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Oxidative stress and DNA damage caused by the urban air pollutant 3-NBA and its isomer 2-NBA in human lung cells analyzed with three independent methods

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Abstract

The air pollutant 3-nitrobenzanthrone (3-NBA), emitted in diesel exhaust, is a potent mutagen and genotoxin. 3-NBA can isomerise to 2-nitrobenzanthrone (2-NBA), which can become more than 70-fold higher in concentration in ambient air. In this study, three independent methods have been employed to evaluate the oxidative stress and genotoxicity of 2-NBA compared to 3-NBA in the human A549 lung cell line. HPLC–EC/UV was applied for measurements of oxidative damage in the form of 8-oxo-2′-deoxyguanosine (8-oxodG), ^{32}P-HPLC for measurements of lipophilic DNA-adducts, and the Comet assay to measure a variety of DNA lesions, including oxidative stress. No significant oxidative damage from either isomer was found regarding formation of 8-oxodG analysed using HPLC–EC/UV. However, the Comet assay (with FPG-treatment), which is more sensitive and detects more types of damages compared to HPLC–EC/UV, showed a significant effect from both 3-NBA and 2-NBA. ^{32}P-HPLC revealed a strong DNA-adduct formation from both 3-NBA and 2-NBA, and also a significant difference between both isomers compared to negative control. These results clearly show that 2-NBA has a genotoxic potential. Even if the DNA-adduct forming capacity and the amount of DNA lesions measured with the ^{32}P-HPLC and Comet assay is about one third of 3-NBA, the high abundance of 2-NBA in ambient air calls for further investigation and evaluation of its health hazard.

Keywords: HPLC–EC/UV; ^{32}P-HPLC; Comet assay; 2-NBA; 3-NBA; Genotoxicity; 8-oxodG; Oxidation; Oxidative stress; DNA-adducts; DNA lesions; FPG-enzyme

1. Introduction

1.1. Oxidative damage

Oxidative stress has been defined “as a disturbance in the pro-/anti-oxidant balance in favor of the former” [1], leading to potential damage, i.e. the level of oxidizing agents, primarily reactive oxygen species (ROS), exceeds the anti-oxidant and DNA-repair capacity of the cells [2]. Analysis of sufficiently stable end-products from oxidation processes can give an estimate of the level of oxidative stress in cells [3]. An increased production of ROS upsets the redox-balance in the cells. ROS are thought to influence the development of cancer and more than 50 other human diseases, and it has been shown that many carcinogens induce the formation of 8-oxodG in liver and/or kidneys in animal studies [2,4]. Radical attack at the 8-position of guanine leads to the oxidation product from 2′-deoxyguanosine (dG), 8-oxodG, which is commonly used as a biomarker for oxidative stress [5,6]. 8-oxodG is pro-mutagenic and can induce a G:C to T:A transversion at DNA replication [7]. Elevated levels of 8-oxodG have been found in a number of disease states related to inflammation and oxidative stress [8,9].

1.2. HPLC–EC/UV method for analysis of oxidative damage

HPLC–EC/UV is a method for measurement of oxidative base damage to DNA. An extensive effort has been done

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within the ESCODD network (European Standards Committee on Oxidative DNA Damage) to optimize and validate different methods for measuring DNA damage [10,11]. GC–MS and HPLC–MS/MS both failed to measure low levels of 8-oxo-dG [12]. In addition, work-up procedures required by these methods elevate oxidation more than HPLC–EC/UV. The absolute background level of 8-oxodG is difficult to estimate because it is easily formed during the isolation and hydrolysis of DNA [2,13]. This artifactual oxidation has been shown to be responsible for overestimated levels of 8-oxodG and there is still no general agreement of basal levels of 8-oxodG in cellular DNA [12,14]. 8-oxodG is more easily oxidized than guanine and can be further oxidized into secondary oxidation products [2,15]. Due to different background levels of 8-oxodG it is difficult to compare results between different laboratories. It is more appropriate to compare data between a control and a treated sample analysed at the same occasion with the same method [5,12].

In order to reduce oxidation during sample preparations, chilled centrifuges as well as high purity chemicals and enzymes should be used and work-up performed on ice. Furthermore, the antioxidant deferoxamine mesylate, which is a Fe^{3+} chelator, also seems to reduce artifactual oxidation and Chelex 100 resin can be used to remove metal ions [13].

1.3. Single cell gel electrophoresis or Comet assay

The single cell gel electrophoresis method (Comet assay) has been evaluated at the International Workshop on Genotoxicity Test Procedures, where it was concluded that the most optimal method of choice was the alkaline (pH above 13) version [16]. The alkaline method is capable of detecting single-strand breaks (SSB), alkali-labile sites (ALS) DNA–DNA/DNA–protein cross-linking and SSB associated with incomplete excision repair sites. In addition, by using formamidopyrimidine-DNA glycosylase (FPG) treatment, the types of damage detected are increased: (i) open ring forms of 7-methylguanine, including 2,6-diamino-4-hydroxy-5-*N*-methylformamidopyrimidine and 4,6-diamino-5-amidopyrimidine, (ii) 8-oxoguanine, (iii) 5-hydroxycytidine, (iv) 5-hydroxyuracil, (v) aflaoxin-bound imidazole-ring-opened guanine and (vi) damage induced by amino compounds such as imidazole-ring-opened-*N*2-aminofluorene-C8-guanine [17–19].

1.4. ^{32}P-HPLC method for DNA-adduct detection

The reaction of carcinogens with DNA is one of the first events in the development of chemical carcinogenesis. Numerous studies have shown that certain agents covalently bind to DNA, thus inducing damages that may lead to a variety of lesions that alter cell functions [20]. Different kinds of methods have been developed for the study of lesions like DNA-adducts, including ^{32}P-post-labelling [21,22], immunoassays [23], fluorescence [24] and accelerated mass spectrometry (AMS) [25]. ^{32}P-post-labelling has a limit of detection (LOD) of DNA-adducts in the range of 0.01–1 fmol but require the handling of radioactive material. Immunoassays offer LOD in the range of 1–40 fmol, thus the lack of sensitivity and, more importantly, the specificity of antibodies can pose a problem [26]. Fluorescence methods with LOD between 1 and 20 fmol require substances with fluoresce and are highly specific for specific types of DNA-adducts, such as benzo[*a*]pyrene-diol-expoxide (BPDE) derived DNA-adducts [27]. AMS alone provides structural information and an even higher sensitivity (0.001 fmol) than post-labelling, but it requires the use of isotopically labeled substances that are specifically searched for during detection [26]. ^{32}P-postlabelling with HPLC-separation (^{32}P-HPLC) is a good method of choice for the detection of DNA-adducts. It yields DNA-adduct patterns of genotoxic substances and characterisations can be achieved by co-chromatography with standards, where the high resolution is beneficial especially for complex mixtures. ^{32}P-HPLC is based on the extraction of DNA, enzymatic digestion to nucleotides, enrichment of adducted nucleotides, enzymatic labelling with ^{32}P-phosphate, chromatographic separation of various adducted and non-adducted nucleotides, and detection of the radioactivity from the labelled compounds. ^{32}P-HPLC was developed in the early 1990s and proved to be somewhat less sensitive than ^{32}P-TLC with autoradiography. However, ^{32}P-HPLC is usually faster, with better separation, versatility and reproducibility compared to ^{32}P-TLC [28]. ^{32}P-HPLC, using only HPLC for separation and online detection, in contrary to variants using TLC for pre-separation and radioactivity measurement on collected eluent fractions, has since then been used to analyse many kinds of DNA-adduct samples, from both in vitro, in vivo and humans [22,29–31].

1.5. 3-NBA versus 2-NBA

3-NBA (Fig. 1A) is most likely activated by the reduction of its nitro group, forming highly reactive metabolites that bind to DNA [32]. The activation pathway of 3-NBA is quite similar to typical activation pathways of nitro-PAHs (Fig. 1B), which include CYP1A1/2A1 expressions and phase II enzymes such as *N*,*S*-tranferases [33,34]. Most of 3-NBA has been suggested to convert into 2-NBA in the atmosphere [35,36], but no toxicity data has been available for 2-NBA so far.

The objective of this study was to compare the two isomers 2-NBA and 3-NBA (Fig. 1A), by three independent methods measuring oxidative stress and genotoxicity. To establish the toxicity, if any, of 2-NBA as compared to its very potent genotoxic isomer 3-NBA three methods, HPLC–EC/UV, ^{32}P-HPLC and the Comet assay with FPG-enzyme treatment, were employed to look at specific damages in exposed A549 type II lung epithelial cells. The lung cells were chosen as an appropriate cell type since the primary exposure to 3-NBA and its isomer is presumed to be through the respiratory tract. The amount of cells needed for analysis by HPLC–EC/UV and ^{32}P-HPLC requires cells that divide fast

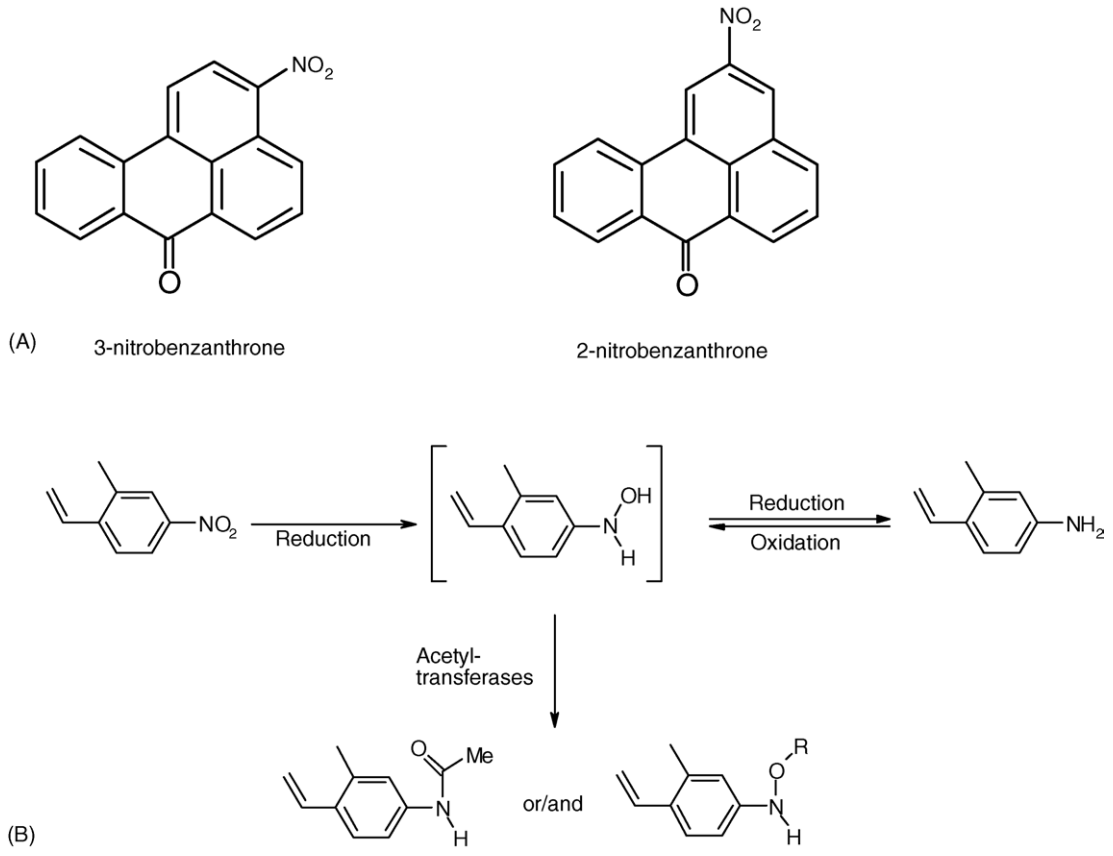

Fig. 1. (A and B) Structure of the two nitrobenazanthrone isomers 3-NBA and 2-NBA (A) and the most common metabolic pathway of nitrated aromatics (B).

and can be harvested in large amounts within a few days. Also, A549 cell lines are sufficient and established for evaluating the metabolic and oxidative processes from exposure to different substances, including nitro-PAHs [37,38].

2. Experimental

2.1. Reagents and chemicals

Reagents and enzymes used were purchased from the following sources: Nuclease P1 and Triton X-100 (Roche Diagnostics, Mannheim, Germany), alkaline phosphatase, deferoxamine mesylate and bovine serum albumin (BSA) (Sigma, St. Louise, MO, USA), RNAse A (from bovine pancreas) and spleen phosphodiestrase (SPD; Boehringer Mannheim GmbG, Mannheim, Germany), RNAse T1 (from *Aspergillus oryzae*) and micrococcal nuclease (MN; Sigma Chemical, Cleveland, OH, USA), *E. coli* formamidopyrimidine-DNA glycosylase (FPG; Nordic BioSite, Täby, Sweden), adenosine 5′-[γ-^{32}P] triphosphate (^{32}P-ATP; ^{32}P-activity of 3000 Ci/mmol; Amersham International, Little Chalfont, UK), dithiothreitol (DTT), Tricin® and HEPES (Merck, Darmstadt, Germany), polynucleotide kinase (PNK; USB, Cleveland, OH, USA), guanidine thiocyanate (GTC) (Fluka Chemie GmbH, Buchs, Switzerland), Chelex 100 resin (Bio-Rad Laboratories, Hercules, CA, USA). Trypsin-EDTA, Dulbeccos minimal essential medium (DMEM), foetal bovine serum (FBS), penicillin–streptomycin and MEM sodium pyruvate (Invitrogen Corporation, Karlsruhe, Germany). All chemicals were of analytical grade.

A549 cells originally obtained from the American Tissue Type Collection was kindly provided by Prof. Ian Cotgreave, the Karolinska Institutet, Department of Environmental Medicine, Stockholm, Sweden.

3-NBA, was synthesized and kindly provided by Prof. Hitomi Suzuki, Department of Chemistry, Graduate School of Science, Kyoto University, Kyoto, Japan and 2-NBA by Dr. Takeji Takamura Enya, Cancer Prevention Division, National Cancer Center Research Institute, Tsukiji, Tokyo, Japan.

Warning: The substances 2-NBA, 3-NBA, phenol and ^{32}P-ATP that were used in the experimental procedures are extremely hazardous and proper precautions and guidelines should be followed when handling and discarding the chemicals.

2.2. Test substances

The solid substances 3-NBA and 2-NBA were dissolved in DMSO to a concentration of 2 mM. Pure DMSO was used as a negative control. Cell exposure was performed by adding a volume of the solutes (2-NBA and 3-NBA) and DMSO,

which corresponded to 1% of the total medium volume and at the same time giving a final concentration of 20 μM of the test substances.

Dose–response was determined only for 3-NBA (since suspected to be the most toxic) at the concentrations of 2, 5, 10 and 20 μM. The cell exposure for 3-NBA was performed by adding 0.2, 0.5, 1 and 2 mM in DMSO to 1% volume of the total medium volume.

2.3. Cell culture

The A549 cells were cultured in DMEM supplemented with 10% FBS, 100 U/ml penicillin–streptomycin and 1 mM MEM sodium pyruvate in a 5% CO_2 atmosphere at 37 °C. Cells were seeded into dishes of 150 cm^2 for HPLC–EC/UV analysis, 75 cm^2 for ^{32}P-HPLC analysis and into 24 well plates for the Comet assay.

Exposure was carried out for 24 h. After exposure the cells were washed twice with sterile PBS and harvested by trypsination. 300, 150 and 80 μl (of which 60 μl was immediately removed) of Trypsine-EDTA (0.05%) × 10 PBS were added to the 150, 75 cm^2 and each well, respectively. Trypsination was performed for 5 min at 37 °C after which enzymatic activities were ceased by the addition of fresh supplemented DMEM. Cells were immediately placed on ice.

Cytotoxicity tests were performed on each harvested batch by Trypan Blue staining. On average 200 cells were counted. The amount of damaged cells out of the total was on average <10% (data not shown).

The concentration of 20 μM of the test substances was selected because pilot studies indicated that this concentration was the minimum required by the method least sensitive in detecting effects by 2-NBA and 3-NBA, and the maximum allowed by those most sensitive.

2.4. HPLC–EC/UV analysis of 8-oxodG

Work-up procedures were performed on ice and as quickly as possible to minimize artifactual oxidation. All aqueous solutions were treated by stirring with Chelex 100 resin for 1 h, to remove metal ions, and then filtered through a CN 0.45 μm filter.

2.4.1. Homogenization and nuclei preparation

Desferoxamine mesylate was added to the isotonic (0.25 M sucrose, 25 mM KCl, 5 mM $MgCl_2$, 2 mM tricine, pH 7.8) and Triton X-100 (0.5% Triton X-100, 20 mM Tris, pH 7.5) buffers to a final concentration of 1 mM, after which the buffers were kept on ice. Harvested cells were centrifuged (2300 × *g*) for 5 min at 4 °C and then the pellets were frozen at −80 °C until preparation. Cells were defrosted and the pellets were washed with PBS and centrifuged again for 6 min. The pellets were homogenized in 900 μl isotonic buffer using a tissue grinder with 25 full strokes. Additional 900 μl isotonic buffer was added and the homogenate was transferred into 2 ml Eppendorf tubes and centrifuged for 5 min (1500 × *g*). The supernatant was discarded and the crude nuclei pellets were washed with isotonic buffer and centrifuged once more. The pellets were then dissolved in 2 × 900 μl Triton X-100 buffer and centrifuged for 5 min (2000 × *g*) and the step was repeated once more.

2.4.2. DNA isolation

The crude nuclei pellets were dissolved in 1 ml 3 M GTC and kept on ice approximately 30 min after which occurring clusters were dissolved using a pipette. Additional 1 ml of 3 M GTC was added and each sample was transferred to a pre-centrifuged 15 ml PLG tube. Two milliliters Sevag was added to each tube and shaken gently for 2 min, followed by centrifugation (2300 × *g*) for 6 min. The Sevag step was repeated once more by adding additional 2 ml Sevag to the same PLG tubes. The upper phases were transferred to new 5 ml tubes and 2.0 ml isopropanol was added. DNA was precipitated at −20 °C for 15 min. The tubes were centrifuged (5500 × *g*) for 20 min. The pellets were washed with 2 ml 70% ethanol (−20 °C), and centrifuged again for 10 min.

2.4.3. DNA hydrolysis

DNA pellets were dissolved in 115 μl of 23 μM deferoxamine mesylate in Milli-Q water. The DNA was hydrolyzed enzymatically with 5 U Nuclease P_1 and 1 U alkaline phosphatase at 50 °C for 60 min (after 40 min occurring clusters were dissolved using a pipette). The DNA hydrolysate was transferred into a Micropure-EZ filter and the filters were centrifuged (14,000 × *g*) for 2 min. The DNA hydrolysates were stored at −80 °C until analysis.

2.4.4. HPLC–EC/UV analysis

The HPLC–EC/UV system consisted of a Waters 717 Autosampler (Waters, Milford, MA, USA), a Scantec 650 pump (Scantec, Partille, Sweden), an Opti-Guard C-18, 15 mm × 1 mm i.d. pre-column (Optimize, Portland, OR, USA), two serial reversed-phase DeltaPak 150 mm × 3.9 mm id, 5 μm 100 A main columns (Waters, Milford, MA, USA), a Coulochem II electrochemical detector (ESA, Chelmsford, MA, USA) for the detection of 8-oxodG and a Waters 486 absorbance detector (Waters, Milford, MA, USA) set to 290 nm for the detection of dG.

Prior to HPLC–EC/UV analyses, the HPLC system was washed (0.08 ml/min) with methanol and Milli-Q water (4:1) overnight and then re-equilibrated with HPLC eluent (methanol and 20 mM sodium acetate in Milli-Q water, 1:10, pH 5.3) at 30 ml/min for at least 30 min. The EC-cell was connected to the HPLC-system and the flow rate was elevated to 0.75 ml/min for approximately 30 min before injection.

8-oxodG and dG-standards were prepared by separate weightings and dilutions. The standards were frozen in 130 μl aliquots. New calibration curves for 8-oxodG and dG were created each day of analysis with an autosampler with an injected volume of 100 μl. Standards and samples were eluted for 20 min using 0.75 ml/min of the HPLC eluent. The EC-

chromatograms were smoothed using the Stavinsky-Golaz method.

The methods are described in detail elsewhere [13].

2.5. Single cell gel electrophoresis (Comet assay) analysis of DNA strand-breakes

Microscopic slides were pre-coated with 0.3% agar at least one day in advance. The harvested cells were centrifuged at (210 × *g*) for 3 min and the supernatant was removed. Cells were washed with 1 ml PBS, centrifuged again and all but 100 μl of the PBS was removed. Twenty-five microliters of the cell suspension were added to 150 μl 0.75% agarose (37 °C) and the mixture was spread over a microscope slide. When the gels hardened the slides were placed in lysis buffer (1% Triton X-100, 2.5 M NaCl, 10 mM Tris, 0.1 M EDTA, pH 10) for 1 h on ice in dark. Following this, DNA unwinding and washing were performed in the alkaline buffer (0.3 M NaOH, 1 mM EDTA) for 40 min, on ice in dark. Electrophoresis was then performed at ∼25 V for 30 min using the same alkaline buffer solution as in the previous step. After electrophoresis the slides were washed twice in 0.4 M Tris–HCl for 5 min and once in water for 5 min. The slides were dried over night, then fixated in methanol for 5 min. Slides were left to dry, then treated with EtBr solution (10 μg/ml in 1 × Tris acetate-EDTA (TAE)) for 5 min. Excess EtBr was washed away in pure 1 × TAE buffer for 5 min. Cover slips were placed over the slides for cell counting.

2.5.1. FPG-enzyme treatment

After the lysis treatment as explained above, the slides were placed in enzyme buffer (0.1 M KCl, 0.5 mM EDTA, 40 mM HEPES and 0.2 mg/ml BSA, pH 7.8) for 3 × 5 min. Following this 30 μl enzyme buffer or 30 μl FPG-enzyme (54.4 ng/μl) was added to each field on the enzyme slides. Incubation was performed in a humidity chamber at 37 °C for 30 min. Following this the slides were alkaline treated and electrophoresis was performed as previously described. The activity of FPG was tested by exposing cells to the enzyme in the presence of H_2O_2.

2.6. ^{32}P-HPLC analysis of DNA-adducts

2.6.1. DNA extraction

The harvested cells were centrifuged (2300 × *g*) for 5 min at 4 °C. The supernatant was discarded and pellets were DNA extracted by a standard phenol–chloroform methodology. Briefly, the cell pellets were suspended in 3 ml 1% SDS–EDTA buffer and 72 μl 1 M Tris–HCl, pH 7.4. The tissues were homogenized and treated with 72 μl RNAse A (10 mg/ml) and 24 μl RNAse T1 (5 U/μl) for 1 h at 37 °C. Then 180 μl protease (10 mg/ml) was added and the mixture was incubated for 1 h more at 37 °C. The mixture was then extracted with 1 vol phenol and centrifuged (2800 × *g*) for 10 min. The aqueous phase was then extracted with 1 vol phenol:Sevag and centrifuged as above. Following this, the aqueous phase was extracted again with 1 vol Sevag and centrifuged once more. The DNA was precipitated using ethanol and NaCl, and the DNA pellet was washed with 70% ethanol and centrifuged at 2800 × *g* for 5 min. After the pellet had dried, it was re-dissolved in water. DNA concentration and purity was determined with ultraviolet spectrometry. Aliquots of 10 μg DNA were taken out and then dried through evaporation and stored in a non-hydrolyzed form at −80 °C for further analysis.

2.6.2. DNA-adduct enrichment and ^{32}P-postlabelling

Briefly, aliquots of DNA were dissolved in Milli-Q purified water and digested by 4 μl micrococcal nuclease (0.2 U/μl) and SPD (1 mU/μl) with the total incubation time of 4 h at 37 °C.

The hydrolysed DNA and nucleotide samples were adduct-enriched by butanol extraction. The phase transfer agent 10 mM tetrabutyl ammonium chloride (TBA), together with 100 mM ammonium formate buffer (pH 3.5), facilitated the transfer of lipophilic adducts to the organic phase, while unmodified hydrophilic nucleotides remained in the water phase. The organic phase was then evaporated to dryness.

The butanol extracted samples were re-dissolved in water. A mixture of 0.25 μl 400 mM PNK buffer, 0.5 μl T4-PNK enzyme (0.5 U/μg DNA) and 1.8 μl (0.06 pmol/μg DNA) ^{32}P-ATP was added and the samples were incubated 30 min at 37 °C. After incubation the samples were stored at −20 °C until analysis.

2.6.3. DNA-adduct analysis

The ^{32}P-HPLC system consisted of a Waters 600 E pump (Waters, Milford, MA, USA), a Hichrom, RP 5-C18, K-100 guard column (Hichrom Ltd., Reading, UK), two serial reversed-phase DeltaPak 150 mm × 3.9 mm i.d., 5 μm 100 A main columns (Waters, Milford, MA, USA), a Packard 500 TR flow scintillation detector (Packard Instrument Co., Meriden, CT, USA). In short, labelled samples were diluted with ∼150 μl of Milli-Q water immediately before injection into the ^{32}P-HPLC system. A plateau gradient with a flow rate of 0.5 ml/min was used as follows: 0–19% of 87.5% acetonitrile:water, during 0–33 min in 2 M ammonium formate and 0.4 M formic acid (pH 4.5). A plateau followed holding this mixture for 15 min. Then the amount of 87.5% acetonitrile:water was increased to 40% during 48–90 min. Most of the polar compounds in the sample were separated by the guard column and removed by a switch valve, which was opened for 1 min after injection.

3. Results

3.1. HPLC–EC/UV analysis of 8-oxodG

Cells exposed to 3-NBA at the concentrations of 5, 10, 20 and 50 μM, using DMSO as negative control, showed no dose–response increase in oxidative damage, measured as 8-

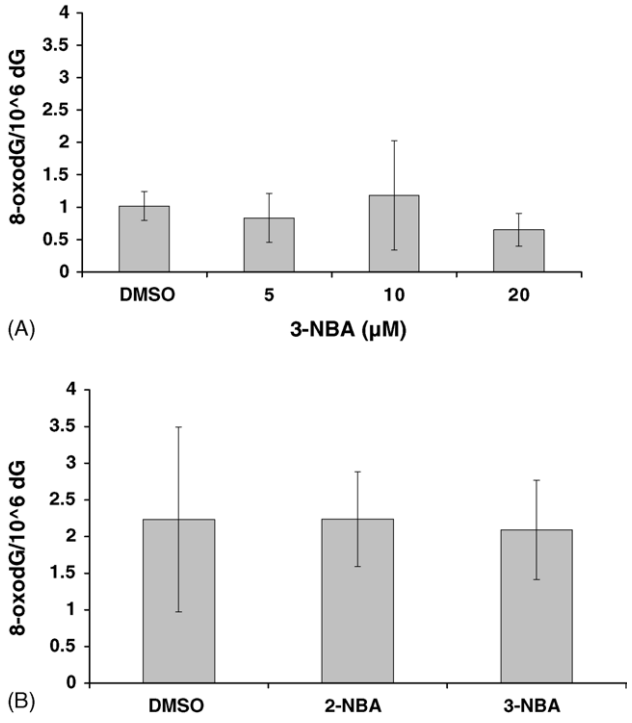

Fig. 2. (A and B) Oxidative damage from 3-NBA was not observed in a dose–response experiment of human A549 lung cells measured as the amount of 8-oxodG/10^6 NN using HPLC–EC/UV analysis (A). The bars represent the mean value of three repeated experiments. No significant differences were seen when 2-NBA and 3-NBA (20 μM) was compared to the DMSO control (B). The bars represent the mean value of five repeated experiments.

oxodG (Fig. 2A). The level of 8-oxodG/10^6 dG for the DMSO control was 0.87 ± 0.37 8-oxodG/10^6 dG, whereas for the highest dose of 50 μm it was 0.89 ± 0.46 8-oxodG/10^6 dG.

There was, in addition, no significant difference observed neither between the two isomers at 20 μM, nor when each isomer was compared to the DMSO control. The oxidative damage caused by DMSO was at a level of 2.23 ± 1.58 8-oxodG/10^6 dG and for the two isomers at 2.24 ± 2.17 and 2.09 ± 1.95 8-oxodG/10^6 dG for 2-NBA and 3-NBA, respectively (Fig. 2B).

3.2. Comet assay analysis of DNA strand-breaks

Dose–response was only performed on cells not treated with FPG-enzymes. The dose–response curve for 3-NBA showed linear increase of DNA damage with increasing concentrations (Fig. 3). There was, however, a slight decrease of the slope between 10 and 20 μM.

There was a significant difference between exposed cells treated with FPG-enzyme and those that were not, with for 3-NBA ($p < 0.01$) and 2-NBA ($p < 0.05$) and DMSO ($p = 0.05$) (Fig. 4A).

The difference between cells with or without FPG-treatment was calculated (Fig. 4B). Significant differences were found between cells exposed to DMSO and 2-NBA ($p < 0.05$), DMSO and 3-NBA ($p < 0.01$) and also between

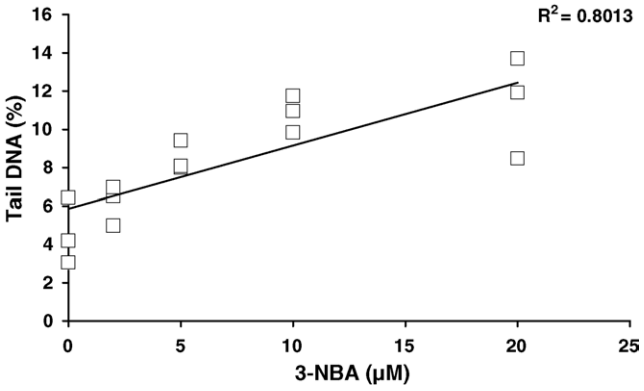

Fig. 3. Amount of DNA damage from 3-NBA in human A549 lung cells, measured as percent tail DNA by the Comet assay. DMSO was used as control. The linear regression in this dose–response curve was based on the mean value of three repeated experiments.

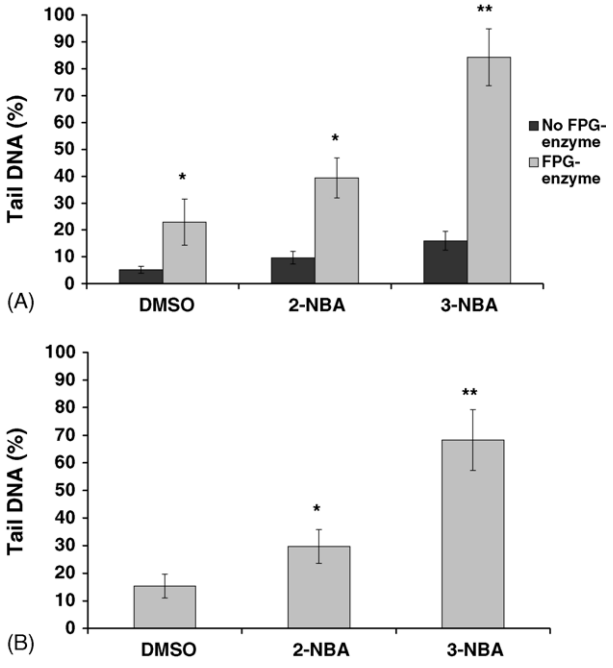

Fig. 4. (A and B) Amount of DNA damage from 3-NBA and 2-NBA compared to DMSO control in human A549 lung cells, measured as percent tail by the Comet assay with and without FPG-enzyme treatment (A). There was a significant difference in elevated damage without and with FPG-enzyme treatment for 3-NBA ($p < 0.01$) and 2-NBA ($p < 0.05$) treated cells, and a borderline significance for DMSO ($p = 0.05$). The bars represent the mean value of three repeated experiments. Each experiment in turn was based on three individual sets of exposed cells. Amount of DNA damage from 3-NBA and 2-NBA (20 μM) compared to DMSO control presented as the difference between the FPG-treated and non-treated cells (B). There was a significant difference between the cells treated with 2-NBA ($p < 0.05$) and 3-NBA ($p < 0.01$), compared to the DMSO control. There was also a significant difference between the two isomers ($p < 0.05$). The bars represent the mean value of three repeated experiments. Each experiment in turn was based on three individual sets of exposed cells.

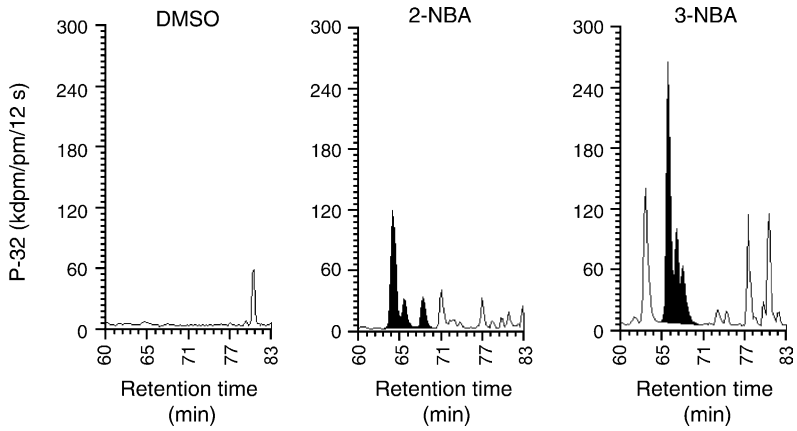

Fig. 5. DNA-adduct patterns from 3-NBA and 2-NBA (20 μM) compared to DMSO control, in human A549 lung cells, measured by ^{32}P-HPLC. The shaded peaks are the major and most prominent DNA-adducts for 2-NBA and 3-NBA, respectively.

2-NBA and 3-NBA ($p < 0.05$). The average percentage in tail DNA for DMSO was 15.4 ± 4.4, for 2-NBA 29.7 ± 6.1 and for 3-NBA 68.3 ± 11.0.

3.3. ^{32}P-HPLC analysis of DNA-adducts

3.3.1. Dose–response

The DNA-adducts selected for the dose–response curve were the major DNA-adducts found between 60 and 70 min (Fig. 5, shaded peaks), because of their stability with regard to changes in concentration and because no endogenous DNA-adducts were present in their vicinity. The dose–response curve with ^{32}P-HPLC was quite similar to that obtained by the Comet assay with respect to linearity and the slight decrease of the slope between 10 and 20 μM (Fig. 6A). The lowest concentration (2 μM) of 3-NBA produced DNA-adducts, which were significantly higher than the DMSO control, which produced on average 4 ± 1 DNA-adducts/10^8 normal nucleotides (NN). All other concentrations tested also gave significant increases in DNA-adduct formation.

3.3.2. 2-NBA versus 3-NBA and DNA-adduct formation

DNA-adducts after treatment with DMSO, 2-NBA and 3-NBA were summarized over a retention time range of 60–83 min (Fig. 6B). The DNA-adducts from both 2-NBA and 3-NBA were significantly higher ($p < 0.001$) than the DMSO control, which in this wider time range formed on average 18 ± 9 adducts/10^8 NN. There was also a significant difference between cells treated with 2-NBA and 3-NBA ($p < 0.005$). On average the amount of DNA-adducts produced by 3-NBA was about three times higher than that produced by 2-NBA.

There were several DNA-adducts from both 2-NBA and 3-NBA that co-chromatographed. However, at the retention times of 60.2, 64.2, 70.6 and 71.8 min, 2-NBA-specific DNA-adducts appeared; i.e. there was no interference with the other isomer. Similar DNA-adducts for 3-NBA were found at the retention times of 60.6, 61.8, 64.8, 67.0 and 77.0 min. At least 15 different DNA-adducts were detected after exposure to 2-NBA and 17 after exposure to 3-NBA. Even with the HPLC plateau method to improve separation, the main DNA-adducts of both 2-NBA and 3-NBA between 60 and 70 min did not separate (Fig. 5).

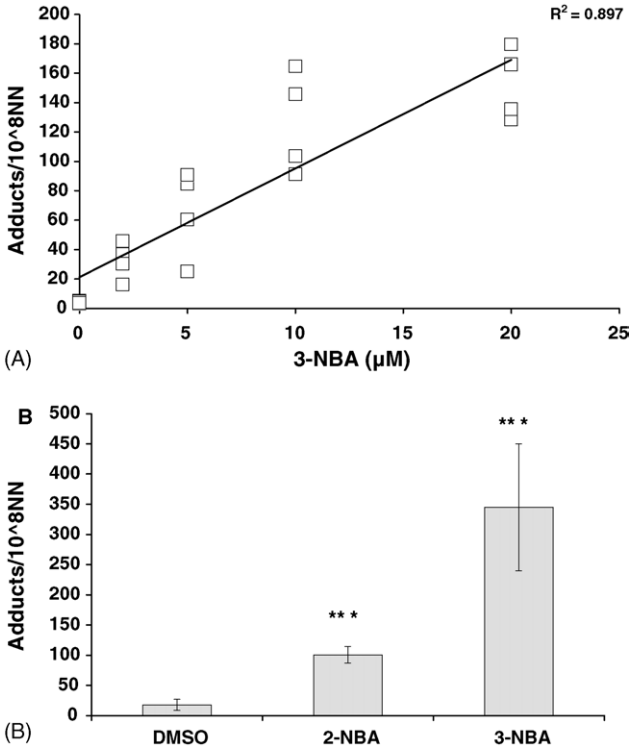

Fig. 6. (A and B) DNA-adducts from 3-NBA in human A549 lung cells, measured by ^{32}P-HPLC. A linear dose response was observed at different concentrations of 3-NBA. DMSO was used as control (A). The regression line is based on the mean value of four repeated experiments. The amount of DNA adducts from 3-NBA and 2-NBA (20 μM) compared to DMSO control, measured in the retention time range of 60–83 min (B). Both 2-NBA and 3-NBA differs significantly ($p < 0.001$) when compared to controls. In addition, there is a significant difference between the two isomers ($p < 0.005$). The bars represent the mean value of six repeated experiments.

4. Discussion

4.1. HPLC–EC/UV analysis

The background level of 8-oxodG/10^6 dG in human DNA is close to the detection limit and it may sometimes be hard to interpret the EC-chromatograms when the 8-oxodG peaks are difficult to distinguish from the background noise. During preparation, samples are susceptible to oxidation, which may elevate 8-oxodG/10^6 dG levels. It has also been suggested that analysis of too small amounts of tissue or cultured cells (<20 μg DNA obtained) generally result in a higher 8-oxodG/dG ratio [2,5]. Thus, results obtained from less than 20 μg of DNA have been excluded from the study since they were considered to be too uncertain. Preparation was performed in a way to minimize artifactual oxidation [13] and all results were within the interval of 0.3–4.2 8-oxodG/10^6 dG, which has been suggested to be the background level of oxidation in normal human cells [12]. There is still no general agreement on background level of 8-oxodG/dG in DNA, hence, it is more important to compare the results with controls used in the experiments, than comparing results between laboratories [5,12].

There was no dose–response for increasing levels of 3-NBA and no significant difference between either 2-NBA or 3-NBA when compared to the DMSO control (Fig. 2B). This points to that the main DNA damaging potentials of these nitrated isomers is work through specific oxidative processes that yield significantly increased levels of 8-oxodG. However, the redox state of the cell and the availability of transition metal ions also influence different types of DNA damage [9,39]. 8-oxodG is also more easily oxidized than dG itself and can be further oxidized, which would not be detected with the EC detection [2,15,40]. Oxidative DNA damage can occur without any changes in 8-oxodG or even with a fall in the level of 8-oxodG/10^6 dG [2]. Some reactive species that attack DNA only produce a minor amount of DNA base damage. Therefore measurement of 8-oxodG may fail to reveal other products of oxidative DNA base damage that is shown with the Comet assay [9,39]. Although 8-oxodG is frequently used as a biomarker for oxidative stress, the results are more reliable if several methods are employed simultaneously.

4.2. The Comet assay (SCGE) analysis

The Comet assay confirmed the genotoxic potential of both 2-NBA and 3-NBA (Fig. 4A and B). Both with and without FPG enzyme treatment, it shows that 3-NBA is two times as potent as 2-NBA in inducing DNA damage detected by the Comet assay (Fig. 4B). There was a significant difference between the DMSO control and the isomers ($p < 0.05$), and also between the two isomers ($p < 0.05$) (Fig. 4B), which is similar to what was observed with ^{32}P-HPLC (Fig. 6B).

FPG-enzyme treatment measures not only oxidative damage on DNA, but a variety of other lesions as well [17–19]. It is reasonable to assume that both 2-NBA and 3-NBA produce a variety of damage, in fact, not even 8-oxodG oxidation can be entirely ruled out, since the Comet assay is more sensitive then the HPLC–EC/UV. Thus, the results from HPLC–EC/UV and the Comet assay with FPG-enzyme are not necessarily contradictory. It has been reported that in the case of peroxynitrite, the levels of 8-oxodG were low and even dropped, whereas other base oxidations increased [15]. Further, the FPG-enzyme probably does not recognize all oxidized bases in compact DNA [41]. FPG-enzyme treatment may also reveal apoptosis, which generates comets [41]. However, each harvested batch of cells was tested with Trypan Blue staining to monitor the viability of the cells. During a pilot dose–response study, the only time elevated levels (>10%) of stained cells were observed was at the 50 μM concentration, which is why experiments at this concentration were not performed. The average amount of stained cells was otherwise between 6 and 10% (data not shown).

Compared to the HPLC–EC/UV method, the Comet assay requires less material and artifactual oxidation is less critical, since DNA is not purified. It is also generally more sensitive than the HPLC–EC/UV method [42].

4.3. ^{32}P-HPLC analysis

The wide range of DNA-adducts, more than 15, seen in this study probably depends on different activation pathways. Several experiments have been conducted to elucidate the reaction pathways of nitrated polyaromatic hydrocarbons, such as 3-NBA. Most findings support the hypothesis that the primary step in activation is the reduction of 3-NBA to 3-aminobenzanthrone (3-ABA), then further oxidation and involvement of phase II enzymes give rise to intermediates that can produce acetylated and non-acetylated adducts [33,43].

Early studies have suggested that reduction of nitroheterocycles in A549 cells occurs mainly through the catalysis by NADPH-requiring enzymes [44]. It has been shown that NADPH:P450 reductase, which is expressed in bronchial and bronchiolar epithelium, alveolar lining cells and alveolar macrophages, does reduce nitrated xenobiotica and thereby activate it [45,46]. Thus, the first step in activation of 2-NBA and 3-NBA in the A549 cells could be through the reduction by NADPH:P450 reductase. The following oxidation steps create reactive intermediates. In hamster lung fibroblasts the strongest expression, due to exposure to 3-NBA, has been observed for CYP1A1, -1A2, -2B6 and -2D6, although others have been seen as well (CYP1B1, -2A6, -2C9, -2E1, -3A4) [33]. The induction of CYP1A1 and -1B1 has been observed in A549 cells treated with different kinds of nitrated polyaromatic hydrocarbons [37]. However, the induction of CYP1A2 was not detected in that study, most likely because in humans this enzyme is said to be liver-specific [47] and the expression of CYP1A2 is exceptionally low, if not absent, in tissue-derived cell lines [48]. Therefore, it is difficult to compare results obtained from different cell lines.

One major DNA-adduct from 3-NBA has been suggested to be dG-N-Ac-ABA [49]. This has been contradicted by a

study, which showed that this DNA-adduct did not form in vivo in rats [50]. In a pilot study, where we compared both dG-N-Ac-ABA and dA-N-Ac-ABA standards to 3-NBA DNA-adduct patterns using comparative chromatography (HPLC), no peak-alignments were seen. However, when comparison was performed on 3-ABA and 3-NBA exposed A549 cells, one prominent 3-ABA derived adduct did align with one of the peaks in the major adduct cluster between 65 and 70 min seen in Fig. 3 (data not shown). This supports previous finding that 3-NBA is reduced to 3-ABA, then followed by further oxidative reactions [33]. Pathways for 2-NBA may be quite similar to those for 3-NBA, but the structural features of its metabolites needs to be further investigated.

2-NBA is significantly less potent than 3-NBA as a genotoxin (Fig. 6B). However, it has been reported that ambient atmospheric concentrations of 2-NBA were 70-fold higher then 3-NBA, whereas measurements at the diesel source showed quite the opposite [35,36]. This suggests that emissions may undergo atmospheric reactions, which is important to take into account. Elevated exposure to 3-NBA due to occupation has been reported [51] and our findings show that 2-NBA does give rise to significant levels of DNA-adducts. Thus, even if 2-NBA produces "only" one third of the DNA-adduct amount compared to 3-NBA, its abundance does urge for further investigation of whether it has a significant impact on health.

5. Conclusion

Neither 3-NBA, nor 2-NBA gave rise to oxidative DNA-damage in the form of elevated levels of 8-oxodG measured by HPLC–EC/UV. On the other hand, the Comet assay gave a strong increase in FPG-sensitive sites for both isomers, which can be oxidative stress-related. In addition, ^{32}P-HPLC have confirmed the genotoxicity of both 3-NBA and 2-NBA through the detection of a variety of DNA adducts. Human exposure to 3-NBA is known, hence it is close at hand to assume that human exposure to 2-NBA occurs as well. It has been shown that 3-NBA is more abundant than 2-NBA near the emission source, but it is quite the opposite in ambient air. In fact, 2-NBA can be more than 70-fold higher in concentration in ambient air. Thus, even if 2-NBA is somewhat less genotoxic than 3-NBA, it is nonetheless important and relevant to evaluate its impact on health.

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