Delayed Radioprotection by NF\(\kappa\)B-Mediated Induction of Sod2 (MnSOD) in SA-NH Tumor Cells after Exposure to Clinically Used Thiol-Containing Drugs

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The ability of thiol-containing reducing agents to activate transcription factors leading to changes in gene expression and enzyme activities provides an additional mechanism to potentially protect against radiation-induced cell killing. Manganese superoxide dismutase (Sod2) is one such gene whose expression levels have been shown to be elevated after exposure to the thiol compounds WR-1065 and N-acetyl-L-cysteine (NAC), resulting in an increase in radiation resistance. To further characterize this effect, SA-NH sarcoma cells, both wild-type and a clone stably transfected with a plasmid containing an IkB\(\kappa\) gene mutated at serines 32 and 36, which prevents the inducible phosphorylation of these residues and the subsequent activation of NF\(\kappa\)B (SA-NH\(^+\)+IkB\(\kappa\)-def), were grown to confluence and then exposed to amifostine's free thiol WR-1065 at a concentration of 4 mM for 30 min. Effects of thiol exposure on NF\(\kappa\)B activation in SA-NH\(^+\)+IkB\(\kappa\)-def cells were determined by a gel shift assay, and changes in Sod2 protein levels in these cells 24 h after exposure to 40 \(\mu\)M or 4 mM WR-1065 were measured by Western blot analysis and compared with wild-type cells exposed to the NF\(\kappa\)B inhibitor BAY 11-7082. Changes in radiation response, measured immediately after thiol exposure or 24 h later, were determined using a colony-forming assay and were correlated with NF\(\kappa\)B activation and Sod2 protein levels. The effects of captopril, mesna and NAC, each at a dose of 4 mM, on radiation response were also determined and contrasted with those of WR-1065. Only WR-1065 and captopril protected SA-NH cells when present during irradiation, i.e. 1.57 and 1.31 times increase in survival at 2 Gy, respectively. All four thiols were protective if irradiation with 2 Gy occurred 24 h later; i.e. increases in survival of 1.40, 1.22, 1.35, and 1.25 times were found for WR-1065, captopril, mesna and NAC, respectively. This delayed radioprotective effect correlated with elevated Sod2 protein levels in wild-type SA-NH tumor cells but was not observed in SA-NH\(^+\)+IkB\(\kappa\)-def cells, indicating that interference with thiol-induced NF\(\kappa\)B activation abrogates this delayed radioprotective effect. Because the delayed radioprotective effect is readily demonstrable at a radiation dose of 2 Gy 24 h after exposure to clinically approved thiol-containing drugs such as amifostine, captopril, mesna and NAC, it suggests a new potential concern regarding the issue of tumor protection and the use of these agents in cancer therapy.

INTRODUCTION

Amifostine (\(\text{S}-2-[3\text{-}3\text{-amino}\text{propylamino}]\text{ethylphosphorothioic acid}\)), captopril ([\(\text{S}\)\(\text{-}1\text{-}[3\text{-mercapto}\text{-2\text{-methyl\text{-}1\text{-oxo}}\text{-propyl}]\text{-L\text{-proline}}\)), mesna (sodium-2-mercaptoethanesulfonate), and N-acetyl-L-cysteine (NAC) are non-protein thiols that are currently in clinical use. While it is possible that patients undergoing treatment for cancer can be exposed to each of these during their therapy, amifostine is the only non-protein thiol that has been approved by the Food and Drug Administration specifically for use as a radioprotector. Its approved indication is for the prevention of xerostomia in head and neck cancer patients treated with radiation therapy (1).

The cytoprotective properties of non-protein thiols are the result of their ability to effectively scavenge highly reactive free radicals formed by oxidative stress-inducing agents such as ionizing radiation. However, the presence of a highly reactive thiol group on these agents also allows them to participate in reductive/oxidative intracellular processes that can affect gene expression and protein activity (2–4). As an example, each of these non-protein thiols is capable of directly activating the redox-sensitive nuclear transcription factor \(\kappa\)B (NF\(\kappa\)B, NIKb), and of enhancing expression of the manganese superoxide dismutase gene (Sod2) (5–8). Sod2 is one of a number of endogenous antioxidant enzymes that protect against reactive oxygen spec-
cies. In particular, Sod2 catalyzes the dismutation of superoxide radicals to oxygen and hydrogen peroxide (9). Catalase and glutathione peroxidase in turn detoxify the resultant hydrogen peroxide into water and oxygen. Sod2 is the only form of superoxide dismutase that has been determined to confer radiation protection (10–15). Specifically, localization of Sod2 within the cytoplasm was ineffective in protecting cells against radiation cytotoxicity, while localization in the mitochondria of Sod1, a form of superoxide dismutase not associated with radiation resistance and normally found in the cytoplasm, led to radiation protection of cells (14, 15). Sod2 also has been reported to protect cells against interleukin 1, tumor necrosis factor, and chemotherapeutic drug-induced cytotoxicity (10–13). These observations have led to the development of novel antioxidant radioprotective approaches, such as the use of gene therapy in which a Sod2 transgene is administered by targeted plasmid/liposomes to protect against esophagitis and pulmonary tissue damage from ionizing radiation (16–21).

We recently reported on a novel phenomenon we identified as ‘‘delayed radioprotection’’ that is induced in SA-NH murine sarcoma cells exposed to WR-1065, the free thiol form of amifostine (22). Delayed cytoprotection refers to the enhanced radiation resistance of cells to a dose of 2 Gy 24 h after their exposure to thiol-containing drugs such as WR-1065, the time when levels of Sod2 are significantly elevated over background levels. The proposed underlying mechanism of action for this effect involves the activation of the redox-sensitive transcription factor NFκB that leads to the enhanced expression of Sod2, followed by a 10- to 20-fold elevation in the levels of active Sod2 protein. This elevation of Sod2 levels gives rise to an increased radiation resistance of cells 24 h after thiol exposure that is comparable in magnitude to that observed immediately after the exposure of cells to the radioprotector WR-1065, i.e. the classical ‘‘immediate radioprotective effect.’’

Classical radioprotection by amifostine is based on its ability to scavenge radiation-induced free radicals, facilitate chemical repair, and/or induce transitory intracellular hypoxia due to auto-oxidation processes. Each of these three mechanisms reflects rapid reactions that require the presence of amifostine at the time of radiation exposure. Since the approved indication of amifostine in radiation oncology involves its daily administration just prior to fractionated 2-Gy doses of radiation, delayed radioprotection, a heretofore unrecognized phenomenon, could lead to a negative impact on tumor response. In this study we further characterize the effects of WR-1065, the active thiol form of amifostine, on the activation of NFκB and Sod2 gene expression as they relate to the delayed radioprotective effect. The magnitude of the delayed effect at a radiation dose of 2 Gy is contrasted to that of the classical immediate radioprotective effect of WR-1065. In addition, other clinically used non-protein thiols (i.e. captopril, mesna and NAC) whose use could also have an impact on tumor response to radiation therapy were examined to determine their ability to induce the delayed radioprotective effect.

METHODS AND MATERIALS

Cells and Culture Conditions

SA-NH cells derived from an SA-NH murine sarcoma tumor and adapted for in vitro growth in culture medium were obtained from the laboratory of Dr. Luka Milas, Department of Experimental Radiation Oncology, M. D. Anderson Cancer Center, Houston, Texas. SA-NH cells were maintained in modified McCoy’s 5A medium (Invitrogen Life Technologies, Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS) (Intergen, Purchase, NY), penicillin and streptomycin (Invitrogen). Cells were subcultured weekly using 0.25% trypsin and 1 mM EDTA (Invitrogen) and were incubated in a humidified atmosphere of 5% CO2 and 95% air at 37°C.

Mutant IκBa Transfection

To assess the possible role of NFκB activation in the delayed radioprotective response, SA-NH cells were transfected with a pcDNA3 plasmid containing a mutant IκBa gene under the control of a CMV promoter in which serines 32 and 36 were mutated (donated by Dr. J. J. Li) and a neomycin resistance gene to allow the selection of stable transfectants using G-418 (Invitrogen) following a method described in detail elsewhere (23). These mutations abolish the inducible phosphorylation of these residues, preventing the subsequent ligand-induced degradation that leads to activation of NFκB (24). Stably transfectants were obtained using Lipofectamine Reagent and Plus Reagent (Invitrogen). Brieﬂy, 15 µg of the plasmid containing the mutant IκBa gene was mixed with 40 µl of Plus Reagent and 435 µl of serum-free, antibiotic-free McCoy’s 5A medium and incubated at room temperature for 15 min. Lipofectamine Reagent (40 µl) was then mixed with 440 µl of serum-free, antibiotic-free McCoy’s 5A medium, combined with the plasmid/Plus Reagent mixture, and incubated for an additional 15 min at room temperature. During this incubation, the cells were washed with phosphate-buffered saline (PBS; 8.1 mM Na2HPO4, 1.5 mM KH2PO4, 140 mM NaCl, Invitrogen) and 1 ml of serum-free, antibiotic-free McCoy’s 5A medium was added. The transfection complex mixture was then added and the cells were incubated at 37°C for 18 h. After transfection, the medium was aspirated and replaced with 10 ml of complete medium. Selection of transfectants was begun 3 days later by adding 600 µg/ml of G-418 to the growth medium. Cells were cultured in this selection medium for 24 h, and then fresh complete medium containing 400 µg/ml G-418 was added. Cells were maintained in the selection medium until the formation of G-418-resistant colonies, approximately 14 days later. One such colony was expanded, and the resulting clone was designated SA-NH+mlkBo1 and used in this study. In all experiments the medium used was identical for both SA-NH and SA-NH+mlkBo1 cells with the exception that growth medium for SA-NH+mlkBo1 cells also contained G-418 at a concentration of 400 µg/ml. All experiments were performed using cells that were grown to confluence (i.e. a monolayer of cells completely covering the surface of the culture dish). After reaching 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.

Drugs

WR-1065 was supplied by the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD. Captopril, mesna and NAC were obtained from Sigma, St. Louis, MO.
Immediately before use, each thiol was dissolved in PBS at a concentration of 1 M. Cells were exposed to each thiol at a concentration of 4 mM for 30 min. These concentrations were not toxic and in the case of WR-1065 represented the dose that afforded a maximal level of cytoprotection under in vitro conditions (25).

**NFkB Inhibitor**

BAY 11-7082 ([(E)-[(4-methylphenyl)-sulfonyl]-2-propenenitrile], a propenenitrile compound that inhibits IkB phosphorylation and NFkB activation, was obtained from Biomol Research Laboratories, Plymouth Meeting, PA. SA-NH cells grown to confluence were exposed for 1 h to 20 μM of BAY 11-7082 prior to treatment with WR-1065.

**Electrophoretic Mobility Shift Assay**

Electrophoretic mobility shift assays (EMSA) were performed using methods that are described in detail elsewhere (5). Briefly, nuclear extracts were prepared from confluent cultures of SA-NH and SA-NH+mlkBα1 cells that were either sham-treated or exposed to 4 mM of WR-1065 for 30 min. Immediately thereafter, cells were washed with PBS to remove WR-1065 and were then maintained in drug-free medium for an additional 30 min. Nuclear protein was then isolated from sham-treated controls and WR-1065-exposed cells and nuclear protein concentrations were determined using the method of Bradford (26) (Bio-Rad, Richmond, CA). Protein samples were adjusted to a final concentration of 2 μg/μl. EMSAs were performed using the Promega gel shift assay system (Madison, WI). The NFkB consensus oligonucleotide (5'-AGTTGAGGGGAATTCGGGC-3') was end labeled with [γ-32P]ATP using T4 polynucleotide kinase and incubated for 10 min at 37°C. All binding reactions were performed using 5 μl of nuclear extract that contained 10 μg of protein as described elsewhere (5). Nonspecific and specific competitor reactions were also performed to characterize binding specificity. HeLa cell nuclear extract was used as a positive control. The competitor reactions contained a 50-fold excess of unlabelled Sp1 consensus sequence oligonucleotide (nonspecific competitor) or unlabelled NFkB consensus sequence oligonucleotide (specific competitor). Samples were electrophoresed at 5 V/cm for 3 h on 8% nondenaturing polyacrylamide gels.

**Western Blotting**

Protein isolation and Western blot analysis were performed following methods described previously (22). Briefly, cell lysates were prepared from SA-NH and SA-NH+mlkBα1 cells that were grown to confluence in 100-mm tissue culture dishes. After isolation and determination of protein concentrations (22, 26), Sod2 protein levels were assessed using the Chemiluminescent Western Blotting Immuno-detection System (Invitrogen). Total cellular proteins were electrophoresed at a concentration of 20 μg on a 12% SDS polyacrylamide gel according to the method of Laemmli (27). 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-MnSOD, catalogue no. 06-984, Upstate, Charlotte, NC) at 4°C overnight. After the blots were washed three times with Washing Buffer (Invitrogen), they were incubated with goat anti-rabbit IgG conjugated with alkaline phosphatase (Invitrogen) for 30 min. Protein bands were visualized using Chemiluminescent Substrate (Invitrogen) as per instructions. The membrane was exposed to X-Omat AR film (Kodak, Rochester, NY) and scanned using an ASTRA 2200 scanner (UMAX, Fremont, CA). Band intensities were analyzed using NIH Image software.

**Irradiation Conditions and Survival Assay**

All survival assays were performed using SA-NH or SA-NH+mlkBα1 cells grown to confluence. Cells were irradiated at room temperature using a Pantak X-ray generator operating at 250 kVp and 15 mA at a dose rate of 1.55 Gy per minute. Radiation doses ranged from 0 to 8 Gy. To assess the “immediate radioprotective” effectiveness of the thiols WR-1065, captopril, mesna and NAC, cells were exposed to a 4 mM concentration for 30 min just prior to irradiation. Immediately after exposure to radiation, the cells were washed free of drug, trypsinized, counted and plated into fresh growth medium at appropriate numbers to give rise to about 100 colonies per dish. Five dishes were used per experimental point, and experiments were repeated two to three times. Cells were incubated under standard growth conditions for 10 days and resultant colonies were stained with 20% crystal violet; colonies containing 50 or more cells were counted. The same procedure was followed to assess the “delayed radioprotective” effects of the thiols with one difference. Cells were exposed to a thiol for 30 min, then washed free of the drug and incubated in growth medium for 24 h before being exposed to ionizing radiation. Survival curves spanning a range of doses from 0 to 8 Gy were derived for both wild-type SA-NH and SA-NH+mlkBα1 cells. The resulting survival curves were analyzed using both an α/β model and a multitarget model. Because Sod2 has been demonstrated to affect primarily the initial slope of the survival curve (11, 13, 22), the α parameters of the survival curves were analyzed and compared. To facilitate a comparison between the various treatment conditions an α protection factor ratio (αPER), defined as αcontrol/αnont, was used. In addition, survival curves were also analyzed using the multitarget model to determine D0 values representing the reciprocals of the slopes of the terminal regions of each survival curve using SigmaPlot 8.0 (SPSS, Chicago, IL) and Microsoft Excel. 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**

**NFkB Activation**

The importance of NFkB activation in affecting the levels of Sod2 protein after exposure of cells to WR-1065 was addressed through the use of both a specific inhibitor of NFkB activation, i.e. BAY 11-7082, and a stably transfected SA-NH+mlkBα1 clone. Presented in Fig. 1 is a representative Western blot that shows the inhibitory effect of BAY 11-7082 on the induction of increased levels of Sod2 after a 30-min exposure to a 4 mM concentration of WR-1065. BAY 11-7082 is a propenenitrile compound that se-
selectively inhibits inducible phosphorylation of IkBα, thereby inhibiting activation of NFκB and its ability to translocate into the nucleus (28). Confluent SA-NH cells were exposed to 20 μM of BAY 11-7082 for 1 h prior to exposure to WR-1065. The medium was then changed, and cells were washed in PBS and exposed to fresh medium for an additional 24 h, a time previously determined to be optimum for maximal enhancement of Sod2 levels (22).

While BAY 11-7082 is a potent inhibitor of IkBα phosphorylation and subsequent NFκB activation, it can also affect other intracellular activities such as stimulation of the stress-activated protein kinases p38 and JNK1 and the activation of tyrosine phosphorylation of a 130–140-kDa protein (28). For this reason we developed a stably transfected SA-NH cell line using a dominant negative IkBα construct in which serines 32 and 36 were mutated so as to abolish inducible phosphorylation of these residues and the subsequent ligand-induced degradation that leads to activation of NFκB (24). We have contrasted the effects of a 30-min exposure to a 4 mM concentration of WR-1065 on the activation of NFκB in both confluent SA-NH+mIkBα1 cells and wild type SA-NH cells. Presented in Fig. 2 is a representative gel shift that describes WR-1065-induced activation of NFκB in SA-NH cells but not SA-NH+mIkBα1 cells.

To assess the effects of WR-1065 exposure on Sod2 levels in SA-NH+mIkBα1 cells, they were exposed to either 40 μM (antimitogenic) or 4 mM (cytoprotective) of WR-1065 for 30 min, then washed free of drug with PBS and cultured for 24 in fresh medium. At that time relative Sod2 protein levels were determined by Western blotting (see Fig. 3). In contrast to a 10-fold enhancement observed after 24 h in SA-NH cells (22), no increase in the levels of Sod2 over control levels were detected in SA-NH+mIkBα1 cells.

**Radiation Response**

The responses of SA-NH and SA-NH+mIkBα1 cells exposed to 0 to 8 Gy of ionizing radiation are presented in Fig. 4 for comparison. Parental SA-NH cells are relatively more radioresistant than are the stably transfected SA-NH+mIkBα1 cells $[\alpha = 0.278 \pm 0.040 \text{ (SEM), } \beta = 0.0962 \pm 0.0073, \alpha/\beta = 2.893 \pm 0.595 \text{ compared to } \alpha = 0.150 \pm 0.021, \beta = 0.0439 \pm 0.003, \alpha/\beta = 3.409 \pm 0.723, \text{respectively}].$ As described in Fig. 5, exposure of SA-NH+mIkBα1 cells to 4 mM of WR-1065 for 30 min prior to irradiation resulted in significant immediate radioprotection. While the data were plotted using the $\alpha/\beta$ model in Fig. 5, relative protection factors were also determined and contrasted using both the ratio of initial slopes, i.e., $\alpha_{\text{control}}/\alpha_{\text{thiol}}$ or $\alpha_{\text{PER}},$ and the traditional ratio of terminal slopes, i.e., $D_0$'s, using the multitarget model (see Tables 1 and 2). These analyses resulted in an $\alpha_{\text{PER}}$ of 4.04 and a protection factor (PF) of 1.8, respectively.

In contrast to a delayed radioprotective effect induced in parental SA-NH cells 24 h after exposure to WR-1065, i.e. $\alpha_{\text{PER}}$ of 2.44 (22), no change in radiation response was observed in SA-NH+mIkBα1 cells treated in the same manner, i.e. $\alpha_{\text{PER}}$ of 0.89. Dose–response curves describing SA-NH+mIkBα1 cells irradiated 24 h after either sham treatment or exposure to 4 mM WR-1065 for 30 min are presented in Fig. 6 for comparison. Radiation survival curve parameters describing the responses of sham- and WR-1065-treated cells were $\alpha = 0.150 \pm 0.021, \beta = 0.0439 \pm 0.0031, \alpha/\beta = 3.409 \pm 0.723$ and $\alpha = 0.169 \pm 0.022, \beta = 0.0423 \pm 0.003, \alpha/\beta = 3.990 \pm 0.800,$ respectively.

The ability to induce activation of the transcription factor NFκB and subsequent enhancement of Sod2 gene expression is not limited to WR-1065. NFκB activation and increased Sod2 gene expression after exposure of cells to non-protein thiols such as captopril, mesna and NAC have also been reported (6, 7, 22). To assess and compare their relative abilities to affect both immediate and delayed radioprotection, SA-NH cells were exposed to 4 mM concentrations of these non-protein thiols for 30 min under conditions described previously for WR-1065. In contrast to WR-1065, mesna and NAC were relatively ineffective as radioprotectors, as shown in Fig. 7. Neither mesna nor NAC had any effect on the immediate radioprotection of SA-NH cells as seen in the lack of an increase in cell survival after exposure to 2 Gy of ionizing radiation, i.e., $PF_{2\gamma} = 1.03, P = 0.72$ and $PF_{2\gamma} = 1.04, P = 0.66,$ respectively. Both WR-1065 and captopril were radioprotective under these conditions resulting in increased numbers of surviving cells, i.e., $PF_{2\gamma} = 1.57, P < 0.005$ and $PF_{2\gamma} = 1.31, P < 0.005,$ respectively.

In contrast, all four non-protein thiols were effective in inducing a delayed radioprotective effect when SA-NH cells were irradiated with 2 Gy 24 h later (see Fig. 8). Non-protein thiols ranked in order of increasing effectiveness were captopril, $PF_{2\gamma} = 1.22; \text{NAC, } PF_{2\gamma} = 1.25; \text{mesna, } PF_{2\gamma} = 1.35; \text{and WR-1065, } PF_{2\gamma} = 1.40.$ All were found to significantly increase the survival of SA-NH tumor cells at 2 Gy over that of control cells that were not treated with non-protein thiols ($P \leq 0.005$).

The ability of captopril, mesna and NAC to affect both immediate and delayed radioprotection of SA-NH+mIkBα1 cells was also evaluated and compared with that of WR-1065. As was observed for SA-NH cells, only WR-1065 and captopril were radioprotective under the immediate exposure conditions, i.e., $PF_{2\gamma} = 1.39, P < 0.001$ and $PF_{2\gamma} = 1.11, P = 0.004,$ respectively (see Fig. 9). In contrast to the delayed radioprotection observed for all four non-protein thiols in the SA-NH cells, neither WR-1065 ($PF_{2\gamma} = 0.99), \text{NAC (PF}_{2\gamma} = 0.97), \text{captopril (PF}_{2\gamma} = 0.97)\text{ nor mesna (PF}_{2\gamma} = 0.96)$ was effective in inducing a delayed radioprotective effect in SA-NH+mIkBα1 cells irradiated with 2 Gy 24 h after drug treatment (see Fig. 10).
**DISCUSSION**

Amifostine, along with other thiol-containing reducing agents, have been observed in a number of experimental systems to be effective in activating NFκB and in enhancing the expression and subsequent translation of the antioxidant gene Sod2 (5–8, 22). Elevation of Sod2 levels in turn leads to increased resistance to the cytotoxic effects of ionizing radiation (10–22). These observations form the basis for the proposed model of delayed radioprotection that we reported on earlier (22). Under this model, exposure of cells to thiol-containing reducing agents results in the activation of the redox-sensitive transcription factor NFκB. The Sod2 gene contains several NFκB binding motifs (6, 7, 11, 12). In particular, there is an intronic NFκB binding site that has been demonstrated to be essential for the induction of Sod2 transcription after exposure of cells to tumor necrosis factor α and interleukin 1β (29). Thus activation of NFκB leads to the enhanced expression of Sod2. This results in 10- to 20-fold elevations in Sod2 protein levels over background levels 16 to 24 h later (22). Cells exposed to ionizing radiation at this time exhibit an enhanced radiation resistance, as seen in higher survival levels, especially at the clinically relevant dose of 2 Gy (22).
The purpose of this study was to test the validity of this model.

To address the role of NFκB activation leading to Sod2 gene expression after exposure to WR-1065, cells were exposed to the specific NFκB inhibitor BAY 11-7082. As shown in Fig. 1, exposure of SA-NH cells to this inhibitor abolished the elevation of Sod2 levels that are routinely observed after exposure of cells to WR-1065. The relationship between WR-1065-induced NFκB activation and elevation in Sod2 gene expression was characterized further through the use of a mutated IκBα plasmid construct that was stably transfected into SA-NH cells (24). IκBα is an isoform of the NFκB inhibitor protein that mediates a rapid and specific activation of NFκB after exposure to stress stimuli such as ionizing radiation (30). Exposure of the stably transfected clone, designated SA-NH+mIκBα1, to WR-1065 failed to demonstrate either an activation of NFκB (Fig. 2) or an elevation of Sod2 protein (Fig. 3), in contrast to results obtained using parental SA-NH cells. However, the basal NFκB activity of untreated control SA-NH+mIκBα1 cells is equal to that of nontransfected SA-NH cells, and this similarity in NFκB activity is also re-
Delayed exposure of stably transfected cells expressing the mutant IkBa such as ionizing radiation (24) transient activation of NFκB and subsequent elevation of Sod2 levels. WR-1065 prevents the subsequent elevation of Sod2 levels and abolishes the delayed radioprotective effect. The demonstration that inhibition of NFκB activation by WR-1065 prevents the subsequent elevation of Sod2 levels and abolishes the delayed radioprotective effect establishes the molecular mechanism of this phenomenon. In assessing the significance of this effect, it is important to note that Sod2, in contrast to the radioprotector WR-1065, a polypeptide-like molecule that concentrates in both the mitochondria and the microenvironment of nuclear DNA and can protect both targets, is present only in the mitochondria (14, 15, 25, 31–33). Therefore, the subcellular targets that are specifically protected by elevated levels of Sod2 are the mitochondria, and it is the protection of this organelle that would account for the enhanced cell survival that is observed when Sod2-based cytoprotective regimens are used (12–22).

IkBa is one of at least three isoforms of the IκB family of NFκB inhibitor proteins that function by masking the nuclear localization sequence of NFκB. In contrast to the IkBβ and IkBε isoforms that are involved in stabilizing the constitutive NFκB response, IkBa mediates the rapid but transient activation of NFκB after exposure to a stress stimulus such as ionizing radiation (24). As discussed earlier, exposure of stably transfected cells expressing the mutant IkBa isoform to WR-1065 does not result in the activation of NFκB or elevation of intracellular Sod2 protein levels. However, it is evident that the presence of WR-1065 at the time of irradiation can confer substantial radioprotection to these cells, as has been described for the parental SA-NH cells, i.e. the immediate radioprotective effect, regardless of the method chosen to analyze the data (see Tables 1 and 2). This is not surprising given the well-described physicochemical-based mechanisms of action of WR-1065 that include free radical scavenging, chemical repair by hydrogen atom donation, and induction of transient intracellular hypoxia, each of which can result in a reduction in the amount of initial radiation damage (34).

In contrast to the qualitatively similar responses of SA-NH and SA-NH+IkBa cells to the immediate radioprotective effects induced by WR-1065 (see Fig. 5) (22), each responded differently when irradiation occurred 24 h after exposure to the radioprotector (see Fig. 6) (22). Intracellular levels of both WR-1065 and its disulfide form rapidly fall to undetectable levels by 1 h after the removal of the exposure medium and the washing of cells with fresh medium (25). Therefore, the response of cells to irradiation 24 h after their exposure to WR-1065 cannot be due to residual intracellular levels of the protector but rather must reflect molecular changes induced by that exposure. As described earlier, it is proposed that those changes reflect the activation of NFκB that leads to enhanced Sod2 gene expression. This conclusion is confirmed by the lack of a delayed radioprotective effect exhibited in SA-NH+IkBa cells.

### Table 1

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<th>Protection Enhancement Ratios* (4 mM WR-1065)</th>
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<td><strong>Cells</strong></td>
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<td>Immediate</td>
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<td>SA-NH</td>
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<td>SA-NH + IkBa</td>
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<tr>
<td>Delayed</td>
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<td>SA-NH</td>
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<td>SA-NH + IkBa</td>
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*αPER = αControl/αTreated.

| Irradiation immediately after 30 min exposure to WR-1065. |

| Data from ref. (22). |

| Irradiation 24 h after 30 min exposure to WR-1065. |

### Table 2

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<th>Protection Factors*</th>
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<td><strong>Cells</strong></td>
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*Ratio of D0’s ± WR-1065.

*Irradiation immediately after 30 min exposure to 4 mM WR-1065.

*Data from ref. (22).

*Irradiation 24 h after 30 min exposure to 4 mM WR-1065.
i.e. αPER of 0.89, in contrast to parental SA-NH cells, i.e. αPER of 2.44, when cells were irradiated 24 h after exposure to WR-1065 (see Fig. 6, Table 1). It is important to recognize that the delayed radioprotective effect is manifested by a broadening of the shoulder region of the radiation response curve that gives rise to an increase in surviving fraction at each radiation dose but does not result in a change in the terminal slope. This is seen in the lack of change or difference in PF determined for SA-NH and SA-NH+mIXBa1 cells from their dose–response curves, i.e. 1.1 and 0.95, respectively (see Table 2). The qualitative differences between the survival responses describing the immediate and delayed radioprotective effect is most probably due to differences in the intracellular localization of the respective protective agents. The immediate radioprotective effect is due to the localization of WR-1065 within two sensitive targets for radiation damage, the mitochondria and the nuclear DNA compartment. Sod2, in contrast, is present only in the mitochondria. It is not surprising, therefore, that the survival response of cells will be different given the disparity in the critical targets that can be protected by these two agents. The immediate radioprotective effect of WR-1065 is clearly a dose-modifying effect in which the relative effect on cell survival increases as the dose of radiation increases. While the delayed radioprotective effect does not increase with increasing radiation dose, the magnitude of the delayed effect is comparable to that of the immediate effect at a radiation dose of 2 Gy, the dose per fraction used in conventional radiotherapy regimens.

While amifostine is the only drug that has been approved specifically as a radioprotector by the FDA for use in the clinic, patients could be exposed to other clinically used thiol-containing drugs such as captopril, mesna and NAC. Each of these drugs has also been demonstrated to activate NFκB and enhance Sod2 gene expression (5–7). For this reason we have characterized the effects of each of these agents, along with WR-1065 for comparative purposes, regarding their ability to induce an immediate and/or delayed radioprotective effect at a radiation dose of 2 Gy. At a concentration of 4 mM, only captopril, along with WR-1065, exhibited immediate radioprotective properties (see Figs. 7 and 9). The difference between the ability of WR-1065 and captopril to provide immediate radioprotection, and the lack of a protective effect by NAC and mesna, is probably related in part to the net charge of each compound (+2 for WR-1065, 0 for captopril, and −1 for both NAC and mesna) which directly affects their ability to concentrate within the microenvironments of the nuclear and mi-
FIG. 8. Bar graph depicting the percentage survival of SA-NH cells exposed to 2 Gy of 250 kVp X rays 24 h after a 30-min treatment with 4 mM of either WR-1065, NAC, captopril or mesna contrasted with that of cells irradiated in the absence of thiol treatment. Each bar represents the mean ± SEM of three separate experiments.

Tochondrial DNA and to scavenge radiation-induced free radicals (35). All four thiols, however, were capable of inducing a delayed radioprotective effect (see Fig. 8) that resulted in a significant increase in survival of SA-NH cells (P < 0.001) at a dose of 2 Gy and a PF$_{2Gy}$ of 1.40, 1.22, 1.35, and 1.25 for WR-1065, captopril, mesna and NAC, respectively. The relationship between the ability of these non-protein thiols to activate NFκB and subsequently enhance Sod2 gene expression and their ability to induce the delayed radioprotective effect was confirmed by the lack of protection observed for SA-NH+mlkBα1 cells exposed to 2 Gy 24 h after drug treatment (see Fig. 10).

These findings suggest that patients taking any one of these four drugs daily during a course of conventional radiation therapy of 2 Gy per fraction could experience elevations in Sod2 levels. If this should occur in their tumors, this phenomenon could lead to enhanced tumor resistance to therapy. Captopril is an angiotensin-converting enzyme inhibitor that is widely used by millions of people in the treatment of hypertension (36). Amifostine is currently approved for use in the treatment of head and neck cancer by radiation therapy to decrease the incidence of moderate to severe xerostomia, while mesna is used as a cytoprotectant to protect against cyclophosphamide-induced damage to the urinary tract (35).

FIG. 9. Bar graph depicting the percentage survival of SA-NH+mlkBα1 cells exposed to 2 Gy of 250 kVp X rays immediately after a 30-min treatment with 4 mM of either WR-1065, NAC, captopril or mesna contrasted with that of cells irradiated in the absence of thiol treatment. Each bar represents the mean ± SEM of two separate experiments.

FIG. 10. Bar graph depicting the percentage survival of SA-NH+mlkBα1 cells exposed to 2 Gy of 250 kVp X rays 24 h after a 30-min treatment with 4 mM of either WR-1065, NAC, captopril or mesna contrasted with that of cells irradiated in the absence of thiol treatment. Each bar represents the mean ± SEM of two separate experiments.
Clinical studies performed to date using amifostine have not shown any evidence for the development of tumor protection (37). However, these studies have been criticized as having lacked sufficient statistical power to detect and quantify a possible tumor protective effect by amifostine (38). As interest in amifostine along with other thiol-based cytoprotective drugs continues to grow, new applications are being developed that will result in a significant expansion of their clinical use. Amifostine in particular has shown preclinical efficacy in inhibiting the development of spontaneous metastases (39) and in stimulating hematopoietic progenitor stem cell growth (40). As the clinical uses of thiols such as amifostine in cancer therapy are expanded, care must be taken to ensure that their use does not compromise treatment efficacy. This is especially important regarding the development of new treatment regimens for neoplastic disease sites where the prognostic outlook for patients is significantly improved over that of advanced ovarian cancer and head and neck cancer patients.

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