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Cellular pathways affected by carbon nanopowder-benzo(α)pyrene complex in human skin fibroblasts identified by proteomics

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ABSTRACT

One of the crucial and unsolved problems of the airborne carbon nanoparticles is the role played by the adsorbed environmental pollutants on their toxicological effect. Indeed, in the urban areas, the carbon nanoparticles usually adsorb some atmospheric contaminants, whose one of the leading representatives is the benzo(α)pyrene. Herein, we used the proteomics to investigate the alteration of toxicological pathways due to the carbon nanopowder-benzo(α)pyrene complex in comparison with the two contaminants administered alone on human skin-derived fibroblasts (hSDFs) exposed for 8 days in semi-static conditions. The preliminary confocal microscopy observations highlighted that carbon-nanopowder was able to pass through the cell membranes and accumulate into the cytoplasm both when administered alone and with the adsorbed benzo(α)pyrene. Proteomics revealed that the effect of carbon nanopowder-benzo(α)pyrene complex seems to be related to a new toxicological behavior instead of simple additive or synergistic effects. In detail, the cellular pathways modulated by the complex were mainly related to energy shift (glycolysis and pentose phosphate pathway), apoptosis, stress response and cellular trafficking.

1. Introduction

One of the most important atmospheric pollutants are the airborne nanosized particles (NSPs; < 100 nm) which affect the human health mainly in the urban areas. Among the NSPs, the carbon nanoparticles (CNPs) occupy a special place as priority pollutants since they are the main component of particulate matter with dimensions lower than 10 μm (PM_{10}) and 2.5 μm ($PM_{2.5}$). CNPs are composed by several natural and engineered classes, including fullerenes, nanotubes, carbon-black and black carbon. The CNPs can be naturally produced as weathering products from graphitic carbon in rocks (Dickens et al., 2004), but also as by-product of incomplete combustion of fossil fuel and vegetation (Cochrane, 2003). The rapid development of nanotechnologies and CNPs' production leads on one side to an increase of exposure, and on the other hand to the understanding of their effects on the health of animals and humans. In the last decade, several studies showed the capability of the CNPs to enter the organism through the respiratory and digestive systems, but also through the skin, in particular for the CNPs with lower dimension (Teow et al., 2011). The main effects whose CNPs are responsible are due to the overproduction of reactive oxygen species (ROS; Souza et al., 2017) that secondarily can induce genotoxicity (Totsuka et al., 2009). Furthermore, ROS induced by nanoparticles has shown to be also involved in asthma, lung cancer, pulmonary fibrosis and systemic cardiovascular disorders (Donaldson et al., 2005). On the other hand, it has been suggested that the toxicity of nanomaterials could be increased by some environmental contaminants adsorbed at the surface (Nowack and Bucheli, 2007) through a Trojan horse mechanism (Limbach et al., 2007). Because of their sources, methods of production and their large surface areas, commercial carbon blacks typically contain varying quantities of adsorbed

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by-products, such as aromatic compounds, derived from the production process (IARC, 2010). In particular, PAHs (polycyclic aromatic hydrocarbons) account about for 39–75% of organic impurities extractable with solvents from CNPs (Watson and Valberg, 2001), whose B(α)P is one of the main component heavily released in urban areas and one of the environmental contaminants more dangerous, so that it is listed as a Group 1 (carcinogenic for humans) by IARC (2010).

Since there are few studies investigating the interactions of CNPs and volatile contaminants, herein we investigated the possible effect of the interaction between a particular carbon black-like powder, the carbon nanopowder (CNPW), and benzo(α)pyrene (B(α)P) on the whole proteome profile in human skin fibroblasts (hSDFs). These cells are very useful for this kind of studies, considering that the skin is one of the major target of these atmospheric pollutants, as suggested by Drakaki et al. (2014) and citation therein) who showed that oxidative stress caused by PM was related to the extrinsic skin aging, while PAHs could induce skin cancer and acneiform eruptions. In addition, the hSDFs used in this study are non-transformed primary cell line, allowing to highlight the eventual effects of selected contaminants on natural physiological processes, while transformed cells might change the physiological effect of nanoparticles, such as exposure time and concentration.

To investigate the toxicity of the CNPW-B(α)P complex and to elucidate the mechanism of action (MoA), we exposed the hSDF to CNPW and B(α)P alone and in co-exposure for 8 days. We applied the high-throughput methodology of proteomics to investigate the effects made by the single contaminants and the CNPW-B(α)P complex because it enables the understanding the structure, function and interactions of the whole protein content of cells. The use of a simple freeware allowed to demonstrate how proteins assemble in larger complexes, identifying common pathways involved in the pollutants' action. Contemporarily, we applied some advanced microscopy techniques to characterize the CNPW and to check its intake in the hSDFs, fortifying the information made by proteomics.

2. Materials and methods

The novelty of our approach lies in the way to administer the two contaminants when in co-exposure. Generally, the two pollutants are administered separated to the selected biological model, making three different components: the two fractions related to the single pollutants and the fraction composed by the complex of them. This generate an interfering situation that makes impossible to distinguish the actual effect exerted by the complex from the ones due to the single contaminants freely dissolved in the medium. In order to solve this confusing aspect, we preliminarily cleaned up the CNPW, eliminating any possible interfering organic chemicals, then we doped the cleaned CNPW with the appropriate concentration of B(α)P. This approach allowed the administration of the CNPW-B(α)P complex to the hSDFs without the confounding presence of the single pollutants.

2.1. Materials

CNPW (CAS no. 7440-44-0) and all solvents used (pesticide grade) were from Sigma-Aldrich (Steinheim, Germany), while B(α)P and PAH (PAH-Mix 14) standards were purchased by Dr. Ehrenstorfer (Augsburg, Germany). CNPW was characterized by high trace metal purity (≥99%) and a particle size < 50 nm.

2.2. CNPW clean-up and B(α)P contamination

CNPW was preliminary cleaned-up with toluene by consecutive washes in a Soxhlet (FALC, Treviglio, Italy) for 92 h. We followed the decreasing trend of the large quantity of PAHs adsorbed on the CNPW particles by the collection of extracts at different times (24, 48, 58 and 92 h) and the following analysis by mass spectroscopy (GC-MS/MS). The cleaned CNPW was dried by Rotavapor and nitrogen flux, and stored in dark glass bottles. The dried CNPW was separated in two glass bottles (500 mL) containing MilliQ® water, maintaining these suspensions in stirring for 48 h, which were previously sonicated by a probe sonicator (Stimin s.a.s, Giussano, Italy) for 15 min at 12 kHz. The first one was doped with 1 mg/L B(α)P, while the second was not contaminated. The two suspensions were then stirred for 48 h in the dark and centrifuged at 3000 ×*g* for 30 min. The supernatants were stored at 4 °C for the check of the possible presence of B(α)P, while the precipitated CNPW was dried for 3–4 days in a muffle furnace. The quantity of B(α)P really adsorbed on CNPW was measured by a gas-chromatographer (GC-MS/MS, Trace GC Ultra, Thermo Finnigan, CA, USA) equipped with a mass spectrometer (Polaris Q, Thermo Finnigan) following the same analytical procedure described above. Lastly, we also measured the amount of B(α)P dissolved in the supernatants by a liquid/liquid extraction and GC-MS/MS.

2.3. CNPW characterization

The DLS (dynamic light scattering; Zetasizer Nano, Malvern, UK) was used to determine the hydrodynamic diameters and surface changes (ζ potentials) of CNPW. The instrument was equipped with a solid-state He-Ne laser operating at a wavelength of 633 nm and detecting the scattered light at a scattering angle of 173°. Each measurement was recorded in quadruplicate. Data were elaborated using Zetasizer Nano Series software, version 7.02 (Particular Sciences, UK). We determined the primary particle diameter and shape of CNPW by TEM and SEM (scanning electron microscopy). The SEM (Zeiss LEO 1430) coupled with a Centaurus detector for energy-dispersive X-ray (EDX) spectroscopy were used to analyze the morphology and purity of the bulk form of CNPW. The powder was mounted onto an aluminum SEM stub and gold-coated. Elemental analysis was performed using Oxford Instruments INCA ver. 4.04 software (Abingdon, UK). Morphology and size distribution of the CNPW were measured by TEM: purified CNPW was suspended in distilled water, stirred and then sonicated. Aliquots of 5 mL were deposited onto Formvar-coated, 300 mesh, copper grids, and the excess water was gently blotted using filter paper. Once dried, the grids were directly inserted into a Zeiss LEO 912 ab Energy Filtering transmission electron microscope operating at 120 kV, and images were collected at a magnification of 25,000 using a CCD-BM/1 K system.

2.4. Cell culture and treatments

Human primary dermal fibroblasts (hSDF) (HuDe/BSPRC41) was provided from Centro Substrati Cellulari, ISZLER (Brescia, Italy) and cultured in EMEM (Euroclone, Milan, Italy) containing 1% L-Glutamine, 1% Penicillin/Streptomycin and 10% FBS (basal medium) at 37 °C in 5% CO_2 for no more than 10 passages. Sub-confluent cells were then incubated for 8 days in semi-static conditions with:

1. basal medium (control)
2. vehicle (dimethyl sulfoxide; DMSO 0.1%)
3. CNPW 50 mg/L
4. B(α)P 20 μg/L (dissolved in DMSO)
5. CNPW 50 mg/L + B(α)P 20 μg/L

The medium containing pollutants, vehicle or basal medium has been changed every day with the same fresh medium, adding the related quantity of contaminants. Results of CNPW and CNPW-B(α)P complex were compared with those of the control on the complete medium, while B(α)P results were compared with those of its carrier (DMSO). Three different flasks with Eagle's minimal essential medium were set up for each experimental group, to which FBS 10% (fetal bovine serum), penicillamine-streptomycin 1% and L-glutamine were added. About 1.5 millions of hSDFs were plated on each flask in