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# Protein carbonylation in human endothelial cells exposed to cigarette smoke extract

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## HIGHLIGHTS

- We show protein carbonylation in human endothelial cells exposed to cigarette smoke.
- The main carbonylated proteins were identified by means of redox proteomics.
- The main carbonylated proteins are cytoskeletal proteins, glycolytic enzymes, Hsp90.
- Fluorescence microscopy evidenced cytoskeletal alterations.
- TEM showed cytoplasm vacuolisation and alteration of mitochondria.

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## ABSTRACT

Cigarette smoke is a significant independent risk factor for vascular diseases and is a leading cause of structural and functional alterations of the vascular endothelium. In this study, we show protein carbonylation in the human umbilical vein endothelial cell line (ECV-304) exposed to whole-phase cigarette smoke extract. The main carbonylated proteins, including cytoskeletal proteins, glycolytic enzymes, xenobiotic metabolizing and antioxidant enzymes, and endoplasmic reticulum proteins, were identified by means of two-dimensional electrophoresis and Matrix-Assisted Laser Desorption/Ionization-Time of Flight (MALDI-TOF) mass spectrometry (redox proteomics). Morphological analyses by fluorescence microscopy evidenced alterations in the microtubule cytoskeleton, especially at longer exposure time to cigarette smoke extract. Morphological analyses by transmission electron microscopy showed vacuolisation of the cytoplasm, alteration of mitochondria ultrastructure, and some enlargement of the perinuclear space. The possible role played by protein carbonylation caused by reactive species contained in cigarette smoke in the cigarette smoke-induced endothelial injury is discussed.



## 1. Introduction

Cigarette smoke (CS) is a significant independent risk factor for cardiovascular disease and is a leading cause of structural and functional alterations of the cardiovascular system. Although the exact pathophysiology of cigarette smoking has not been unveiled, CS causes injury to the vascular endothelium, produces reactive oxygen and nitrogen species (ROS and RNS), reduces production and bioavailability of nitric oxide, increases production and release of endothelin, causes endothelial dysfunction, thrombosis, atherosclerosis, infarction, coronary artery disease, and stroke. CS is a complex mixture containing more than 7000 different constituents, including ROS and α,β-unsaturated aldehydes (Rodgman and Perfetti, 2009). Furthermore, ROS production mediated through inflammatory processes may exacerbate those produced through direct exposure (van der Vaart et al., 2004).

The mechanism by which CS contributes to injury remains unclear; however, CS-induced oxidative damage may be a key pathogenic factor. One of the prominent deleterious effects of CS is the oxidative damage of biological macromolecules, including

*Abbreviations:* CS, cigarette smoke; CSE, whole-phase cigarette smoke extract; DAPI, 4′,6-diamidino-2-phenylindole; DCF, dichlorofluorescein; DCFH-DA, 2,7-dichlorodihydrofluorescein diacetate; DNP, dinitrophenyl; DNPH, 2,4-dinitrophenylhydrazine; DTT, dithiothreitol; ECL, enhanced chemiluminescence; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LDL, low-density lipoprotein; MALDI-TOF, Matrix-Assisted Laser Desorption/Ionization-Time of Flight; PCO, protein carbonyls (carbonylated proteins); PPP, pentose phosphate pathway; RCS, reactive carbonyl species; RNS, reactive nitrogen species; ROS, reactive oxygen species; TEM, transmission electron microscopy.

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proteins. The most widely studied biomarker of protein oxidation is protein carbonylation, which is also the most widely used biomarker of oxidative stress/damage (Dalle-Donne et al., 2006a). Protein carbonyl groups can be formed by direct oxidation of the side chains of Lys, Pro, Arg, and Thr residues. In addition, carbonyl groups can be introduced into proteins by addition reactions of α,β-unsaturated aldehydes, such as acrolein and crotonaldehyde, to His, Lys or Cys residues (Dalle-Donne et al., 2006a; Colombo et al., 2010).

A large body of evidence points to the endothelium as a primary target of CS-induced insult, preceding plaque formation and vascular injury. CS has been shown to affect endothelial cells lining the vasculature causing endothelial dysfunction, increased endothelial permeability, cellular injury, and the binding of inflammatory cells initiating the formation of atherosclerotic plaques (Rahman and Laher, 2007; Cacciola et al., 2007). Whole-phase cigarette smoke extract (CSE), a widely used model system for studying *in vitro* effects of CS (*e.g.*, Orosz et al., 2007; Kode et al., 2008; Colombo et al., 2010, 2012a; Lemaître et al., 2011), induces tubulin carbonylation in endothelial cells (Bernhard et al., 2005). *In vitro* data have confirmed the detrimental effects of CSE on endothelial cell growth, migration, nitric oxide production, endothelial permeability, and angiogenesis (Michaud et al., 2006; Barbieri and Weksler, 2007; Edirisinghe et al., 2008, 2010). These studies indicate that CS can act directly on endothelial cells to promote endothelial dysfunction and some proteins involved in the CS-induced insult of endothelial cells have been identified (Hoshino et al., 2005; Barbieri et al., 2008). However, the overall response of endothelial cells to CS is not fully understood.

The main purposes of the present study were to investigate CSE-induced morphological alterations and protein carbonylation in the human umbilical vein endothelial cell line (ECV-304) and to identify the major carbonylated proteins by redox proteomics/mass spectrometry (MS) techniques (Dalle-Donne et al., 2006b; Colombo et al., 2012b).

## 2. Materials and methods

### 2.1. Materials

Research-grade cigarettes (3R4F) were purchased from the College of Agriculture, Kentucky Tobacco Research and Development Center, University of Kentucky (USA). 2,7-Dichlorodihydrofluorescein diacetate (DCFH-DA), Neutral Red, rhodamine-phalloidin, and mouse anti-α-tubulin antibody were obtained from Sigma Aldrich (Milan, Italy). Cell Proliferation Kit (XTT, *i.e.*, sodium 3,3′-(1((phenylamino)carbonyl)-3,4-tetrazolium)-bis(4-methoxy-6-nitro) benzene sulphonic acid hydrate, based) was obtained from PBI International (Milan, Italy). CellTiter-Glo Luminescent Cell Viability Assay was purchased from Promega Italia (Milan, Italy). Medium 199, fetal bovine serum, and tissue culture reagents were obtained from Sigma Aldrich (Milan, Italy). Anti-dinitrophenyl-KLH (anti-DNP) antibodies, rabbit IgG fraction, goat anti-rabbit IgG, and goat anti-mouse antibodies conjugated with Alexa 488 were purchased from Molecular Probes (Eugene, OR, USA). ECL Plus Western blotting detection reagents were obtained from GE Healthcare (Milan, Italy). Precision Plus Protein All Blue Standards, ranging from 10 to 250 kDa, were obtained from Bio-Rad Laboratories s.r.l. (Segrate, Italy). Mouse anti-vimentin antibody was obtained from Novocastra (Leica Microsystems, Milan, Italy). All other reagents were of analytical grade (Sigma–Aldrich, Milan, Italy).

### 2.2. Preparation of whole-phase cigarette smoke extract (CSE)

Whole-phase CSE from Kentucky 3RF4 reference cigarettes was prepared as previously described (Colombo et al., 2010, 2012a). Mainstream smoke from one cigarette (10 puffs) was allowed to dissolve (for 10 s each puff) in 1 ml of 50 mM potassium phosphate buffer (PBS), pH 7.4. The resultant dark yellow solution was defined as 100% whole-phase CSE and was filtered through a 0.22-mm Millipore filter (Bedford, MA) to remove bacteria and large particles. The pH of the whole-phase CSE was adjusted to 7.4 by addition of 2 M sodium hydroxide solution. To ensure standardization between experiments and batches of CSE, CSE preparations were made uniform by measurement of absorbance at 340 nm. CSE was freshly prepared immediately before use for each experiment and diluted to an appropriate concentration with 50 mM PBS, pH 7.4.

### 2.3. Cell culture

The human ECV-304 cell line was maintained as adherent cells in Medium 199, supplemented with 10% fetal bovine serum and 1.4 mM L-glutamine, in a humidified 5% $CO_2$ atmosphere, at 37 °C. Cells were routinely passaged using 0.5% trypsin–EDTA and grown to 90% confluency.

### 2.4. Exposure of ECV-304 cells to CSE

Before treatment, $2 \times 10^4$ cells/well were seeded into 96-well assay plates and cultured for 24 h at 37 °C in a humidified atmosphere containing 5% $CO_2$ to allow equilibration and adhesion. Cells were then exposed, for 1 h and 6 h,–0, 2.5, 5 or 10% CSE in PBS.

### 2.5. Detection of intracellular ROS formation

$2 \times 10^4$ cells/well were seeded into 96-well assay plates and cultured for 24 h at 37 °C in a humidified atmosphere containing 5% $CO_2$ to allow equilibration and adhesion. Cells were then washed with PBS and incubated for 1 h with 5 μM DCFH-DA (prepared as a 5 mM stock solution in methanol) to estimate the production of ROS. To remove non-incorporated DCFH-DA, cells were washed with PBS and exposed for 1 h–0%, 2.5%, 5% or 10% CSE in PBS. The DCF fluorescence intensity was recorded at $\lambda_{exc}$ = 485 nm (band width 20 nm) and at $\lambda_{em}$ = 535 nm (band width 25 nm) using the Infinite F200 (Tecan Group, Switzerland) plate reader. The data were normalized and expressed as fold increase in fluorescence intensity relative to the controls.

### 2.6. Cell viability measurement

Cell viability was measured using the Neutral Red Uptake method, whose key component is the vital dye Basic Red 5, Toluylene Red. Viable cells will take up the dye by active transport and incorporate the dye into lysosomes. The incorporated dye is then liberated from the cells in a destaining solution (50% ethanol, 49% $H_2O_2$, 1% acetic acid), giving rise to a coloured solution, whose absorbance is measured at 540 nm against background (destaining solution).

The neutral red uptake assay resulted to be the most sensitive and reliable for assessing the cytotoxic potential of CS and CS condensate/extract and has been recommended for inclusion in a battery of *in vitro* assays to evaluate the biological activity of CS and CSC/CSE (Andreoli et al., 2003). Moreover, protocols of this assay have been subjected to the critical assessment by the most important international agencies for *in vitro* testing, such as The European Centre for the Validation of Alternative Methods (ECVAM) (http://www.hc-sc.gc.ca/hc-ps/tobac-tabac/legislation/reg/indust/method/tox-eng.php).

### 2.7. Transmission electron microscopy

For transmission electron microscopy (TEM) observations, cells were harvested, fixed with 2% glutaraldehyde in 0.1 M Na-cacodylate buffer (pH 7.2) for 1 h at room temperature, washed in the same buffer, and post-fixed for 1 h with 1% osmium tetroxide in 0.1 M Na-cacodylate buffer (pH 7.2) at room temperature. After standard steps of serial ethanol dehydration, samples were embedded in an Epon-Araldite 812 mixture. Thin sections, obtained with a Reichert Ultracut S Ultratome (Leica, Nussloch, Germany), were stained with uranyl acetate and lead citrate and observed with a Jeol 1010 electron microscope (Jeol, Tokyo, Japan) operating at 80 kV.

### 2.8. Detection of protein carbonylation by SDS-PAGE and Western blotting with anti-DNP antibody

Whole cell lysates were obtained by addition of ice-cold lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100; $1 \times 10^6$ cells/100 μl) supplemented with protease inhibitors. Cell lysates were incubated on ice for 30 min and centrifuged at 14000 *g*, for 10 min, at 4 °C, to remove cell debris; supernatants were recovered and analyzed for protein concentration using the bicinchoninic acid (BCA) assay. Protein samples were mixed with an equal volume of standard reducing 2 × Laemmli buffer, electrophoresed on 10% Tris–HCl resolving SDS-PAGE gels and electroblotted on to an Immobilon P polyvinylidene difluoride (PVDF) membrane. Protein carbonylation was evaluated by means of Western blot analysis with anti-DNP antibody specific for the 2,4-dinitrophenyl hydrazone–protein adduct after derivatization with 2,4-dinitrophenylhydrazine (DNPH) as previously reported (Dalle-Donne et al., 2007; Colombo et al., 2010, 2012b) and visualized with ECL detection.

### 2.9. Detection of carbonylated proteins by two-dimensional gel electrophoresis (2D-GE) and Western blotting with anti-DNP antibody

Each sample containing 200 μg proteins was precipitated using a chloroform/methanol protocol and resuspended in a solution containing 7 M urea, 2 M thiourea, and 4% CHAPS. Solubilized samples were used to rehydrate IPG strips just before isoelectrofocusing.

For the first dimensional electrophoresis, samples were applied to immobilized pH gradient (IPG) strips (11 cm, pH 3–10 linear gradient; GE Healthcare). Strips were