

Protective Effect of Lecithinized SOD on Reactive Oxygen Species-Induced Xerostomia

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Reactive oxygen species (ROS) are believed to be involved in radiation-induced xerostomia, and the application of antioxidants may be a promising method for treating patients suffering from salivary gland dysfunction. In this study, we examined the ability of the antioxidant superoxide dismutase (SOD) to restore radiation-induced salivary gland dysfunction using a mouse model of radiation-induced salivary gland hypofunction and ultraviolet B (UVB)-irradiated human salivary gland cells. We administered lecithinized SOD (PC-SOD) prior to and after irradiation and measured the amount of saliva secreted. To confirm ROS generation, flow cytometry was performed using an oxidant-sensitive fluorescent dye, dihydroethidium, and CM-H₂DCFDA. While no significant decrease in saliva secretion was observed after irradiation in the mice that were treated with PC-SOD, a significant reduction in saliva secretion was noted in the irradiated mice that were not treated with PC-SOD. Furthermore, flow cytometry clearly revealed that PC-SOD eliminated superoxide (O₂⁻) induced by UVB radiation. These results suggested that PC-SOD may protect against exocrine gland dysfunction induced by radiation, presumably by rapidly converting O₂⁻ to hydrogen peroxide. We believe that our results may advance the potential application of antioxidants for the prevention of ROS-induced xerostomia. © 2009 by Radiation

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INTRODUCTION

Oxidative stress arises from the strong cellular oxidizing potential of excess reactive oxygen species (ROS), such as superoxide (O₂⁻), hydroxyl radicals (·OH) and hydrogen peroxide (H₂O₂). These ROS are constantly being generated within the cell by metabolic processes and can react with, and thereby damage, cellular components, including important

enzymes, proteins, carbohydrates, DNA and lipids that contribute to the maintenance of the structure and function of the living body. Despite their cytotoxic effects, O₂⁻ and H₂O₂ play important physiological roles at low concentrations: They may act as secondary messengers to stimulate apoptosis or necrosis (1).

Radiation is directly toxic to tissue and induces various tissue damage factors, including ROS. Damage from ionizing radiation is caused mostly by the ionization of water resulting in the production of free radicals, such as hydrogen atoms, OH and hydrated electrons. These highly reactive radicals react rapidly with other cellular constituents, causing damage and generating secondary free radicals, including O₂⁻. This increase in oxidative stress is accompanied by an increase in the number of dysfunctional mitochondria and an increase in genomic instability.

Increased levels of nitric oxide (NO) have been reported in salivary gland tissue after X irradiation (2). NO is produced by NO synthase (NOS) from the essential amino acid L-arginine. Although the toxicity of NO by itself is modest, NO reacts rapidly with O₂⁻ to form peroxynitrite (ONOO⁻), which has been identified as a potent oxidant and potential mediator of tissue injury (3,4). ONOO⁻ affects cell metabolism by inducing lipid peroxidation (5), damaging DNA, and interfering with mitochondrial function (6). UVB-radiation exposure induces the generation of ROS, including O₂⁻ and H₂O₂, and genomic instability in mammalian cells (7). In addition, recent studies have reported that UVB radiation induces the generation of NO and ONOO⁻ in human keratinocytes (4). Thus X and UVB radiation generate similar ROS.

ROS have been reported to be associated with the appearance and exacerbation of various diseases, including diabetes mellitus (8), hypertension (9), atherosclerosis (10), inflammation (11), neurodegenerative diseases (12), carcinogenesis (13), ischemia-reperfusion injury (14), radiation injury (15), and aging (16).

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Oxidative stress is also thought to be involved in the development of radiation-induced xerostomia. The stable nitroxide tempol (4-hydroxy-2,2,6,6-tetramethylpiperidine-*N*-oxyl) is a superoxide dismutase (SOD) mimetic that specifically reacts with O_2^- and prevents radiation-induced salivary gland dysfunction (17). The administration of N^G -monomethyl-L-arginine acetate (L-NMMA), an inhibitor of NOS, has also been shown to prevent radiation-induced xerostomia (2). In addition, increased levels of 8-hydroxy-2'-deoxyguanosine (8-OHdG), a biomarker of oxidative stress, have been found in the saliva of patients with Sjogren's syndrome, which is characterized by the distressing symptom of dry mouth, but not in the saliva of healthy individuals (18). Therefore, ROS are believed to be involved in the pathogenesis of xerostomia. However, the mechanisms underlying radiation-induced secretory dysfunction and the effects of antioxidants have not been investigated adequately.

Antioxidants might be useful as therapeutic agents against oxidative stress-related diseases. SOD is an antioxidant enzyme that eliminates O_2^- , thereby inhibiting $ONOO^-$. $ONOO^-$ is well known as a powerful oxidant that is regulated mainly by SOD and NOS. Because SOD is believed to be an important antioxidant enzyme against ROS, several clinical applications of SOD have been attempted, but the results have not been encouraging (19). The main limitation is the extremely short half-life of SOD in the blood. To obtain good clinical effects, therefore, the half-life of SOD in the blood must be prolonged and its affinity to the cell membrane (the main site of ROS production) must be increased. Lecithinized SOD [phosphatidylcholine (PC)-SOD] is a chemically modified SOD that is synthesized by the covalent bonding of a lecithin derivative, PC, to recombinant human Cu-Zn SOD, thereby increasing the cellular affinity of SOD; the pharmacological potency of PC-SOD is 100 to 200 times greater than that of unmodified SOD (20). Additionally, SOD concentrations in the brain, lungs, heart, liver and serum were all higher in rats treated with PC-SOD than in rats treated with unmodified SOD (21). We assumed in the present study that the administration of PC-SOD would result in an adequate distribution of SOD to the mouse salivary glands. Together, the above findings suggest that PC-SOD may be effective against a variety of diseases mediated by oxidative stresses (22).

In this study, we analyzed the effects of antioxidants, such as PC-SOD and *N*-acetyl-L-cysteine (NAC), on salivary gland dysfunction and elucidated the pathogenesis underlying the secretory dysfunction induced by ROS. For this purpose, we used a mouse model of radiation-induced salivary hypofunction and cells of a human salivary gland cell line (HSY).

MATERIALS AND METHODS

Animals

Six-week-old male C57BL/6J mice (body weight 20–25 g; Clea Japan Inc., Tokyo) were used. The mice were housed in polycarbonate cages in a specific-pathogen-free mouse colony and were given food and water *ad libitum*. All animal experimental procedures were approved by the animal welfare committee of Tsurumi University (Kanagawa, Japan).

Irradiation

Each mouse was anesthetized with an intraperitoneal injection of 60 mg/kg sodium pentobarbital. A single acute exposure of 10 MV X rays (MEVATRON74 DX40; Toshiba Medical Systems, Tokyo) at a dose rate of 3 Gy/min and a distance of 1000 mm was then administered. The effective radiation dose to the salivary gland was set using the percentage depth dose and was over 95% of the maximum dose delivered.

Chemicals

PC-SOD was provided by LTT Bio-Pharma (Tokyo, Japan) and was dissolved in 5% mannitol solution. Lecithinization has been reported to increase the cell membrane affinity and extends the blood half-life of SOD. NAC (Sigma, St. Louis, MO) was diluted with physiological saline and adjusted to pH 7.0 before use. Pilocarpine-HCl (1% sanpilo) was purchased from Santen Pharmaceutical Co., Ltd. Carbachol (CCh) and PC were purchased from Sigma. L-NMMA was purchased from Dojindo, Japan. Lecithinized catalase (PC-CAT) was kindly provided by Dr. Yu Imai (Keio University).

Administration of Antioxidants

PC-SOD was first administered intravenously 3 h prior to irradiation and then once a day from the next day for 2 weeks at doses of 1 mg/kg or 3 mg/kg. Mannitol (the solvent used for the PC-SOD solution) and PC (0.15 mg/kg, a dose equivalent to that used in the PC-SOD solution) were used as controls. PC-SOD was administered intravenously to attain the required blood concentration of SOD. NAC (500 mg/kg) was administered intraperitoneally 1 h prior to irradiation and then once a day from the next day for 2 weeks. Intraperitoneal administration of NAC was sufficient to obtain the required effects (23). To examine the additive effect of the concurrent administration of PC-SOD and L-NMMA, we injected PC-SOD (3 mg/kg) and L-NMMA (3 mg/kg) intravenously 3 h prior to irradiation and once a day from the next day for 2 weeks. L-NMMA alone was also administered intravenously.

Measurement of Saliva Secretion

Saliva secretion was measured 1 week prior to irradiation, and the animals were assigned to groups so that each group had an equivalent distribution of saliva secretion. The mice were weighed and then anesthetized with an intraperitoneal injection of a mixture of xylazine (24 mg/kg) and ketamine (36 mg/kg). To stimulate salivation, pilocarpine (0.1 mg/kg) was injected intraperitoneally. The saliva secreted into the oral cavity during 1-min periods after the injection of pilocarpine was collected carefully using capillaries (Ringcaps; Hirschmann Laborgeräte GmbH & Co. KG, Eberstadt, Germany). The total amount of saliva produced in 15 min was then divided by the weight of the mouse.

Measurement of SOD Enzymatic Activity in Salivary Glands

Two weeks after irradiation, the total protein was extracted from salivary glands isolated from irradiated mice that had been treated with 5% mannitol, PC or PC-SOD (1 mg/kg or 3 mg/kg). Salivary

gland tissue samples were homogenized in 10 volumes of ice-cold 20 mM Hepes buffer (pH 7.2) containing 1 mM EGTA, 210 mM mannitol, and 70 mM sucrose, then sonicated. After 15 min of centrifugation at 10,000g, aliquots of the supernatant were stored at -80°C for subsequent batch analysis of the total SOD activity. The total protein concentration was measured using the BCA Kit reagent (Thermo Fisher Scientific). SOD activity was assayed using the SOD Assay Kit-WST (Dojindo, Japan). Enzyme-specific activities were expressed as units/mg of protein. One unit of SOD activity was defined as the amount of enzyme required to inhibit 50% of the WST-1 formazan per minute.

Submandibular Gland Cell Preparation

Each mouse was anesthetized with a mixture of xylazine and ketamine and then killed humanely. The bilateral submandibular gland (SMG) cells were immediately removed and placed in ice-cold balanced salt solution (BSS) containing 115 mM NaCl, 5.4 mM KCl, 2 mM Ca^{2+} , 1 mM Mg^{2+} , 20 mM Hepes, and 10 mM glucose (pH 7.4), supplemented with 1.25% bovine serum albumin (BSS-BSA), and then minced rapidly. The material was then digested at 37°C with 2 mg/ml of collagenase type 2 (Worthington, Malvern, PA) in BSS-BSA for 20 min. After digestion, the preparation was centrifuged at 70 g for 2 min, and the pellet was resuspended in 10 ml of BSS-BSA, rinsed twice and filtered through a 100- μm nylon mesh (Cell Strainer 100 μm ; BD Biosciences, Bedford, MA) to generate a batch of SMG cells.

Measurement of Intracellular Ca^{2+} Concentration

The isolated SMG cell preparations were loaded with fura-2 and incubated for 1 h at room temperature with 3 μM fura-2/acetoxymethyl ester (Dojindo, Kumamoto, Japan) suspended in BSS-BSA, rinsed twice, resuspended in 4 ml of BSS-BSA, and stored at 4°C . Ratiometric measurements of the fura-2 fluorescence were then made using a spectrofluorometer (CAF-110; Jasco, Tokyo, Japan). Fura-2-loaded SMG cells were transferred to a glass cuvette and were alternately illuminated with 340- and 380-nm excitation light; the resultant fluorescence (510 \pm 10 nm) was measured at 25 Hz. CCh (4 μM) was added directly to the cell suspension during the fluorescence measurement. At the end of each experiment, the R_{max} was determined by adding 0.2% Triton X-100 to the cuvette; then the R_{min} was determined by adding 10 mM EGTA. The fluorescence intensities excited by the 340- and 380-nm wavelengths of light (F_{340} and F_{380} , respectively) and the ratios (F_{340}/F_{380}) were digitized using 12-bit resolution and stored and displayed in a personal computer using the MacLab4/s system (AD Instruments-Japan, Tokyo, Japan).

Measurement of Protein Carbonyls

Protein carbonyls were detected using the dinitrophenylhydrazine (DNPH) derivatization method and the Oxyblot protein oxidation detection kit (Chemicon, Temecula, CA). Six micrograms of protein extract was incubated with an equal volume of 12% SDS and 2 volumes of DNPH solution for 15 min at room temperature, then with 1.5 volumes of neutralization solution to stop the reaction. The samples were electrophoresed on SDS-PAGE gels and electrotransferred to a PVDF membrane. The membranes were analyzed by Western blotting with an antibody against DNP to detect protein carbonyls that had been derivatized by DNPH. The bands were visualized using chemiluminescent chemicals and captured on film. A densitometric analysis was performed using Quantity One (Bio-Rad).

Cell Cultures

HSY cells derived from an adenocarcinoma of the parotid gland were used. HSY cells were cultured in a growth medium comprised of DMEM and high glucose (D6429; Sigma) plus 10% fetal bovine

serum (Sigma) and 1% penicillin or streptomycin (Invitrogen); the cultures were maintained in 100-mm culture dishes in a humidified atmosphere containing 95% air/5% CO_2 at 37°C . The cells were allowed to grow to 80% confluence, released by a trypsin-EDTA solution, reseeded in a 100-mm culture dish, and grown for 24 h before their use in experiments.

UVB Irradiation

Cells were exposed to 27.0–270.0 mJ/cm² UVB radiation using a Benchtop UV transilluminator [LM-20E (2UV); Funakosi, Tokyo, Japan] as a light source. The exposure was measured using a UV Meter (J-221; Analog) calibrated at 365 nm.

FACS Measurement of ROS

We assessed ROS generation using two fluorescent probes: dihydroethidium (DHE, 10 μM), which specifically reacts with intracellular O_2^- , and CM-H₂DCFDA (5 μM), a dye that is sensitive to a broad range of ROS, including H_2O_2 and $\cdot\text{OH}$ (24). DHE specifically reacts with intracellular O_2^- and is converted to the red fluorescent compound ethidium, which then binds irreversibly to double-stranded DNA and appears as punctuate nuclear staining. CM-H₂DCFDA is a cell permeant indicator for ROS that is non-fluorescent until the removal of acetate groups during oxidation within the cell. Cells that had been grown at a density of 1×10^6 cells per 12-well plate and had been maintained for 24 h in growth medium were incubated for 1 h with PC-SOD (50 $\mu\text{g}/\text{ml}$), NAC (10 mM) or PC-CAT (200 $\mu\text{g}/\text{ml}$) and then exposed for 15 min to one of the two fluorescent probes. After UVB irradiation, the medium was removed and the cells were washed with PBS and collected after the application of 0.02% EDTA and 0.25% trypsin, then analyzed. HSY cells were also exposed to 1.0 mM H_2O_2 for 30 min to analyze the intracellular ROS level induced by H_2O_2 . The analyses were carried out using a FACSCaliber flow cytometer and Cell Quest software (Becton Dickinson).

Statistics

Each experiment was repeated at least three times. The significance of the differences between groups was evaluated using a Student's paired two-tailed *t* test. A value of $P < 0.05$ was regarded as being statistically significant. All values were expressed as means \pm SE.

RESULTS

Effects of Antioxidants on Radiation-Induced Salivary Dysfunction

A previous study showed that saliva production in C57BL/6J mice was sensitive to radiation in a dose-dependent manner (2), and Vitolo reported a constant reduction in saliva flow until 8 weeks after irradiation (17). We also found a decrease in the saliva flow rate from a baseline value of 13.6 ± 1.8 $\mu\text{l}/\text{g}$ to an 8-week postirradiation value of 7.6 ± 1.7 $\mu\text{l}/\text{g}$, representing a reduction of about 50% (for 15 Gy) ($P < 0.05$; Fig. 1C). These results are consistent with those of previous studies. To evaluate the effects of SOD and NAC on saliva secretion, we measured the cumulative amounts of pilocarpine-induced saliva secretion for 15 min after administration. At 2 weeks after irradiation, the average saliva flow rates in mice treated with 1 and 3 mg of PC-SOD (12.1 ± 1.7 $\mu\text{l}/\text{g}$ and 12.7 ± 1.8 $\mu\text{l}/\text{g}$, respectively)

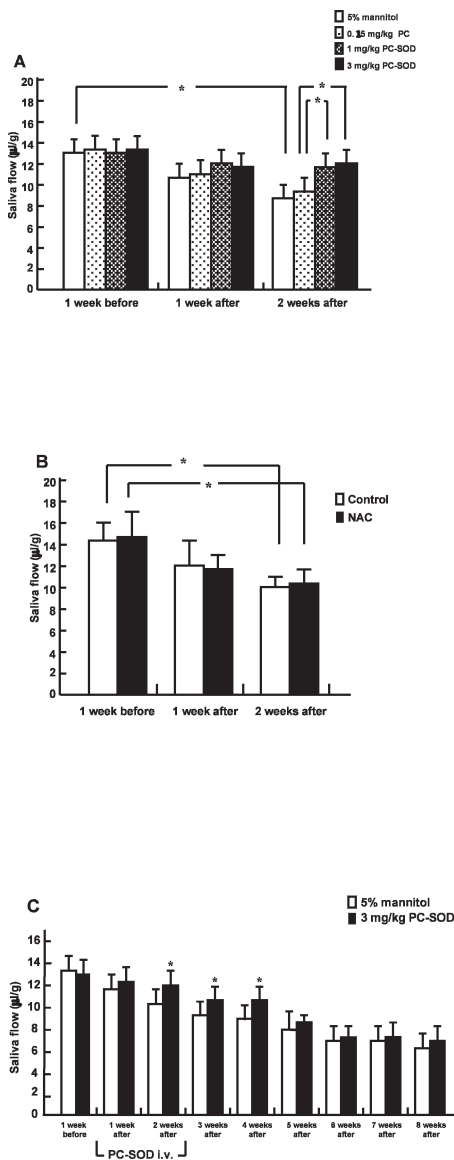


FIG. 1. Effects of PC-SOD and NAC on salivary gland secretion. Panel A: PC-SOD at a dose of 1 mg/kg or 3 mg/kg was administered intravenously 3 h prior to irradiation and once a day from the next day for 2 weeks. Mannitol, the solvent used to administer the PC-SOD and PC, was used as a control. Panel B: NAC (500 mg/kg) was administered intraperitoneally 1 h prior to irradiation and once a day from the next day for 2 weeks. Physiological saline, the solvent used to administer the NAC, was used as a control. Saliva flow was expressed as the total output of saliva during the first 15 min after pilocarpine stimulation, normalized to the body weight. Five to six mice were analyzed in each group. The error bars indicate the standard errors of the mean. The asterisks indicate a significant decrease ($P < 0.05$) in comparison to the respective control. Panel C: PC-SOD at a dose of 3 mg/kg was administered intravenously 3 h prior to irradiation and once a day from the next day for 2 weeks. Mannitol was used for the control. Saliva secretion was measured 8 weeks after irradiation.

were significantly higher than those observed after the administration of 5% mannitol or PC ($9.2 \pm 1.1 \mu\text{l/g}$ and $9.9 \pm 1.7 \mu\text{l/g}$, respectively). These results showed that radiation-induced salivary dysfunction was restored by

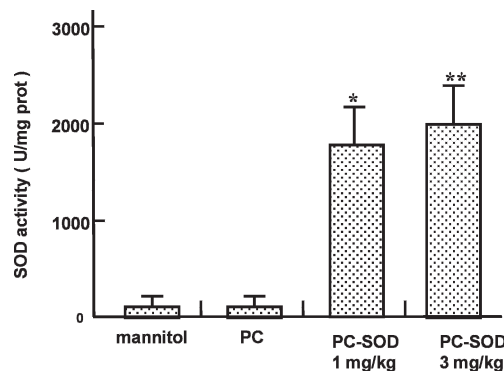


FIG. 2. Enzymatic activity of SOD in salivary glands. Two weeks after irradiation, SMG tissue was extracted from each treatment group. The total protein from the SMG tissue was assessed for SOD activity. Data are the means \pm SD from three independent experiments. * $P < 0.05$, ** $P < 0.05$, PC-SOD compared to mannitol and PC, respectively.

the administration of PC-SOD. In addition, although PC-SOD was administered for only 2 weeks, the improvement in saliva production after treatment with PC-SOD persisted until 4 weeks after irradiation (Fig. 1C).

On the other hand, the average saliva flow rates in the NAC-administered mice and the control mice (physiological saline) at 2 weeks after irradiation were significantly lower than those observed before irradiation (Fig. 1B). No adverse effects of NAC treatment on the radiation-induced reductions in saliva secretion were observed.

Enzymatic Activity of SOD in Salivary Glands

A dramatic increase in SOD activity was observed in the salivary glands isolated from mice treated with PC-SOD, compared with the activity level in the control group ($P < 0.05$; Fig. 2). Therefore, we assumed that the continuous administration of SOD for 2 weeks had resulted in the accumulation of SOD in the salivary glands.

[Ca²⁺]_i Measurement

In salivary acinar cells, the intracellular Ca²⁺ concentration ($[\text{Ca}^{2+}]_i$) is closely involved in the regulation of fluid secretion by muscarinic receptor stimulation (25). Thus, to study the effect of radiation on salivary gland secretory function, we measured $[\text{Ca}^{2+}]_i$ induced by cholinergic stimulation in SMG cells. Radiometric measurements using fura-2 were conducted in SMG cells obtained from nonirradiated and irradiated mice 2 weeks after irradiation (26). The results showed a typical increase in $[\text{Ca}^{2+}]_i$ in SMG cells isolated from the control mice in response to stimulation with CCh ($4 \mu\text{M}$), a nonselective cholinergic agonist. The CCh-induced $[\text{Ca}^{2+}]_i$ increase was significantly reduced in irradiated mice compared with that in the nonirradiated mice (Fig. 3A). These results showed that the elevation of

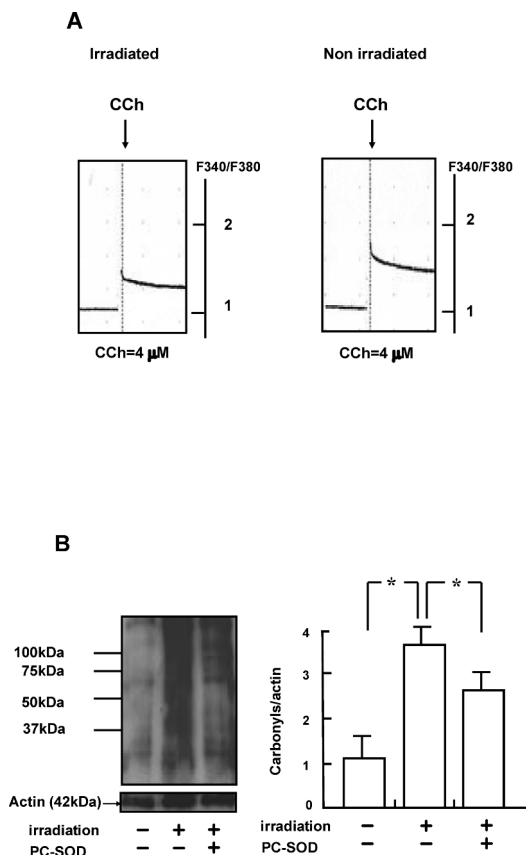


FIG. 3. Analysis of SMG cells from nonirradiated and irradiated mice. Panel A. Calcium assay: The CCh-induced $[Ca^{2+}]_i$ in the SMG cells from control and irradiated mice were measured. CCh was applied to Fura-2-loaded SMG cells at the time indicated by the arrows. Panel B: Detection of protein carbonylation using Western blotting for DNP-modified protein carbonyls: Protein carbonylation was detected by DNPH derivatization. Protein extracts from the cells were treated with DNPH to derivatize the oxidized amino acids. SDS-PAGE-separated proteins were probed with anti-DNP polyclonal antibody. Approximately 6 μ g of each protein was loaded per lane. Actin was used as a loading control. Representative blot (left panel) and densitometric analysis normalized to actin (right panel). * $P < 0.05$.

$[Ca^{2+}]_i$ induced by CCh was inhibited in the SMG cells obtained from irradiated mice, suggesting radiation-induced exocrine dysfunction.

Detection of Protein Carbonylation

ROS production in cells can result in a number of protein modifications, including carbonylation. We therefore investigated protein carbonylation in salivary glands derived from nonirradiated, irradiated and irradiated plus PC-SOD-treated (3 mg/kg) mice. An increase in protein carbonylation was noted in the SMG cells obtained from the irradiated mice, and PC-SOD pretreatment reduced this protein carbonylation to the control level (Fig. 3B). These changes paralleled the variations in saliva secretion that were observed. Moreover, a quantitative analysis showed that PC-

SOD significantly reduced radiation-induced protein carbonylation.

Flow Cytometry of Intracellular ROS Generated in HSY Cells Pretreated with PC-SOD or NAC Prior to UVB Irradiation and PC-CAT prior to H_2O_2 Exposure

We analyzed the intracellular ROS levels in UVB-irradiated HSY cells to elucidate the mechanism of radiation-induced salivary gland dysfunction and to examine the effects of treatment with PC-SOD. The intracellular ROS levels in HSY cells were tested after UVB irradiation using the oxidant-sensitive fluorescent dye DHE, which specifically reacts with intracellular O_2^- , and CM- H_2DCFDA , a dye that is sensitive to several ROS, including H_2O_2 , and $\cdot OH$. The mean fluorescence intensity (MFI) values of DHE and CM- H_2DCFDA showed dose-dependent increases after UVB irradiation (30–270 mJ/cm²) (data not shown). In addition, at a UVB-radiation dose of 90 mJ/cm², increases in the MFIs of DHE and CM- H_2DCFDA were noted in the absence of any microscopic changes in the HSY cells. Thus we used a UVB-radiation dose of 90 mJ/cm² in subsequent experiments. Pretreatment with PC-SOD (50 μ g/ml) significantly reduced the MFI of DHE in HSY cells compared with that observed after irradiation without PC-SOD pretreatment (Fig. 4A). In contrast, pretreatment with NAC (10 mM) did not change the MFI of DHE. These results suggested that PC-SOD, but not NAC, promptly eliminated the O_2^- generated by UVB radiation.

We then tested the broad intracellular levels of other ROS, including H_2O_2 and $\cdot OH$. In the cells that were pretreated with PC-SOD (50 μ g/ml), the MFI of CM- H_2DCFDA was similar to that observed after irradiation without PC-SOD pretreatment. In contrast, pretreatment with NAC (10 mM) decreased the MFI to the control level (Fig. 4B). This effect was stronger than the effect induced by PC-CAT, producing a complete elimination of the MFI of CM- H_2DCFDA induced by 1.0 mM H_2O_2 (Fig. 4C). These results indicated that PC-SOD mainly eliminated the O_2^- that had been induced by UVB radiation, whereas NAC may have removed ROS other than O_2^- , such as H_2O_2 and $\cdot OH$. These results also suggest that salivary gland dysfunction is caused mainly by radiation-induced O_2^- production and that the effects of H_2O_2 and $\cdot OH$ on salivary gland dysfunction are negligible. Therefore, PC-SOD may be effective for protecting against exocrine gland dysfunction induced by UVB radiation, presumably by rapidly converting O_2^- to H_2O_2 and O_2 .

Effect of Concurrent Administration of PC-SOD and L-NMMA

Because O_2^- reacts rapidly with NO to form stable ONOO⁻ (3) and Takeda *et al.* reported previously that

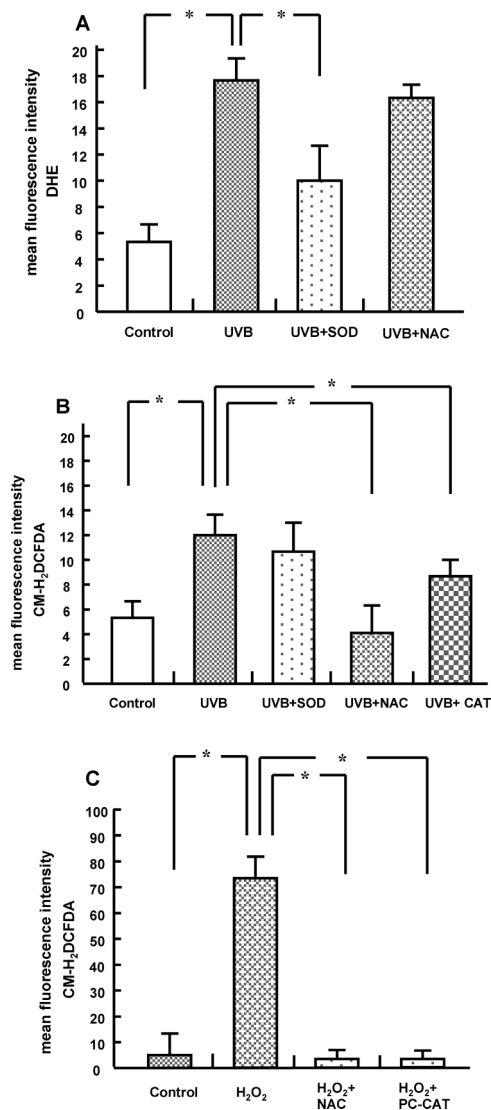


FIG. 4. Flow cytometry analysis of reactive oxygen species. ROS generation was assessed using the fluorescent probes DHE (panel A) and CM-H₂DCFDA (panels B, C). The HSY cells were incubated with PC-SOD (50 μ g/ml), NAC (10 mM) or PC-CAT (200 μ g/ml) prior to UVB irradiation or H₂O₂ stimulation. The error bars are SE. The asterisks indicate significant decreases in comparison with the controls (* $P < 0.05$).

NO was involved in radiation-induced salivary dysfunction (2), we also studied the effect of L-NMMA. At 2 weeks after irradiation, the average saliva flow rate in mice treated with L-NMMA was significantly higher than the values observed after the administration of either 5% mannitol or PC. The effect of L-NMMA was similar to that of PC-SOD. Moreover, the concurrent administration of PC-SOD and L-NMMA showed an additive improvement in saliva secretion at 6 weeks after irradiation. These results suggested that ONOO⁻ might also be involved in radiation-induced exocrine dysfunction.

DISCUSSION

Various causes of salivary gland dysfunction have been studied. These causes include high doses of radiation, certain diseases such as Sjogren's syndrome, and the use of certain systemic medications. High-dose radiotherapy for head and neck tumors often results in severe xerostomia (27). Sjogren's syndrome is an autoimmune disease characterized by dry mouth and dry eyes caused by the progressive destruction of salivary and lacrimal glands (28). However, there are also cases with unexplained salivary gland dysfunction, in which the deterioration in the salivary glands' environment and aging have been implicated. Significantly increased concentrations of 8-OHdG have been reported in the saliva of patients with SS compared with in the saliva of matched controls (18). An examination of the molecular mechanisms involved in saliva secretion revealed that the CCh-induced [Ca²⁺]_i in SMG cells was significantly reduced by irradiation (Fig. 3A), even though the cells remained morphologically intact. Therefore, ROS are believed to be involved in the mechanism of salivary gland dysfunction.

ROS can cause a number of nonenzymatic modifications of proteins, including carbonylation. Protein carbonylation can occur through direct oxidation of amino acid side chains with ROS (29,30) and has been studied predominantly in association with aging (16,31). We found that protein carbonylation was increased in irradiated mice and that this reaction was attenuated by treatment with PC-SOD (Fig. 3B). Protein carbonylation can alter the rate of protein degradation, increasing the turnover of some proteins and reducing the rate of others (32). Perhaps most significantly, the carbonylation of a protein can also reduce its activity (33). Consequently, cells that have accumulated a large number of protein carbonyls may also exhibit impaired functions. Thus protein carbonylation may be involved in radiation-induced salivary gland dysfunction and symptom relief after PC-SOD administration.

We confirmed the presence of radiation-induced salivary gland dysfunction in irradiated mice. While PC-SOD provided protection against radiation-induced salivary gland dysfunction (Fig. 1A, C), NAC did not (Fig. 1B). This difference might be attributable to the difference in the species of ROS that are eliminated by PC-SOD and NAC. SOD is a ubiquitously expressed family of enzymes that catalyze the efficient dismutation of O₂⁻. In contrast, the antioxidant effect of NAC is mediated by the regeneration of glutathione that scavenges many ROS, including H₂O₂ and [•]OH (34). In this study, we showed that O₂⁻ generation induced by UVB radiation was decreased by PC-SOD but not by NAC (Fig. 4A).

Takeda *et al.* demonstrated the partial recovery of saliva secretion in irradiated mice by blocking NOS (2),

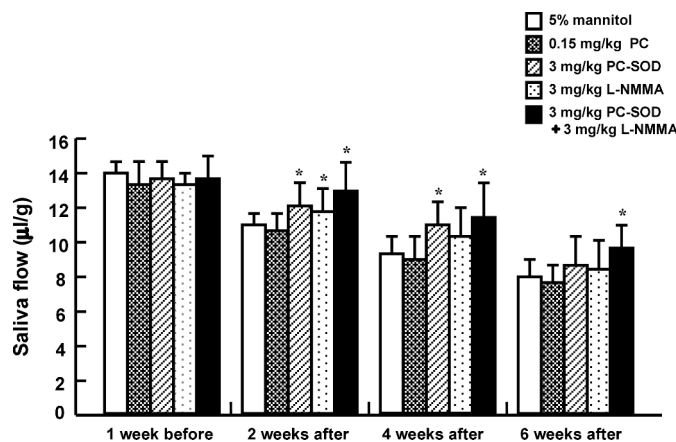


FIG. 5. Concurrent administration of PC-SOD and L-NMMA. PC-SOD (3 mg/kg) and L-NMMA (3 mg/kg) were injected intravenously in the same mouse 3 h prior to irradiation and once a day from the next day for 2 weeks. Saliva flow was expressed as the total output of saliva during the first 15 min after pilocarpine stimulation and was normalized to the total body weight. Five to six mice in each group were analyzed. The error bars indicate the standard errors of the means. * $P < 0.05$ compared with the mannitol and PC control groups each week.

indicating that NO might also be involved in dysfunctional saliva secretion. NO reacts rapidly with O_2^- to form stable ONOO⁻, which decomposes to generate a strong oxidant with a reactivity similar to hydroxyl radicals (3). Hanaue *et al.* showed that ONOO⁻ might be an important pathogenic factor in radiation-induced salivary gland dysfunction (35). We hypothesized that PC-SOD attenuated the dysfunctional saliva secretion induced by radiation by preventing the formation of ONOO⁻ through the exclusive elimination of radiation-induced O_2^- production. Moreover, we confirmed an additive protect effect against radiation injury by the concurrent administration of PC-SOD and L-NMMA (Fig. 5). Thus ONOO⁻ might be an important factor in radiation-induced salivary gland dysfunction.

Meanwhile, NO signal transduction has been shown to have a crucial role in Ca^{2+} homeostasis in the M3 agonist-stimulated increase in Aquaporin5 (AQP5) expression in the apical plasma membrane (APM) of rat parotid glands (36). AQP5, which forms water channels that selectively transport water across the plasma membrane, was cloned from rat submandibular gland tissue (37). Salivary fluid secretion was found to be defective in transgenic mice lacking AQP5, indicating that AQP5 is important for normal salivary gland function (38). Thus NO might play an important role in saliva secretion. We propose that PC-SOD improves saliva secretion by diminishing O_2^- production, thereby enabling NO to accumulate.

PC-SOD has many advantages, such as a longer half-life in the blood *in vivo*, greater tissue affinity, and better drug delivery, resulting in a pharmacological potency 100 to 200 times greater than that of unmodified SOD

(21). The intravenous administration of PC-SOD promoted recovery from spinal cord injury-induced motor dysfunction in a dose-dependent manner in a rat model (39). Furthermore, Takenaga *et al.* demonstrated the inhibitory effects of PC-SOD on the development of bleomycin (BLM)-induced pulmonary fibrosis in mice (22). PC-SOD may also exert beneficial effects when used as an adjunct to conventional clinical treatments in diseases that are pathogenetically mediated by an oxidant/antioxidant imbalance. PC-SOD has also been shown to be active against a variety of diseases mediated by oxidative stresses.

ROS are believed to be involved in the pathogenesis of salivary gland dysfunction (2,17,18,40). Xerostomia, which causes a secondary increase in the incidence of dental caries, difficulty in chewing, swallowing and speaking, and an increased incidence of oral candidiasis, can have significant adverse effects on quality of life. In this study, we demonstrated that the administration of PC-SOD restored the salivary gland dysfunction caused by ROS. At present, no treatment for oxidative stress-induced xerostomia exists. We believe that our results may advance the potential application of antioxidants for the prevention of oxidative stress-induced salivary gland dysfunction.

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