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Inactivation of Primary Antioxidant Enzymes in Mouse Keratinocytes by Photodynamically Generated Singlet Oxygen

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ABSTRACT

Cellular antioxidant enzymes protect against damage caused by exposure to endogenous or exogenous prooxidants. Singlet oxygen ($^1O_2$) is a reactive form of oxygen that can be produced *in vivo* either in normal and pathophysiologic conditions or by photosensitizing chemicals, as during photodynamic treatment. We hypothesized that photodynamically generated $^1O_2$ would decrease the enzymatic activities of cellular antioxidants. To test this hypothesis, we treated cultured mouse epidermal keratinocytes with the photosensitizer Photofrin plus visible light to produce $^1O_2$, and then measured CuZnSOD, MnSOD, and catalase activities with both in-gel and spectrophotometric enzyme activity assays. Our results demonstrated that the enzymatic activities of cellular CuZnSOD, MnSOD, and catalase were significantly decreased after keratinocytes were treated with Photofrin plus visible light. By contrast, the enzymatic activities of cellular CuZnSOD, MnSOD, and catalase were unaffected in control cells treated with Photofrin only or visible light only. Despite the decreased levels of enzymatic activities, the protein levels of all three primary antioxidant enzymes remained constant after photodynamic treatment, as determined by Western blotting. L-Histidine, a $^1O_2$ quencher, protected against the inactivation of cellular CuZnSOD, MnSOD, and catalase enzymes induced by photodynamically generated $^1O_2$. The conclusion from these experiments is that the primary cellular antioxidant enzymes CuZnSOD, MnSOD, and catalase can be inactivated by photodynamically generated $^1O_2$ in nucleated mammalian cells. These findings may be useful in the future development of antineoplastic adjuvant therapies that use photodynamic generation of $^1O_2$ to inactivate antioxidant defenses with a goal of sensitizing tumor cells to prooxidant-generating drugs. *Antioxid. Redox Signal.* 8, 1307–1314.

INTRODUCTION

LIVING CELLS ARE EXPOSED to reactive oxygen species (ROS) that can be derived from both endogenous biologic processes and from exogenous environmental sources. Primary antioxidant enzymes CuZnSOD, MnSOD, and catalase are normally present in living systems and provide a substantial defense network against the cellular accumulation of such ROS (37). The enzymatic activities of the SODs catalyze the dismutation of toxic $O_2^{\bullet -}$ to H_2O_2 and O_2. Catalase acts both catalytically ($2H_2O_2 \rightarrow 2\ H_2O + O_2$) and peroxidatically ($RH_2 + H_2O_2 \rightarrow R + 2\ H_2O$) to scavenge H_2O_2 (8,30). The primary antioxidant enzymes CuZnSOD, MnSOD, and catalase, together with other antioxidant enzymes such as glutathione peroxidase, keep the $O_2^{\bullet -}$ and H_2O_2 at low steady-state concentrations in cells and tissues; $O_2^{\bullet -}$ is maintained at 10^{-11} to 10^{-9} *M* by SOD, and H_2O_2 is maintained at 10^{-9} to 10^{-7} *M* (7, 8, 13, 30). Whereas $O_2^{\bullet -}$ and H_2O_2 are often derived from one-electron reduction of oxygen near the mitochondrial electron-transport chain, another form of ROS, singlet oxygen, can be produced in cells by exposure to photosensitizers and light, or by long-wave ultraviolet light (UVA). The latter exposure is particularly relevant for cells of the skin, including epidermal keratinocytes.

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Singlet oxygen ($^1O_2$) is an excited state of triplet ground state dioxygen (O_2). $^1O_2$ has extra energy because of the rearrangement of its orbital electrons from two unpaired electrons to paired electrons (3, 13). The two forms of $^1O_2$ are $^1\Sigma gO_2$ and $^1\Delta gO_2$. The higher-energy excited $^1\Sigma gO_2$ is extremely short-lived and is deactivated to $^1\Delta gO_2$ so rapidly that it has little chance to participate in chemical reactions. However, $^1\Delta gO_2$ is relatively long-lived, and its lifetime varies from 4 μs in water to >1 ms in solvents lacking hydrogen and deuterium atoms, which is long enough to cause chemical reactions. Although the energy of $^1O_2$ is only 94 kJ/mol above that of ground-state oxygen, it is very reactive and can cause various types of cellular damage leading to lipid peroxidation, nucleic acid oxidation, protein oxidation and enzyme inactivation, DNA base modifications, and DNA strand breaks (7, 8). Singlet oxygen is one of the reactive ROS produced in mammalian cells in both normal and pathophysiologic conditions, such as in the environmental exposure of keratinocytes to UVA light.

Besides exposure to UVA light, singlet oxygen can also be produced by visible light in the presence of photosensitizers (such as methylene blue, rose bengal and Photofrin) and molecular oxygen both *in vitro* and *in vivo*. Escobar *et al.* (11) reported that rose bengal plus red light generated $^1O_2$ that inactivated purified SOD and catalase *in vitro* from bovine erythrocytes and liver, respectively. These authors found that both SOD and catalase were readily inactivated by $^1O_2$ and that the rate constants for the loss of enzymatic activity induced by $^1O_2$ were 3.9×10^7 M/sec for SOD and 2.5×10^7 M/sec for catalase (11). Kim *et al.* (20) also reported that methylene blue or rose bengal in the presence of white light generated $^1O_2$ which caused oxidative modification and damage in bovine erythrocytes CuZnSOD and bovine liver catalase and inactivated the enzymatic activities of CuZnSOD and catalase *in vitro*. All of these experiments were done on proteins *in vitro*. Thus an interesting question raised from these experiments is whether antioxidants such as CuZnSOD, MnSOD, and catalase can be inactivated by photodynamically generated $^1O_2$ in the context of living cells. Therefore the purpose of this study was to determine whether primary cellular antioxidants could be inactivated in living cells by exposure to $^1O_2$. We chose epidermal keratinocytes as a model because their environmental exposure to $^1O_2$-producing UVA radiation makes them a highly relevant model system.

MATERIALS AND METHODS

Cell culture

The immortalized nontumorigenic mouse epidermal keratinocyte cell line 308 was grown as monolayers in DMEM medium supplemented with 10% fetal bovine serum, 100 μg/ml penicillin, 100 μg/ml streptomycin, and maintained at 37°C in a humidified incubator with 5% CO_2.

Photodynamic treatment

Photofrin (porfimer sodium) was kindly provided by QLT Phototherapeutics, Inc., Vancouver, BC, Canada. It was reconstituted in 5% aqueous dextrose solution (pH 7.4) and frozen until immediately before use. A fluorescent light source was used for light treatment. The power density at the illuminated cell monolayer was 5 J/m^{2}s. Controls consisted of no treatment, light alone, or Photofrin alone at a dose of 6 μg/ml.

Cytotoxicity assays

The 308 cells were incubated with Photofrin for 24 h and then treated with visible light (5 J/m^{2}s). The 308 cells were trypsinized and resuspended, and 0.4% trypan blue was added to the cells. Two minutes later, trypan blue–negative cells were counted by hemocytometer under the microscope.

CuZnSOD, MnSOD, and catalase zymography

Cellular CuZnSOD, MnSOD, and catalase activities were measured by electrophoretic enzymography and then visualized as described by Beauchamp and Fridovich (1). In brief, the gels were pre-electrophoresed at 40-mA current for 1 h at 4°C. Then 200 μg of total cellular proteins from cell lysates were loaded into each well, and electrophoresis was performed at 40 mA until the bromphenol blue marker dye had swept through the gel, and then continued for 1 more hour. CuZnSOD and MnSOD were localized by soaking the gels in NBT (nitroblue tetrazolium) for 20 min in the dark. The gels were then exposed to light on a fluorescent transilluminator until the bands showed maximal resolution. To detect MnSOD alone, NaCN was added to inhibit CuZnSOD. For catalase, the gel was rinsed and incubated in 0.003% H_2O_2 for 10 min. The gel was rinsed and stained with 2% ferric chloride and 2% potassium ferricyanide. Achromatic bands demonstrated the presence of catalase activity.

Superoxide dismutase activity assay

Because gel zymography studies are not quantitative, SOD enzyme activity was also measured by using an indirect competition assay between SOD and an indicator molecule NBT. This was performed in the crude homogenate according to the method of Spitz and Oberley (29, 33). In brief, SOD activities were determined spectrophotometrically at 560 nm by measuring the reduction of NBT. The $O_2^{\bullet -}$ generated from the xanthine and xanthine oxidase system reduced NBT. However, in the presence of SOD, the reduction of NBT is competitively inhibited. By measuring the amount of protein necessary for half-maximal inhibition, the SOD activity in the sample was determined. Then 5 m*M* NaCN was used to inhibit CuZnSOD activity, and therefore the SOD activity in the presence of NaCN indicated MnSOD activity. The difference between total SOD activity and MnSOD was taken as CuZnSOD activity. The activity values were expressed in units per milligram protein (U/mg protein).

Catalase activity assay

Catalase activity was determined by a spectrophotometric assay based on the catalyzed decomposition of H_2O_2 (2). The peroxide decomposition rate is directly proportional to the enzyme activity in a pseudo first-order reaction. To determine catalase activity, 2.0 ml phosphate buffer (50 m*M*, pH 7.0) and 1.0 ml 30 m*M* H_2O_2 were added to a quartz cuvette. After

reading the initial absorbance at 240 nm, 50 µl of a catalase-containing cell lysate was added and the change in absorbance was monitored during 60 sec. The slope of the absorbance versus time plot is directly proportional to the activity of the sample. Catalase activity was expressed in *k* units.

Western blot analysis

The 308 cells were lysed in a buffer consisting of 0.25 *M* Tris-HCl (pH 7.6) and sonicated after photodynamic treatment. Lysate, 20 µg, was separated by sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose membrane, and incubated overnight at 4°C with primary polyclonal rabbit antibodies to the various antioxidant enzymes. After washing in TBST buffer, the membranes were incubated with peroxidase conjugated, goat–anti-rabbit immunoglobulin G (IgG) for 1 h at room temperature. The SOD and catalase protein recognized by the antibody was detected with ECL chemiluminescent substrate.

Statistical analysis

All data were calculated as mean ± standard deviation from three independent experiments. Differences between mean values of multiple groups were analyzed by using one-way or two-way analysis of variance (ANOVA). Bonferroni's multiple comparison correction was used for pair-wise comparisons to control for type I error rate per experiment, and the significant differences were determined at $p < 0.05$.

RESULTS

Photodynamically generated $^1O_2$ inactivates cellular antioxidant enzymes CuZnSOD, MnSOD and catalase

To determine initially whether $^1O_2$ could inactivate primary cellular antioxidant enzymes, activity gel electrophoresis was performed to measure the enzymatic activities after photodynamic treatment. Results of the CuZnSOD activity gel, shown in Fig. 1A, suggest that CuZnSOD enzyme activity underwent a dose-dependent decrease in activity after treatment with Photofrin plus visible light. In addition, as shown in Fig. 1B, MnSOD enzyme activity was significantly decreased in 308 cells that were treated with Photofrin plus visible light. In a subsequent experiment, 308 cells were infected with 100 multiplicity of infection (MOI) of adenovirus encoding human SOD2 to augment MnSOD activity. Although MnSOD enzyme activity increased only slightly in the infected cells, this MnSOD activity was nevertheless inhibited by singlet oxygen in a dose-dependent manner, as shown in Fig. 1C. Catalase activity was also decreased in a dose-dependent fashion after 308 cells were treated with Photofrin plus visible light, as shown in Fig. 1D.

Gel activity assays provide an estimate of antioxidant enzyme activity, but they are not quantitative; therefore we also used biochemical spectrophotometric assays to measure the enzyme activities of CuZnSOD, MnSOD, and catalase in 308 cells that were treated with Photofrin plus visible light. Figure 2 (A–C) shows that CuZnSOD, MnSOD, and catalase enzyme activities were quantitatively decreased in a dose-dependent manner after 308 cells were treated with Photofrin plus visible light. These results were consistent with enzyme activity gel results, thus confirming and extending our initial observations. We also used L-histidine, a $^1O_2$ quencher, to determine whether L-histidine could protect against the inactivation of cellular antioxidants by photodynamic treatment. L-Histidine was added to the cell culture 2 h before illumination and remained present during illumination. Figure 3 (A–C) shows that L-histidine protected against the inactivation of cellular antioxidants CuZnSOD, MnSOD, and catalase by photodynamic treatment in a dose-dependent manner. Because L-histidine quenches the effects of singlet oxygen but not those of other ROS, we concluded from these results that the inactivation of cellular antioxidants CuZnSOD, MnSOD, and catalase was caused predominantly by singlet oxygen generated by Photofrin plus visible light.

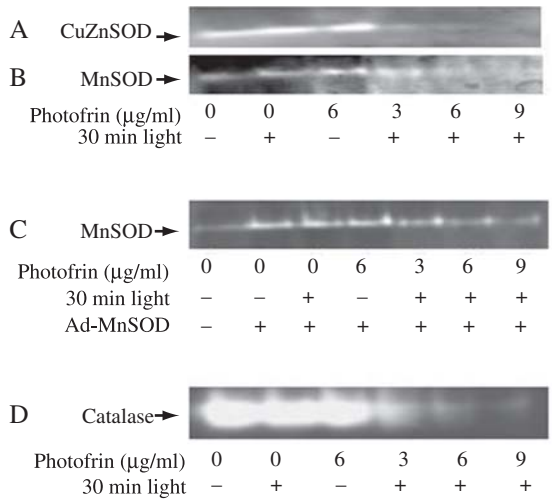

FIG. 1. Cellular primary antioxidant enzyme activities were decreased after 308 cells were treated with Photofrin plus visible light, as measured by activity gels. The 308 cells were incubated with Photofrin for 24 h and then treated with visible light (5 J/m²s) in 1 × PBS. The polyacrylamide gel was pre-electrophoresed at 40-mA current for 1 h at 4°C; 200 µg of total cellular proteins was electrophoresed at 40 mA for 1 h after the bromphenol blue marker dye had swept through the gel. (**A**) CuZnSOD was visualized by soaking the gels in NBT (nitroblue tetrazolium) for 20 min in the dark. The gels were then exposed to moderately intense light until the bands developed. (**B**) MnSOD was localized by soaking the gels in NBT and (nitroblue tetrazolium) and NaCN for 20 min in the dark. NaCN (sodium cyanide) was added to inhibit CuZnSOD. The gels were then exposed to moderately intense light until the bands developed. (**C**) The 308 cells were infected with 100 MOI adenovirus encoding human SOD2 24 h before photodynamic treatment. (**D**) Catalase activity was detected by rinsing the gel and soaking in 0.003% H_2O_2 for 10 min. The gel was then rinsed and stained with 2% ferric chloride and 2% potassium ferricyanide. Achromatic bands demonstrated the presence of catalase activity.

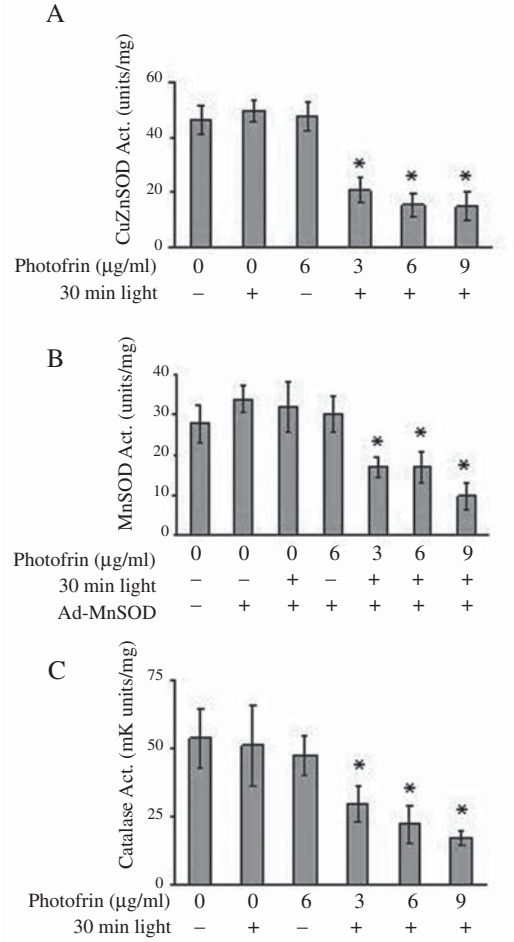

FIG. 2. Cellular primary antioxidant enzyme activities were decreased after 308 cells were treated with Photofrin plus visible light, as measured by spectrophotometric assay. The 308 cells were incubated with Photofrin for 24 h and then treated with visible light (5 J/m²s) in 1 × PBS. Total SOD activity was determined spectrophotometrically at 560 nm by measuring the reduction of NBT, and 5 mM NaCN was used to inhibit CuZnSOD activity, and therefore the SOD activity in the presence of NaCN indicated MnSOD activity (**B**). The difference between total SOD activity and MnSOD was taken as CuZnSOD activity (**A**). The SOD activity values were expressed in units per milligram protein (U/mg protein). (**C**) Catalase activity was determined by a spectrophotometric assay based on the catalyzed decomposition of H_2O_2. Catalase activity was expressed in *k* units. *$p < 0.05$ compared with untreated control.

Photodynamic treatment is cytotoxic to 308 cells

To determine the cytotoxicity in 308 cells induced by photodynamic treatment, cell viability was measured by trypan blue exclusion. Figure 4 shows that the cell viability was decreased in a dose-dependent manner after 308 cells were treated with Photofrin plus visible light. These results demonstrated that photodynamic treatment caused cellular toxicity in 308 cells.

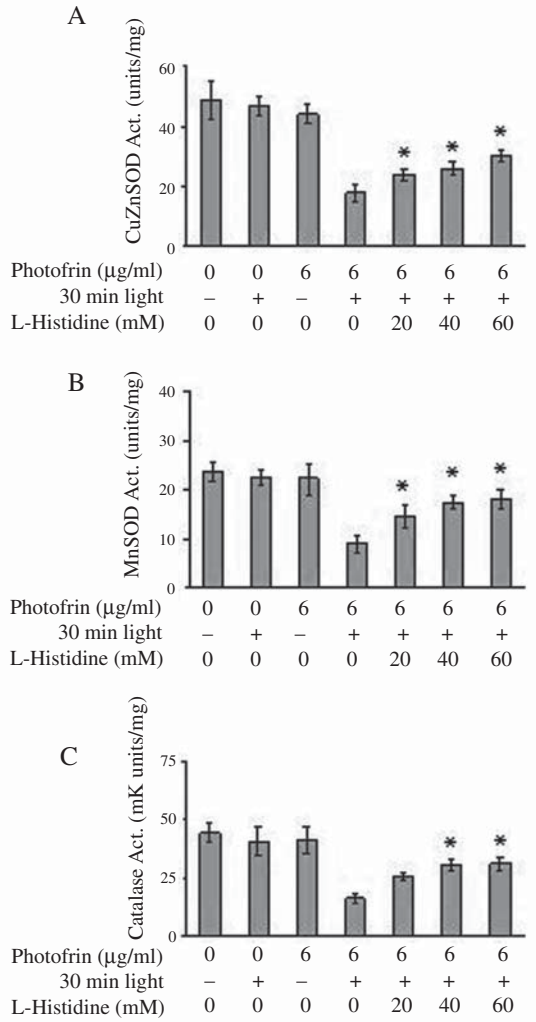

FIG. 3. L-Histidine, a $^1O_2$ quencher, protected against the photodynamic inactivation of cellular primary antioxidant enzyme activities as measured by spectrophotometric assay. Before illumination, 308 cells were incubated with Photofrin for 24 h and then incubated with various concentrations of L-histidine as indicated for 2 h. Cellular CuZnSOD activity was measured by spectrophotometric assay as described before. **A:** CuZnSOD activity. **B:** MnSOD activity. **C:** Catalase activity. *$p < 0.05$ compared with Photofrin-plus-light group.

The protein levels of cellular antioxidant enzymes remained constant after photodynamic treatment

A decrease in antioxidant enzyme activities in response to singlet oxygen as demonstrated earlier could be the result of decreased levels of the respective proteins. To determine whether photodynamic treatment affected the protein levels of cellular antioxidants CuZnSOD, MnSOD, and catalase, Western blots were performed to determine the protein levels of these antioxidant enzymes after photodynamic treatment. Because 308 cells have relatively low levels of endogenous MnSOD, 308 cells were infected with 100 MOI adenovirus encoding human SOD2 in an attempt to increase their MnSOD immunoreactive protein levels. Figure 5 (A–C)

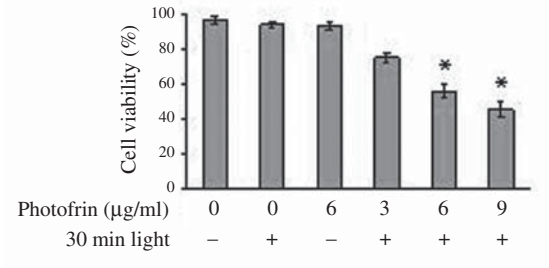

FIG. 4. Photodynamic treatment (PDT) was cytotoxic to mouse epidermal keratinocyte cell line 308 in a dose-dependent manner after treatment with photosensitizer Photofrin plus visible light (5 J/m² s) as measured by trypan blue exclusion assay. Cell viability was determined by trypan blue exclusion assay. The 308 cells were incubated with Photofrin for 24 h and then treated with visible light (5 J/m²s) in 1 × PBS. Cells were trypsinized and resuspended in 1 χ PBS, and 0.4% trypan blue was added to the cell suspension. After 2 min, trypan blue–negative cells were counted under a microscope with a hemocytometer. Percentage of dye exclusion was calculated as the blue-negative cells/total cells. Each data point was a mean of three independent experiments; *error bars* represent ± SEM. *$p < 0.05$ compared with untreated, Photofrin-alone, and light-alone controls.

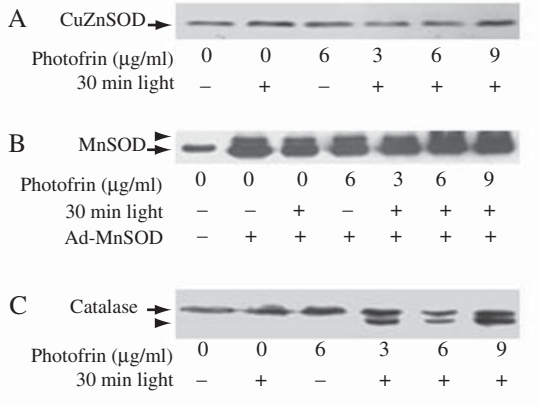

FIG. 5. Cellular primary antioxidant enzyme protein levels were unaffected by photodynamic treatment. The 308 cells were incubated with Photofrin for 24 h and then treated with visible light (5 J/M²S) in 1 × PBS. The cells were lysed, and 20 μg of total cellular proteins was separated by SDS-PAGE, transferred to nitrocellulose membrane, and incubated overnight at 4°C with primary antibodies specific to each protein. After washing in TBST buffer, the membranes were incubated with peroxidase-conjugated, goat-anti-rabbit IgG for 1 h at room temperature. The proteins were detected with ECL chemiluminescent substrate. (**A**) CuZnSOD immunoreactive protein. (**B**) MnSOD immunoreactive protein (*arrow*); *arrowhead*, pre-form of MnSOD. (**C**) Catalase immunoreactive protein. *Arrow,* native catalase; *arrowhead,* presumptive oxidized catalase.

shows that the protein levels of cellular CuZnSOD, MnSOD, and catalase remained constant after photodynamic treatment. Taken together with the previous results, these data imply that photodynamic treatment inactivated the enzymatic activities of cellular CuZnSOD, MnSOD, and catalase, whereas the protein levels of these antioxidants remained unchanged after exposure to photodynamic treatment. The MnSOD Western blot shown in Fig. 5B revealed two bands in the 308 cells that were infected with 100 MOI adenovirus, whereas only one band was present if the cells were not infected with MnSOD adenovirus. The upper band (arrowhead) is a preprocessed form of MnSOD that has not yet had the mitochondrial targeting sequence removed. This form is often present in cells that have very high forced MnSOD expression. Two bands were apparent in the catalase Western blot shown in Fig. 5C after the cells were treated with Photofrin plus visible light, whereas only one band was seen if the cells were treated with Photofrin only or visible light only. The lower band is believed to be an oxidized form of catalase.

DISCUSSION

Cellular primary antioxidant enzymes CuZnSOD, MnSOD, and catalase constitute a system that keeps cellular toxic byproducts such as $O_2^{\bullet -}$ and H_2O_2 at low steady-state concentrations in cells and tissues (8). CuZnSOD, a dimer, is found mainly in the cytosol in eukaryotic cells; MnSOD, a homotetramer, is found in the mitochondrial matrix; and catalase, a tetramer, is located mainly in peroxisomes but is also present in endoplasmic reticulum and cytoplasm. These enzymes act in concert and normally enable cells to cope with biologically and environmentally derived prooxidants and oxidative stress. Thus, normal cells are protected by cellular antioxidant enzymes from ROS generated during normal cellular metabolism as well as the toxic effects of ROS from environmental sources. If cellular antioxidant enzyme activities are inactivated by increased endogenous cellular $^1O_2$ or by exogenous sources such as UVA or photodynamically generated $^1O_2$, cellular antioxidants enzyme activities will be decreased. The decreased enzyme activities of CuZnSOD, MnSOD, and catalase, coupled with a subsequently increased oxidant species, will lead to increased oxidative stress (24). Oxidative stress will further damage cellular antioxidant enzymes, which will cause increased cytotoxic effects.

Singlet oxygen is one of the ROS produced in mammalian cells under oxidative stress conditions, either from decomposition of lipid peroxides or by spontaneous dismutation of $O_2^{\bullet -}$ (22). Singlet oxygen can also be produced by naturally occurring compounds such as riboflavin and xenobiotics such as psoralene, porphyrins, and tetracyclins when they are illuminated with visible light (10, 15, 18, 38). Photofrin, a partially purified preparation of hematoporphyrins, was used as a photosensitizer in our experiments. The photodynamic action of Photofrin relies on the absorption of visible light to form its excited triplet state. This excitation energy is transferred to molecular oxygen, producing $^1O_2$ (19). The lipophylic character of Photofrin causes it to localize in plasma and subcellular membranes where $^1O_2$ is produced.

Both Escobar *et al.* (11) and Kim *et al.* (20) reported that the enzymatic activities of purified bovine erythrocytes CuZnSOD and bovine liver catalase proteins were inactivated by photodynamically generated $^1O_2$ *in vitro*. Our results demonstrated that the activities of all three primary cellular an-

tioxidant enzymes (CuZnSOD, MnSOD, and catalase) were inactivated by photodynamic treatment in nucleated mammalian cells. To our knowledge, this is the first report to demonstrate that all three cellular primary antioxidants can be inactivated by photodynamic treatment in nucleated mammalian cells. Although $^1O_2$ may not be the only ROS involved, the inactivation of cellular antioxidant enzymes was likely due primarily to the production of $^1O_2$ by photodynamic treatment because our results demonstrated that the $^1O_2$ quencher L-histidine protected against the inactivation of cellular antioxidant enzymes.

The specific targets of antioxidant enzyme inhibition *in vitro* by photodynamically generated $^1O_2$ were investigated by both Kim *et al.* (20) and Nakatani *et al.* (28) by using amino acid analysis after acid hydrolysis of the modified proteins. Kim *et al.* found that when purified CuZnSOD and catalase proteins were treated with 25 μ*M* methylene blue and 1 h of illumination, histidine residues were significantly lost with a frequency of 48% and 32%, respectively. Nakatani *et al.* (28) found that when purified bovine calatase protein was treated with 0.02% of methylene blue plus 120-min visible light *in vitro,* catalase enzyme activity was decreased to 3% of the original value. Meanwhile, the histidine, tyrosine, and tryptophan contents of the oxidized catalase were decreased to 12%, 70%, and 96%, respectively. These results were perhaps not surprising because $^1O_2$ has already been shown to be particularly reactive toward histidine, tyrosine, methionine, cysteine, and tryptophan residues (3, 5, 31).

The main reaction centers for $^1O_2$ are R-S (methionine and cysteine) and C=C groups (retinoids, unsaturated fatty acids, and cholesterol). Structural analyses of CuZnSOD and MnSOD show that four histidine residues (His 44, His 46, His 61, and His 118) are located in the active site in each monomer of CuZnSOD, and three histidine residues (His 26, His 74, and His 163) bind to the manganese in the active site of each monomer of MnSOD (4, 35). His 30 and Tyr 34 are also located in the active site of MnSOD. When His 30 was substituted by Asn, or Tyr 34 was substituted by Phe, these substitutions were responsible for a 10-fold decrease in enzyme activity (23). Moreover, Tyr 34 in MnSOD can be oxidized and nitrated by peroxynitrite ($ONOO^-$) to form dityrosine and nitrotyrosine, respectively, both of which compromise MnSOD enzyme activity (14, 27). Other $^1O_2$ -sensitive amino acids, including Tyr 45, Tyr 166, Tyr 193, Trp 123, and Trp 161, are also located in the active site of MnSOD (17, 26). The structural analysis of catalase showed that each monomer contains His 74 and Tyr 357 located in the active site. These amino acids are considered to be essential in the catalytic action of catalase (12, 32). Therefore $^1O_2$ produced by photodynamic treatment can oxidize these $^1O_2$-sensitive amino acids, which may cause structural alterations and unfolding in these antioxidant enzymes. This may then allow $^1O_2$ more accessibility to the active site to react with other $^1O_2$-sensitive amino acids in the active site of these antioxidants. Thus singlet oxygen will cause a significant decrease in enzymatic activities of cellular CuZnSOD, MnSOD, and catalase. Among these $^1O_2$-sensitive amino acids, histidine seems the most important amino acid targeted by $^1O_2$. It seems that the histidine residue is not only the target of $^1O_2$, but also the target of H_2O_2 because both Bray *et al.* (6) and Hogson *et al.* (16) found that histidine in CuZnSOD was the primary target when CuZnSOD was treated with H_2O_2. It is thought that cellular antioxidants are inactivated not only by $^1O_2$, but also by other ROS such as $O_2^{\bullet -}$ and H_2O_2. For example, CuZnSOD can be inactivated by H_2O_2, whereas catalase can be inactivated by $O_2^{\bullet -}$ (6, 16, 21). $^1O_2$ not only inactivates CuZnSOD, MnSOD, and catalase *in vitro* and *in vivo*, but also can inactivate other enzyme activities. For example, $^1O_2$ can inactivate acetylcholinesterase, bacterial respiratory chain enzymes, blood coagulation factor I (fibrinogen), factor V, factor VIII, and factor X (9, 34, 36). We can infer from these results that a complicated relation exists between cellular antioxidants and cellular ROS. Cellular antioxidants can scavenge cellular ROS. However, cellular ROS can also inactivate cellular antioxidant enzyme activities.

Photodynamically generated $^1O_2$ may also cause structural alterations and oxidative modification of cellular antioxidants. Kim *et al.* (20) reported that purified bovine erythrocyte CuZnSOD and bovine liver catalase protein were oxidized, modified, and damaged, and their structures were altered when treated photodynamically. Lledias *et al.* (25) also reported that human catalase was oxidized by photodynamically generated $^1O_2$, giving rise to more acidic conformers detected in zymograms after electrophoresis in polyacrylamide gels. Our results shown in Fig. 5C demonstrated that cellular catalase appears to be oxidized by photodynamically generated $^1O_2$. The catalase Western blot showed that two bands were present only after the cells had been treated with Photofrin plus visible light, whereas only one band was present if the cells were treated with Photofrin only or visible light only. The lower band is believed to be an oxidized form of catalase. No visibly altered forms of CuZnSOD and MnSOD were seen in our Western blots (except the preprocessed form described in Results) when cells were photodynamically treated, which suggests that catalase may be easier to oxidize than CuZnSOD and MnSOD. Alternatively, oxidation of these latter antioxidant enzymes does not result in forms of the proteins with altered electrophoretic mobility.

In conclusion, our results indicated that all three cellular primary antioxidant enzymes, CuZnSOD, MnSOD, and catalase, were inactivated by photodynamic treatment, and the inactivation of cellular antioxidants was caused mainly by photodynamically generated $^1O_2$. These findings may be used in developing future antineoplastic adjuvant therapies that use photodynamic generation of $^1O_2$ to inactivate cellular antioxidant defenses and sensitize tumor cells to killing by prooxidant-generating drugs.

ACKNOWLEDGMENTS

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ABBREVIATIONS

CuZnSOD, Copper zinc superoxide dismutase; DMEM, Dulbecco's modified Eagle medium; MnSOD, manganese superoxide dismutase; MOI, multiplicity of infectivity; NBT, ni-

troblue tetrazolium; O_2, molecular oxygen; $^1O_2$, singlet oxygen; $O_2^{\bullet -}$, superoxide; PBS, phosphate-buffered saline; RH_2, ethanol or methanol; ROS, reactive oxygen species; SDS-PAGE, sodium dodecylsufate–polyacrylamide gel electrophoresis.

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