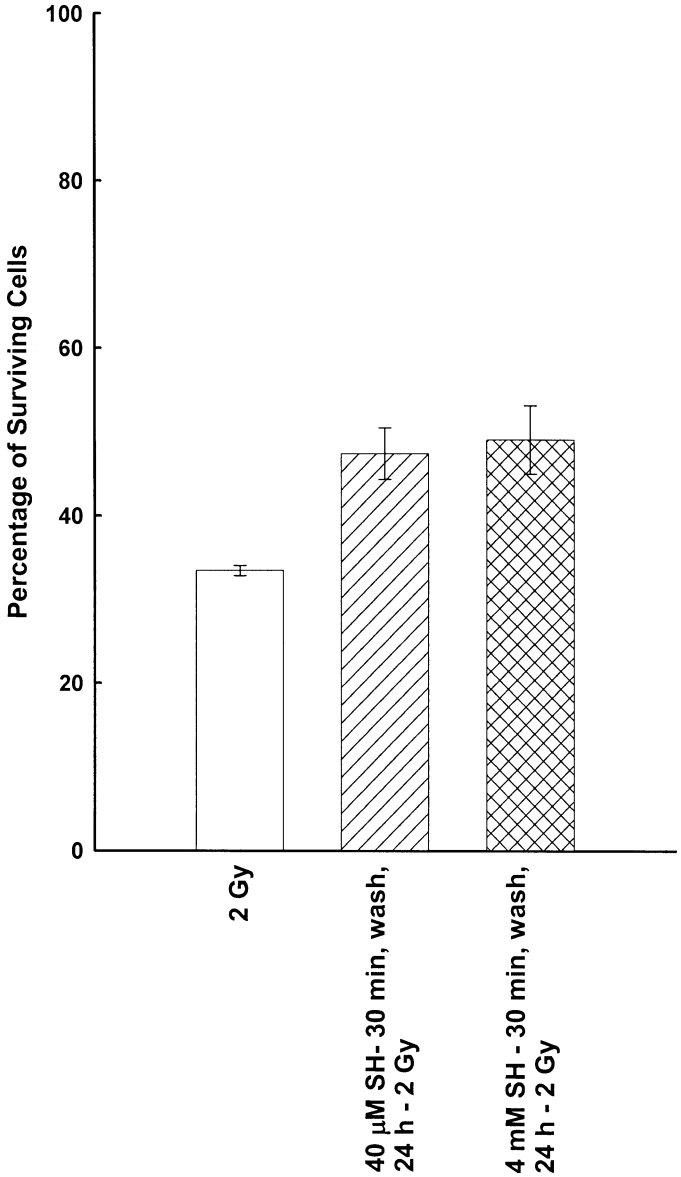


**FIG. 7.** Survival of RKO36 cells exposed to 2 Gy of 250 kVp X rays 24 h after a 30-min treatment with either 40 μ*M* or 4 m*M* WR-1065 (SH) and of cells irradiated in the absence of thiol treatment. Each bar represents the mean ± SEM of three separate experiments. Compared to the radiation only group, protection ratios 1.42, $P = 0.004$, and 1.48, $P = 0.006$, were calculated for the 40 μ*M* and 4 m*M* WR-1065 treatment conditions, respectively. *P* values were calculated using a two-tailed *t* test.

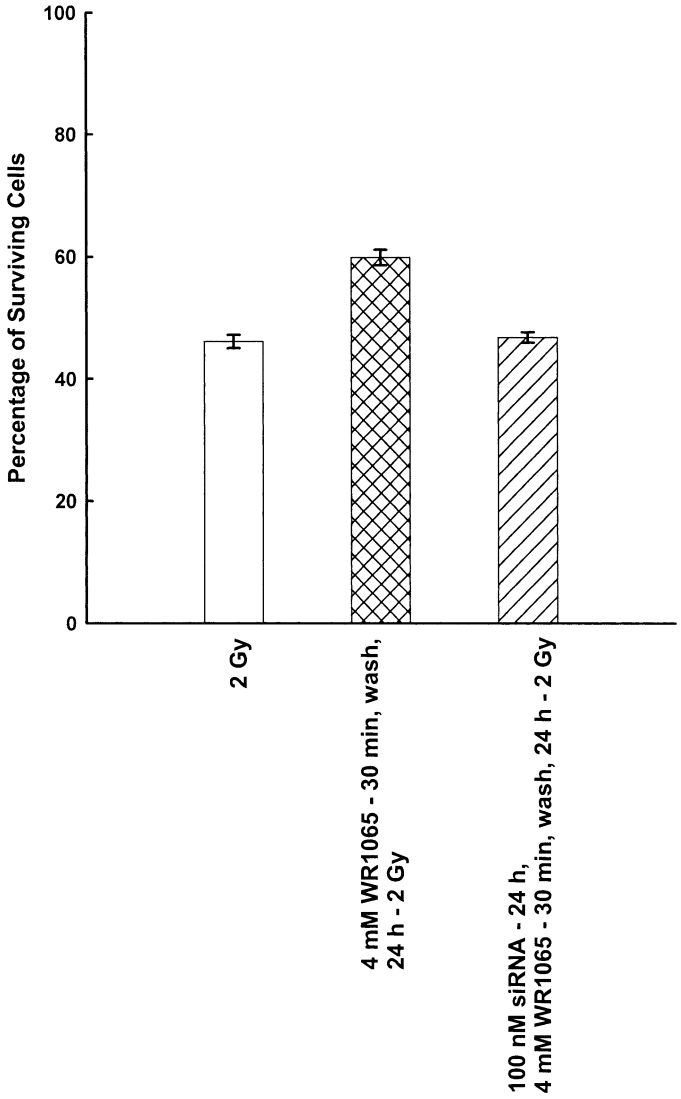


**FIG. 8.** Survival of RKO36 cells transfected with SOD2 siRNA and exposed to 2 Gy of 250 kVp X rays 24 h after a 30-min treatment with 4 m*M* WR-1065 and of non-transfected cells treated with 4 m*M* WR-1065 and irradiated 24 h later and cells irradiated in the absence of thiol treatment. Each bar represents the mean ± SEM of three separate experiments. Compared to the radiation only group, protection ratios of 1.02, $P = 0.666$, and 1.30, $P < 0.001$, were calculated for the SOD2 siRNA + 4 m*M* WR-1065 and the 4 m*M* WR0165 treatment conditions, respectively. *P* values were calculated using a two-tailed *t* test.

induced damage. TNFα is a pleiotropic cytokine that not only is an effective inducer of SOD2 (*4, 7*) but also has been identified as an effective radiation protector (*29–32*) and a radiation sensitizer (*26–28*). In the present study RKO36 cells were irradiated either immediately after or 20 h after a 30-min exposure to TNFα, the time at which SOD2 was elevated to its maximum value (see Fig. 3). TNFα at a dose of 10 ng/ml was not cytotoxic, nor did it exert either a protective or sensitizing effect when it was present for only 30 min prior to radiation exposure (see Fig. 9). However, if exposure of RKO36 cells to 2 Gy occurred 20 h later, when SOD2 was elevated over 20-fold above background, cellular resistance to radiation was increased significantly by 36% (see Fig. 10). Regardless of the inducing agent, elevated resistance to radiation toxicity is directly correlated with the increased levels of SOD2. These observations are in strong agreement with those of Neta (*30, 31*) and Moreb and Zucali (*29*), who described significant enhancement in the survival of mice administered TNFα 18 to 24 h before exposure to potentially lethal doses of whole-body radiation. This protective effect was attributed to an induction of elevated levels of SOD2.

In contrast to these studies, others have reported the ef-

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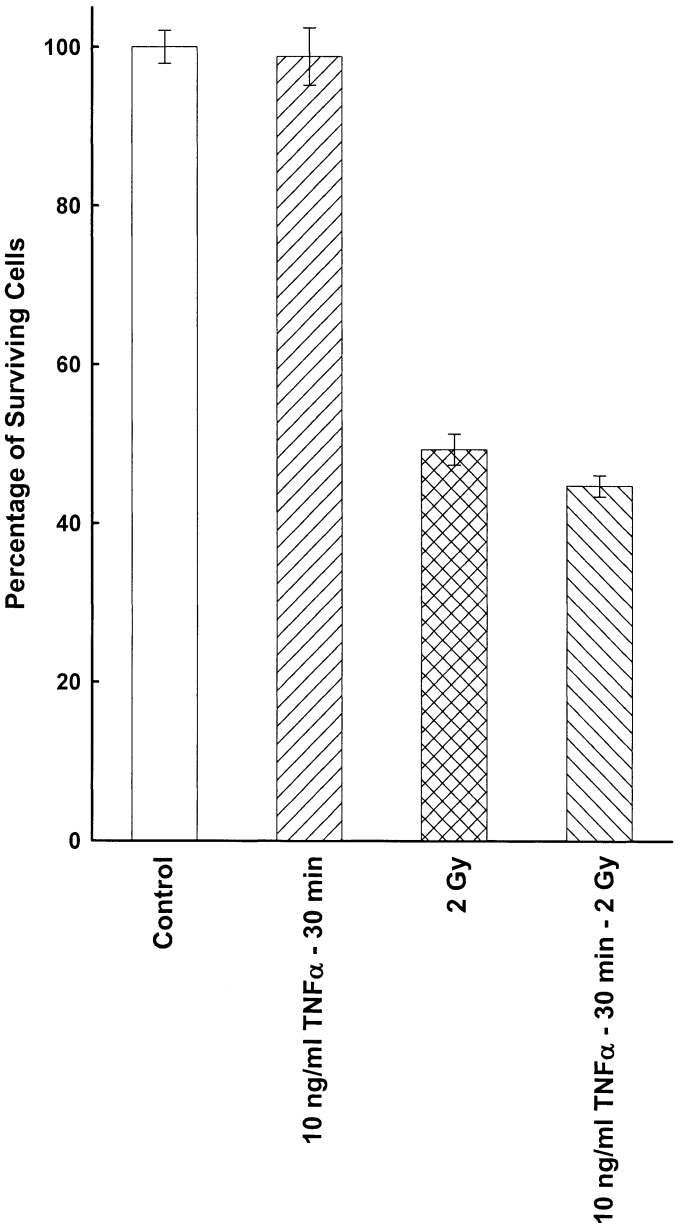


**FIG. 9.** Survival of RKO36 cells exposed to 10 ng/ml of the cytokine TNFα alone or 2 Gy of 250 kVp X rays either alone or immediately after a 30-min treatment with TNFα. Each bar represents the mean ± SEM of three separate experiments. Compared to the control group, a protection ratio of 0.99, $P = 0.772$ was calculated for the TNFα alone treatment. A protection ratio of 0.92, $P = 0.056$ was calculated for the group treated with TNFα prior to radiation exposure compared to the radiation only group. $P$ values were calculated using a two-tailed $t$ test.

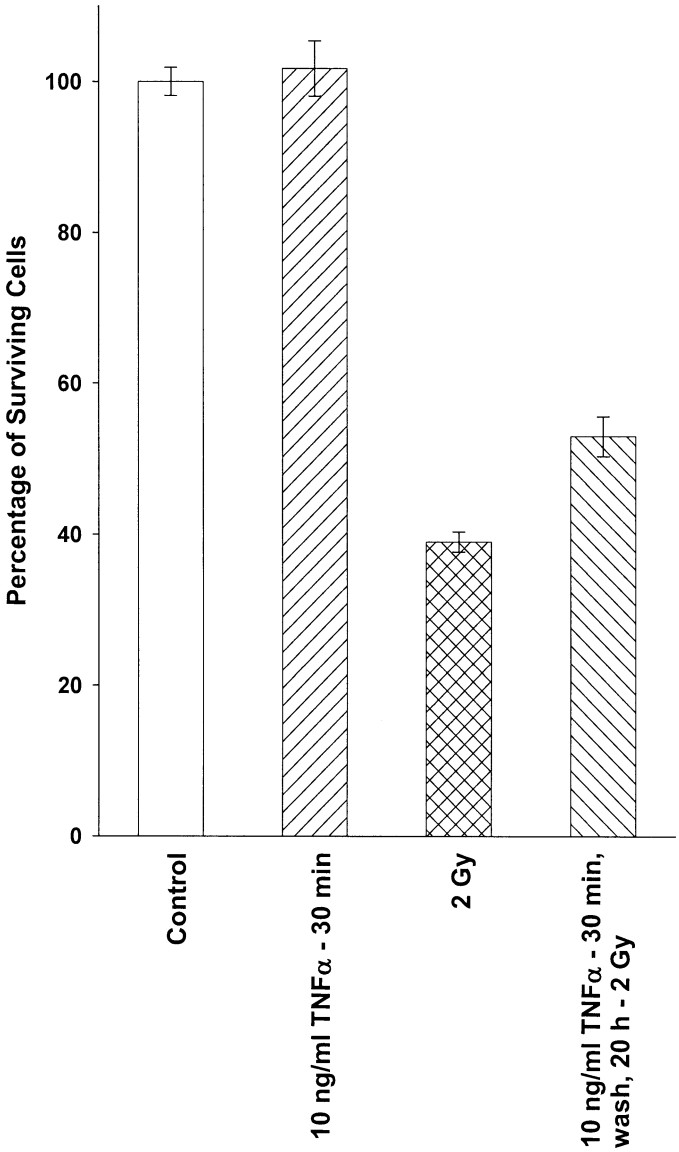


**FIG. 10.** Survival of RKO36 cells 20 h after exposure to 10 ng/ml of the cytokine TNFα alone, or 2 Gy of 250 kVp X rays either alone or 20 h after treatment with TNFα. Each bar represents the mean ± SEM of three separate experiments. Compared to the control group, a protection ratio of 1.02, $P = 0.407$ was calculated for the TNFα alone treatment. A protection ratio of 1.36, $P < 0.001$ was calculated for the group treated with TNFα 20 h prior to radiation exposure compared to the radiation only group. $P$ values were calculated using a two-tailed $t$ test.

fectiveness of TNFα as a radiation sensitizer (*26–28*). TNFα can induce ROS and affect cellular pathways leading to cell proliferation, cell death and inflammation (*33*). In the studies demonstrating the radiation-sensitizing effects of TNFα, cells were exposed to the cytokine for prolonged periods, with a 12-h exposure prior to irradiation being the most effective to induce sensitization (*26–28*). Under the *in vitro* conditions used in those studies, it was suggested that TNFα induced oxidative damage that contributed to ROS produced by ionizing radiation in an additive or synergistic manner that resulted in an overall greater toxicity to cells. Differences between the experimental design of these studies and the one described here may account for the discrepancy in the effects of TNFα observed regarding radiation response. In the present study, TNFα was administered to cells for 30 min, at which time it was removed rather than allowing the full dose of the cytokine to remain in the growth medium for a prolonged period prior to irradiation. More importantly, SOD2 values remained relatively low 12 h after TNFα exposure in the present study,

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rising to their peak value at 20 h, the time at which the cells were irradiated. While TNFα can elicit many different cellular responses, it appears not to function as either a radioprotector or a radiosensitizer if administered just prior to irradiation (see Fig. 9). Rather, the downstream responses it elicits can function directly in these two diverse processes. Specifically, TNFα can induce a SOD2-mediated delayed radioprotective effect like the nonprotein thiol WR-1065. Therefore, whether it acts as a protector or sensitizer may be a function of the time it is administered/induced prior to radiation exposure.

The delayed radioprotective effect that is elicited by nonprotein thiols like WR-1065 and TNFα is clearly mediated by downstream changes in *SOD2* expression (*15–17*). Not only can SOD2 participate in antioxidant processes to protect directly against ROS-induced damage to cells, but overexpression of *SOD2* has also been implicated in affecting the expression of additional genes that are proposed to participate in other radiation-induced adaptive responses. *MYC*, p21,14-3-3 zeta, cyclin A, cyclin B1, and *GADD153* gene expression were each stimulated in cells of a MCF-7 human breast adenocarcinoma cell line engineered to overexpress *SOD2*, suggesting that its increased expression could affect additional downstream signaling pathways involved in the activation of these radioresistance genes (*34*). Regardless, *SOD2* appears to be an important antioxidant gene whose inducible expression is a key element in adaptive responses in general, and the delayed radioprotective effect in particular. Furthermore, while both nonprotein thiols such as WR-1065 and the cytokine TNFα are effective inducers of *SOD2* gene expression, WR-1065, in contrast to TNFα, has an advantage in that it does not induce the expression of inflammatory genes known to have NFκB binding motifs such as intercellular adhesion molecule 1 (*ICAM1*) (*13, 35*).

The SOD2-mediated delayed radioprotective effect can be considered as an example of an adaptive response. The adaptive response was first observed using an *E. coli* system in 1977 (*36*). These observations led to a restricted description of the adaptive response in that it referred to a process by which cells exposed to a low dose of a deleterious agent such as one capable of inducing a mutagenic or clastogenic effect would become more resistant to a subsequent higher dose when it was administered at some later time. The adaptive response, in the context of radiation biology, was considered to be a phenomenon by which exposure of certain types of cells to a relatively low dose of radiation induces an enhanced resistance to the deleterious effects of a second but much larger dose of radiation (*18*). This response has been linked to the requirement that *de novo* protein synthesis must occur after the initiating dose since exposure of cells to the protein synthesis inhibitor cycloheximide will abrogate the effect (*37*).

The radiation-induced adaptive response has been studied extensively since that time using a variety of end points that include chromosomal aberrations, mutation induction and cell survival (*38*). The implication of this phenomenon to the field of low-dose radiation risk assessment has been a major driving force for its continued study. However, taken in a more global context, the adaptive response can be considered as any coordinated response of cells and tissues to a stimulus that elicits a downstream robust response to subsequent more intense stimuli. Presumably this process results from inducing signals of the initial stimulus that leads to a signal transduction-mediated response that generates a coordinated adaptive response to better prepare cells to cope with more intense additional exposures (*19*). In the case of the radiation-induced adaptive response, the initial priming dose is in the range of 0.01–0.2 Gy (*38*), and it is not a universal phenomenon but is cell type specific. In contrast, the nonprotein thiol-induced SOD2-mediated delayed radioprotective effect appears be a more general phenomenon. It has been demonstrated to occur in mouse SANH tumor cells, HMEC and human colorectal RKO36 cells, and it can be induced by nonprotein thiols that include *N*-acetylcysteine, captopril, mesna and WR-1065 (*13, 15–17*). Both the radiation-induced adaptive response and the thiol-induced delayed radioprotective effect clearly fit within the global context of adaptive responses, but only the latter readily lends itself as a novel paradigm for application to the field of radiation protection.

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