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SOD2-Mediated Effects Induced by WR1065 and Low-Dose Ionizing Radiation on Micronucleus Formation in RKO Human Colon Carcinoma Cells

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Abstract

RKO36 cells exposed to either WR1065 or 10 cGy X rays show elevated *SOD2* gene expression and SOD2 enzymatic activity. Cells challenged at this time with 2 Gy exhibit enhanced radiation resistance. This phenomenon has been identified as a delayed radioprotective effect or an adaptive response when induced by thiols or low-dose radiation, respectively. In this study we investigated the relative effectiveness of both WR1065 and low-dose radiation in reducing the incidence of radiation-induced micronucleus formation in binucleated RKO36 human colon carcinoma cells. The role of SOD2 in this process was assessed by measuring changes in enzymatic activity as a function of the inducing agent used, the level of protection afforded, and the inhibitory effects of short interfering RNA (*SOD2* siRNA). Both WR1065 and 10 cGy X rays effectively induced a greater than threefold elevation in SOD2 activity 24 h after exposure. Cells irradiated at this time with 2 Gy exhibited a significant resistance to micronucleus formation ($P < 0.05$; Student's two-tailed *t* test). This protective effect was significantly inhibited in cells transfected with *SOD2* siRNA. SOD2 played an important role in the adaptive/delayed radioprotective response by inhibiting the initiation of a superoxide anion-induced ROS cascade leading to enhanced mitochondrial and nuclear damages.

INTRODUCTION

The exposure of cells to ionizing radiation leads to the formation of reactive oxygen species (ROS) that are a major determinant of radiation-induced biological damage, e.g., the indirect effect. A variety of ROS can form as a result of the decomposition of water resulting in the formation of highly reactive and damaging molecules such as hydroxyl radical, superoxide anion and hydrogen peroxide (1). Theoretical calculations regarding the magnitude of such ionizations induced by radiation are lower by orders of magnitude than those actually observed for ROS produced by normal cellular metabolic processes (2). To account for this discrepancy, amplification mechanisms have been proposed that involve both a biological reaction to radiation-induced ROS and subsequent participation of mitochondria in a ROS

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propagation process(es) (3,4). It has been proposed that radiation-induced changes in mitochondrial membrane permeability could lead via a Ca^{2+} -mediated process to intermitochondrial communication and amplification of ROS and reactive nitrogen species generation (3). Such an amplification would in turn offer a rapid and robust mechanism by which to activate cellular damage response signaling pathways (2,4). One such model has been proposed for cardiac cells responding to ischemia and reperfusion and has been named ROS-induced ROS release or the RIRR model (5–7). Elevated levels of ROS released from one mitochondrion could cause ROS-induced ROS release in neighboring mitochondria, potentially culminating in substantial mitochondrial, nuclear and cellular damage.

Endogenous antioxidant enzymes such as superoxide dismutase (SOD), catalase and glutathione peroxidase (GPx) can protect against ROS formation. SOD is a class of metalloproteins that catalyze the dismutation of superoxide anion to oxygen and hydrogen peroxide. CuZnSOD (SOD1) is located primarily in the cytoplasm while MnSOD (SOD2) is localized only within the mitochondria, where it effectively scavenges superoxide anion generated as a result of normal respiration processes at the NADH-Q segment and the QH₂ segment of the respiratory chain in mitochondria (8) as well as from stress-inducing ionizing radiation (8–10). SOD2, possibly because of its localization within the mitochondria, is the most effective member of the SOD family of enzymes as a protector against radiation-induced toxicity (11).

Thiols are capable of inducing elevated *SOD2* gene expression (12–14). The underlying mechanism of action for this process is most likely linked to the ability of such reducing agents to reduce disulfide bonds of cysteine residues (15) in the p50 and p65 subunits of NF κ B that result in its activation and migration to the nucleus and its subsequent binding to an intronic NF κ B binding site that has been demonstrated to be essential for the rapid induction of *SOD2* gene expression (16). WR1065, the active free thiol form of amifostine, at a dose as low as 40 μM induced a 10- to 20-fold increase in SOD2 protein levels 24 h later as determined by Western blotting in mouse SA-NH sarcoma cells (17), human RKO colorectal carcinoma cells (18), and nonmalignant human microvascular endothelial cells (HMEC) (19). Increased intracellular SOD2 protein levels were accompanied by an associated increase in enzymatic activity that correlated with a 20 to 40% increase in cell survival when cells were exposed at that time to a 2-Gy challenge dose (17–19). Elevated levels of SOD2 and associated increased radiation resistance could be prolonged by continued treatment with WR1065 (20). Treatment of cells with NF κ B inhibitors or *SOD2* siRNA prior to exposure to WR1065 completely inhibited the thiol-induced increases in intracellular SOD2 levels and activities in mouse SA-NH tumor cells, human microvascular endothelial cells (HMEC), and RKO human carcinoma cells. The specific inhibition of thiol-induced elevation of SOD2 activity was directly correlated with subsequent decreases in radiation resistance as shown by decreased cell survival (17–20).

Mitochondrial dysfunction can lead to radiation-induced genomic instability (21). Enzymatic activity of SOD2 in such unstable clones is reduced compared to corresponding control cells. Biological markers that have been identified and chosen for assessment of such radiation-induced damage include an increased frequency of chromosomal aberrations and mutations and the formation of micronuclei. Micronuclei are of special importance given the underlying biological mechanism leading to their formation and the ease of their detection. These structures result from either chromosome fragments or whole chromosomes that lag behind at anaphase during the division process (22). Micronuclei are easily discernable in the cytoplasm of binucleated cells formed after a cytokinesis block (23). We have expanded our thiol-induced SOD2 studies to include the genomic instability end point of micronucleus formation along with a comparison to a classical low-dose (e.g. 10 cGy) radiation-induced adaptive effect in the same cell line.

MATERIALS AND METHODS

WR1065

WR1065, 2-[(aminopropyl)amino] ethanethiol, is the active free thiol form of amifostine. It was supplied by the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD. Immediately before its use, a 1 *M* stock solution was made by dissolving the drug in phosphate-buffered saline (PBS; 8.1 mM Na₂HPO₄, 1.5 mM KH₂PO₄, 140 mM NaCl; Invitrogen Life Technologies, Carlsbad, CA). Final concentrations were chosen to reflect the optimum dose affording either the maximal level of immediate radioprotection as measured by increased survival (4 mM) or the prevention of elevated mutation induction (40 μ M) (24).

Cell Culture Conditions

RKO36 cells are a subclone of RKO colorectal carcinoma cells. They were grown in Dulbecco's modified Eagle's medium (Invitrogen Life Technologies) supplemented with 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA) at 37°C in 5% CO₂ and 95% air (18). In all experiments cells were grown to confluence and then refed with fresh medium and maintained for an additional 3 days. Cultures were again refed with fresh medium 1 day prior to each experiment.

Irradiation Conditions

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 0.368 Gy/min to deliver a dose of 10 cGy or 1.65 Gy/min to deliver a dose of 2 Gy.

Micronucleus Assay

The effects of WR1065 and/or radiation exposure on chromosomal integrity in RKO36 cells were measured by determining the frequency of micronucleus formation using a cytokinesis block technique described in detail elsewhere (22). Briefly, 10⁶ sham- or drug/radiation-treated cells were plated in T-25 culture flasks in the presence of 1.5 μ g/ml cytochalasin B and incubated for 48 h at 37°C. At this concentration cytochalasin B is not toxic to RKO36 cells (25). After incubation, cells were harvested by trypsinization, fixed with cold methanol:acetic acid (3:1), and stored at -20°C. Slides were prepared and stained with acridine orange (0.01% in PBS) and examined under a fluorescence microscope (60 $\times$, dual-band filter). At least 1000 binucleated cells with well-defined cytoplasm were scored for the presence of micronuclei. The frequency of micronucleus formation was calculated as the ratio of the number of binucleate cells containing micronuclei relative to the total number of binucleate cells scored.

SOD2 siRNA Transfection

RKO36 cells grown to confluence in 150-mm dishes were transfected with 100 nM *SOD2* or negative control (NC) short interfering RNA (siRNA, Applied Biosystems/Ambion, Foster City, CA). The sequence for human *SOD2* (Catalog no. 4392422, 40 nmol) is: sense (5' CAA CAG GCC UUA UUC CAC UTT 3') and antisense (5' AGU GGA AUA AGG CCU GUU GTT 3'). The negative control is a scrambled sequence of bases (Catalog no. AM4635, 40 nmol). Transfection was performed using Lipofectamine 2000 reagent (Invitrogen Life Technologies) per the manufacturer's instructions. *SOD2* and negative control siRNA oligomers (100 nM final concentration) were diluted in 3 ml serum-free antibiotic-free medium, mixed gently, and then incubated for 5 min at room temperature. The siRNA oligomer was then combined with the diluted Lipofectamine 2000, mixed gently, 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 appropriate dishes and gently swirled to ensure a uniform distribution. The cells were incubated for 24 h with the transfection complexes under their normal growth conditions of 37°C and 95% air/5% CO₂. The cells were then washed with PBS at 37°C and fresh complete growth medium was added. RKO36 cells, nontransfected or transfected with negative control (NC) siRNA or SOD2 siRNA, were treated in subsequent experiments with WR1065, 40 μ M or 4 mM exposure for 30 min, or irradiated with 10 cGy. After this treatment the culture medium was aspirated and the cells were washed twice with PBS and maintained at 37°C. Fresh complete medium was added to cells and they were incubated for an additional 24 h. Transfected cells were then irradiated with 2 Gy X rays and prepared for micronucleus or SOD activity analyses, and the resulting data were contrasted to those obtained using non-SOD2 siRNA-transfected cells exposed to the same drug and/or irradiation conditions.

SOD Enzyme Activity Assay

SOD 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 trypsinized and lysed with 500 μ l of lysis solution. The resulting suspension was centrifuged at 14,000g for 5 min at 4°C and then transferred to a clean 1.5-ml tube. Protein concentration was determined using the Bradford assay and adjusted to 5 μ g/ μ l with lysis buffer. Activity was determined at room temperature using a colorimetric assay based on the ability of SOD to form H₂O₂ from superoxide radicals generated by an exogenous reaction involving xanthine and xanthine oxidase that 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 total SOD activity. Activity was measured using 50–500 μ g of total cellular protein in the presence of 5 mM sodium cyanide (NaCN) (Sigma-Aldrich, St. Louis, MO) to inhibit CuZnSOD (SOD1) activity. Absorbance changes were recorded for 5 min, and the rate of increase in absorbance units per minute was calculated for each experimental sample and a negative control, which contained all of the reaction components except cell lysate. The percentage inhibition was then calculated and plotted as a function of protein concentration for each treatment condition. The highest maximum percentage inhibition for each group of sample curves to be compared was determined and used to calculate the amount of protein that inhibited NBT reduction by 50% of this maximum value. Total SOD (reactions not containing NaCN) and SOD2 activities (reactions containing 5 mM NaCN) in U/mg protein were then calculated. SOD1 activities were determined by subtracting the SOD2 activity in each experimental sample from the total SOD activity.

Statistical Analysis

Means and standard errors were calculated for all data points from at least three independent experiments. Pairwise comparisons of SOD2 activities and micronucleus frequencies between each of the experimental conditions were performed using a Student's two-tailed *t* test.

RESULTS

Effects of WR1065 Exposure on SOD Enzymatic Activity

Thiol-containing compounds such as WR1065 are known to induce elevated SOD2 activity (12–14). Presented in Fig. 1 are bar graphs describing the change in both SOD1 and SOD2 enzymatic activities as a function of time after exposure of RKO36 cells to 2 Gy of ionizing radiation or 40 μ M or 4 mM WR1065. Both SOD1 and SOD2 activity remained relatively

unchanged compared to untreated control levels when measured immediately after irradiation or 30 min after exposure to either concentration of WR1065. SOD1 activity 24 h after WR1065 treatment was actually diminished in RKO36 cells compared to untreated control levels, reaching significance for cells exposed to 4 mM WR1065 ($P = 0.033$). In contrast, SOD2 activity was elevated over threefold above background levels 24 h after exposure of cells to either 40 μM or 4 mM of WR1065, reaching significance levels of $P = 0.014$ and 0.002, respectively, an observation consistent with previous results (18). These data demonstrate that SOD2 appears to be preferentially susceptible to modulation by WR1065 exposure.

Effects of SOD2 siRNA on Subsequent SOD2 Enzymatic Activity

To assist in the characterization of the WR1065-induced SOD2-mediated adaptive response, *SOD2* siRNA was used to selectively interfere with the expression of *SOD2*. To determine the relative efficiency of this siRNA in inhibiting SOD2 translation, oligomers of either a negative control (NC) containing a scrambled sequence of bases or *SOD2* specific siRNA were transfected into RKO36 cells and incubated 24 h prior to exposure of cells to either 40 μM or 4 mM of WR1065 for 30 min. Twenty-four hours later SOD2 activity was assessed; the results are presented in Fig. 2. *SOD2* siRNA transfection resulted in a small and nonsignificant change in the constitutive background level of SOD2 activity compared to control and NC-treated cells. In contrast, *SOD2* siRNA transfection significantly inhibited SOD2 activity in WR1065-exposed cells. Both 40 μM and 4 mM WR1065 significantly induced SOD2 activity more than threefold over background levels ($P = 0.017$ and 0.009, respectively) when measured 24 h after treatment in NC-transfected cells. RKO36 cells transfected with *SOD2* siRNA and exposed to 40 μM or 4 mM WR1065 and evaluated 24 h later exhibited only a slight and nonsignificant elevation in SOD2 activity ($P = 0.27$ and 0.28, respectively), indicating that *SOD2* siRNA transfection under the conditions used was effective in inhibiting the inducible levels of SOD2 routinely observed after exposure of cells to WR1065.

WR1065-Induced Adaptive Response Micronucleus Formation

Having established that transfection of RKO36 cells with *SOD2* siRNA significantly inhibited the elevation in SOD2 activity that could be induced by exposure of cells to WR1065, the roles of WR1065-induced elevation in SOD2 activity and the subsequent induction of micronucleus formation by ionizing radiation were determined. The frequency of micronuclei detected in untreated control cells was found to be similar to that in mock-transfected, NC siRNA-transfected, and *SOD2* siRNA-transfected RKO36 cells (see Fig. 3). A 2-Gy dose induced an equal and approximately threefold increase in the percentage of micronuclei in all four groups of cells. RKO36 cells that were mock-transfected or transfected with NC siRNA were protected against radiation-induced micronucleus formation if they were irradiated 24 h after exposure to either 40 μM or 4 mM of WR1065, experimental conditions that are known to lead to a threefold or more buildup in SOD2 activity. In contrast, RKO36 cells transfected with *SOD2* siRNA exhibited only a modest reduction in radiation-induced micronucleus formation when irradiation occurred 24 h after exposure to WR1065, an experimental condition that results in a relatively low elevation in SOD2 activity (see Fig. 2).

Effect of Ionizing Radiation on SOD2 Activity

The radiation-induced adaptive response is characterized as a protective phenomenon that can result when cells are first exposed to a very low dose of ionizing radiation and then challenged at a later time with a significantly higher dose of radiation. SOD2 activity was measured and compared in control and irradiated RKO36 cells. When measured immediately or 4 h after irradiation with 10 cGy or immediately after 2 Gy, the level of

SOD2 activity was unchanged from that of untreated control cells (see Fig. 4). SOD2 activity, however, was found to be increased over threefold ($P < 0.001$) when measurements were made 24 h after irradiation with 10 cGy. SOD2 activity remained significantly elevated at 32 h ($P = 0.003$) and was reduced to almost background levels 144 h later ($P = 0.283$). The significant elevation of SOD2 activity 24 h after 10 cGy irradiation parallels the effect observed after exposure of cells to WR1065 (see Fig. 1).

Radiation-Induced Adaptive Response: Micronucleus Formation

The frequency of radiation-induced micronucleus formation as a function of time between an “adaptive” dose of 10 cGy and a challenge dose of 2 Gy is presented in Fig. 5. Maximum induction of micronuclei occurred after a dose of 2 Gy. However, as the time between an initiating dose of 10 cGy and a second dose of 2 Gy increased to 48 h, the frequencies of micronuclei fell to background levels and then began to rise to levels approximating those obtained with 2 Gy of radiation only. The maximum protective effect of 10 cGy on a subsequent dose of 2 Gy, e.g., the maximum adaptive response, appeared to occur in the interval of 20 to 48 h.

SOD2 and the Radiation-Induced Adaptive Response

A positive correlation was observed between WR1065-induced SOD2 activity and protection against radiation-induced micronucleus formation. To test whether a similar relationship exists in a radiation-induced adaptive response, RKO36 cells were again transfected with either NC siRNA or *SOD2* siRNA and then exposed to 10 cGy, 2 Gy or 10 cGy followed 24 h later with 2 Gy. The frequency of cells containing micronuclei was similar in all control and 10 cGy-irradiated cohorts of cells (see Fig. 6). Nontransfected control cells and both NC and *SOD2* siRNA-transfected RKO36 cells exhibited similar twofold or greater elevated frequencies of micronuclei when exposed to 2 Gy only. Control and NC-transfected cells exposed to 10 cGy 24 h prior to the 2-Gy challenge dose exhibited micronucleus frequencies similar to those observed in the unirradiated control cells. Cells transfected with *SOD2* siRNA, however, exhibited no reduction in micronucleus formation when exposed to 2 Gy 24 h after an exposure to 10 cGy compared to cells exposed only to 2 Gy. Thus treatment of cells with *SOD2* siRNA resulted in the abrogation of this radiation-induced adaptive response.

DISCUSSION

We have extensively characterized a thiol-induced and SOD2-mediated delayed radioprotective effect using cell survival as an end point that in many ways parallels a phenomenon referred to as the radiation-induced adaptive response (17–20,28–30). In the current study we have expanded our investigation not only to include a marker for chromosome damage, e.g., the micronucleus assay, but also to contrast the relative magnitudes of the thiol- and radiation-induced “adaptive” responses with each other and to determine the role of SOD2 in the mediation of each of these phenomena.

While micronucleus formation in cells may be a reflection of genomic instability along a potential pathway to neoplastic transformation or simply an indication of a normal cellular damage elimination process (23), it is considered to be an important biomarker for genotoxicity testing (22). WR1065 is well known to exhibit both anti-mutational and anti-transformational protection against ionizing radiation along with its ability to induce elevated SOD2 activity (26,31). The delayed protection exhibited by WR1065 is directly correlated to elevated levels of the mitochondrially localized enzyme SOD2. As demonstrated in Fig. 1, and consistent with previously published results (12–14), SOD2 is unique in its susceptibility to induction through the action of thiol-containing reducing

agents such as WR1065. In particular, the mechanism of action has been characterized as comprising the thiol-induced reduction of cysteine disulfide bonds in the p50 and p65 subunits of NF κ B (14,15), resulting in its activation and translocation into the nucleus (12–14). Its subsequent binding to an intronic NF κ B element in the *SOD2* gene results in elevated gene expression (16) and a buildup of active SOD2 enzyme within the mitochondria (18,20). The kinetics of this process appears to be consistent in human and murine cell systems, with SOD2 levels increasing at about 4 h after thiol exposure and reaching maximal levels between 24 and 32 h later (14,17–20). This induction of and subsequent elevation in SOD2 activity can be significantly inhibited through the use of NF κ B inhibitors (17–19) and, more importantly, specific inhibitors such as short interfering RNA to *SOD2* (*SOD2* siRNA), as shown in Fig. 2. Cells challenged at this time with ionizing radiation exhibit an enhanced resistance to both cell killing (17–20) and micronucleus formation as described herein. Transfection of cells with *SOD2* siRNA not only inhibits the thiol-induced elevation of SOD2 activity but also inhibits the associated delayed radioprotective or low-dose radiation-induced “adaptive” response (see Fig. 3). These data clearly demonstrate the linkage between subsequent changes in SOD2 activity and both the thiol-induced delayed radioprotective effect and the low-dose radiation-induced adaptive response.

The radiation-induced adaptive response appears to be the result of a more complex signaling pathway that shares some common elements with the thiol-induced delayed radioprotective effect, both of which appear to be mediated by elevated SOD2 activity. However, a major difference between these two phenomena is that the delayed radioprotective effect is initiated through the reductive action of thiols while the low-dose radiation-induced adaptive response results from oxidative stress (2–4). The maximum elevation in SOD2 activity occurs between 18 and 20 h after TNF α treatment and 22 to 24 h after thiol or low-dose ionizing radiation exposure. If cells are challenged at this time of maximum increase of SOD2 protein with a 2-Gy dose of ionizing radiation, they exhibit an associated elevation in radiation resistance (18,32,33). An example of this is demonstrated using a 10-cGy dose of radiation that induced over a fourfold increase in SOD2 activity over background, as shown in Fig. 4. Elevated SOD2 activity correlated directly with protection against radiation-induced micronucleus formation, as shown in Fig. 5. The strong association between this radiation-induced adaptive response and elevated SOD2 activity is further supported by the specific inhibitory effect of *SOD2* siRNA transfection on both SOD2 enzymatic activity and associated protection against micronucleus formation, as shown in Fig. 6.

The similarity between the thiol-induced delayed radioprotective effect and the low-dose radiation-induced adaptive response in RKO36 cells appears to be confined to the molecular signaling steps occurring between the activation of NF κ B and the elevation of active SOD2 enzyme. The low-dose radiation-induced adaptive response, however, involves a much more complex set of signaling processes that occur after the initial deposition of energy and involve TNF α signaling (34,35).

The role of SOD2 in the expression of a delayed or adaptive response has several important implications, most importantly the identification of the mitochondria as the major cellular organelle involved in these processes. SOD2 is known to be localized only within the mitochondria, while SOD1 is distributed throughout the cytoplasm and SOD3 is found in extracellular regions (36). Furthermore, SOD2, like its SOD1 and 3 counterparts, has only one function and that is to dismutate superoxide anion into hydrogen peroxide, which in turn can be converted into water and oxygen through the action of catalase or glutathione peroxidase. While superoxide anion is not a strong oxidant, it is known to be the precursor of other ROS and a potent initiator of an ROS cascade process(es) (37). A phenomenon

known as ROS-induced ROS release has been identified in which mitochondria respond to elevated ROS concentrations by increasing their own ROS production (5–7). Increased ROS leads to mitochondrial depolarization via activation of the mitochondrial permeability transition pore, which in turn can produce a short-lived burst of ROS originating from the mitochondrial transport chain (7). A second possible mechanism involves the ROS-induced opening of the inner mitochondrial membrane anion channel that results in a brief enhanced level of mitochondrial electron transport chain derived ROS. Both of these modes of ROS-induced ROS release result in ROS released into the cytosol, which in turn can propagate ROS-induced ROS release in neighboring mitochondria and eventually lead to enhanced ROS damage to nuclear targets (5–7). While not addressed in this study, these potential mechanisms offer a working hypothesis with regard to the role of mitochondrial processes and subsequent damage in the response of the cell to ionizing radiation-induced damage.

Another important implication of this model is that superoxide anion is the prime initiator of the ROS-induced ROS release effect as it relates to both the thiol- and low-dose radiation-induced adaptive responses. If, after either thiol or low-dose radiation exposure, the level of SOD2 is sufficiently elevated within the mitochondria at the time of exposure of cells to a subsequent large challenge dose, it will effectively prevent the initiation of a superoxide anion-induced ROS cascade that could contribute via the ROS-induced ROS release process to the overall level of radiation-induced toxicity. SOD1 and SOD3, like SOD2, are also effective in dismutating superoxide anion, but because of their localization within the cytoplasm and extracellular spaces where superoxide will represent only a very small component of the subsequent ROS cascade emanating from the mitochondria through the process of ROS-induced ROS release, the removal of superoxide will have little or no effect on the overall ROS burden to the cell. It is therefore not surprising that both the thiol- and low-dose radiation-induced adaptive responses described in this study were found to correlate only with an elevation in SOD2 enzymatic activity and that the use of the specific inhibitor of SOD2 translation, *SOD2* siRNA, was sufficient to inhibit these responses. SOD2 plays an important role in both the low-dose radiation-induced adaptive response and the thiol-induced delayed radioprotective effect by dismutating superoxide anion and thereby inhibiting a subsequent superoxide anion-induced ROS cascade initiated in mitochondria that can lead to genomic instability as measured by micronucleus formation as well as changes in cell survival.

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FIG. 1.

Effect of WR1065 exposure on SOD1 and SOD2 enzymatic activity in RKO36 cells. Cells were exposed for 30 min to either 40 μ M or 4 mM WR1065 and then assayed for SOD activity either 30 min or 24 h later. P values above the bar compare the treatment groups with the corresponding unirradiated control cells. Each experiment was performed three times, and the error bars represent the SEM.

FIG. 2.

Effect of *SOD2* siRNA transfection of RKO36 cells on subsequent SOD2 activity measured 24 h after exposure to either 40 μ M or 4 mM WR1065 for 30 min. Background SOD2 activities were also determined for non-thiol-treated control cells transfected with either negative control (NC) siRNA or *SOD2* siRNA. Each experiment was repeated three times, and error bars represent the SEM. *P* values comparing cells treated with WR1065 to their respective negative controls or *SOD2* siRNA-transfected controls are presented.

FIG. 3.

Effect of *SOD2* siRNA transfection on micronucleus formation in RKO36 cells irradiated with 2 Gy 24 h after exposure to 40 μ M or 4 mM WR1065. The percentage of micronuclei was determined in non-pretreated cells (Control), mock-transfected cells (MT), cells transfected with negative control (NC) siRNA, and cells transfected with *SOD2* siRNA. Comparisons of micronucleus percentages of non-pretreated RKO36 cells irradiated with 2 Gy and of selected mock-transfected (MT) and *SOD2* siRNA irradiated experimental groups exposed to 40 μ M and 4 mM WR1065 are identified with resulting *P* values. Each experiment was repeated three times, and error bars represent the SEM.

FIG. 4.

Effect of 10 cGy X rays on the induction of SOD2 activity in RKO36 cells as a function of time after irradiation. *P* values compare treatment groups with untreated control cells. Each experiment was repeated three times, and error bars represent the SEM.

FIG. 5.

Effect of 10 cGy X rays on micronucleus induction in RKO36 cells by a 2-Gy challenge dose as a function of the time between radiation doses. Comparisons of micronucleus frequencies in unirradiated control cells and cells irradiated with either 10 cGy or 2 Gy only are presented. The resulting P values are included above the corresponding treatment groups. All experiments were repeated three times, and error bars represent the SEM.

FIG. 6.

Effect of *SOD2* siRNA transfection on micronucleus formation in RKO36 cells irradiated with 10 cGy, 2 Gy and 10 cGy followed 24 h later with 2 Gy. Cells were either not transfected, transfected with negative control (NC) siRNA, or transfected with *SOD2* siRNA 24 h prior to irradiation. Comparisons of micronucleus frequencies induced by 10 cGy and followed 24 h later with 2 Gy in nontransfected or *SOD2* siRNA-transfected cells were compared to the frequency of micronucleus formation induced by 2 Gy alone in nontransfected cells. The resulting *P* values are included above the corresponding treatment groups. All experiments were repeated three times, and error bars represent the SEM.