Protein Carbonylation in THP-1 Cells Induced by Cigarette Smoke Extract via a Copper-Catalyzed Pathway

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Cigarette smoke is a mixture of chemicals that cause direct or indirect oxidative stress in different cell lines. We investigated the effect of nonfractionated cigarette smoke extract (CSE) on protein carbonylation in human THP-1 cells. Cells were exposed to various concentrations (2.5−20%) of CSE for 30 min, and protein carbonylation was assessed by use of the sensitive 2,4-dinitrophenylhydrazine immuno-dot blot assay. CSE-induced protein carbonylation exhibited a dose−response relation with CSE concentrations. However, with prolonged exposure to CSE, significant decrements were observed when compared with the 30 min exposure. Cotreatment of THP-1 cells with antioxidants (*N*-acetyl-cysteine, *S*-allyl-cysteine, and α-tocopherol) and copper(II) ion chelators (D-penicillamine) during CSE exposure significantly reduced protein carbonylation, whereas cotreatment with antioxidants (vitamin C and trolox) and a metal chelator (EDTA), iron chelator (1,10-phenanthroline), or copper(I) chelator (neocuprin) did not decrease CSE-induced protein carbonylation in THP-1 cells. These results suggest that protein carbonylation is induced by CSE in THP-1 cells via a copper(II)-catalyzed reaction and not an iron-catalyzed reaction. Furthermore, the copper(II) ions involved in this CSE-induced protein carbonylation are derived from the intracellular pool, not via uptake from the extracellular medium. We speculate that natural copper(II) chelators may prevent some of the health problems caused by cigarette smoking, including lung disease, renal failure, and diabetes.

Introduction

Cigarette smoking is a major risk factor influencing the health of humans. Cigarette smokers have a higher prevalence of common diseases such as atherosclerosis and emphysema, chronic obstructive pulmonary disease, and lung cancer (*1*−*3*). One of the prominent deleterious effects of cigarette smoke is the oxidative damage it does to biological macromolecules, including proteins and DNA (*4*), which actually accelerates aging processes (*5*).

Cigarette smoke is a complex mixture with more than 5000 identified constituents (*6*); it contains compounds such as polycyclic aromatic hydrocarbons (*7*), reactive glycation products (*8*), and transition metals (*9*). Some of these compounds have been shown to have genotoxic or carcinogenic properties along with a large constituent of free radical substances (*10*−*13*). Free radicals in the particulate component of cigarette smoke appear to be relatively stable semiquinones, whereas the gas phase of cigarette smoke contains short-lived radicals that are potent oxidants (*14*). Free radical-induced oxidative damage has been implicated as an important mechanism responsible for the toxicity of smoking.

Cigarette smoke extract (CSE) directly oxidizes human plasma proteins, bovine serum albumins, and amino acid homopolymers and also causes extensive oxidative degradation of microsomal proteins (*15, 16*). Cigarette smoke-induced protein oxidation and proteolysis are exclusively caused by the tar phase and are completely blocked by vitamin C (VitC) addition (*17*). In addition, CSE induces protein oxidation in endothelial cells and in animals (*18, 19*). In a cell system, CSE induces protein carbonylation via a metal-catalyzed pathway that is blocked by the addition of thiol group-containing agents and cation chelators; by contrast, the addition of antioxidants has only marginal effects on CSE-induced protein carbonylation (*18*). CSE-induced protein oxidation may proceed down different pathways in cell-dependent and cell-free systems. In a cell-free system, the proteins are mostly oxidized by oxidants in the CSE directly, whereas in a cell-dependent system, the proteins are indirectly oxidized by an intracellular transition metal-catalyzed oxidative mechanism (*15, 17, 20*).

Chronic inflammatory lung disease, kidney disease, and atherosclerosis are associated with monocyte and macrophage dysfunction. Smoking alters alveolar macrophage recognition, phagocytic ability, and apoptosis (*21, 22*) and increases CD11b-dependent monocyte adhesiveness in humans (*23*). The mechanism by which cigarette smoke contributes to inflammatory diseases like chronic inflammatory diseases remains unclear; however, cigarette smoke-induced oxidative stress in cellular systems may be the key pathogenic factor. Protein carbonylation is the most widely used marker of oxidative stress and has been predominantly studied in aging (*24*).

Recent studies further indicate that protein carbonylation is a means of signal transduction that closely relates to human chronic diseases including chronic lung disease and atherosclerosis (*25*). However, few studies have been made of cigarette smoking-mediated oxidative stress in monocytes. Thus, the main purpose of the present study was to determine whether CSE could induce protein carbonylation in human monocytic THP-1

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cells and to explore the possible mechanism of CSE-induced protein oxidation.

Experimental Procedures

Chemicals. Cell culture medium (RPMI 1640), fetal bovine serum, trypsin−EDTA, and penicillin−streptomycin were purchased from Gibco/Invitrogen (Carlsbad, CA). *S*-Allyl-cysteine (SAC) was purchased from Tokyo Chemical Industry (Tokyo, Japan), polyvinylidene difluoride (PVDF) membranes were from Millipore Corp. (Bedford, MA), and 40% acrylamide (37.5:1) was purchased from GenePure TM (Foster City, CA). Anti-β-actin mouse monoclonal antibody, horseradish peroxidase (HRP)-labeled antirabbit IgG polyclonal antibody, and HRP-labeled antimouse IgG polyclonal antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA); anti-DNPH (2,4-dinitrophenylhydrazone) rabbit polyclonal antibody was from Zymed Laboratories, Inc. (San Francisco, CA); and anti-PARP [poly(ADP-ribose) polymerase] rabbit polyclonal antibody was from Roche Molecular Biochemicals (Mannheim, Germany). Coomassie plus protein assay reagent kits were purchased from Pierce Biotech Inc. (Rockford, IL), enhanced chemiluminescence detection kits were from PerkinElmer Life Science (Wellesley, MA), and nuclear/cytosol fractionation kits were from BioVision (Mountain View, CA). All other chemical reagents were from Sigma-Aldrich Inc. (St. Louis, MO).

Cell Culture and Treatment. Human THP-1 monocytic cells were maintained in RPMI 1640 medium containing heat-inactivated 10% fetal calf serum, 50 U/mL of penicillin G, and 50 mg/mL of streptomycin sulfate in a humidified incubator at 37 °C and with 5% CO_2. Cells were seeded at a density of 5×10^5 cells/cm^2 in six well plates, and the overnight culture was used for the experiment. For carbonylation protective experiments, THP-1 cells were preloaded with different doses of antioxidants [VitC, trolox (TRO), SAC, and α-tocopherol (ATOC)] or metal chelators [1,10-phenanthroline (PHE), neocuproine (NC), *N*-acetyl-cysteine (NAC), D-penicillamine (DPEN), EDTA] for 30 min before they were treated with 20% CSE. After CSE addition, the cells were incubated for an additional 30 min in the presence of antioxidants or metal chelators before they were collected for the protein carbonylation assay. For experiments aimed at determining the localization of protein carbonylation, the cells were treated with various concentrations for indicated times and were collected for preparation of whole cell lysate, cytosol, and nuclear subfraction extracts. The level of protein carbonylation and the band pattern of carbonylated protein were determined by immuno-dot blot assay and Western blot assay, respectively.

Preparation of CSE. A nonfractionated CSE was prepared according to the procedures described by Su et al. (*26*) and Chen et al. (*27*). Briefly, commercial filter-tipped cigarettes (Marlboro, Philip Morris, Inc., Richmond, VA) were smoked continuously in the apparatus designed by Su et al. Mainstream smoke was forced through 15 mL of PBS by the application of a water pump vacuum. Each cigarette was smoked for 5 min, and three cigarettes were used per 15 mL of PBS to generate a CSE−PBS solution. The CSE solution was diluted with culture medium and used immediately. The final concentrations of these solutions are expressed as percentage values.

Whole Cell Lysate Preparation and Subcellular Fractionation. Cells were collected after centrifugation at 110*g* for 6 min and were rinsed twice with PBS. Cell pellets were added to lysis buffer containing 1 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), and 10 mM Tris-HCl, pH 7.4, and were sonicated for 10 cycles (20 s on and 20 s off) in a cup-horn sonicator. After sonication, the cellular detritus was removed by centrifugation at 25400*g* for 30 min, and the supernatant served as the whole cell extract. Cytosolic and nuclear fractions were prepared by using the nuclear/cytosol fractionation kit (BioVision) according to the manufacturer's instructions. The protein concentrations of the whole cell lysates and the subcellular fractions were determined by use of Coomassie plus protein assay reagent kits (Pierce) using bovine serum albumin as the standard, and small aliquots were stored at −70 °C for protein carbonylation analysis.

Protein Carbonylation Detection. The content of protein carbonyls was assessed as a measure of oxidative stress by use of the sensitive DNPH immuno-dot blot assay as described by Robinson et al. (*28*). After the protein contents were determined, equal aliquots (5 μg) were applied to a 24 well dot-blot apparatus (Bio-Rad, Hercules, CA) and were then transferred to a PVDF membrane by vacuum filtration. The carbonyl groups in the protein side chains were derived first by reaction with DNPH. After it was blocked with 5% nonfat dry milk for 30 min at room temperature, the PVDF membrane was treated with the HRP-conjugated anti-DNPH antibody diluted 1:20000 in PBST [10 mM Na-phosphate buffer, pH 7.2, 0.9% (w/v) NaCl, and 0.1% (v/v) Tween 20], and development was performed with an enhanced chemiluminescence detection kit. The blots were scanned and quantified by use of the AlphaImager system (Alpha Innotech, San Leandro, CA). For comparison, a membrane was loaded with an equal amount of proteins and stained with Coomassie brilliant blue R-250.

Protein Carbonylation Detection by SDS-PAGE and Western Blot. Alternatively, the reactions of protein carbonyls with DNPH were assayed by SDS-PAGE and Western blot as described by Dalle-Donne et al. (*29*). Protein samples were run in an 8% SDS-PAGE system, blotted to a PVDF membrane, and sequentially incubated in 2 N HCl and DNPH (0.1 mg/mL in 2 N HCl) for 5 min each. The membrane was then washed three times in 2 N HCl and seven times in 100% methanol for 5 min each, followed by one wash in PBST, and was then blocked for 1 h in 5% (w/v) nonfat dried milk in PBST. After it was washed three times with PBST for 5 min each, immunologic evaluation of carbonyl formation was performed for 2 h in 5% milk/PBST containing anti-DNPH antibodies. As an additional control, replicate blots were incubated in 5% milk/PBST without the primary antibody to DNPH, which was expected to prevent the appearance of carbonyl bands in the immunoblots. After three washes with PBST for 5 min each, the membrane was incubated with the secondary antibody linked to HRP in PBST containing 5% milk for 1 h. After it was washed three times with PBST for 5 min each, development was performed with an enhanced chemiluminescence detection kit. The blots were scanned and quantified by use of the AlphaImager system as described above. Immunoblotting with anti-β-actin and PARP antibodies confirmed equivalent protein loading.

Statistical Analysis. Data are expressed as the means ± SEs from at least three independent experiments. Statistical analysis was performed with commercially available software (SAS Institute Inc., Cary, NC). Data were analyzed by Student's *t* test. A value of $P < 0.05$ was considered to be significant.

Results

CSE-Induced Protein Carbonylation. As shown in Figure 1, CSE induced protein carbonylation in THP-1 cells in a dose-dependent manner. Significant protein carbonylation was observed at 30 min with 10, 15, and 20% CSE exposure, and the fold inductions were 3.26, 4.03, and 4.71, respectively. The time course of protein carbonylation induction by CSE is shown in Figure 2. Addition of 20% CSE resulted in a transient increase in protein carbonylation in THP-1 cells, and this increase reached a peak within 30 min and fell progressively over the next half hour. These results suggest that 20% CSE with a 30 min exposure time was an appropriate condition for inducing protein carbonylation, and this condition was selected for the further experiments in THP-1 cells.

A test tube study of CSE-induced bovine serum albumin carbonylation is also shown in Figure 2. In the test tube system, bovine serum albumin carbonylation rapidly increased at 30 min of CSE exposure and reached its maximum at about 3 h. Prolonged incubation to 6 or 24 h did not decrease the level of protein carbonylation. These results clearly indicate that there was a strict difference in CSE-induced protein carbonylation between the cell and the test tube models.

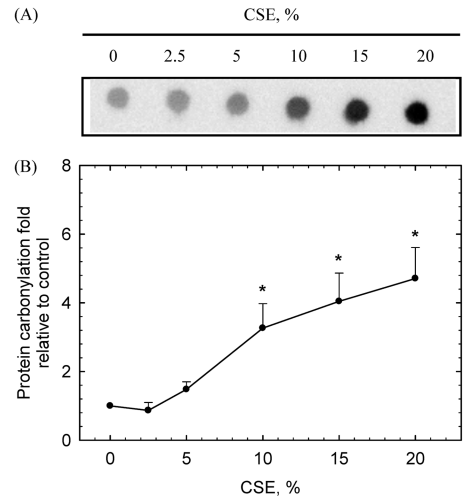

Figure 1. Dose-dependent response of protein carbonylation formation by CSE in THP-1 cells. Cells were treated with a series of doses of CSE (2.5−20%) for 30 min, and the level of protein carbonylation was determined by a sensitive DNPH immuno-dot blot assay as described in the Experimental Procedures. Data are presented as the means ± SEs of three independent experiments. $^{*}P < 0.05$ relative to control as evaluated by Student's *t* test. (A) A representative immuno-dot blot of protein carbonylation. (B) Levels of protein carbonylation expressed as the fold induction relative to control.

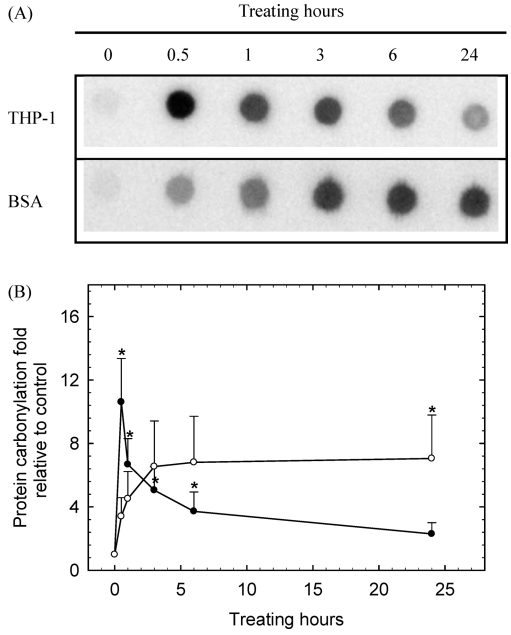

Figure 2. Time-dependent response of protein carbonylation formation by CSE. Cells (●) or bovine serum albumin (○) was treated with 20% CSE for different lengths of time (0.5−24 h), and the levels of protein carbonylation were determined by a sensitive DNPH immuno-dot blot assay as described in the Experimental Procedures. Data are presented as the means ± SEs of three independent experiments. $^{*}P < 0.05$ relative to control as evaluated by Student's *t* test. (A) A representative immuno-dot blot of protein carbonylation. (B) Levels of protein carbonylation expressed as the fold induction relative to control.

Protective Effect of Antioxidants and Metal Chelators on CSE-Induced Protein Carbonylation. To assess whether transition metals play a role in protein carbonylation induced by CSE exposure, we compared the degree of CSE-induced protein carbonylation with or without chelators of copper or iron ions. These data are shown in Figure 3. Cotreatment with the cell-permeable Cu(II) chelator DPEN could completely block CSE-induced (20% for 30 min) protein carbonylation in THP-1 cells; however, there was no significant reducing effect of the

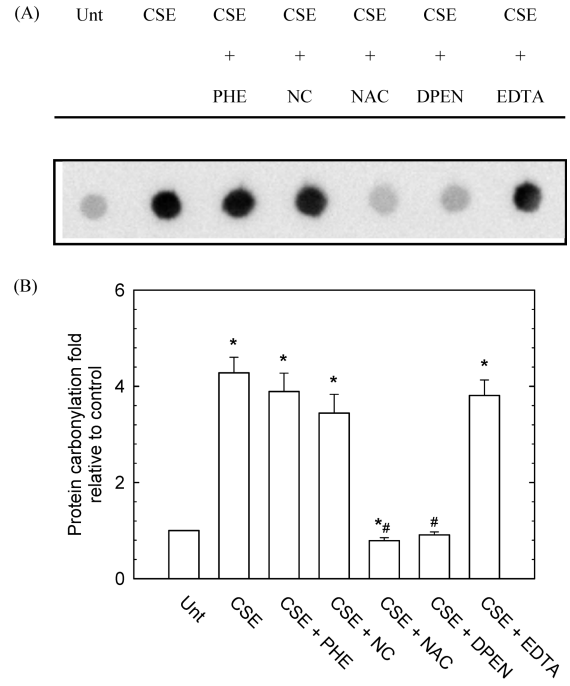

Figure 3. Effects of metal chelators on CSE-induced protein carbonylation. Cells were pretreated with 25 μM PHE, 5 μM NC, 5 mM NAC, 1.5 mM DPEN, and 200 mM EDTA for 30 min and were then treated with 20% CSE for another 30 min. Protein carbonylation levels were determined by immuno-dot blot assay as described in the Experimental Procedures. Data are presented as the means ± SEs of three independent experiments. $^{*}P < 0.05$ relative to control as evaluated by Student's *t* test. $^{\#}P < 0.05$ relative to the CSE-treated group as evaluated by Student's *t* test. (A) A representative immuno-dot blot of protein carbonylation. (B) Levels of protein carbonylation expressed as the fold induction relative to control.

cell-permeable Cu(I) chelator NC or the Fe(II) chelator PHE. In contrast with the cell-permeable copper(II) ion chelator, EDTA (a cell-impermeable divalent cation chelator) did not reverse the effect of CSE-induced protein carbonylation in THP-1 cells. Furthermore, NAC (both a free radical scavenger and a potent metal chelating agent) also completely blocked CSE-induced protein carbonylation in THP-1 cells.

Lower doses of DPEN and NAC could also partially block CSE-induced protein carbonylation (Figure 4). The percent inhibitions of CSE-induced protein carbonylation at doses of 10, 50, and 100 μM were 11.3, 33.2, and 43.8% for NAC and 9.4, 46.2, and 49.8% for DPEN, respectively. In addition, the inhibitory effect of NAC on the CSE-induced protein carbonylation in THP-1 cells had a better dose-dependent effect than in the DPEN group within a range of 10−100 μM. These results indicate that both intracellular copper ions and free radicals may be the key mediators for CSE-induced protein carbonylation in THP-1 cells.

To further understand the role of free radicals, we tested the protective effect of several antioxidants on CSE-induced protein carbonylation in THP-1 cells. These results are shown in Figure 5. A garlic thiol-containing compound, SAC (10 mM), and an active form of vitamin E, ATOC (100 μM), could partially block CSE-induced protein carbonylation. The inhibition percentages were 49.2 and 31.0%, respectively. By contrast, the antioxidants VitC and a water-soluble derivative of vitamin E (TRO) did not block CSE-induced protein carbonylation in THP-1 cells. To avoid the possible bias resulting from single-dose protocol, we further analyzed the dose effect of VitC (50−250 μM) and TRO (100 μM to 5 mM) on CSE-treated THP-1 cells. Once again, the results showed that VitC and TRO did not cause a significant dose-related decrease in the level of protein carbonylation induced by CSE in THP-1 cells (Figure 6).

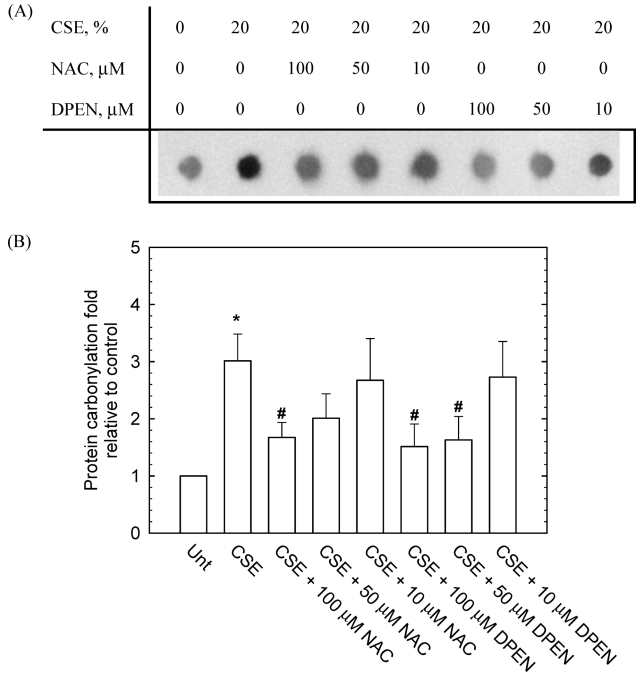

Figure 4. Dose-dependent inhibition by NAC and DPEN of protein carbonylation induced by CSE. Cells were pretreated with different doses of NAC (10, 50, or 100 μM) or DPEN (10, 50, or 100 μM) for 30 min and were then treated with 20% CSE for 30 min. Protein carbonylation levels were determined by immuno-dot blot assay as described in the Experimental Procedures. Data are presented as the means ± SEs of three independent experiments. $^{*}P < 0.05$ relative to control as evaluated by Student's *t* test. $^{\#}P < 0.05$ relative to the CSE-treated group as evaluated by Student's *t* test. (A) A representative immuno-dot blot of protein carbonylation. (B) Levels of protein carbonylation expressed as the fold induction relative to control.

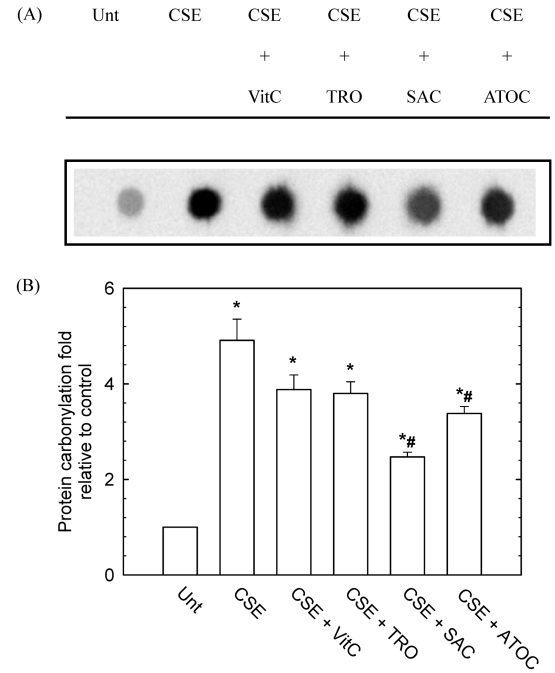

Figure 5. Effect of antioxidants on CSE-induced protein carbonylation. Cells were pretreated with 100 μM VitC, 5 mM TRO, 10 mM SAC, and 100 μM ATOC for 30 min and were then treated with 20% CSE for another 30 min. Protein carbonylation levels were determined by immuno-dot blot assay as described in the Experimental Procedures. Data are presented as the means ± SEs of three independent experiments. $^{*}P < 0.05$ relative to control as evaluated by Student's *t* test. $^{\#}P < 0.05$ relative to the CSE-treated group as evaluated by Student's *t* test. (A) A representative immuno-dot blot of protein carbonylation. (B) Levels of protein carbonylation expressed as the fold induction relative to control.

Subcellular Distribution of CSE-Induced Protein Carbonylation. To determine whether the carbonylated proteins were confined to a specific area, cells were incubated for 1 h in the absence or presence of 20% CSE, followed by subcellular fractionation and analysis of protein carbonylation by immuno-dot blot and Western blot. As shown in Figure 6, there was a marked increase in protein carbonylation in both the cytosolic and the nuclear fractions. The induction levels of protein carbonylation related to untreated cells after CSE exposure were 2.1-, 3.0-, and 2.9-fold in the whole cell, cytosolic, and nuclear fractions, respectively. In addition, the carbonylated protein profiles of the whole cell, cytosolic, and nuclear fractions after exposure to CSE were analyzed by Western blot. These data are shown in Figure 7C. A significant portion of the carbonylated proteins induced by CSE exposure was found in the regions corresponding to molecular masses from about 73 to 211 kDa. Although different sets of carbonylated proteins were detected in the cytosolic and nuclear fractions by Western blot, in general, the band patterns of carbonylated proteins in the cytosolic and nuclear fractions were similar between CSE-treated and untreated cells.

These results suggest that CSE exposure could quickly (<30 min) carbonylate multiple proteins in both cytosolic and nuclear fractions in THP-1 cells. Furthermore, CSE exposure did not significantly change the profile of carbonylated proteins but only enhanced the carbonylated level of many sensitive protein targets in the cytosolic and nuclear fractions under normal, nonstressful conditions.

Discussion

We clearly showed in the present study that CSE induced protein carbonylation through two different mechanisms in a cell system or an in vitro system. In THP-1 cells, CSE-induced carbonylated proteins could be degraded quickly by intracellular systems. Previous studies indicated that proteasomes and lysosomes are the major organelles involved in the degradation of oxidized proteins (*30*). Our recent data showed that the proteasome inhibitor MG132 could partially enhance CSE-induced protein carbonylation in CHO-K1 cells (data not shown). A similar finding was reported by Bernhard et al. (*18*). Those authors found that epoxomicine, a specific inhibitor of the proteasome, potently inhibits tubulin degradation in response to CSE treatment. In the present study, CSE induced higher levels of protein carbonylation in the THP-1 cell system than in the in vitro system. Taken together, our results indicate that CSE-induced protein carbonylation in THP-1 cells may not be mediated solely via direct damage by the stable free radical substances contained in the CSE.

We also found in the present study that the simple antioxidants VitC and TRO (a water-soluble form of vitamin E) did not block CSE-induced protein carbonylation. However, the antioxidants NAC, SAC, and ATOC could completely or partially block CSE-induced protein carbonylation in THP-1 cells. According to these findings, intracellular reactive oxygen species (ROS) generation may play a role in CSE-induced protein carbonylation. In addition, ATOC (but not TRO) partially blocked CSE-induced protein carbonylation. This implies that the CSE-induced production of ROS may partially occur in lipophilic environments (e.g., the mitochondrial membrane) due to lipid peroxidation. Evidence to support this claim is derived from the following data: (1) main mitochondrial ROS generators have been localized in the inner membrane (*31*) and (2) vitamin E (ATOC) addition inhibits lipid peroxidation in inner mitochondrial membranes and downregulates mito-

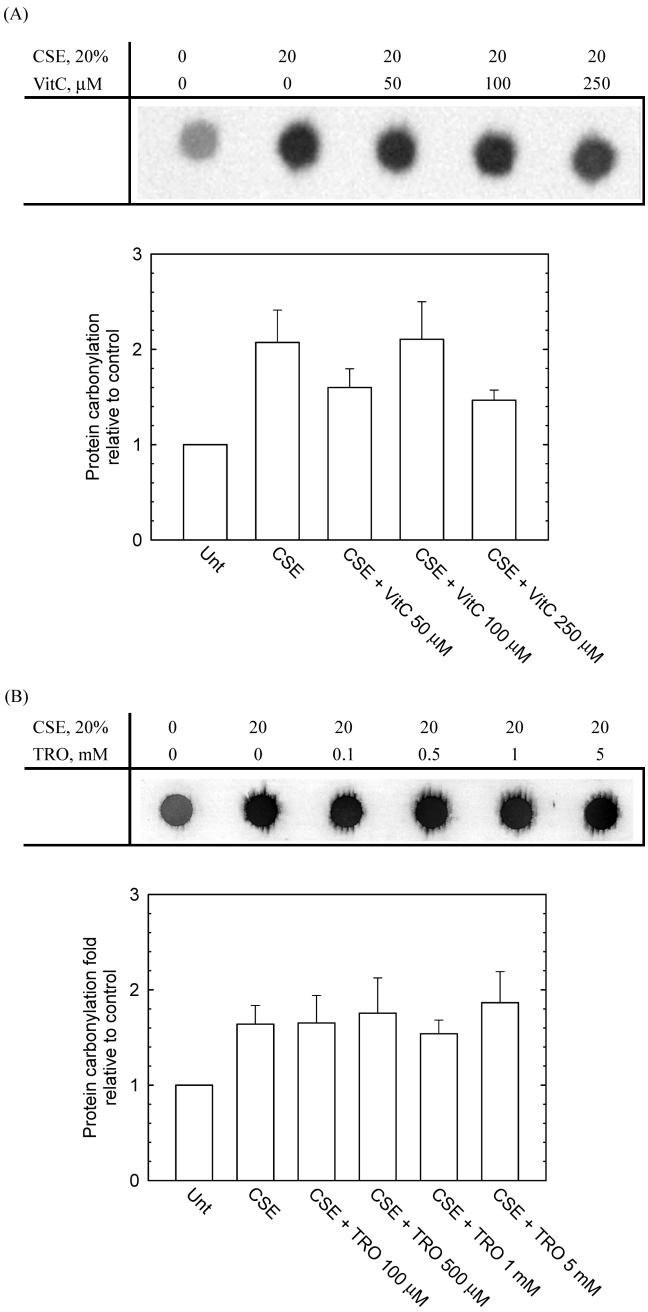

Figure 6. Dose-dependent effect of VitC and TRO on protein carbonylation induced by CSE. Cells were pretreated with different doses of VitC (50, 100, or 250 μM) or TRO (0.1, 0.5, 1, or 5 mM) for 30 min and were then treated with 20% CSE for 30 min. Protein carbonylation levels were determined by immuno-dot blot assay as described in Experimental Procedures. Data are presented as the means ± SEs of three independent experiments. A representative immuno-dot blot of protein carbonylation and levels of protein carbonylation expressed as the fold induction relative to control for VitC (A) and TRO (B).

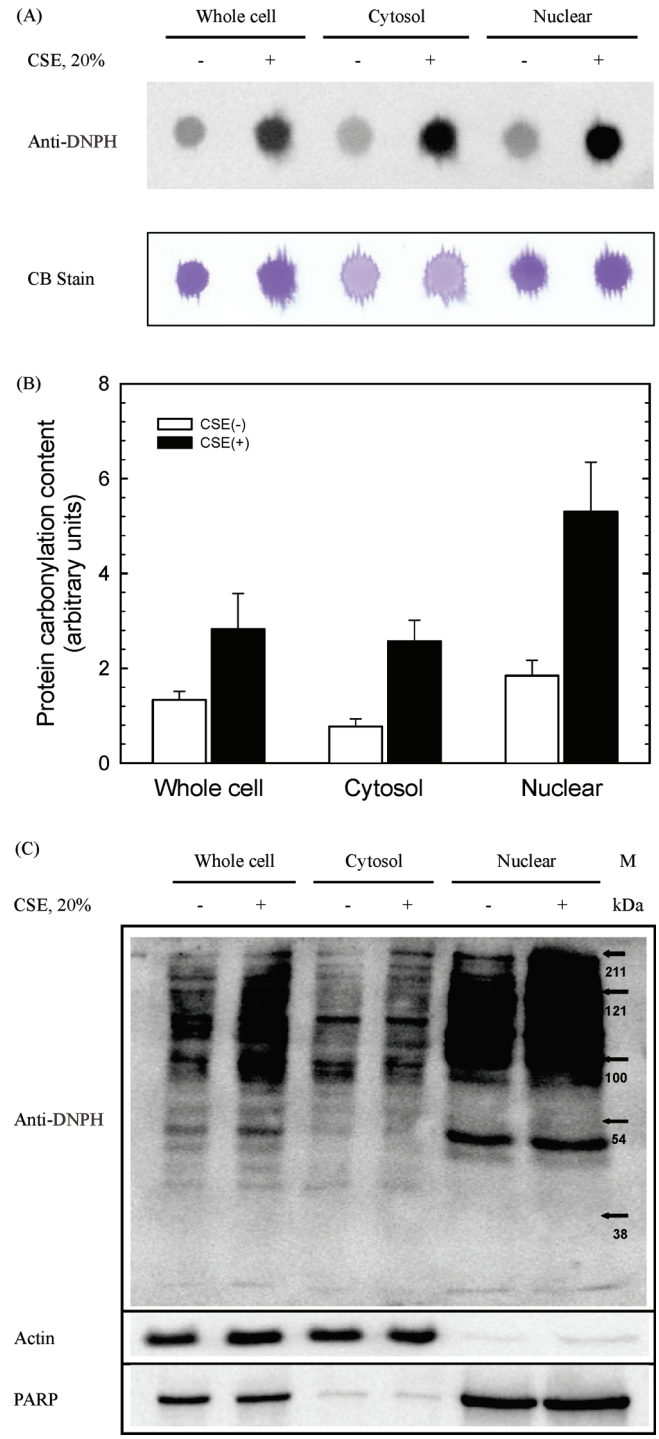

Figure 7. Subcellular distribution of protein carbonylation in CSE-treated THP-1 cells. Cells were treated with 20% CSE for 30 min. After incubation, cells were collected, and the whole cell lysate, cytosolic, and nuclear fractions were prepared. Protein carbonylation levels of the whole cell lysate and subcelluar fractions were determined by immuno-dot blot assay and Western blot assay as described in the Experimental Procedures. (A) A representative immuno-dot blot of protein carbonylation and Coomassie protein staining. (B) Levels of protein carbonylation expressed as arbitrary units per protein depending on Coomassie blue stain intensity. (C) A representative Western blot of carbonylation patterns of proteins in the subcellular fraction. The internal loading control and purity of the fractions were controlled by immunoblotting for β-actin and PARP proteins, which were localized to the cytosolic and nuclear fractions, respectively. Values represent the means ± SEs of eight independent experiments.

chondrial generation of superoxide and hydrogen peroxide (*32, 33*). In addition, our finding of no protective effect of VitC on CSE-induced protein carbonylation may also be consistent with this possibility.

In addition to ROS scavenging, previous reports indicated NAC or SAC to be copper chelating agents (*34*−*36*). The protective effects of NAC and SAC would therefore be predominantly by their copper chelating activity and not by their ROS scavenging activity. The previous report showing that copper-mediated protein damage is not effectively prevented by hydroxyl radical scavengers such as dimethyl sulfoxide or mannitol (*37*) also supports our notion of copper ion being the limiting factor for CSE-induced protein carbonylation.

Cotreatment with the copper(II) chelator DPEN blocked CSE-induced protein carbonylation more potently than antioxidants did in THP-1 cells. In contrast with DPEN, however, the copper(I) chelator NC and the iron(II) chelator 1,10-phenanthriline could not effectively block CSE-induced protein carbonylation in cells. These results indicate that CSE exposure may rapidly (<30 min) and selectively elevate the intracellular redox-active copper ion pool and result in the generation of large amounts of ROS and protein carbonylation. Uptake of copper from the extracellular medium as the free ion does not seem to occur in CSE-treated cells, because addition of a membrane-impermeable metals chelator, EDTA, did not reduce the level of CSE-induced protein carbonylation. These results indicated that CSE-induced copper ion releasing was from the intracellular pools. Furthermore, this evidence supports our postulation above that the inhibition of CSE-induced protein carbonylation by NAC and SAC may predominantly be through their copper-chelating activity and not their free radical scavenging activity.

A previous study showed strong evidence for a kinetically labile copper pool localized predominantly in the mitochondria and the Golgi apparatus (*38*). Atomic absorption spectroscopy studies showed that the order for copper binding was mitochondria > microsomes > nuclei in rat liver (*39*). The mitochondria are known to be a sensitive target to cigarette smoke exposure (*40*−*42*). A previous report indicated that CSE-induced damage to mouse brain mitochondria was due to the direct action of CSE on enzymatic activity rather than through oxygen free radical injury (*43*). That finding is supported by the result in the present study that VitC addition did not block CSE-induced protein carbonylation in THP-1 cells. Taken together, these findings suggest that a possible mechanism for the CSE toxicity occurring in the early phase in cells is the release of redox-active copper ions from damaged mitochondria or Golgi apparatus. The transient intracellular increase in copper ions could also be due to their release from copper binding proteins by CSE exposure. Metallothionein is the major copper binding protein in higher animals and was found to be most concentrated in cytosols, followed by nuclei, mitochondria, and microsomal fractions (*44*). Mitochondrial metallothionein has also been localized in the intermembrane space of rat liver mitochondria (*45*). Furthermore, oxidative stress causes nuclear translocation of metallothionein as the result of oxidation of its cytosolic partner (*46*). Importantly, previous studies have shown that oxidants such as nitric oxide, hypochlorous acid, and superoxide anion can cause the release of redox-active copper ions from metallothionein (*47, 48*). CSE increases intracellular oxidant activity, including that of peroxynitrite, in many different cell lines (*49*). This increase in oxidant activity may result from the stable oxidants in CSE directly or the oxidants induced by CSE indirectly. These observations nevertheless suggest that CSE may induce the release of copper ions from metallothionein due to disruption of metallothionein copper binding thiolate clusters.

The copper ions released by CSE exposure may cause a copper-specific induction of protein carbonylation. A previous report showed that copper-mediated protein damage is not effectively prevented by hydroxyl radical scavengers such as DMSO or mannitol (*37*). These observations are consistent with the concept that a copper−oxygen complex, instead of hydroxyl radicals, is perhaps directly involved in the protein oxidation by a copper-containing system and also support why VitC did not effectively inhibit CSE-induced protein carbonylation in the present study. Surprisingly, our previous result showed that the addition of VitC could completely block CSE-induced DNA strand breaks in human umbilical vein endothelial cells (*27*);

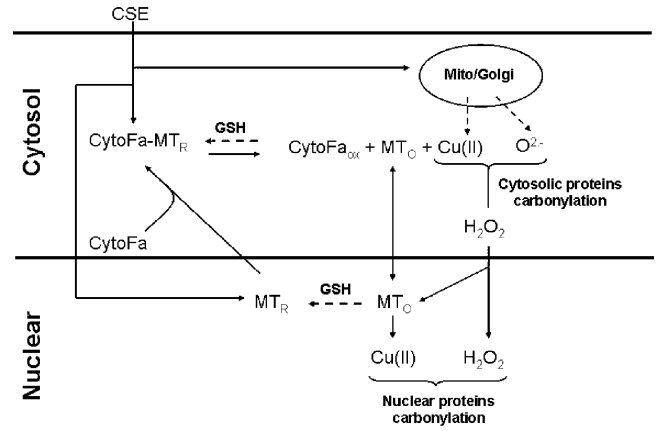

Figure 8. Proposed mechanism of CSE-induced protein carbonylation in THP-1 cells. See the text for a discussion. CytoFa, cytosol factor; MT_R, reduced form of metallothionein; MT_o, oxidized form of metallothionein; Mito, mitochondria; Golgi, Golgi apparatus; and GSH, glutathione.

this suggests that CSE may induce protein carbonylation and DNA strand breaks through different pathways. CSE-induced protein carbonylation was a copper-predominant condition; CSE-induced DNA damage was an oxidant-predominant condition. The previous report showed that stable compounds of CSE induced endothelial superoxide anion production via the activation of NADPH oxidase (*50*). These findings are proposed to provide a possible explanation for our observation that CSE-induced DNA strand breaks were oxidant-mediated through the activation of nucleus-specific NADPH oxidase and were not copper ion-mediated. There is also evidence that NADPH oxidase is found in the nucleus (*51*).

In conclusion, we propose the following working hypothesis to explain the pathway of CSE-induced protein carbonylation in THP-1 cells (Figure 8). In our postulated pathway, CSE oxidants attack intracellular copper ion pools (mitochondrial, Golgi apparatus) and copper binding proteins (metallothionein) and cause an increase in redox-active copper ions. This oxidative stress may also trigger a cytosol factor-mediated metallothionein nuclear transport to increase the distribution and utility of intracellular redox-active copper ions and produce cytosol and nuclear protein carbonylation. More investigations, however, are necessary to prove this postulation, especially to elucidate where and how the copper ions are released by CSE exposure.

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