Free Radical Research, 2002 Vol. 36 (1), pp. 99–106

Toxicity of Cadmium in Tobacco Smoke: Protection by Antioxidants and Chelating Resins

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Accepted by Professor H. Sies

(Received 14 February 2001; In revised form 3 August 2001)

The effects of cadmium, an environmental toxin present in tobacco smoke, were studied *in vitro* in human monocytes and compared to those of tobacco smoke. Overexpression of the 72 kDa heat shock/stress protein Hsp70 and cell death occurred with a similar time-course and to a similar extent in human monocytes exposed to either cadmium or tobacco smoke. Cadmium and tobacco smoke-mediated toxicity were associated with a decrease in the cellular content of glutathione and ATP and the glutathione precursor N-acetyl-L-cysteine prevented both cadmium and tobacco smoke-mediated toxicity. Furthermore, tobacco smoke-mediated toxicity was prevented by pretreatment with the cadmium chelator resin Chelex-100, supporting the conclusion that cadmium plays a major role in tobacco smoke-mediated toxicity.

Keywords: Cadmium; Tobacco smoke; Glutathione; N-acetyl-L-cysteine; Chelex-100

INTRODUCTION

Cadmium (Cd^{2+}) is a highly toxic environmental pollutant that causes damage to a variety of organs, in particular lungs, liver and kidneys.[1–3] Tobacco smoke (TS) is a major source of Cd^{2+} exposure in the general population and Cd^{2+} concentrations have been reported to increase in various body fluids and tissues of smokers as compared to non-smokers.[4,5] TS inhaled during cigarette smoking consists of a solid phase (particulate matter of respirable size) and a gas phase.[6] Most of the Cd^{2+} content of TS is present in the particulate phase[7] of which only 15–20% is non-selectively retained in the filter tip.[8,9] Thus during cigarette smoking most of the Cd^{2+} content passes out in the inhaled smoke.

The exact chemical/biochemical mechanisms underlying TS biological effects are as yet incompletely understood, though it has been proposed that reactive oxygen species (ROS) present in TS may contribute to TS-related diseases through a direct oxidative-mediated mechanism. Indeed, in conjunction with heavy metals, nicotine and specific carcinogens as benzo(a)pyrene, TS also contains relatively stable semiquinone radicals (in equilibrium with quinone and hydroquinone) that continuously produce superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and (with iron catalysis) hydroxyl radical ($^{\cdot}OH$). Metals, particularly Cd^{2+} and iron (Fe^{2+}) are also considered to participate in the oxidative injury mediated by TS and an increased concern has developed about the potential role of Cd^{2+} in TS-associated diseases. In this context, the International Agency for Research on Cancer (IARC) has recently classified Cd^{2+} as group I carcinogen in humans.[10]

TS exposure induces a significant decrease of intracellular reduced glutathione (GSH), a thiol

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ISSN 1071-5762 © 2002 Taylor & Francis Ltd
DOI: 10.1080/10715760290001218

containing tripeptide that constitutes one of the major antioxidant defence systems.[11,12] Maintenance of high levels of intracellular GSH is crucial to normal physiological processes and involves both γ-glutamyl-cysteine synthase as a rate limiting-enzyme and L-cysteine as a rate-limiting substrate.[13] GSH has also been involved in sequestering Cd^{2+}, particularly in the absence of the endogenous metal-binding protein, metallothionein.[14] We and others have reported that acute effects of TS-exposure are prevented by pretreatment with N-acetyl-L-cysteine (NAC), a cysteine donating drug.[15]

Mitochondria are well established targets for oxidative injury resulting from GSH depletion and we have previously shown that TS induces mitochondrial membrane depolarization, which is known to precede nuclear and cytosolic changes leading to cell death.[15] In agreement, our studies have shown that mitochondrial depolarization precedes TS-induced cell death in human monocytes.[15] TS exposure also induces the synthesis of heat shock/stress proteins (HSP), particularly the highly inducible 72 kD Hsp70, as part of an adaptive and protective response to oxidative stress.[16,17]

Our purpose in this study was two-fold. Firstly, we aimed at defining the contribution of Cd^{2+} in TS-mediated toxicity. To this end, we investigated whether TS-mediated toxicity could be reproduced and to what extent, following *in vitro* exposure of human monocytes to cadmium chloride ($CdCl_2$). Secondly, we examined whether TS/Cd^{2+} effects could be overcome by antioxidants and metal chelators, notably NAC and Chelex-100, the chelating ion-exchange resin which has been used to adsorb divalent metal ions such as Cd^{2+}.[18]

The results reported here indicate that Cd^{2+} mimics TS effects such as inducing Hsp70 expression and cell death through a mechanism involving GSH and ATP depletion. NAC and Chelex-100 significantly prevented both Cd^{2+} and TS-mediated effects, thus illustrating the importance of Cd^{2+} in TS-mediated toxicity.

MATERIALS AND METHODS

Reagents and Antibodies

Culture medium (RPMI 1640), fetal calf serum (FCS), L-glutamine, HEPES, phosphate buffered saline (PBS), trypsin-EDTA (0.05% trypsin–0.02% EDTA) and bovine serum albumin (BSA, fraction V), were from GibcoBRL, (Paisley, UK). Ficoll-Paque was from Pharmacia (Uppsala, Sweden). Research cigarettes (2R1) were purchased from the University of Kentucky. Cadmium chloride ($CdCl_2$), Ferrous sulfate ($FeSO_4$), Chelex-100 (50/100 mesh, sodium form). N-acetyl-L-cysteine (NAC), deferoxamine mesylate, 5′,5′-dithiobis (2-nitro benzoic acid), paraformaldehyde and saponin were from Sigma (Saint Louis, MO). Hydroethidine was purchased from Molecular Probes (Eugene, OR). Annexin-V-fluorescein isothiocyanate (FITC), propidium iodide (PI) viability determination kit, lactate dehydrogenase (LDH) viability determination kit and ATP Bioluminescence Assay kit HSII were from Boehringer (Mannheim, Germany). Human superoxide dismutase (rh Cu/Zn SOD) was supplied by Bio-Technology General Corporation, Iselin, NJ. The monoclonal antibody against Hsp70 (mouse IgG1, SPA-810) was from StressGen (Victoria, Canada). The $F(ab')_2$ fragment of rabbit anti-mouse lgG conjugated to FITC, used as secondary antibody was from Dako (Glostrup, Denmark). Reagents for immunoelectron microscopy were from TAAB (Aldermaston, UK).

Cells

Human monocytes were isolated from buffy coats obtained from healthy donors and further purified by 16 h adherence on plastic Petri dishes. Incubations were performed in RPMI culture medium supplemented with 10% FCS, 2 mM glutamine and 25 mM HEPES, at 37°C in a CO_2 incubator.

Cell Treatments

Tobacco Smoke

A peristaltic pump (MR 1G, Heinrich Borgwaldt, Hamburg, Germany) was used to generate TS extract through a puffing mechanism mimicking the human smoking pattern.[6] Smoke from one research cigarette, corresponding to 10 puffs of 35 ml gas phase, aspirated one per minute during 2 s, was bubbled in a side arm flask containing 5 ml PBS. Aliquots of this aqueous extract were added to the culture medium and final concentrations were expressed as puff/ml as previously described.[15] Considering the Cd^{2+} content of one cigarette (1–2 μg)[8,19] and that 70% of the Cd^{2+} content passes out in TS,[8] the final Cd^{2+} concentration of this aqueous TS extract could not exceed 1–3 μM.

Cadmium Chloride

Exposure to $CdCl_2$ was carried out by adding $CdCl_2$ at concentrations ranging from 1 to 100 μM to the culture medium. These concentrations, though higher than those present in TS extract, were previously found to be efficient in inducing Hsp70 expression.

Exposure to Protective Agents

For Chelex-100 treatments, fresh TS extracts or $CdCl_2$ solution (100 μM) were stirred for 15 min on a rotating platform (4 ml TS extract or $CdCl_2$ solution / g Chelex-100) before addition to the cell cultures. Chelex-100 beads were removed by a brief low speed centrifugation. A slight decoloration of TS extracts was detectable following Chelex-100 treatment. In some experiments designed to analyze whether the effect of Chelex-l00 could be reversed, 100 μM $CdCl_2$ or 500 μM $FeSO_4$ were added to the Chelex-l00 treated TS extract (0.5 g / 4 ml TS) before cell exposure.

NAC (10 mM) or rhCu / ZnSOD (2500 U / ml) and deferoxamine mesylate (200 μM), were added to the culture medium 1 h prior exposure to TS or $CdCl_2$. Deferoxamine strongly chelates ferric ions but also leads to Fe^{2+} depletion through a prooxidant mechanism.

Determination of GSH and ATP Content

Total GSH was evaluated spectrophotometrically as previously described.[20] Briefly, monocytes were treated with metaphosphoric acid (6% final concentration) to yield protein-free supernatants that were stored at −80°C before analysis. Changes in absorbance corresponding to the reduction of 5′,5′-dithiobis (2-nitro benzoic acid) were followed at 412 nm. GSSG was also measured after reduction to GSH, using glutathione reductase and NADPH, the thiol function being blocked by 2-vinyl pyridine. Values were determined by comparing the reduction rate against a standard curve of GSH and expressed as μmol / g protein.

Intracellular ATP was evaluated luminometrically, using the ATP Bioluminescence Assay kit HSII. Monocytes were resuspended in Tris 100 mM-EDTA 4 mM pH 7.5 and boiled for 2 min to yield supernatants that were stored at −80°C before analysis. Aliquots of the supernatants (10 μl) were mixed with 90 μl luciferase reagent that catalyzes the oxidation of luciferin in the presence of ATP. ATP concentrations were calculated from a log–log standard curve and expressed as ng / mg protein.

Determination of the Intracellular Production of Superoxide Anions

Intracellular production of $O_2^{\cdot -}$ was measured by flow cytometry as the oxidation of hydroethidine to ethidium.[21] Cell fluorescence was analyzed in a Coulter EPICS XL fluorescence activated cell sorter (Coulter, Miami FL) through a 575 nm filter and results were expressed as the percentage of labeled cells.

Determination of Hsp70 Expression by Flow Cytometry

Hsp70 expression was determined by flow cytometry as previously described.[22] Briefly, cells were detached from Petri dishes with Trypsin-EDTA and immediately fixed in 3% paraformaldehyde in PBS. Incubations with the primary antibody (SPA-810 diluted to 1:100) were performed for 10 min at room temperature in the presence of the permeabilizing agent saponin (0.6% final concentration). Following incubation with the secondary antibody (FITC-conjugated anti-mouse IgG diluted to 1 / 30) for 10 min in the darkness, cell fluorescence was analyzed in a Coulter EPICS XL fluorescence activated cell sorter through a 525 nm filter. Results are expressed as the mean fluorescence intensity.

Determination of Hsp70 Expression by Immunoelectron Microscopy

Cells were fixed as pellets in 2% paraformaldehyde–0.4% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4 (2 h, 4°C). After washing, cells were post-fixed in 0.2% osmium tetroxide (30 min at 4°C). Cells were partially dehydrated in 70% ethanol and embedded in LR white resin (medium grade). Polymerization was carried out at 55°C for 36 h. Ultrathin sections were collected on naked gold grids. Incubation with the primary anti-Hsp70 antibody was performed overnight at 4°C (SPA-810 diluted to 1:200 in PBS-0.5% BSA-0.05% Tween-20). Incubation with the secondary antibody was carried out for 1.5 h at room temperature (10 nm gold-labeled goat $F(ab')_2$ anti-mouse IgG diluted to 1:40). Sections were washed in distilled water, dried in filter paper, post stained in aqueous uranyl acetate and lead citrate and examined in a Phillips EM 201 electron microscopy (Eindhoven, The Netherlands).

Analysis of Cell Death

Cell death by apoptosis or by necrosis was determined using the Annexin-V-FITC-PI viability determination kit as described by the manufacturer. Fluorescence was analyzed in a Coulter EPICS XL fluorescence activated cell sorter through a 525 nm filter for green fluorescence and a 575 nm filter for red fluorescence. Unlabeled cells were considered as live, Annexin-V-FITC monolabeled cells were considered as apoptotic and PI labeled and double positive cells as necrotic. Results are expressed as the percentage of cells labeled with different fluorochromes.

Necrotic or lytic cell death was also monitored, by measuring the release of LDH using a commercial kit as specified by the manufacturer. Results are expressed as the percent of LDH released from the total LDH content.

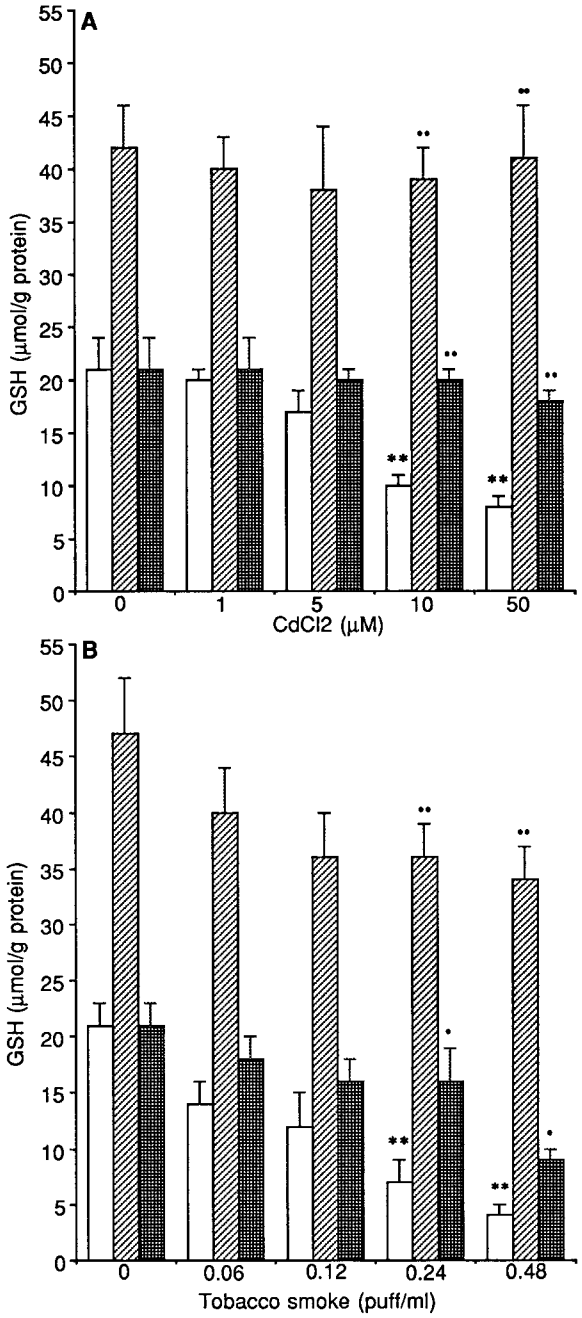

FIGURE 1 Dose–response decrease in GSH content induced by $CdCl_2$ or TS: protection by NAC or Chelex-100. Intracellular GSH was measured in untreated human monocytes or monocytes exposed for 3 h to increasing concentrations of $CdCl_2$ (1–50 µM), (A) or TS (0.06–0.48 puff/ml), (B) (open bars). NAC (10 mM) was added to the cell cultures 1 h before $CdCl_2$ or TS addition (hatched bars). $CdCl_2$ solution or TS extract were pretreated with Chelex-100 prior to cell exposure as detailed under "Materials and Methods", (dotted bars). Each value (mean ±SEM, n = 6–7) represents µmol GSH/g total protein. **p0.01 vs untreated monocytes, ●● p0.0l vs $CdCl_2$ or TS-treated monocytes, ● p0.05 vs $CdCl_2$ treated cells.

Statistics

Results are expressed as means ± SEM and compared using the Mann.Whitney U test. A p-value 0.05 was considered significant.

RESULTS

Effects of $CdCl_2$ and TS in Intracellular GSH Content: Modulation by NAC and Chelex-100

Changes in GSH content were investigated in human monocytes following 3 h incubation with different concentrations of $CdCl_2$ or TS. Both $CdCl_2$ and TS treatments induced a concentration-dependent decrease in intracellular GSH that reached 58 and 79% decrease for the highest dose of $CdCl_2$ and TS, respectively (Fig. 1A, B). Control cells incubated with the GSH precursor NAC alone exhibited a two-fold increase in GSH, while the Cd^{2+} chelator Chelex-100 was ineffective. However, NAC and Chelex-100 were highly effective in preventing both $CdCl_2$ and TS-mediated GSH depletion (Fig. 1A, B).

Effects of $CdCl_2$ and TS in Intracellular ATP Content: Modulation by NAC and Chelex-100

As compared to GSH, a similar trend was observed concerning ATP levels (Fig. 2A, B). Negligible effects are observed below 10 µM $CdCl_2$ or 024 puff/ml TS while higher concentrations resulted in a significant drop of cell ATP that reached 58 and 65% decrease in $CdCl_2$ and TS-exposed monocytes, respectively. ATP decrease was significantly prevented by pretreatment with either NAC or Chelex-100 (Fig. 2A, B).

Generation of Intracellular ROS by $CdCl_2$

Because TS itself contains high amounts of $O_2^{\cdot-}$ it cannot be resolved by flow cytometry whether intracellular $O_2^{\cdot-}$ is generated by monocytes or arises from TS species entering the cytoplasm. Experiments with $CdCl_2$ revealed an increased generation of $O_2^{\cdot-}$ in monocytes exposed to 25 µM $CdCl_2$ for 3 h: 5.5 ± 0.7% of untreated cells were labeled and this value increased up to 13.5 ± 0.9% after $CdCl_2$ exposure ($p < 0.01$, $n = 5$). Thus, basal levels of intracellular $O_2^{\cdot-}$ reached a maximal increase of 2.4 fold in $CdCl_2$-exposed monocytes.

Effects of $CdCl_2$ and TS in Hsp70 Expression: Modulation by NAC and Chelex-100

TS induced a concentration-dependent increase of the expression of Hsp70 that was completely reversed by pretreatment with both Chelex-100 and NAC. Table I shows that 4 h exposure of monocytes to $CdCl_2$ also induced Hsp70 overexpression that strongly depended on the $CdCl_2$ concentration. lmmunoelectron microscopy analysis confirmed that both TS and $CdCl_2$ induced Hsp70 expression within monocytes with a similar

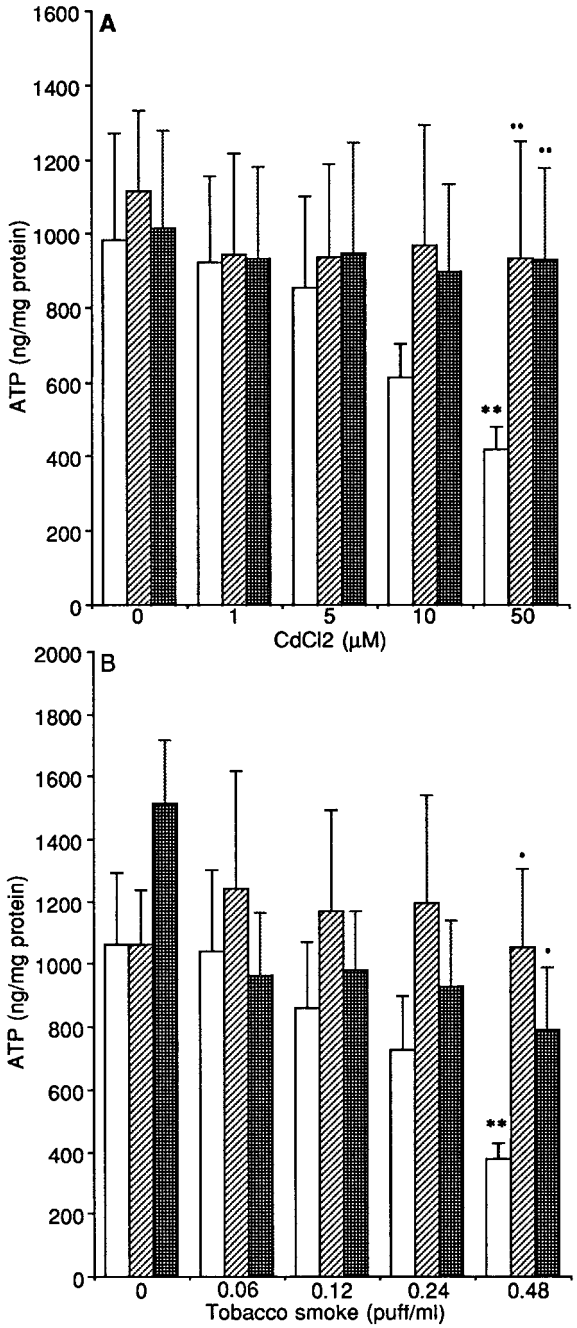

FIGURE 2 Dose–response decrease in ATP content induced by $CdCl_2$ or TS: protection by NAC or Chelex-100. Intracellular ATP was measured in untreated human monocytes or monocytes exposed for 3 h to increasing concentrations of $CdCl_2$ (1–50 μM), (A) or TS (0.06–0.48 puff / ml), (B) (open bars). NAC (10 mM) was added to the cell cultures 1 h before $CdCl_2$ or TS addition (hatched bars). $CdCl_2$ solution or TS extract were treated with Chelex-100 prior to cell exposure as detailed under "Materials and Methods" (dotted bars). Each value (mean ± SEM, n = 6–7) represents ng ATP / mg protein. **p0.01 vs untreated monocytes. ●●p0.01 vs $CdC1_2$-treated monocytes. ●p0.05 vs TS treated moncytes.

cytoplasmic and nuclear localization. The protein appeared evenly distributed within the cytoplasm while in the nucleus it was mainly localized in the less condensed euchromatin region where most of the RNA synthesis occurs (data not shown).

Effects of TS and $CdCl_2$ in Cell Viability: Modulation by Antioxidants and Metal Chelators

We have previously shown that TS induces a time and concentration-dependent cell death in human monocytes and in different mammalian cell lines. TS-induced cell death proceeded either by apoptosis or by necrosis, depending on the dose applied, low concentrations inducing apoptosis and high concentrations, necrosis.[15,17] Flow cytometric analysis revealed that $CdCl_2$-induced cell death was also time and concentration-dependent but proceeded mainly by necrosis (Fig. 3). Cell death occurred only following long incubations (16 h) and was negligible with low $CdCl_2$ concentrations (1–10 μM) while with 25 or 50 μM viability was reduced by 2.5 and 4.8 fold, respectively, as compared to control cells. Preincubation of monocytes with 10 mM NAC significantly prevented $CdCl_2$-mediated cell death: the percent of necrotic cells was reduced by NAC from 26.7 ± 7.6 to 12.9 ± 2.4% and from 51.4 ± 4.1 to 16.1 ± 1.1% ($p = 0.0l$, $n = 4$) in 25 μM $CdCl_2$ and 50 μM $CdCl_2$-treated monocytes, respectively.

In distinct experiments we examined whether TS-mediated necrotic cell death was related to its $CdCl_2$ content. Cell lysis attested by LDH leakage was determined in the presence of TS alone, or following pretreatment with NAC, rhCu / ZnSOD, deferoxamine and Chelex-100. As illustrated in Fig. 4, NAC and Chelex-100 prevented necrotic cell death while deferoxamine and rhCu / Zn SOD were ineffective. Indeed, deferoxamine slightly increased cell death while rhCu / ZnSOD significantly increased TS-mediated necrotic cell death suggesting that neither $O_2^{\cdot-}$ nor iron play an important role in TS-mediated cell death. Reversibility of the protective effects of Chelex-100 against TS toxicity was also demonstrated by adding $CdCl_2$ to the Chelex-treated TS extract. In these experiments, cell lysis measured in untreated monocytes (14.7 ± 2.3%) increased to reach 71.0 ± 6.0% ($n = 4$) in cells exposed for 16 h to 0.48 puff / ml TS. In monocytes exposed to the Chelex-treated TS extract, cell lysis decreased to 29.9 ± 2.9% ($n = 4$) attesting for 73.2% protective effect exerted by Chelex-100. When $CdCl_2$ (100 μM) was added to the Chelex-treated TS extract before cell exposure the Chelex-mediated protective effect was reversed since cell lysis reached 49.2 ± 9.2% ($n = 3$), thus providing only 39.1% protection against TS-mediated lytic effect. Addition of $FeSO_4$ to the Chelex-treated TS extract did not reverse the protective effect exerted by Chelex-100.

DISCUSSION

In the present study we observed that Cd^{2+} behaves like TS in several models of cell injury, including

TABLE I Effects of cadmium and tobacco smoke in Hsp70 expression. Modulation by NAC and Chelex-100. Results represent mean ± SEM from four separate experiments and are expressed as mean fluorescence intensity.*$p < 0.05$ vs untreated cells, **$p < 0.01$ vs untreated cells, •$p < 0.05$ vs TS-exposed cells, ••$p < 0.01$ vs TS-exposed cells

Untreated cells	$CdCl_2$ 1 μM	$CdCl_2$ 5 μM	$CdCl_2$ 10 μM	$CdCl_2$ 50 μM
2.5 ± 0.5	3.6 ± 1 5	4.7 ± 0.8**	6.5 ± 1.9**	9.1 ± 3**
Untreated cells	TS 0.06 puff / ml	TS 0.12 puff / ml	TS 0.18 puff / ml	TS 0.24 puff / ml
2.5 ± 0.5	3.3 ± 0.3	5.1 ± 1.1*	8.8 ± 2.1*	12.4 ± 3.1**
NAC	TS 0.06 + NAC	TS 0.12 + NAC	TS 0.18 ± NAC	TS 0.24 + NAC
2.2 ± 0.8	3.8 ± 0.7	3.1 ± 0.2••	3.4 ± 0.5••	4.3 ± 0.7••
Chelex-100	TS 0.06 + Chelex	TS 0.12 + Chelex	TS 0.18 + Chelex	TS 0.24 + Chelex
2.9 ± 0.6	3.1 ± 1.9	3.2 ± 0.4•	3.2 ± 0.8••	3.3 ± 1.1••

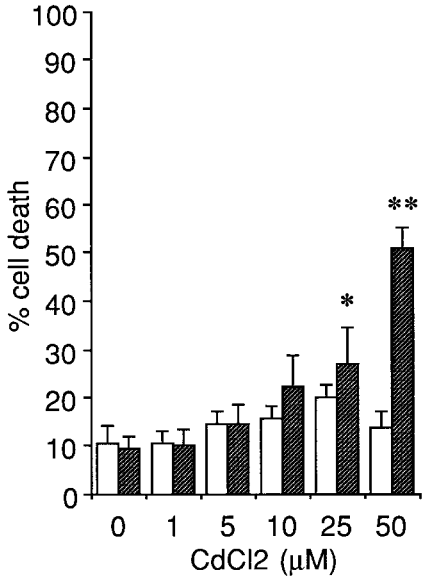

FIGURE 3 Flow cytometric analysis of cell death in human monocytes exposed to $CdCl_2$. Human monocytes were exposed to increasing concentrations of $CdCl_2$ (1–50 μM) for 16 h and stained with the AnnexinV-FITC-PI viability determination kit as recommended by the manufacturer. Cell fluorescence was analyzed by fow cytometry as detailed in "Materials and Methods". Values (mean ± SEM, $n = 5$) represent percentage cells labeled with different fluorochromes. Annexin V-FITC monolabeled cells were considered as apoptotic (open bars) while PI labeled calls and double positive were considered as necrotic (black bars). * p0.05 vs untreated cells, ** p0.0l vs untreated cells.

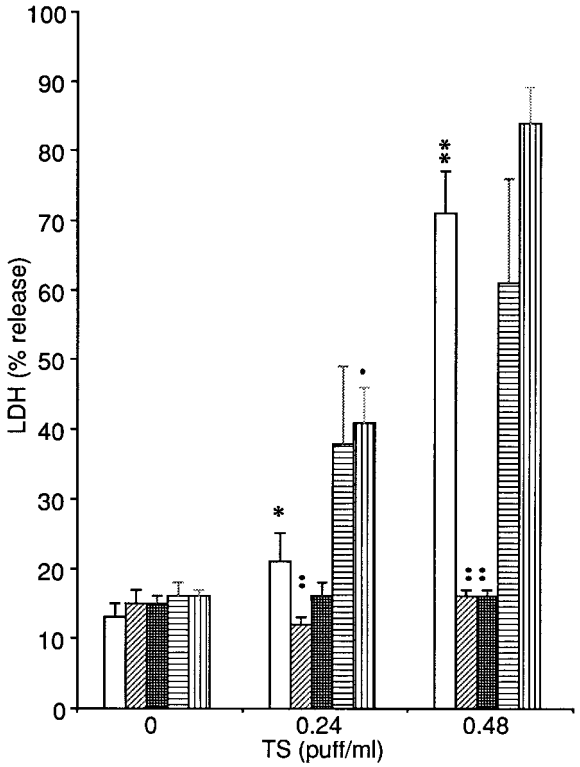

FIGURE 4 Analysis of necrotic cell death in human monocytes exposed to TS: modulation by antioxidants or metal chelators. Necrotic cell death as attested by the release of LDH was measured in untreated monocytes or monocytes exposed for 16 h to TS (0.24–0.48 puff / ml) (open bars). NAC (10 mM) was added to the cell culture 1 h prior TS addition (sloped hatched bars). TS extracts were pretreated with Chelex-100 prior to cell exposure as detailed in "Materials and Methods" (dotted bars). Deferoxamine (200 μM) was added to the cell culture 1 h prior TS addition (horizontally hatched bars). rhCu / Zn SOD (2500 U / ml) was added to the cell culture 1 h prior TS addition (vertically hatched bars). Each value (mean ± SEM, $n = 4$) represents percentage LDH release from the total LDH content. ** p0.0l vs untreated cells. * p0.05 vs untreated cells. •• p0.01 vs monocytes treated with TS alone. • p0.05 vs monocytes treated with TS alone.

Hsp70 overexpression and cell death. These effects were related to a marked decrease in intracellular GSH and ATP content, comparably induced by either Cd^{2+} or TS, suggesting that Cd^{2+} is an important mediator of TS-induced cell damage, through a GSH-sensitive mechanism. Two compounds were particularly effective in preventing Cd^{2+} and TS-mediated effects: the GSH precursor NAC; and the Cd^{2+} chelating resin Chelex-100.

The importance of GSH depletion in TS and Cd^{2+}-mediated toxicity has been previously recognized.[3,11,12,23] We found that the effects of both agents may be prevented by NAC, an antioxidant molecule with protective potential against GSH depletion. In addition, we found that pretreatment of TS with Chelex-100 mimicked the protective effect of NAC, suggesting that metal chelators could be also useful to protect against TS. Though a number of research efforts have been directed toward overcoming heavy metal toxicity problems, no studies have been conducted yet to analyze the potential role of Chelex-100 in the prevention of TS-mediated toxicity. Chelex-100 has been used to selectively adsorb divalent metal cations, which are mainly present as particulate matter in TS. Chelex-100 likely prevents adverse TS effects by removing the

injurious agent and its capacity to reduce the toxicity of Cd^{2+} has been previously reported.[18]

The exact mechanism responsible for GSH and ATP depletion was not investigated in our study, but is likely dependent on mitochondrial alterations and ROS-mediated oxidative injury. As previously observed with TS,[24] Cd^{2+} did not cause the release of $O_2^{\cdot-}$ from monocytes (data not shown), while basal levels of intracellular $O_2^{\cdot-}$ increased of 2.4 fold in $CdCl_2$-exposed monocytes. The levels of oxidized GSH (GSSG) observed were below the limit of sensitivity of the colorimetric assay employed, but previous studies have shown that Cd^{2+} as well as TS do not cause direct oxidation of GSH but interact with sulfhydryl groups,[11,25] thereby exhausting the reducing environment within the cell. GSH plays a critical role in the maintenance of mitochondrial membrane potential that stabilizes mitochondria, preventing thereby loss of ATP and cell viability. Using the fluorescent probe JC-1, which allows to detect changes of the mitochondrial membrane potential, we recently demonstrated that TS may be added to the list of compounds that promote mitochondrial membrane depolarization in human monocytes.[15] Disruption of the mitochondrial membrane potential also occurred with $CdCl_2$ (our unpublished results), suggesting that mitochondrial alterations are also involved in the mechanism responsible for $CdCl_2$-mediated toxicity.

Hsp70 was found to be significantly overexpressed in monocytes exposed to Cd^{2+} or TS, likely to exert protective functions by virtue of its chaperone activity that enables cells to recover and survive after stressful exposure like oxidative injury. In line with this concept, we previously demonstrated protective effects of Hsp70 against necrotic cell death induced by TS in different mammalian cell lines.[17] Immunoelectron microscopy experiments further illustrated Cd^{2+} and TS-mediated Hsp70 overexpression. Concentrations of 10 μM Cd^{2+} or 0.12 puff/ml TS were sufficient to induce Hsp70 overexpression whereas higher doses were necessary to induce GSH/ATP drop and cell death, thus suggesting that Hsp70 is the most sensitive index for TS/Cd^{2+} toxicity.

When monocytes were exposed to $CdCl_2$ or TS, changes in GSH/ATP levels and Hsp70 expression were evident at 3–4 h whereas more severe cytotoxic changes as cell death only developed several hours later (16 h). $CdCl_2$-mediated cell death occurred mainly by necrosis and was prevented by NAC while TS-mediated cell death was prevented by both NAC and Chelex-100. Our data, notably with Chelex-100 provide further support to the contribution of divalent heavy metal constituents in TS-mediated toxicity. Some of these metals, such as nickel, lead and Cd^{2+} are known to induce GSH depletion, while $Fe^{2+}$and copper undergo redox cycling and actively participate to the production of ROS.[26] However, it appears from our data and evidence in the literature that among heavy metals Cd^{2+} is the most susceptible contributor to TS-induced necrotic cell death.[7,28] Cell death was preceded by GSH depletion and mitochondrial alterations leading to energy (ATP) deprivation. Progression to apoptosis or necrosis depends on the availability of ATP.[27,29] ATP is required for the execution of apoptosis and therefore only cells affected by a limited ATP loss may undergo apoptosis while severe ATP loss leads to necrosis. In our experiments, ATP rapidly decreased to approximately one half of control values and should reach lower levels after longer exposure when necrotic killing predominantly developed instead of apoptosis.

Our data using rhCu/ZnSOD suggest that besides Cd^{2+} something other exerts adverse activity and mediates necrotic cell death in TS-exposed monocytes. SOD catalyzes the reaction that dismutates $O_2^{\cdot-}$ to H_2O_2, thus potentially increasing the concentration of H_2O_2, suggesting that besides Cd^{2+}, H_2O_2 may be a causal factor of TS-mediated biological damage. In agreement, known adverse health effects of TS like inactivation of α-1-proteinase inhibitor, that causes emphysematous lesions, was shown to be strongly inhibited by catalase, which detoxifies H_2O_2.[30] Furthermore, NAC is capable of scavenging ROS like H_2O_2,[31] and antioxidant effects of GSH include its ability to act as a substrate for GSH peroxidase so as to remove H_2O_2, which may lead to additional protective properties of both drugs against specific damage caused by H_2O_2. A typical cigarette weights about 1 g and contains 1–2 μg Cd^{2+}, depending on the area of production of tobacco plants. Though Cd^{2+} content may be much higher in cigarettes from Cd^{2+} polluted areas,[19] the amount of Cd^{2+} used in our experiments was higher than that reported to be contained in TS. It seemed therefore plausible that at least in short-term exposure situations, TS-containing divalent heavy metals as Cd^{2+} and ROS as H_2O_2 exert synergistic lytic effects through oxidative stress. However, because of the wide variety of TS constituents it seems unlikely that any single molecule or mechanism can totally explain TS-mediated cytotoxicity.

Although avoidance of smoking is the best approach to preventing TS-related diseases, many individuals unfortunately continue to smoke and the search for protective agents remains a relevant challenge. The efficacy of NAC as an *in vivo* antioxidant has not been convincingly established to date.[12] Our findings that Chelex-100 suppressed TS-mediated cytotoxicity deserves consideration: inasmuch available commercial filter tips do not result in a significant reduction of Cd^{2+} present in TS, a cigarette filter containing Cd^{2+} chelating resins

could be tested for its potential to reduce the risk of TS-associated toxicity and diseases.

Acknowledgements

We are grateful to J.M. Davis (Winthrop University Hospital, Mineola, NY) and Bio-Technology General Corporation (Iselin, NJ), for providing rhCu/Zn SOD. We also thank the Centre de Microscopie Electronique (IFR Jussieu) for providing access to the electron microscope. F.P. was supported by Serono Laboratories (Boulogne, France). This study was partially supported by CISC, Switzerland.

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