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Comparative study of the formation of oxidative damage marker 8-hydroxy-2′-deoxyguanosine (8-OHdG) adduct from the nucleoside 2′-deoxyguanosine by transition metals and suspensions of particulate matter in relation to metal content and redox reactivity

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Abstract

An association between exposure to ambient particulate matter (PM) and increased incidence of mortality and morbidity due to lung cancer and cardiovascular diseases has been demonstrated by recent epidemiological studies. Reactive oxygen species (ROS), especially hydroxyl radicals, generated by PM, have been suggested by many studies as an important factor in the oxidative damage of DNA by PM. The purpose of this study was to characterize quantitatively hydroxyl radical generation by various transition metals in the presence of H_2O_2 in aqueous buffer solution (pH 7.4) and hydroxylation of 2′-deoxyguanosine (dG) to 8-hydroxy-2′-deoxyguanosine (8-OHdG) under similar conditions. The order of metals' redox reactivity and hydroxyl radical production was Fe(II), V(IV), Cu(I), Cr(III), Ni(II), Co(II), Pb(II), Cd(II). Then, we investigated the generation of hydroxyl radicals in the presence of H_2O_2 by various airborne PM samples, such as total suspended particulate (TSP), PM_{10}, $PM_{2.5}$ (PM with aerodynamic diameter 10 and 2.5 μm), diesel exhaust particles (DEP), gasoline exhaust particles (GEP) and woodsmoke soot under the same conditions. When suspensions of PMs were incubated with H_2O_2 and dG at pH 7.4, all particles induced hydroxylation of dG and formation of 8-OHdG in a dose–dependent increase. Our findings demonstrated that PM's hydroxyl radical (HO·) generating ability and subsequent dG hydroxylation is associated with the concentration of water-soluble metals, especially Fe and V and other redox or ionizable transition metals and not their total metal content, or insoluble metal oxides, via a Fenton-driven reaction of H_2O_2 with metals. Additionally, we observed, by Electron paramagnetic resonance (EPR), that PM suspensions in the presence of H_2O_2 generated radical species with dG, which were spin–trapped by 2-methyl-2-nitroso–propane (MNP).

Keywords: *Airborne particulate matter, Fenton reaction, hydrogen peroxide, hydroxyl radicals, 8-hydroxyl-2′-deoxyguanosine, reactive oxygen species*

Abbreviations: *DMPO, 5,5-Dimethyl-1-pyrroline–N-oxide; 8-OHdG, 8-hydroxy-2′-deoxyguanosine; MNP, 2-methyl-2-nitroso–propane; dG, 2′-deoxyguanosine; TSP, total suspended particulates; DEP, diesel exhaust particles; GEP, gasoline exhaust particles*

Introduction

In recent years, many epidemiological studies showed that exposure to ambient particulate matter (PM) is associated with the incidence of pulmonary cancer, increased cardiovascular mortality and morbidity and inflammatory effects on the heart and lungs [1,2]. Studies proved that the most cytotoxic of PM were particles with an aerodynamic diameter less than 10 μm (PM_{10}) and especially 2.5 μm ($PM_{2.5}$) [3].

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Studies investigating the possible mechanisms of cytotoxic, proinflammatory and carcinogenic potential of PM have focused on various aspects of their action on cellular constituents [4,5]. It is now generally accepted that the potential of PM for cellular damage is mainly the result of generation of reactive oxygen species (ROS), especially hydroxyl radicals (HO·), which have a pivotal role in PM–induced adverse health effects to humans [6,7].

The critical issues of research with inhalable PM are the size of the particles and their chemical composition, and the potential of biologic mechanisms that underlie the epidemiological associations. Although biochemical mechanisms for particulate–induced cytotoxicity remain unclear, it has been suggested that metal–mediated generation of ROS is an important factor in oxidative damage to biomolecules, especially nuclear DNA [8,9]. The emphasis of metal–induced oxidative DNA damage has been placed on Fenton–driven reactions, especially by iron and copper which are the most abundant metals in PM [10,11]. Both metals have been associated with the formation of 8-hydroxy-2′-deoxyguanosine (8-OHdG), recognized as an important oxidative and mutagenic DNA damage marker, and various strand breaks of DNA [12].

Lloyd and co–workers observed differences in the formation of 8-OHdG, as well as single– and double–strand breaks in DNA mediated by Fenton reactions of various metals. They suggested that the differences observed are due to the well–known binding specificity of metals and their ability to complex with different nucleotide moieties of DNA [13]. Although, the biochemical mechanisms of metal mediated cytotoxicity and carcinogenicity (Fe, V, Cr, Ni, etc) are not fully understood, studies have indicated that these metals mediate in the generation of ROS and induce significant oxidative DNA damage [14–16].

The ability of PM to generate ROS in the pulmonary environment is suggested to be a series of reactions where quinone mixtures shuffle electrons (enzymatically or nonenzymatically) among their reduced/oxidized and semiquinone forms in redox cycles, generating superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and ultimately with the Fenton reaction by metals hydroxyl radicals (HO·) causing oxidative damage [17,18]. PM from various air pollution sources was found to contain large amounts of persistent radicals, characteristic of carbon-centered radicals and semiquinone radicals chemisorbed in carbon particles [19]. Quinones, such as hydroquinone and catechol (found mainly in cigarette tar), in the presence of endogenous NADH and Cu(II) induced DNA damage in guanosine and thymine [20]. Redox mechanisms by quinones have been proposed in order to explain that aqueous extracts of airborne fine PM (PM_{10} and $PM_{2.5}$) induced damage to DNA in human cells and supercoiled phage DNA [21].

Diesel exhaust particles (DEP) have been investigated thoroughly in the last decade for their cytotoxic and carcinogenic potential. It has been reported that cellular DNA damage, due to ROS generated by DEP, leads to the formation of the most critical oxidative lesion 8-OHdG of deoxyguanosine residue in DNA [22]. The formation of 8-OHdG in lung DNA from mice treated with DEP showed a dose dependent increase and significantly correlated with the lung tumour incidence [23]. Similar studies were performed with inhalable PM in the urban environment. Experiments *in vitro* with calf thymus DNA showed that generation of 8-OHdG by coarse and fine PM was significantly correlated to hydroxyl radical generation [24]. A recent study with fine subway particles (PM_{10}) found increased ability (compared to street inhalable particles) to induce oxidative stress and genotoxicity in lung cells, measured by HPLC as 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) and by the comet assay, due to their redox active solid metals [25].

Recent occupational and epidemiologic studies concerning exposure to fine PM (PM_{10}, $PM_{2.5}$, oil fly ash) found that oxidative DNA damage, expressed with the urinary marker 8-OHdG or 8-oxodG levels was correlated with exposure to PM, especially with the concentrations of their metals (vanadium, manganese, nickel and lead) [26–28].

The present study was undertaken to investigate the quantitative aspects of hydroxyl radical production by transition metals [Fe(II), Cu(I), Cr(III), V(IV), Co(II), Ni(II), Cd(II)] with H_2O_2 in aqueous solutions at pH 7.4 by spin-trapping with DMPO and EPR spectroscopy. Then, we extended these measurements to the generation of 8-OHdG from 2′-deoxyguanosine (dG) under similar conditions, determined by EPR (spin-trapped by MNP) and quantitatively by HPLC with electrochemical detection and spectrophotometerically. Finally, we measured the formation of 8-OHdG under physiological conditions by six different types of airborne PM in aqueous suspensions with H_2O_2 (total suspended particulate (TSP), diesel exhaust particles (DEP), gasoline exhaust particles (GEP), fresh woodsmoke soot, PM_{10} and $PM_{2.5}$) from dG, in relation the their metal content, which were determined by atomic absorption spectroscopy (AA).

Materials and methods

Chemicals

Ferrous sulfate ($FeSO_4 \cdot 7H_2O$), Copper chloride (CuCl), Chromium (III) chloride ($CrCl_3 \cdot 6H_2O$), Vanadyl sulfate hydrate ($VOSO_4 \cdot H_2O$), Nickel chloride ($NiCl_2$), Cobalt chloride ($CoCl_2$) and Cadmium chloride ($CdCl_2{\cdot}2H_2O$) were purchased from Aldrich. Spin-traps DMPO (5,5-dimethyl-1-pyrroline-N-oxide), MNP (2-methyl-2-nitroso-propane, dimer), dG (2-deoxyguanosine), 8-HOdG standard (8-hydroxy-2′-deoxyguanosine) and deferoxamine mesylate were purchased from Sigma. EDTA-Na_2 (ethylenediamine tetraacetic acid, disodium salt), phosphate buffer (pH 7.4) were purchased from Merck. All other chemicals used were of analytical quality.

Samples of airborne suspended particulates

PM were collected as follows:

(a) TSP in the period 2003–2004, in the centre of Athens (Division of Atmospheric Pollution and Noise Control of the Ministry of Environment, Regional Planning and Public Works), by high volume pumps ($80\,m^3h^{-1}$) on a 24 h basis and collected using pre-weighed glass microfibre filters (Whatman GF/A 20.3 × 25.4 cm) with 99% collection efficiency for particles with 0.3 μm diameter. The location of the sampling site was in the centre of Athens mainly influenced by local traffic.

(b) Samples of soot from diesel vehicles were collected on annealed glass–fibre filters (Ø 5 cm), using a low volume air sampler, at a distance of 0.5 m from the exhaust pipe of a diesel taxi (2.0 L engine, GM Astra model, 5 years old). Vehicle exhaust emissions sampling lasted for about 30 min in a test laboratory and used as "fresh" soot to distinguish from soot deposited in the exhaust pipes. Vehicle exhaust emissions sampling represent warm-engine operation at 800 (idle mode) and 2500 rpm (simulating urban driving conditions)

(c) Samples of gasoline exhaust particles were collected from a gasoline on annealed glass–fibre filters (Ø 5 cm), using a low volume air sampler, at a distance of 0.5 m from a gasoline exhaust engine (1.6 L, passenger vehicle Ford, 10 years old, without catalytic converter). Vehicle exhaust emissions sampling lasted 1 h and was used as "fresh" soot in our experiments.

(d) Samples of soot from wood combustion (pine, olive oil tree, beech) were collected on a glass microfibre filters from a domestic fireplace exhaust chimney at a distance of 1 m with a low volume air sampler.

(e) PM_{10} were collected in the same site as in the case of TSP with a high volume sampling system equipped with a six-stage slit cascade impactor (Andersen) with back-up filter, which effectively separates the PM into seven fractions, with the following cut-off diameters at 50% efficiency: >10.2, 10.2–4.2, 4.2–2.1, 2.1–1.4, 1.4–0.73, 0.73–0.41, <0.41 μm. Unloaded and loaded filters were kept in a darkened desiccator for 48 h before weighing for gravimetric analysis. Loaded filters were wrapped in solvent-rinsed aluminium foils and kept refrigerated until extraction (within 3–7 days).

(f) $PM_{2.5}$ were collected with the same high-volume cascade impactor (Andersen) system. We used the fraction with aerodynamic diameter 2.1 μm and lower (we used the notation $PM_{2.5}$ which is common in most studies)

All samples were kept at −20°C for 3–7 days until used in experiments.

Extraction of PM from filters of solid samples of PM

Filters were cut into smaller pieces and added in a conical flask with 50 ml of deionised water. PM was extracted, first by vigorous shaking (20 min) and subsequently by ultrasonication in a water bath at 25°C for another 20 min. The cleaned filters were removed carefully and because the obtained particle suspension was partly contaminated by small filter fibres (3–5% on mass basis) a blank filter was treated the same way and was used as a control in all experiments. To test for the amount of material extracted the suspension was centrifuged for 20 min and the precipitate was dried in a high vacuum desiccator, under these conditions 0.01–0.05 g of solid (TSP) was obtained. Dried samples were kept at −20°C in the dark until used.

Characterization of PM samples for trace metals

Trace metals, especially Fe, Cu, Co, Cr, Pb, Ni, V, Cd, Zn and Mn were determined in PM samples by atomic absorption spectroscopy (AA). Samples were extracted by vigorous shaking and evaporated to dryness. After gravimetric analysis, PMs were ultrasonically treated with concentrated HNO_3/HCl and analysis was performed by atomic AA spectrometry. Two types of AA were used according to the concentrations of metals in MP samples. For higher metal concentrations flame AA (Varian Spectr AA-200) for metals such as Fe, Zn, Mn etc. For lower concentrations flameless AA was used, graphite furnace atomic absorption spectrometer (GFAAS) (Varian Spectr AA-640Z) with Zeeman background correction, equipped with GTA autosampler (for Cu, Cd, Pb, Co, Ni, etc).

Trace metals calculated as μg/g of PM. Fe, Zn, Pb and Cu were found in measurable amounts, Cr, V, Ni, Mn, Co, Cd were found at lower concentrations while other metals were below the detection limits.

Generation of HO· by metals in aqueous buffer solution in the presence of H_2O_2 with and without the metal chelate EDTA

Reaction mixtures were prepared by mixing 1 ml of 0.02 M of metal with 1 ml of DMPO (0.08 M) and 1 ml of 0.2 M of H_2O_2 in aqueous buffer solution (pH 7.4). The aqueous buffer solution was degassed by sparging with dry nitrogen in order to minimize the rapid air oxidation of metals from the lower valence to a higher one. Total volume for all solutions was 5 ml. The same reactions were carried out, but with the addition of 1 ml of 0.02 M EDTA-Na_2 at room temperature. After the mixture was shaken gently for 3 min it was transferred into a quartz flat cell and electron paramagnetic resonance (EPR) was recorded in the next 10 min. The EPR signal was recorded as a four line spectrum (1:2:2:1, quartet, $a_N = a_H = 14.9$ G). Instrumental conditions (Varian E-4) were: microwave power, 20 mW; modulation amplitude 1.0 G; scan time 8 min; receiver gain $6 \times 10^3 - 1.25 \times 10^4$.

Generation of HO· by suspensions of PM in aqueous buffer solutions

Reaction mixtures of PM contained two different amounts of suspensions (20 and 40 mg) of PM, 1 ml of DMPO (0.08 M), 1 ml of 0.2 M H_2O_2 and 3 ml of aqueous phosphate buffer solution. Also, experiments were repeated with the addition in the mixture of 1 ml of 0.01 M EDTA-Na_2. The mixture was shaken gently for 3 min, degassed by dry nitrogen and then filtered. The EPR spectrum was recorded at room temperature with instrumental conditions similar as above. Each reaction was performed twice and measurements of EPR signal intensity (adding the 4 lines, arbitrary units) are reported as average of the two trials ± S.D.

EPR identification of radical adducts by spin-trapping with MNP

The radical adduct formed by the Fenton reaction in the presence of dG was studied by EPR in aqueous buffer solutions (pH 7.4) following the methodology of Davies et al. [29] The radical adduct was spin-trapped by the MNP ($Me_3C{-}N{=}O$). The MNP solution contained 10–15% acetonitrile (having been left to stir for 2–5 h to bring about the dissolution of the spin-trap). Concentrations of the reactants in the final solution were: metal 0.02 M, MNP 10^{-2} mol dm^{-3}, 0.02 M H_2O_2, 0.05 M dG and 0.02 M EDTA. Mixtures were deoxygenated by purging with nitrogen for 2 min and then agitated gently for 10 min (at room temperature in the dark) and transferred in a flat-type quartz cell. EPR instrument parameters (Varian E-4): modulation amplitude 1.0 G; microwave power 20 mW; scan time 8 min; receiver gain $5 \times 10^3 - 1.25 \times 10^4$. Similarly, PM samples were used for a series of experiments. 50 mg of PM were suspended in aqueous buffer solution which contained 1 ml of MN and 1 ml of H_2O_2 (0.2 M). The mixture was shaken gently for 15 min in the dark, filtered and placed in a quartz flat cell for EPR measurements.

HPLC quantitative determination of 8-OHdG

We followed two methods for the HPLC analysis of 8-OHdG with small changes to those described earlier. In the first method, we used 7% methanol-93% buffer solution (KH_2PO_4, 50 mM, pH 7.4), flow rate 1.2 ml/min and UV-EC detection [30], and in the second method with eluting solution of 3% acetonitrile and 0.1% aqueous acetic acid, flow rate 1.0 ml/min and monitoring was performed only by UV–Vis at 293 nm [31].

Samples were prepared by mixing aqueous buffered solutions 1 ml of 0.02 M of transition metals and 1 ml of EDTA-Na_2 (0.02 M). This solution was degassed gently with dry nitrogen to prevent oxidation of the metal. Then, 1 ml of dG (0.05 M) was added with 1 ml of 0.2 M H_2O_2 and 1 ml of phosphate buffer at pH 7.4. The concentration of 2′-deoxyguanosine in the mixture of 5 ml was 0.01 M. The samples were shaken gently in the dark for 1 h at room temperature, while the incubation mixture was kept open under ambient air to allow air exchange, indicating the important role of oxygen in the hydroxylation process. Then, the mixture was filtered prior to HPLC analysis using Gelman Nylon Acrodisc 0.2 μm syringe filters. Each reaction was performed twice, and 8-OHdG amounts are reported as average of the two trials ± S.D. A control containing only dG was analyzed in parallel with the reaction mixture to be certain that 8-OHdG was not arising merely from decomposition of the dG with time. HPLC (Hewlett-Packard, Agilent 1100 series), reverse phase-HPLC. Column Lichro 250 × 4 mm, Lichrospher 100 RP-18, 5 μm column (25 cm × 4.6 mm) under isocratic conditions. The mobile phase was 7% methanol-93% buffer solution KH_2PO_4, 50 mM (pH 7.4), with a flow rate of 1.2 ml/min. The 8-OHdG and dG were monitored at 254 nm, and by an electrochemical detector (Coulochem II EC, ESA, Inc., Chelmsford, MA) set at 400 mV and 20 nA full scale. The concentrations of 8-OHdG are high enough to be detected also only by UV–Vis (with the appropriate expansion) by the second method with eluting solution of 3% acetonitrile, the monitoring was performed at 293 nm (Hewlett-Packard, Agilent 1100 Series).

The content of Deoxyguanosine and 8-OHdG was calculated by comparison to the peak areas obtained with respective standards. A calibration curve was constructed from 8-OHdG solutions with concentrations ranging from 1 mM to 3 μM in aqueous buffer solution and exhibited linearity with an R^2 value of 0.991. The detection limit of 8-OHdG is estimated to be, approximately, 2–3 μM.

It is well known that following oxidative attack on nuclear and mitochondrial DNA by hydroxyl radicals, guanosine base undergoes 8-hydroxylation, forming the 8-hydroxy-2′-deoxyguanosine (8-OHdG) adduct, that upon oxidation gives the stable product 8-oxo-2′-deoxyguanosine (8-oxodG). The first name is used still in many papers on oxidative DNA damage, but the second is more common nowdays in medical and biological journal, especially in papers for quantitative analysis of biomarkers of oxidative DNA damage in humans [32].

Results

Hydroxyl radical generation from metal/H_2O_2 with and without EDTA

The generation of hydroxyl radicals (HO·) by metal/H_2O_2 mixtures with and without EDTA chelator was determined by EPR measurements of spin trapped radicals with DMPO. The heights of the 4-line EPR spectrum (1:2:2:1) were added to increase accuracy and the mean value is expressed in arbitrary units as a quantitative expression of hydroxyl radical production. The following results are presented in Table I.

Generation of HO· radicals by suspensions of PM in aqueous buffer solution

The intensities of the EPR signal (1:2:2:1) of DMPO-OH spin adduct, generated by PM suspensions (at two different amounts) in aqueous buffer was quantified (arbitrary units). These comparative results represent mainly a measure of the ability of PM to release hydroxyl radicals by the Fenton reaction, through the redox capabilities of their transition metals. The results are presented in Table II.

These results indicate that PM suspensions in aqueous buffer solutions with H_2O_2 generate hydroxyl radicals by a Fenton reaction in a dose-dependent mode. In all cases the addition of EDTA increases the HO· formation, indicating that complexation of metals with this particular chelator, such as Fe(II), V(IV), etc, are critical in its generation. Whereas, the addition of well known metal chelator deferoxamine mesylate inhibits substantially the formation of HO· by blocking all available valences (results not shown).

EPR evidence for the spin adducts formed by the reaction of 2′-dG with metal ions and H_2O_2 at pH 7.4

2′-Deoxyguanosine (dG) was incubated with metal ions and H_2O_2 and the spin adduct formed by the attacking HO· was spin-trapped by MNP. The EPR spectrum is dominated by a triplet of doublets with hyperfine splittings of $\alpha(\alpha - N) = 15.0–15.2$ G (or 1.5–1.52 mT) and $\alpha(\beta\text{-N}) = 3.0–3.2$ G. After careful analysis the EPR spectrum is believed to be composed of three radical adducts with MNP: (a) C_4—OH spin adduct with splitting parameters: $\alpha(\alpha\text{-N}) = 15.2$ G and $\alpha(\beta\text{-N}) = 3.2$ G; (b) C_5—OH with $\alpha(\alpha\text{-N}) = 15.0$ G; and (c) C_8—OH with $\alpha(\beta\text{-N}) = 15.0$ G. Details on EPR spectra, derived from incubation of guanine and their ribose and deoxyribose derivatives with Fe^{2+}/EDTA/H_2O_2 and MNP at pH 7, were presented by Davies et al. [29] Radical adducts of 2-dG with MNP in Figure 1. Although most papers assume that the main attack of HO· is on the C_8—OH position, it seems that C_4 − and C_5 − positions are also susceptible to hydroxyl attack. The N-7 -centered radical is regarded as a precursor of 8-OHdG [33].

Representative EPR spectra of TSP, DEP, GEP, PM10, PM2.5 and woodsmoke with MNP are presented in Figure 2.

HPLC quantitative determination of 8-OHdG from dG in aqueous buffer solution by metal/H_2O_2 and by PM/H_2O_2

The quantitative measurements of 8-OHdG produced by the Fenton reaction, mixtures of metals with H_2O_2, and by various suspensions of airborne particulates in the presence of H_2O_2, under ambient air, at room

Table I. Quantitative measurements of HO· generation by metal/H_2O_2 with and without EDTA. Relative peak heights signal intensity of the 4-line EPR spectrum (arbitrary units) of DMPO-OH adduct.

Metal ion (0.04 M, final concentration of metal and H_2O_2)	Signal intensity height (arbitrary units ± SD)	
	without EDTA	with EDTA (0.004 M final concentration)
Fe(II)	183 ± 12	increase by 60–70%
V(IV)	83 ± 6.5	increase by 100–120%
Cr(III)	25.5 ± 3.6	decrease by 10–15%
Cu(I)	25 ± 2.5	decrease by 70–80%
Co (II)	20 ± 2.2	decrease by 30–40%
Ni(II)	10.7 ± 1.8	decrease by 50–60%
Pb(II)	8.5 ± 1.5	decrease by 80–100%
Cd(II)	3.5 ± 0.5	No change

*Experiments with Sb(II), Mn(II) and Zn(II) with H_2O_2 in aqueous buffer solution (pH 7.4) under similar conditions did not produce hydroxyl radicals (results not shown).

Table II. Comparative and quantitative measurements of generation of HO· by PM and H_2O_2 in aqueous buffer solution (pH 7.4) with and without the addition of EDTA.

Particulate matter (PM) (mg/5 mL)	Signal intensity height (arbitrary units ± SD) without EDTA	Signal intensity height (arbitrary units ± SD) with EDTA
TSP (total suspended particulates)		
20 mg	24 ± 3.5*	33 ± 4.0
40 mg	42 ± 4.8	60 ± 5.5
DEP (diesel exhaust particles)		
20 mg	20 ± 4.2	35 ± 6.2
40 mg	38 ± 5.5	62 ± 7.5
GEP (gasoline exhaust particles)		
20 mg	18 ± 2.4	27 ± 4.0
40 mg	32 ± 3.8	43 ± 3.3
Woodsmoke soot		
20 mg	12 ± 1.6	22 ± 1.7
40 mg	25 ± 3.0	33 ± 2.4
PM_{10}		
20 mg	25 ± 2.5	38 ± 3.5
**$PM_{2.5}$		
20 mg	28 ± 3.0	42 ± 4.6

* All data are expressed as mean value ± SD ($n = 2$). ** This fraction of particulate matter is actually $PM_{2.1}$, with aerodynamic diameter from 2.1 μm and smaller. But we keep the common notation of $PM_{2.5}$

temperature and at pH 7.4 (for 1 h) are presented in Table III.

Representative HPLC/UV-EC chromatogram profiles obtained from the reaction of freshly prepared metal ions (0.04 mM in the final volume) and various airborne PM and dG (0.1 mM) in phosphate buffer (pH 7.4) are presented in Figure 3. (A) and (B) method with 7% methanol under UV–VIS and EC detection, (C) method with 3% acetonitrile and UV–Vis detection at 293 nm.

Concentrations of transition metals in various airborne PM samples

Most emphasis has been placed on Fenton-driven Fe and Cu reactions. Also, the transition metals Ni, V, Co

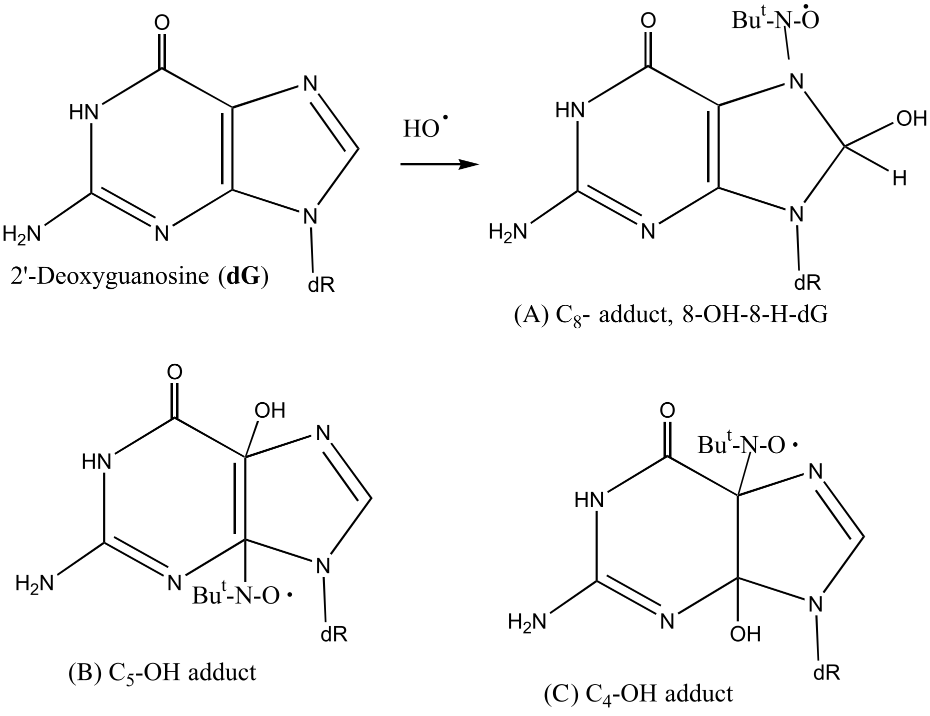

Figure 1. Molecular structures of 2′-dG radicals produced by the attack of hydroxyl radical (HO·) and spin-trapped by MNP (Bu^t—N-O). (A) C_8-OH spin adduct; (B) C_5-OH spin adduct and (C) C_4-OH spin adduct.

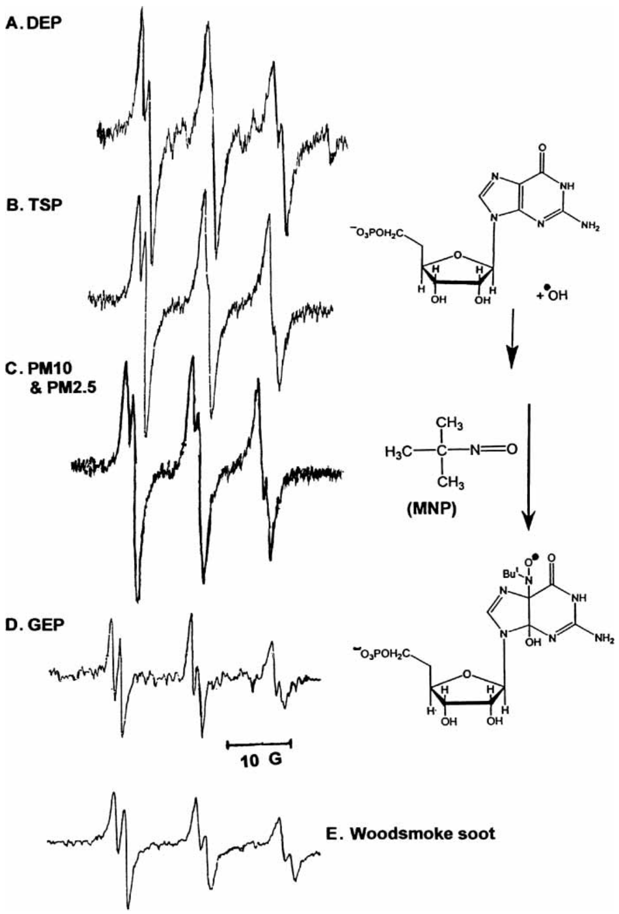

Figure 2. EPR spectra obtained from the reaction of HO· with 2′-deoxyguanosine at pH 7.4 in the presence of spin-trapping MNP. The hydroxyl radical was generated from various suspensions of airborne particulates with H_2O_2 in buffered solutions (pH 7.4). (A) diesel exhaust particles (DEP), (B) total suspended particulates (TSP), (C) PM_{10} and $PM_{2.5}$ (particulate matter), (D), gasoline exhaust particles (GEP), (E) woodsmoke.

and Cr are capable of redox cycling electrons utilizing multiple valence states, which initiate the production of ROS, such as $O_2^{\cdot-}$, H_2O_2 and the highly reactive HO·, by reduction of molecular oxygen (metal concentrations in Table IV) [12].

Discussion

The association between adverse health effects and inhalable airborne PM, is supported by numerous epidemiologic research findings [1–3]. Although precise mechanisms remain unclear, most studies suggest that a pivotal role for PM's cytotoxicity is mainly associated with their ability to generate ROS and the resulting oxidative stress [34].

In the present study, we investigated the comparative hydroxyl radical generation capability of various metals at physiological pH (7.4) and the ability of various samples of airborne PM to generate hydroxyl radicals, in relation to their metal content in the water soluble and insoluble part. Then we extended our investigation in quantitative terms for their ability to induce 2′-deoxyguanosine (dG) hydroxylation under the same conditions in order to compare the relationship with their metal redox reactivity.

Table III. Quantitative HPLC measurements of 8-OHdG formation from dG by the mixture: metals/H_2O_2 and PM/H_2O_2, in µg 8-OHdG/10^6 dG.

Metals / H_2O_2	8-OHdG (µg/10^6 dG)
Fe(II)	270.0 ± 32.0*
V(IV)	58.8 ± 8.5
Cr(III)	40.7 ± 6.4
Cu(I)	32.2 ± 4.5
Pb(II)	28.0 ± 4.3
Cd(II)	21.9 ± 3.7
Co(II)	18.8 ± 2.6
Ni(II)	15.4 ± 2.2
Suspensions of airbone particulate matter/H_2O_2	
Total suspended particulates (TSP)	48.5 ± 5.5
Diesel exhaust particles (DEP)	115 ± 10.8
Gasoline exhaust particles (GEP)	42.0 ± 4.9
Woodsmoke soot	35.3 ± 3.2
PM_{10}	85 ± 4
$PM_{2.5}$**	100.4 ± 5.5

*All results are expressed as means ± SD ($n = 2$). ** This fraction of PM is actually $PM_{2.1}$.

Most of the emphasis in our investigation, as in other studies, has been placed on the Fenton-driven reactions, not only of Fe and Cu, which are the most abundant metals in PM, but also for the transition metals V, Ni, Co, Cr and Pb, which are capable of redox cycling of electrons utilizing multiple valence states leading to the production of ROS and especially the highly reactive radical HO·, by reduction of molecular oxygen [35]. Metals, such as Zn(II) and Sb(II) and Mn(II), with H_2O_2 did not produce hydroxyl radicals in our experiments (results not shown). Similarly, metal oxides, such as Fe_2O_3, where all six coordination sites are occupied and are therefore unavailable to support shuffling and redox reactions do not generate hydroxyl radicals. In other cases, such as Cu(II), reductants may be required to drive free radical generation[9,36].

The results of our comparative Fenton reactions for the generation of HO· radicals with the couple metal/H_2O_2, with and without EDTA, were as expected. Metals can be arranged in a decreasing order of their ability to generate hydroxyl radicals in aqueous buffer solution at pH 7.4, as follows:

$$\mathrm{Fe(II)} >> \mathrm{V(IV)} > \mathrm{Cr(III)} > \mathrm{Cu(I)} > \mathrm{Co(II)}$$
$$> \mathrm{Ni(II)} > \mathrm{Pb(II)} > \mathrm{Cd(II)}$$

Ferrous ions have the highest potential to generate hydroxyl radicals, under these conditions, almost double than V(IV) and 4-times than Cr(II) and Cu(I). The metal ions Co(II), Ni(II), Pb(II) and Cd(II) are at the lower end of the scale. Metal ions and their oxides, such as Sb(II), Zn(II), Mn(II) and insoluble Fe_2O_3 did not generate hydroxyl radicals (results not shown).

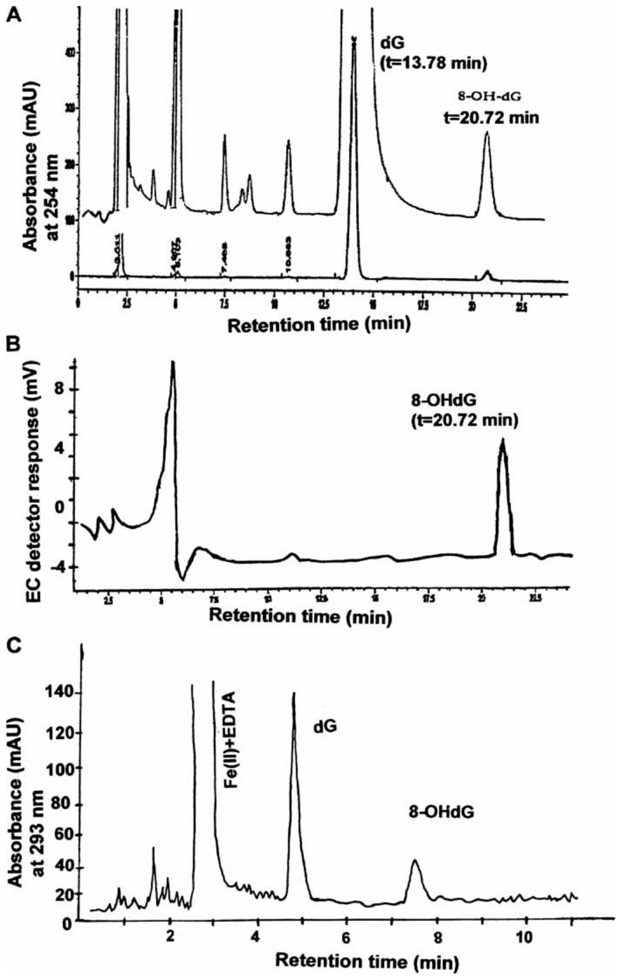

Figure 3. Representative HPLC/UV-EC profiles of 8-OHdG obtained from the reaction of freshly prepared metal ions and dG in phosphate buffer (pH 7.4): lower profiles, UV(A_{253}); upper profiles, EC detector response. See Materials and methods for HPLC conditions. The sample was injected after incubation for 1 h at room temperature.

Previous studies on oxidant generation by metal species demonstrated that soluble and reduced metal ions, such as vanadyl and ferrous [V(IV) and Fe(II)] exhibited relatively enhanced hydroxyl radical generation and similarly enhanced dG hydroxylation, compared to vanadate and ferric [V(V) and Fe(III)] [8].

In our experiments (Table I), the generation of HO· (spin-trapped by DMPO) by the couple of Fe(II)/H_2O_2 was almost double when compared to the similar reaction of the couple V(IV)/H_2O_2. Similar results were observed by Carmichael [37]. Various studies in the last decade showed that metal ions and their complexes in their lower oxidation states, have oxidative features of the Fenton reagent leading to the production of hydroxyl radicals [38,39].

The presence of metal chelates in equimolar concentrations in the solution, such as EDTA-Na_2, increased the ability for the HO· generation only for the Fe(II) and V(IV), whereas in the case of other metal ions

Table IV. Transition metal concentrations, capable for redox cycling, in various airborne PM samples (TSP, PM_{10}, $PM_{2.5}$ in Athens area, 2003–2004).

Airborne Particulate matter	Total particle mass (μg/m^3)	Fe (μg/g) (min-max)	V (μg/g)	Cu (μg/g)	Cr (μg/g)	Co (μg/g)	Ni (μg/g)	Mn (μg/g)	Pb (μg/g)	Zn (μg/g)	Cd (μg/g)
TSP	180–240 (winter) 150–180 (summer)	8.100–14.000	50–130	700–900	350–450	20–30	340–420	700–750	2700–3.100	2.260–2.700	150–270
PM_{10}	80 ± 25 (summer) 95 ± 10 (winter)	7.000–10.000	60–110	850–900	410–540	Below detection limit	410–480	580–640	560–750	1.880–2.130	BDL
$PM_{2.5}$	40 ± 5 (summer) 65 ± 5 (winter)	6.500–8.000	40–85	430–570	125–400	BDL	450–520	380–650	580–720	1.700–1.900	BDL
DEP		750–1.850	25–45	45–60	170–190	10–15	180–245	30–45	–	–	BDL
GEP		1.550–1.840	–	140–155	–	–	210–240	–	17.500	5.800–6.000	50–60
Wood smoke Soot		750–800	240–260	120–160	–	15–25	–	–	–;	3.700–4.200	9–12

* It was calculated that PM10 was the 50–60% of TSP, ** BDL = below detection level.

we observed decreases in the ability to generate hydroxyl radical or no change, as in the case of Cd (Table I). Three other metals ions, namely Sb(II), Zn(II) and Mn(II), which are found at lower amounts in airborne PM, gave negative results. These results are supported from previous research investigations [40,41].

The ability for the generation of HO· by suspensions of airborne PM in aqueous buffered solution, in the presence of H_2O_2, was expected to be associated mainly with the concentrations of certain transition metal ions in these PM samples, as it was indicated by other research investigations [6,11,12].

Our results showed that PM with small aerodynamic diameter, such as PM_{10} and $PM_{2.5}$, which can reach airways of the lower respiratory system have the highest ability for the generation of HO· (Table II). These results are consistent with their higher concentrations of the highly redox active and water-soluble or ionizable metals, such as Fe, V, Cu, Cr and Ni[6].

TSP, which contains coarse, insoluble and fine particles (up to 0.3 μm) showed lower reactivity than PM_{10} and $PM_{2.5}$. Although TSP has high concentrations of Fe, Cu, Cr and other metals, most of its iron is found in the insoluble Fe_2O_3 state without redox reactivity. Also, various studies showed that TSP contains high amounts of coarse particles of insoluble metal oxides, such as SiO_2, Al_2O_3, MgO, CaO, BaO [42]. Also, Zn and Mn which are found at high concentrations in TSP are not redox active.

Diesel exhaust particles (DEP), despite comparatively lower Fe, V and Ni concentrations, showed high capacity for HO· generation. The collection of "fresh" DEP from the exhaust pipe and its immediate use in our experiments means that most of its Fe and other metals are in the lower valence state and capable for redox cycling. Diesel driven vehicles and other local combustion processes are known to contribute to a great extent to $PM_{2.5}$ in urban atmospheres [43,44].

GEP exhibited lower reactivity compared to TSP and DEP. Fe, Ni, Cu and Cd are found in GEP at similar concentrations with DEP, but metals such as V, Cr or Co are absent.

Woodsmoke soot, which is very low in Fe and other metals (but high in Zn) showed the lowest reactivity of all samples.

Similar results (generation of hydroxyl radical) among airborne particle matter, such as TSP, PM_{10}, $PM_{2.5}$, oil and coal fly ash have been observed by other studies. Most of these studies were focused on the role of redox active and water-soluble metals. The concentrations of these metals and their bioreactivity were related with hydroxyl radical generation and oxidative DNA damage [16,19,24]. Special attention was paid to iron (Fe) which is the most abundant in PM. The mobilization of iron from urban air particulates is considered an important factor in the cytotoxicity of PM and its pathological effects in the lungs after inhalation [45].

In the second part of our comparative study we focused on the ability of metal ions and PM samples to hydroxylate dG towards 8-OHdG in aqueous buffer solutions (pH 7.4). We investigated different time reactions and we observed that incubation of metals with dG for 15, 30 min and 1 h. The 1 h duration reaction gave the maximum yield (results not shown) which is the preferred time reaction in other investigations [16,24].

The results of the hydroxylation reaction of dG showed the following order:

$$\mathrm{Fe(II)} >> \mathrm{V(IV)},\ \mathrm{Cr(III)} > \mathrm{Cu(I)} > \mathrm{Pb(II)} > \mathrm{Cd(II)} > \mathrm{Co(II)} > \mathrm{Ni(II)}$$

Preincubation of metal species, such as Fe(II), V(IV) and Ni(II), with equimolar concentrations of metal chelator EDTA-Na_2 in the reaction with dG resulted in inhibition of 8-OHdG formation (results not shown). Similar results were noted by Prahalad et al. [8] The preincubation of Fe(II) with EDTA before the addition of dG and H_2O_2 increased the yield of 8-OHdG but not as expected [8]. Colwell and Morris investigated extensively the Fe(II) + EDTA + H_2O_2 reaction, under various buffered conditions and order of addition of reagents into the mixture. Some important changes in the yield of 8-OHdG were observed. They concluded that 8-OHdG formation form the nucleoside dG via the Fenton reaction is highly variable depending on the reaction conditions and the binding ability of the metal [31].

Experimental evidence for oxidative damage by metal ions in different forms of DNA is obviously very different than naked nucleosides, such as dG. But, we have to consider experimental evidence which suggest that DNA has the capability of binding metal ions, and reaction with H_2O_2 at or close to the site of metal binding would account for the selective nature of hydroxyl radical damage. A recent paper observed that Fe(II) interacts with guanine at the N7 position site for metal binding [46]. Other studies suggested that site-selective nature of hydroxyl radical damage leads to different type of damages (double-strand breaks in DNA, intrastand cross-links and formation of 8-OHdG). The site of metal binding can be different, metals can bind to both DNA bases and phosphate groups. Metal ions that are poor DNA-binders, such as Ni(II) and Cr(VI) would be less likely to generate high concentration of hydroxyl radicals [13,47].

Consistent with metal ion-mediated dG hydroxylation, TSP, PM_{10} and $PM_{2.5}$ airborne particles, rich in soluble or ionizable metals, such as Fe, V and Cu, exhibited a dose-dependent increase in the formation of 8-OHdG. The DEP (diesel) sample showed higher hydroxylation activity compared to its concentration in Fe, V, Ni and Cu. These data might be explained if we consider that sampling "fresh" airborne particles contain higher proportion of metals in the lower valence state. The soluble and reduced metal ions vanadyl V(IV) and ferrous Fe(II) ions exhibited enhanced formation of hydroxyl radicals. GEP (gasoline) and woodsmoke showed the lowest ability for dG hydroxylation among the PM samples, but higher than their respective concentrations of soluble metal, when compared to TSP, PM_{10} and $PM_{2.5}$.

Our data suggest that particle size and metal composition have profound effects on their oxidant activity *in vitro* as measured by our assay and that it may be achieved by affecting solubility, offering catalyzing surfaces and binding abilities to DNA nucleosides, as was suggested by Shi et al. [24] The concentrations of metals of various PMs and their readox activities are important factors for a quantitative calculation of "total" oxidative reactivity, but the composition of airborne PM is much more complex. Particle's size and ability to adsorb metal ions, as well as proportion of insoluble metals and oxides of Fe (such as Fe_2O_3) make a quantitative estimation relatively difficult. Prahalad and co-workers [8,16] noted in their papers that, despite the high sensitivity of the analytical methods employed in the determination of hydroxylation of dG, coal and oil fly ash containing high concentrations of insoluble Fe (Fe_2O_3) failed to induce significant hydroxylation of dG [12,22]. They suggested that soluble Fe(II) is a very critical factor in dG hydroxylation reactions, soluble V(IV) plays a secondary role, whereas Ni(II) is a poor hydroxylation agent compared to Fe and V [8].

The hydroxylation of dG to form 8-OHdG adduct, is an intermediary leading after oxidation to the stable product of 8-oxo-2′-deoxyguanosine (8-oxodG). Although there are mixed references in various papers to both names, the 8-oxodG has become more common in molecular epidemiology studies which use the quantitative measurements 8-oxodG as an important biomarker of oxidative DNA damage and a risk assessment indicator of exposure to carcinogenic substances [48,49]. The importance of measurements of 8-oxodG has become a very important scientific issue. The European standard committee on oxidative DNA damage (ESCODD) was funded by the European Commission to examine the precision and accuracy of methods for measuring 8-oxodG and their methodological differences of 27 laboratories. Their results were published recently [50].

Taken together, experimental data support the conclusion that in the presense of H_2O_2 soluble and catalytically redox active metals in various samples of inhalable PM are mainly responsible for the formation of hydroxyl radicals. The second part of our experimental data showed that the hydroxylation at physiological pH of dG to form 8-OHdG adduct, [leading after oxidation to the stable product of 8-oxo-2′-deoxyguanosine] is proportional to the hydroxyl radical formation through a Fenton redox reaction of transition metals adsorbed in PM.

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