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Effects of cigarette smoking on the human urinary proteome

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ABSTRACT

In this pilot study we used a proteomic approach to compare the urinary protein patterns of healthy smokers and non-smokers. Proteins were resolved by two-dimensional gel electrophoresis and identified by mass spectrometry. The relative abundance of three inflammatory proteins (S100A8, inter-alpha-trypsin inhibitor heavy chain 4, CD59) and that of two isoforms of pancreatic alpha amylase was significantly higher in smokers. Zinc-alpha-2-glycoprotein was the only protein down-regulated in smokers. Its abundance was significantly correlated with urinary glucocorticoids. Most of the proteins identified may be non-specific biomarkers of tobacco effects, since they are involved in inflammatory responses associated with several diseases. Of greater interest are the changes in abundance of pancreatic alpha amylase and zinc-alpha-2-glycoprotein, which after proper validation, might be candidate biomarkers of diseases resulting from exposure to tobacco smoke. The data also show for the first time that smoking can affect the expression profile of urinary proteins.

Introduction

Cigarette smoking attributable deaths are projected to rise by from 5.4 million in 2005 to 6.4 million in 2015 under the present scenario [1]. Because of the wide spread of the smoking habit and its consequent impact on human health, interventions to reduce tobacco-related diseases have high priority.

Obviously, quitting smoking is usually the best preventive action, but most smokers are unable or unwilling to do this, so a number of biomarkers have been used to detect exposure and/or individual susceptibility to tobacco smoke carcinogens, with the ultimate goal of avoiding health problems.

It is assumed that exogenous compounds may become toxic or carcinogenic by interacting with receptors, proteins, gene regulatory elements, DNA, and various biological processes. The result may be dysregulation of the expression of proteins on which cell physiological functions depend. Establishing a relationship between exposure to toxic compounds and protein markers might provide information to lead to new or refined hypotheses on the mechanisms of toxic or carcinogenic effects of xenobiotics and help prevent adverse health effects. Therefore studying the proteome of readily obtainable human biological samples, such as body fluids, is challenging. Urine, the most accessible body fluid, has been previously studied to search for protein markers of a pathological status or exposure to toxic compounds [2, and references herein]. Lifestyle, however, although recognized as having important effects on protein expression, has been only marginally considered [3].

This pilot study assessed whether the human urinary proteome is affected by cigarette smoking and whether the proteins whose expression was changed could be candidate biomarkers with potential clinical value in relation to tobacco-related diseases. Proteins were separated by two-dimensional gel electrophoresis (2DE) and identified by mass spectrometry (MS) coupled to a database search.

Materials and methods (see Supplementary materials and methods for details)

Recruitment of subjects

Urine specimens were collected from twenty healthy male donors, ten smokers and ten non-smokers, with no history of kidney or extra-renal disease. Each subject signed an informed consent and answered a questionnaire about personal data, smoking, alcoholic drinks and coffee consumption, and use of prescription or over-the-counter medications.

Sample preparation

The second morning urine (about 150–200 mL) was collected from consenting individuals on one tablet of Complete Protease Inhibitor Cocktail (Roche Diagnostics Italia, Milan, Italy). Urine

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specimens were immediately cooled on ice, filtered through filter paper, and centrifuged at 1000g for 5 min at 4 °C to remove cell debris and particulate matter. The supernatant protein concentration was measured using the Bio-Rad protein assay reagent (Bio-Rad Laboratories). Creatinine was measured using the kit from Chema Diagnostica, Jesi, Italy. Proteome analysis was done on pooled urine specimens only from subjects smoking more than 15 cigarettes/day (four), and four age-matched non-smokers. Smoker and non-smoker pools were created by mixing urine samples containing equal amounts of proteins. The samples were then divided into 20 mL aliquots and stored at −20 °C until analysis. Three replicates were run for each pool. Individual urine samples were also stored in 20 mL aliquots at −20 °C.

Removal of serum albumin

Serum albumin was removed by immunoaffinity chromatography. Anti-human albumin polyclonal antibodies (Sigma–Aldrich, Italy) were immobilized on CNBr-activated Sepharose 4B, following the manufacturer's instructions (GE Healthcare). Urine (20 mL) was loaded on the immunoaffinity column and left to percolate at 1 mL/min. The flow-through fractions containing non-retained proteins were collected, desalted by ultrafiltration and lyophilized. The dry residue was dissolved in 400 μL of water, transferred to an Eppendorf tube and dried again using a vacuum centrifuge.

Proteome analysis

Two-dimensional gel electrophoresis. The dry residue was dissolved in the rehydration solution containing 5M urea (Sigma–Aldrich), 2M thiourea (Sigma–Aldrich), 2% CHAPS, 2% Zwittergent (Calbiochem, La Jolla, CA, USA), and 100 mM DeStreak reagent (GE Healthcare) added to protect cysteinyl groups and prevent non-specific oxidation during the IEF run, and analyzed for protein content.

Two-hundred microgram of total proteins were used for 2DE separation, which was essentially as previously described [4], with minor modifications as reported in Supplementary materials and methods.

Gel image analysis. Gels were stained with colloidal Coomassie blue (Pierce, Rockford, IL, USA) as previously described [4]. Gel images were acquired at 16-bit (Expression 1680 Pro, Epson) and analyzed using PG240 (v2006, Nonlinear Dynamics, Newcastle upon Tyne, UK, http://www.nonlinear.com). Spot detection comes after the image has been processed using the Intelligent Noise Correction Algorithm (INCA), which discriminates true signal from high noise levels. Proteins expressed differently in smokers and non-smokers were identified by an unpaired two-tailed *t*-test using the statistical tools available in the PG240 software. Control for false discovery rate (FDR) in multiple testing was performed, setting the significance at $p < 0.05$. We considered only novel spots, if any, or spots present in all three replicate gels and showing a volume difference >1.5.

In-gel digestion. The spots of interest were manually excised from the gels, destained, sequentially reduced and alkylated, and digested overnight with sequencing-grade modified trypsin (Roche), as previously described [4]. Aliquots of the sample containing tryptic peptides were directly analysed by MALDI-TOF MS for Peptide mass fingerprinting (PMF) analysis, and LC–ESI–MS/MS to generate amino acid sequence information for additional confirmation of the PMF-derived identification.

MALDI-TOF–MS. PMF analysis was run on a REFLEX III MALDI-TOF mass spectrometer (Bruker Daltonics GmbH, Bremen, Germany) equipped with a SCOUT 384 inlet and a 337 nm nitrogen laser and used in the positive ion reflectron mode with delayed extraction (accelerating voltage, 20 kV). Samples were loaded onto an AnchorChip™ MALDI target (Bruker Daltonics GmbH) using the thin-layer affinity preparation procedure described by Bruker Daltonics GmbH (see Supplementary materials and methods). Peptide mass lists were fed into the Mascot search engine (http://www.matrixscience.com) and searched against the National Center for Biotechnology Information non-redundant (NCBInr, 20061027) and/or Swiss-Prot (v. 54.0) databases. Detailed main search parameters are reported in Supplementary materials and methods. Proteins listed as significant matches in the Mascot search results were further considered as candidates for identification if the following conditions were met: (a) >20% sequence coverage by tryptic peptides, (b) ±25% concordance with the theoretical MW, and ±10% pI with the respective experimental values deduced from gel protein spot position.

LC–ESI–MS/MS. Protein identification by peptide sequencing was done by reversed-phase microbore-LC using a Surveyor system (autosampler and MS pump) coupled to an ion trap mass spectrometer LCQ Deca XP^{Plus} (Thermo Finnigan) equipped with a standard electrospray source, operated in positive ion mode, with an ion sprayer voltage of 4.6 kV and capillary temperature 220 °C. Peptide separation was done using a packed capillary column Aquasil C18 Kappa 100 × 0.5 mm, 3 μm (Thermo Electron Corp.). The HPLC linear gradient was from 10% to 40% solvent B in solvent A in 51 min. (Solvent A, 0.1% formic acid in water; solvent B, acetonitrile). See Supplementary materials and methods for details.

Data were acquired sequentially in MS mode (400–2000 amu), and in data-dependent mode, recording the MS/MS spectra of the three most intense ions of each MS scan.

Tandem mass spectra were analyzed using Phenyx (GeneBio, Switzerland, www.phenyx-ms.com, phenyx@genebio.com) against the Uniprot-Swiss-Prot database (v.54.0). Information on the main submission parameters for database interrogation using the search engine Phenyx, including algorithm, scoring models, and thresholds is given in Supplementary Table S1.

Western blot analysis

Pooled and individual urine extracts were analyzed in triplicate by Western blotting as detailed in Supplementary materials and methods. Mouse monoclonal antibodies (ZAG 1E2 and Amylase G-10, Santa Cruz Biotechnology, CA, USA) diluted, respectively 1:400 and 1:200, were used. The secondary antibody (goat anti-mouse peroxidase-conjugated antibody, Santa Cruz Biotechnology) was at a dilution of 1:1000. Proteins detection was by enhanced chemiluminescence (ECL) reaction (ECL detection kit GE, Healthcare).

Network analysis

The list of the identified differentially expressed proteins was uploaded as their Swiss-Prot IDs to MetaCore (GeneGo, St. Joseph, MI, USA) to map the proteins into biological networks. The biological process enrichment was analyzed based on GO Ontology processes. A network of these proteins was generated using the shortest paths algorithm.

Glucocorticoids analysis

Individual urine samples were analyzed for cortisol, cortisone, tetrahydrocortisol (THF), allo-tetrahydrocortisol (a THF), and tethahydrocortisone (THE) content by LC–MS/MS with selected reaction monitoring (SRM) using fludrocortisone as internal standard. SRM transitions are given in Supplementary Table S2.