Radical mechanisms in nitrosamine- and nitrosamide-induced whole-genome gene expression modulations in Caco-2 cells.

TOXICOLOGICAL SCIENCES **116(1)**, 194–205 (2010)
doi:10.1093/toxsci/kfq121
Advance Access publication April 19, 2010

Radical Mechanisms in Nitrosamine- and Nitrosamide-Induced Whole-Genome Gene Expression Modulations in Caco-2 Cells

Dennie G. A. J. Hebels,[1] Jacob J. Briedé, Roongnapa Khampang, Jos C. S. Kleinjans, and Theo M. C. M. de Kok

Department of Health Risk Analysis and Toxicology, Maastricht University, Maastricht 6200 MD, The Netherlands

[1]To whom correspondence should be addressed at Department of Health Risk Analysis and Toxicology, Maastricht University, PO Box 616, Universiteitssingel 50, 6200 MD Maastricht, The Netherlands. Fax: 31-43-3884146. E-mail: d.hebels@grat.unimaas.nl.

Received March 22, 2010; accepted April 15, 2010

N-nitroso compounds (NOCs) may be implicated in human colon carcinogenesis, but the toxicological mechanisms involved have not been elucidated. Because it was previously demonstrated that nitrosamines and nitrosamides, representing two classes of NOC, induce distinct gene expression effects in colon cells that are particularly related to oxidative stress, we hypothesized that different radical mechanisms are involved. Using electron spin resonance spectroscopy, we investigated the radical-generating properties of genotoxic NOC concentrations in human colon adenocarcinoma cells (Caco-2). Cells were exposed to nitrosamides (N-methyl-N′-nitro-N-nitrosoguanidine and N-methyl-N-nitrosourea) or nitrosamines (N-nitrosodiethylamine, N-nitrosodimethylamine, N-nitrosopiperidine, and N-nitrosopyrrolidine). Nitrosamines caused formation of reactive oxygen species (ROS) and carbon-centered radicals, which was further stimulated in the presence of Caco-2 cells. N-methyl-N-nitrosourea exposure resulted in a small ROS signal, and formation of nitrogen-centered radicals (NCRs), also stimulated by Caco-2 cells. N-methyl-N′-nitro-N-nitrosoguanidine did not cause radical formation at genotoxic concentrations, but at increased exposure levels, both ROS and NCR formation was observed. By associating gene expression patterns with ROS formation, several cellular processes responding to nitrosamine exposure were identified, including apoptosis, cell cycle blockage, DNA repair, and oxidative stress. These findings suggest that following NOC exposure in Caco-2 cells, ROS formation plays an important role in deregulation of gene expression patterns that may be relevant for the process of chemical carcinogenesis in the human colon, in addition to the role of DNA alkylation.

Key Words: **nitrosamines; nitrosamides; N-nitroso compounds; free radicals; toxicogenomics; colon carcinogenesis.**

INTRODUCTION

N-nitroso compounds (NOCs) have long been known to be mutagenic in bacterial mutagenicity studies and carcinogenic in test animals (Lijinsky, 1992), which has lead to the classification of dozens of NOC as potential human carcinogen (U.S. Department of Health and Human Services, 2005). NOCs have been found in the environment and certain types of food, such as beer, fish, and nitrite-preserved meat (Tricker and Preussmann, 1991), but can also be formed endogenously by nitrosation of dietary precursors, a process that occurs predominantly in the gastrointestinal tract (Kuhnle *et al.*, 2007; Mirvish, 1995; Sen *et al.*, 2000). Although a direct link with human carcinogenicity is still lacking, NOCs have been suggested to play a role in the development of several types of cancers, including those of the brain, head and neck, gastric, and colorectal cancer (Knekt *et al.*, 1999; Lijinsky, 1992).

NOC can be divided into two main subclasses, nitrosamines and nitrosamides (Preussmann and Eisenbrand, 1984), which are both capable of inducing alkylating DNA damage by formation of a highly reactive diazonium ion (Mirvish, 1995; Tricker and Preussmann, 1991). O^6-alkylguanine adducts, e.g., have been identified as the main premutagenic lesion and cause GC-AT transition mutations (Mirvish, 1995). Cyclic NOCs may also form similarly reactive cyclic oxonium ions, capable of causing heterocyclic DNA adducts (Young-Sciame *et al.*, 1995). There are, however, important differences in the modes of action between nitrosamines and nitrosamides. Whereas nitrosamides can spontaneously decompose to form a diazonium or oxonium ion, nitrosamines require metabolic activation through α-hydroxylation by several cytochrome P450 (CYP) enzymes (Mirvish, 1995).

The genotoxic aspects of NOCs have been studied in detail, but data on gene expression modulation associated with these effects were, until recently, lacking. In a recent toxicogenomics study, we provided a detailed analysis of gene expression changes in the human colon adenocarcinoma cell line Caco-2 following exposure to comparable genotoxic concentrations of six NOC, and this revealed strong differences in transcriptional responses between nitrosamides and nitrosamines (Hebels *et al.*, 2009). An interesting observation was the induction of several oxidative stress– and radical scavenging–related pathways and gene groups by the nitrosamines, whereas this response was absent following nitrosamide exposure. These transcriptomic responses suggest a difference in the radical-generating capability

between nitrosamines and nitrosamides. Although both classes of compounds are known to be capable of inducing oxidative stress, the molecular mechanisms may be different (Arranz *et al*., 2007; Bai *et al*., 2007; Mabrouk *et al*., 2002). Several studies have reported the formation of short-lived radicals from both nitrosamines and nitrosamides by liver microsomes, and upon UV radiation or presence of Fenton reagent, however, only under nonphysiological conditions and in the absence of a cellular system (Bai *et al*., 2007; Floyd *et al*., 1978; Grover *et al*., 1987; Hiramoto *et al*., 2002). The available data suggest that nitrosamines and nitrosamides form different types of radicals, but it is uncertain whether these two classes of compounds differ with respect to the type of radical formation in a cellular system.

Based on the strong discrimination in gene expression modifications between nitrosamides and nitrosamines following exposure in the human Caco-2 cell line as found in our previous study, we hypothesize that these two classes of NOCs display a difference in the radical-generating ability. To investigate this, we performed electron spin resonance (ESR), also known as electron paramagnetic resonance (EPR), spectroscopy on Caco-2 cells exposed to six NOCs (Fig. 1) that have been used frequently in literature: the nitrosamides N-methyl-N′-nitro-N-nitrosoguanidine (MNNG) and N-methyl-N-nitrosourea (MNU) and the nitrosamines N-nitrosodiethylamine (NDEA), N-nitrosodimethylamine (NDMA), N-nitrosopiperidine (NPIP), and N-nitrosopyrrolidine (NPYR). These nitrosamines have been demonstrated to occur in the colon, whereas MNU can be formed under gastric conditions and subsequently reach the colon (Sen *et al*., 2000; Tricker and Preussmann, 1991). MNNG is widely used as a model nitrosamide compound, and although it is less relevant in human exposure, it has been used to induce malignant transformation of human colonic epithelial cells (Paraskeva *et al*., 1990). For all compounds, we applied concentrations that induced comparable levels of genotoxicity in the comet assay, to detect differences in radical generation at doses known to result in distinct gene expression patterns (Hebels *et al*., 2009). In addition, equimolar NOC concentrations were tested to determine cellular differences at equal doses. Furthermore, we hypothesize that NOC-induced radical generation is associated with changes in the gene expression pattern. To test this, gene expression modifications at two time points (1 and 24 h) were separately associated with ESR data to functionally anchor specific pathways to the formation of radicals during the exposure period and to investigate the development of the gene expression response over time. Because the Caco-2 cell line is widely used as an *in vitro* model for the human colon, this study will clarify the cellular radical-generating properties of NOCs that may be relevant for human colorectal cancer development.

MATERIALS AND METHODS

Cell culture. The human colon adenocarcinoma cell line Caco-2 was cultured in Dulbecco's Modified Eagle's Medium (Sigma-Aldrich, Zwijndrecht, The Netherlands) with 4.5 g/l glucose, L-glutamine, $NaHCO_3$, and pyridoxine HCl supplemented with 1% (vol/vol) nonessential amino acids, 1% Na-pyruvate, 1% penicillin/streptomycin, and 10% (vol/vol) heat-inactivated fetal calf serum, all purchased from GIBCO BRL (Breda, The Netherlands). Cell cultures were transferred weekly by trypsinization and incubated at 37°C in a humidified incubator containing 5% CO_2.

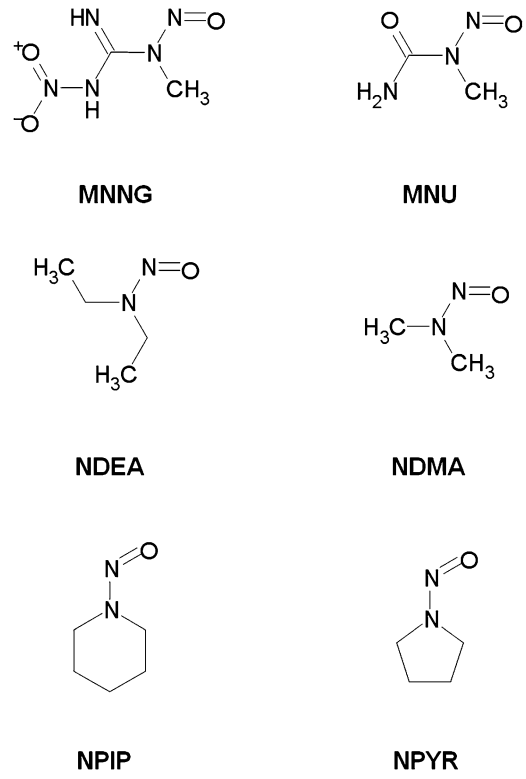

FIG. 1. Molecular structures of the two nitrosamides and four nitrosamines used in this study.

Chemicals and exposure concentrations. Caco-2 cells were exposed to six NOCs, i.e., MNNG, MNU, NDEA, NDMA, NPIP, and NPYR, or the corresponding solvent control (MilliQ for the nitrosamines or dimethyl sulfoxide [0.1%] for the nitrosamides). All NOCs were obtained from Sigma-Aldrich (the respective product numbers and reported purity were 15427LO [97%], N4766 [11% H_2O, 3% acetic acid], N0756, N7756, N6007 [99.8%], and 158240 [98.9%]). Cells were exposed to comparably genotoxic concentrations, as determined by comet assay analysis described previously (Hebels *et al*., 2009), or equimolar concentrations (1mM). The comparably genotoxic concentrations were 1μM (MNNG), 1mM (MNU), 50mM (NDEA), 100mM (NDMA), 40mM (NPIP), and 100mM (NPYR). Solutions of the spin trap 5,5-dimethyl-1-pyrroline-N-oxide (DMPO; Sigma-Aldrich) diluted in nitrogen-flushed MilliQ water were further purified by charcoal treatment. Stock concentrations of DMPO were determined spectrophotometrically by using the extinction coefficient $\varepsilon = 7700/M/cm$. H_2O_2 (35%, wt/vol) was obtained from Merck Sharp & Dohme (Haarlem, The Netherlands).

ESR spectroscopy measurements. For ESR experiments Caco-2 cells were plated out 3 days before and grown to 90–100% confluence in 20-cm^2 culture dishes. To guarantee comparability of individual experiments, cell numbers were in the same range for all dishes ($3.38 \pm 0.32 \times 10^6$ cells per dish). At the start of the experiments, dishes were washed twice with Hank's Balanced Salt Solution (HBSS; GIBCO BRL), followed by incubation of the cells in 2-ml 50mM DMPO in HBSS in a CO_2 incubator at 37°C for 30 min. This was followed by adding the required amount of a comparably genotoxic or equimolar dose of MNNG, MNU, NDEA, NDMA, NPIP, or NPYR to the HBSS and incubation for 30 min. This incubation time was found to result in

the highest DMPO-radical adduct concentration. Longer incubations led to lower signals because of the intracellular depletion of DMPO as the decay of the DMPO-radical adduct does not result in the regeneration of free DMPO. Cells were subsequently harvested by scraping and homogenized by pipetting. For each sample, a 100-µl glass capillary (Brand, Wertheim, Germany) was filled with the suspension and sealed. After sealing, the capillary was immediately placed in the resonator of the ESR spectrometer. During exposure and measurement of samples, light exposure was kept to a minimum. ESR spectra were recorded at room temperature on a Bruker EMX 1273 spectrometer equipped with an ER 4119HS high-sensitivity resonator and 12 kW power supply operating at X band frequencies. The modulation frequency of the spectrometer was 100 kHz. Instrumental conditions for the recorded spectra were as follows: magnetic field, 3450 G; scan range, 200 G; modulation amplitude, 1 G; receiver gain, 1×10^4; microwave frequency, 9.85 GHz; power, 50 mW; time constant, 40.96 ms; scan time, 20.97 s; and number of scans, 35. Quantification of the spectra was performed by peak surface measurements through double integration of the ESR spectrum using the WIN-EPR spectrum manipulation program (Bruker BioSpin, Wormer, The Netherlands). Spectra were created at identical intensity scales to enable visual comparison of the different conditions in each figure. Number of cells and viability were determined by trypan blue exclusion analysis. Viability (> 90%) was not significantly different from control samples. All experiments were performed in triplicate, and statistical analysis was performed using an unpaired two-tailed *t*-test with a 95% confidence interval.

Microarray hybridization and data analysis. Gene expression modifications, following a 1- and 24-h exposure to comparably genotoxic NOC concentrations, were measured to investigate the link with radical formation found after 30 min. The 1-h microarray hybridizations (two biological replicates) were hybridized against Agilent 4x44K Whole Human Genome microarrays (Agilent Technologies, Amstelveen, The Netherlands), as described previously (Hebels *et al.*, 2009). The biological replicates of the NOC and reference samples were dye swapped in the hybridization protocol.

After scanning the microarray slides, using settings described before (Hebels *et al.*, 2009), the data were flagged manually and automatically using the GenePix Pro software (version 6.0; Molecular Devices, Sunnyvale, CA). Following a quality control in the statistical software environment R (version 2.10.1; The R Foundation for Statistical Computing, Vienna, Austria), regions of lower quality were identified and subsequently flagged in Spotfire DecisionSite (version 9.1; TIBCO, Somerville, MA). LOWESS normalization and subsequent data analysis was performed in ArrayTrack (version 3.4; National Center for Toxicological Research, Jefferson, AR). The expression difference for each spot was calculated by subtracting the $\log_2$-transformed mean intensity of the control sample from the $\log_2$-transformed mean intensity of the treated sample, resulting in a $\log_2$ ratio. Gene expression data, following a 24-h exposure period were taken from our previous study (Hebels *et al.*, 2009).

Gene expression data from both the 1- and 24-h exposures were subsequently used to perform correlation analyses with reactive oxygen species (ROS) levels. Ratios of ROS versus their respective controls were first calculated and subsequently $\log_2$ transformed to obtain a data distribution similar to the $\log_2$ gene expression values. Spearman's rank correlations between gene expression and levels of ROS were calculated using the online Gene Expression Profile Analysis Suite (http://gepas.bioinfo.cipf.es/). Significantly correlating genes (p value < 0.05) were subsequently analyzed using MetaCore (GeneGo, San Diego, CA). MetaCore is an online software suite that identifies and visualizes the involvement of differentially expressed genes in specific cellular pathways, which is subsequently related to the total amount of genes involved in the particular pathway and in all the available pathways combined. This results in a set of significantly modulated pathways (p value < 0.05).

The gene expression data discussed in this publication have been deposited in National Center for Biotechnology Information's Gene Expression Omnibus (GEO) and are available through GEO Series accession numbers GSE14284 (for 24-h data) and GSE20993 (for 1-h data), accessible via http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE14284 and http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE20993.

RESULTS

Radical Generation at Genotoxic Concentrations

To investigate the difference in radical-generating properties between nitrosamides and nitrosamines in a cellular system, we first tested the effects of these compounds after exposure of Caco-2 cells to genotoxic concentrations for 30 min. DMPO was used as a spin-trapping agent because it is widely used for the detection of ROS-induced ESR signals and can also pick up carbon- and nitrogen-centered radicals (CCRs and NCRs, respectively), which have been shown to be formed in previous studies (Floyd *et al.*, 1978; Grover *et al.*, 1987).

Figure 2 shows representative ESR signals induced by NDEA under different conditions. In the presence of Caco-2 cells, NDEA induces a characteristic four-line 1:2:2:1 ESR signal with a hyperfine splitting constant (HFSC) of $a_N = a_H = 14.7$ G (Fig. 2C), identified as a ROS-induced DMPO-OH signal (Rosen and Klebanoff, 1979), and a NOC radical consisting of a clear six-line pattern ($a_N = 15.4$ and $a_H = 23.6$, Fig. 2C). Identical signals were generated after exposure with the other three nitrosamines (results not shown). As further explained in the "Discussion" section this six-line pattern is indicative of a trapped CCR (Floyd *et al.*, 1978). Although there is also a DMPO-OH and NOC radical pattern visible in the incubation without cells (Fig. 2A), incubation with cells clearly increases the signal intensity, indicating a stimulating effect in a cellular system. Caco-2 cells also display a small DMPO-OH signal spontaneously (Fig. 2B). The induction of nitrosamine-induced cellular ROS formation was confirmed in control experiments with H_2O_2 (Fig. 2D), showing a characteristic ROS signal.

Exposure of Caco-2 cells for 30 min to nitrosamides resulted in different ESR spectra as compared with those induced by the nitrosamines. MNU displays a more complex signal, which consists of a DMPO-OH signal, and an additional 18-line MNU-derived NOC radical spectrum, which is visible after incubation both with and without cells (Figs. 3A and C). Non-NOC–exposed solvent control cells only displayed a small background DMPO-OH signal (Fig. 3B). A spectrum was simulated using HFSCs reported in literature (Grover *et al.*, 1987) for MNU following UV light exposure to identify the NOC radical. We included a DMPO-OH signal with the simulated spectrum, which resulted in a composite simulated pattern (Fig. 3D) similar to the results obtained from MNU after cellular exposure (Fig. 3C). As discussed later on, the NOC radical detected is probably an NCR. MNNG on the other hand only displayed a small DMPO-OH signal, but this does not appear to be higher as compared with the solvent control (Fig. 3E). An NOC radical, as observed with MNU, was not present.

Altogether, the ESR study showed that at comparable genotoxic concentrations, nitrosamines and nitrosamides strongly differ with respect to radical generation. In Caco-2

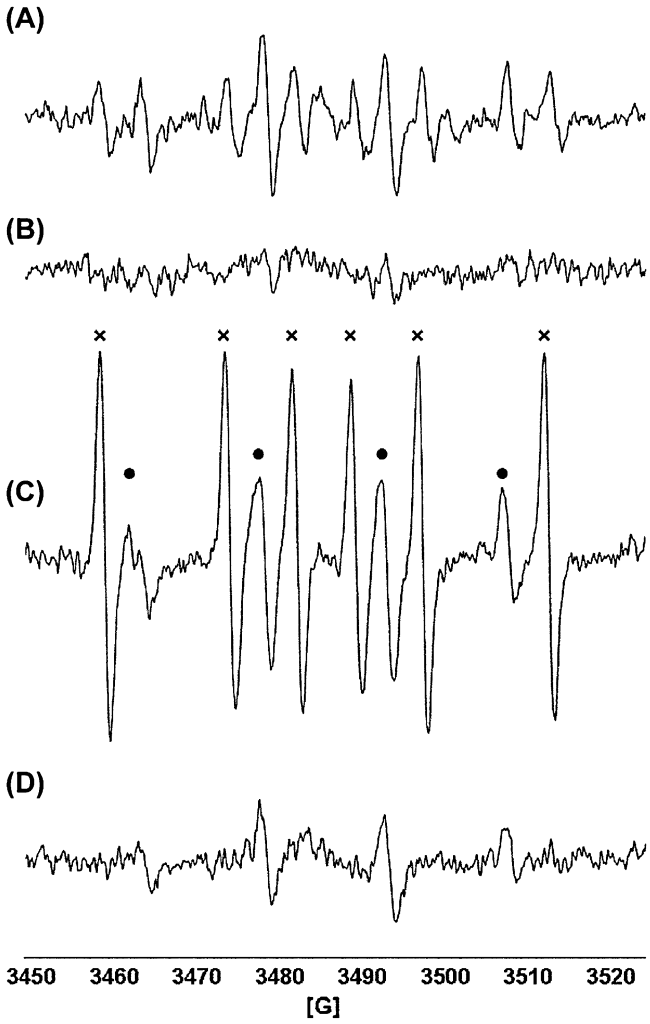

FIG. 2. ESR spectra recorded after a 30-min incubation with 50mM NDEA and 50mM DMPO without cells present (A), or incubation in the presence of DMPO pretreated Caco-2 cells with solvent control (B), with 50mM NDEA (C) or with 0.5mM H_2O_2 (D). Identical signals were generated after exposure with the other three nitrosamines. Representative spectra of triplicate experiments are shown. Spectra are shown at identical intensity scale; cross symbols, DMPO-trapped CCR signal; bullet symbols, DMPO-trapped ROS signal.

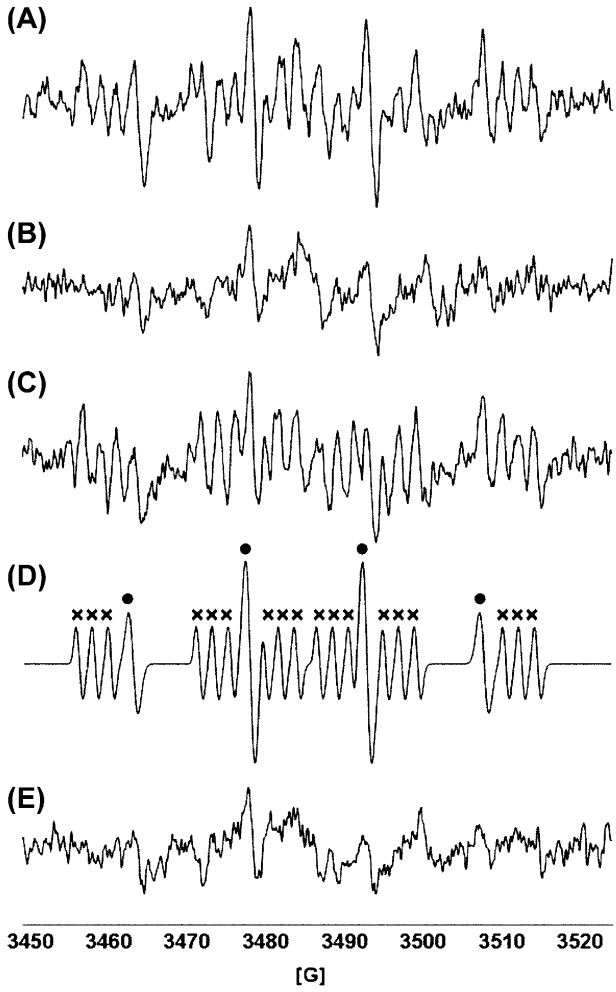

FIG. 3. ESR spectra recorded after a 30-min incubation with 1mM MNU and 50mM DMPO without cells present (A), or incubation in the presence of DMPO pretreated Caco-2 cells with solvent control (B), with 1mM MNU (C), a simulation of an MNU-derived NCR (a_{NO} = 15.1 G, a_N = 2.0 G, and a_β^H = 23.4 G) in combination with ROS (DMPO-OH, $a_H = a_N$ = 14.7 G) (D), and incubation in the presence of DMPO pretreated Caco-2 cells with 1μM MNNG (E). Representative spectra of triplicate experiments are shown; cross symbols, DMPO-trapped NCR signal; bullet symbols, DMPO-trapped ROS signal.

cells, all nitrosamines and MNU induced significant ROS formation (Fig. 4). Presence of cells significantly increases DMPO-OH levels following nitrosamine exposure. Within the nitrosamine group, NDEA, NPIP, and NPYR induce a significantly higher ROS production than NDMA. Without cells, no ROS formation above normal background levels was detected. MNNG, in the presence of cells, displays no apparent increase in ROS production compared with its control.

In addition to ROS formation, a compound-specific NOC radical was detected following nitrosamine exposure (Table 1). NOC radical levels were significantly increased in the presence of cells, again indicating a cellular stimulating effect. The NOC radical formed from MNU was also significantly higher in the presence of cells. No NOC radical signals were found following MNNG exposure.

Radical Generation at Equimolar Concentrations

We subsequently tested all six NOCs at an equimolar concentration to investigate differences in radical formation between these compounds at similar doses. All compounds were tested at a 1mM exposure concentration for 30 min, which corresponded with an increase in concentration from genotoxic levels for MNNG and a decrease for the four nitrosamines. This concentration was selected because it can be expected that MNNG will generate a detectable ESR spectrum at this concentration, similar to the other nitrosamide, MNU. In addition, nitrosamide concentrations higher than 1mM are

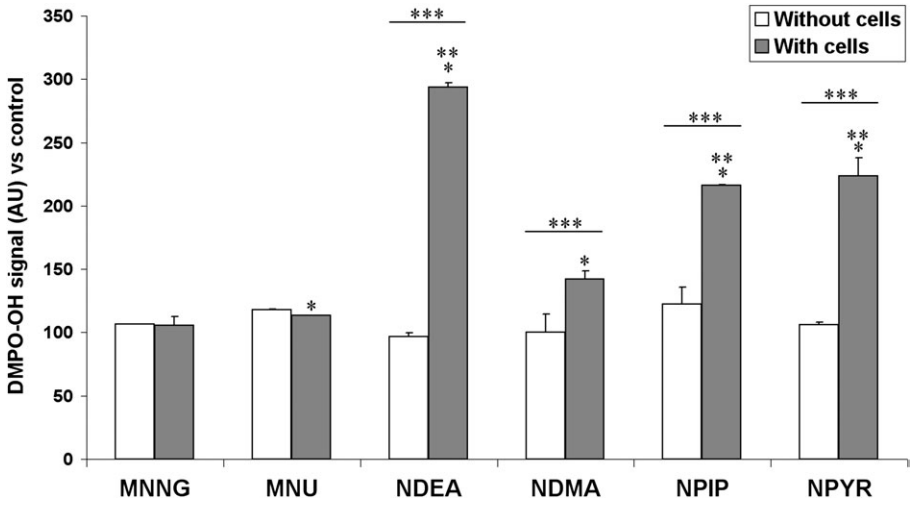

FIG. 4. Intensities of the ESR-detected ROS formation after 30-min incubations in the presence or absence of Caco-2 cells and relative to solvent control levels. *Significantly higher versus solvent control level (p value < 0.01), **significantly higher versus NDMA ESR signal (p value < 0.001), ***significantly higher versus without cells condition (p value < 0.05), AU = arbitrary units.

likely to result in cytotoxicity, which needs to be avoided. At 1mM, all four nitrosamines no longer displayed the spectrum seen at genotoxic concentrations (Fig. 5A). Only a small DMPO-OH signal was present, which was not significantly different from control cells (Fig. 2B). MNNG on the other hand showed a 16-line ESR spectrum (Fig. 5B). Because the spectrum for MNU at 1mM turned out to be a composite signal from a compound-specific NOC radical (identified as an MNU NCR) and DMPO-OH signal, we used the same approach to simulate a spectrum for MNNG. Based on HFSCs reported by Grover *et al.* (1987) for an MNNG-derived NCR, we could simulate a comparable composite signal including a DMPO-OH signal (Fig. 5C). ROS and NOC radical formation at an equal dose of 1mM of the nitrosamides is shown in Table 2. ROS formation is significantly higher following MNNG incubations compared with MNU.

Gene Expression Correlation Analysis

We subsequently investigated changes in the gene expression profile during incubation that can be attributed to the formation of radicals, by measuring the strength of the gene expression response at 1 and 24 h of exposure. Because the ESR experiments require measurements to be performed at the beginning of the exposure because of instability of the spin-trapped adduct, we first tested whether NOCs were still present in a sufficient amount after 24 h of exposure to continue the formation of radicals. NDMA, a model nitrosamine and the most volatile NOC, was shown to be present at 90% of the original concentration after 24 h. To link differences in ROS formation induced by nitrosamides and nitrosamines, with transcriptomic responses, we performed Spearman's rank correlation analyses with gene expression modulations across the six compounds in two separate analyses for two different time points (1 and 24 h). Significantly correlating genes (p value < 0.05), i.e., genes whose $\log_2$ expression ratios across the six compounds correlate with the induced ROS levels by these compounds, were subsequently used for a pathway analysis in MetaCore. Because the NCRs and CCRs identified by ESR analysis all represent compound-specific radicals, and, thus, are different at the molecular level, gene expression correlation analyses were not performed with NOC radical levels.

Table 3 shows a selection of cellular processes that are correlated with ROS levels following NOC exposure at

TABLE 1
Intensities of the ESR-Detected Compound-Specific NOC Radicals in the Presence or Absence of Caco-2 Cells after 30-min Incubations at Genotoxic Concentrations

	MNNG-derived NCR	MNU-derived NCR	NDEA-derived CCR	NDMA-derived CCR	NPIP-derived CCR	NPYR-derived CCR
Without cells	ND	1.28 ± 0.003	1.30 ± 0.04	0.57 ± 0.08	1.07 ± 0.18	0.61 ± 0.002
With cells	ND	1.86 ± 0.02[a,b]	2.71 ± 0.45[a,b]	1.53 ± 0.24[a,b]	2.11 ± 0.04[a,b]	2.20 ± 0.34[a,b]

Note. Values represent arbitrary units ($\times 10^6$). ND, not detectable.
[a]Significantly higher versus solvent control level (p value < 0.01).
[b]Significantly higher versus without cells condition (p value < 0.05).

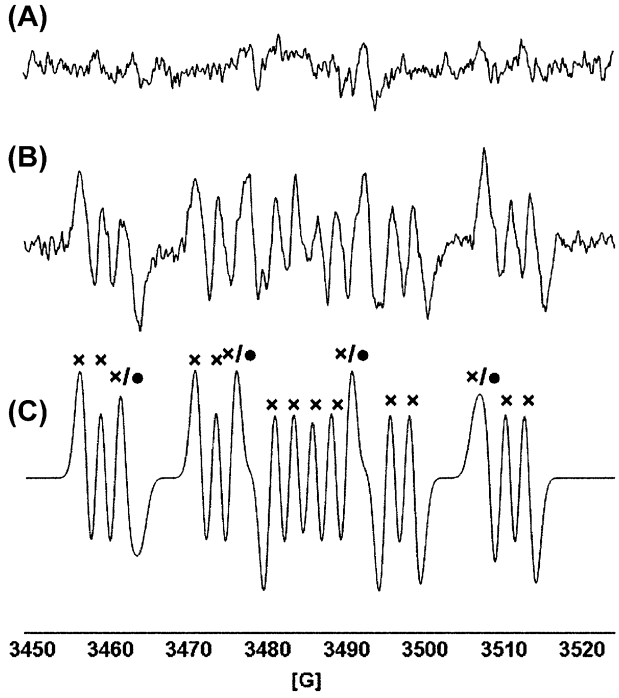

FIG. 5. ESR spectra recorded after a 30-min incubation in Caco-2 cells at an equimolar concentration of 1mM. As exemplified here by the spectrum of NDEA (A), none of the nitrosamines displayed a signal different from solvent control cells (Figure 2B). The pattern displayed by MNNG (B) matches a simulation of an MNNG-derived NCR (a_{NO} = 15.1 G, a_N = 2.5 G, and a_β^H = 22.1 G) together with ROS (DMPO-OH, $a_H = a_N$ = 14.7 G) (C). Representative spectra of triplicate experiments are shown; cross symbols, DMPO-trapped NCR signal; bullet symbols, DMPO-trapped ROS signal.

genotoxic concentrations. Affected cellular processes after 1 h of exposure include apoptosis, cell cycle regulation, DNA repair, and the oxidative stress response. Because the number of significantly affected genes for each pathway was relatively small at this time point, the expected biological effect for the cellular processes was difficult to assess. At 24 h mostly the same biological processes were found to be modulated as after 1 h. The effects were more pronounced, though, as demonstrated by the higher number of significantly modulated genes, allowing for the direction of the expected biological effects (up- or down-regulation) to be determined for most processes, although the modulated pathways contributing to these processes were different. The total number of gene expression modifications found to correlate with ROS levels observed at the beginning of the exposure period (30 min.), was also higher after 24 h, corresponding with approximately 23% of the data set (vs 7% at 1 h). Analogous to the significantly higher ROS levels that we found following nitrosamine exposure compared with nitrosamides, at 24 h, modulated pathways were only found to be associated with nitrosamine exposure. A full overview of modulated pathways can be found in the Supplementary Excel file. Among the most commonly affected cellular processes were apoptosis, cell cycle regulation, nucleotide metabolism, and cytoskeleton remodeling. Apoptosis

TABLE 2
ROS Formation and Compound-Specific NOC Radicals in Caco-2 Cells after 30-min Incubations at an Equimolar Concentration versus Control Levels

	ROS level		MNNG-derived NCR	MNU-derived NCR
	MNNG	MNU		
Solvent control	0.58 ± 0.02	0.55 ± 0.003	ND	ND
NOC exposed	3.02 ± 0.47[a,c]	0.62 ± 0.001[a]	3.04 ± 0.61[a]	1.86 ± 0.02[a]

Values represent arbitrary units ($\times 10^6$). ND, not detectable.
[a]Significantly higher versus solvent control level (p value < 0.01).
[c]Significantly higher MNNG-induced ROS level compared with MNU.

was mainly stimulated, whereas cell cycle progression was inhibited during the G1/S phase transition and mitosis. Changes in nucleotide metabolism are indicative of a DNA replication block and modifications of the nucleotide turnover. The cytoskeleton remodeling pathways can be linked to cytoskeletal rearrangements associated with the processes of apoptosis, cell cycle progression, and cell movement and adhesion. In addition, DNA repair and oxidative stress pathways were affected. In most cellular processes, all four nitrosamines were involved, except for the apoptosis and oxidative stress processes, which were not modulated by NDMA. Furthermore, a number of immune response, G-protein signaling, and developmental pathways were modified (Supplementary data), indicating modulations in the inflammatory response, G-protein–controlled processes, and cell differentiation.

DISCUSSION

Although the radical-generating capacity of NOCs has received some attention (Arranz *et al.*, 2006, 2007; Kumaraguruparan *et al.*, 2005; Mabrouk *et al.*, 2002), no studies have been performed to establish radical formation processes in a cellular system. Our recently performed toxicogenomics study demonstrated the importance of radical processes in cellular responses to NOC exposures, with nitrosamines and nitrosamides showing different transcriptomic profiles (Hebels *et al.*, 2009). In the present study, we established for the first time differences between nitrosamines and nitrosamides with respect to radical generation in a cellular system.

Investigating cellular radical formation at comparably genotoxic concentrations, all four nitrosamines displayed a similar ESR spectrum, showing both a typical DMPO-OH and a compound-specific NOC radical signal (Figs. 2C and 4 and Table 1). The generation of DMPO-OH adducts is indicative of ROS formation, which can lead to oxidative DNA damage. It has indeed been demonstrated that apart from the induction of alkylating DNA damage, oxidative damage is

TABLE 3
A Selection of Affected Cellular Processes Significantly Modulated by a Set of ROS-Correlating Genes Found after MetaCore Pathway Analysis at 1 and 24 h of NOC Exposure

1-h Exposure		Genes in Pathway[a]		24-h Exposure		Genes in Pathway[a]	
Modulated Pathways[b]	NOC[c]	Significant	Total	Modulated Pathways[b]	NOC[c]	Significant	Total
Apoptosis (stimulation of apoptotic process)[d]							
Apoptotic TNF-family pathways	4	2	41	Beta-2 adrenergic receptor anti-apoptotic action	3/6	7	15
DNA damage–induced apoptosis	3	2	15	HTR1A signaling	3/6	12	38
p53-Dependent apoptosis	3	4	29	BAD phosphorylation	3/6	11	35
EPO-induced Jak-STAT pathway	2	3	35	EPO-induced Jak-STAT pathway	3/6	11	35
p53 Signaling pathway	3	8	39				
Cell cycle regulation (cell cycle block in S and G2 phase)							
ESR1 regulation of G1/S transition	1/3	6	32	Spindle assembly and chromosome separation	5/6	11	32
Regulation of G1/S transition (part 2)	3	5	26	Initiation of mitosis	3/5	9	25
Role of SCF complex in cell cycle regulation	3	3	29	Regulation of G1/S transition (part 1)	3/5/6	12	38
p53 Signaling pathway	3	8	39	Transition and termination of DNA replication	4	7	26
Cell adhesion (diverse effects related to apoptosis and cell cycle regulation)[d]							
Cadherin-mediated cell adhesion	6	5	26	Integrin-mediated cell adhesion and migration	3/5/6	20	45
Endothelial cell contacts by junctional mechanisms	6	3	26	Histamine H1 receptor signaling in the interruption of cell barrier integrity	3/4/5/6	16	37
Ephrins signaling	3	5	41	Integrin inside-out signaling	5	11	44
Histamine H1 receptor signaling in the interruption of cell barrier integrity	1	8	37	Chemokines and adhesion	3	22	93
PLAU signaling	3	3	38				
Role of tetraspanins in the integrin-mediated cell adhesion	6	6	37				
Cytoskeleton remodeling (diverse effects related to apoptosis and cell cycle regulation)[d]							
CDC42 in cellular processes	6	3	22	TGF, WNT, and cytoskeletal remodeling	3/4/5/6	35	107
Cytoskeleton remodeling	3	12	96	Cytoskeleton remodeling	3/5/6	31	96
Integrin outside-in signaling	3/6	8	46	Keratin filaments	4/5	10	36
Keratin filaments	1/3/6	9	36	Reverse signaling by ephrin B	3/4/5	9	30
RalA regulation pathway	6	2	26	Neurofilaments	3/4/5/6	12	25
Reverse signaling by ephrin B	3/4/6	9	30	Role of PKA in cytoskeleton reorganization	3/5/6	13	31
Role of activin A in cytoskeleton remodeling	3	2	20	Regulation of actin cytoskeleton by Rho GTPases	3/4/5	10	23
Role of PKA in cytoskeleton reorganization	3/4	6	31	Role of activin A in cytoskeleton remodeling	3	7	20
TGF, WNT, and cytoskeletal remodeling	3	14	107	FAK signaling	3/5/6	16	47
				Integrin outside-in signaling	3/5/6	14	46
				Slit-Robo signaling	3	10	30
				Alpha-1A adrenergic receptor–dependent inhibition of PI3K	3/5/6	5	12
DNA damage repair (stimulation of DNA repair)[d]							
ATM/ATR regulation of G1/S checkpoint	1/3	4	32	NHEJ mechanisms of DSBs repair	3/4/5/6	10	19
Brca1 as a transcription regulator	1/3	6	30	Role of Brca1 and Brca2 in DNA repair	4	6	30
DNA damage–induced apoptosis	3	2	15				
p53 Signaling pathway	3	8	39				
Nucleotide metabolism (DNA replication block/DNA repair)							
TTP metabolism	2	3	38	GTP-XTP metabolism	3/4/5/6	22	59
				CTP/UTP metabolism	3/4/5/6	23	65
				ATP/ITP metabolism	3/4/5/6	28	85
				dCTP/dUTP metabolism	3/5/6	16	46
				dATP/dITP metabolism	3/5/6	18	54
				TTP metabolism	3/5	12	38
				dGTP metabolism	3/5	12	38

TABLE 3—*Continued*

1-h Exposure		Genes in Pathway[a]		24-h Exposure		Genes in Pathway[a]	
Modulated Pathways[b]	NOC[c]	Significant	Total	Modulated Pathways[b]	NOC[c]	Significant	Total
				Oxidative stress (stimulation of anti-oxidant response)[d]			
Delta-type opioid receptor–mediated cardioprotection	2/3	5	25	Delta-type opioid receptor–mediated cardioprotection	3/5/6	8	25
(L)-selenoaminoacids incorporation in proteins during translation	2/3/4/5/6	2	36	Role of ASK1 under oxidative stress	3/5/6	9	22
				Development (diverse effects)			
25 Pathways involved (see Supplementary data)	1/2/3/4/6			24 Pathways involved (see Supplementary data)	3/4/5/6		
				G-protein signaling (diverse effects)			
6 Pathways involved (see Supplementary data)	1/2/3			7 Pathways involved (see Supplementary data)	3/5/6		
				Immune response (diverse effects)			
22 Pathways involved (see Supplementary data)	1/2/3/4/6			7 Pathways involved (see Supplementary data)	3/4/5/6		

Note. BAD, bcl-2-associated death promoter; DSB, double-strand break; EPO, erythropoietin; FAK, focal adhesion kinase; NHEJ, non-homologous end joining; PI3K, phosphoinositide-3-kinase; PKA, protein kinase A; PLAU, plasminogen activator, urokinase; SCF, skp, cullin, f-box containing; TGF, transforming growth factor; TNF, tumor necrosis factor; WNT, wingless-type MMTV integration site family.

[a]Significantly modulated genes (p value < 0.05) versus total number of genes in pathway.

[b]Significantly modulated (p value < 0.05).

[c]NOC involved denoted by 1 (MNNG), 2 (MNU), 3 (NDEA), 4 (NDMA), 5 (NPIP), and 6 (NPYR).

[d]Expected effects (between parentheses) of cellular processes are only based on pathways found after 24 h.

an important second type of DNA lesion that may be induced by nitrosamine exposure (Aiub *et al*., 2009; Arranz *et al*., 2006). The origin of ROS following nitrosamine exposure is most likely related to their metabolic activation by CYP enzymes (Fig. 6A) (Bartsch *et al*., 1989; Lewis, 2002), and particularly CYP2E1, known to be involved in nitrosamine metabolism (Kushida *et al*., 2000), is capable of forming ROS by redox cycling (Lewis, 2002).

The NOC radical signal we detected was identified as originating from a CCR, and its formation has been suggested to involve a short-lived intermediate in the α-hydroxylation step (the α-nitrosamino radical, Fig. 6A) (Bartsch *et al*., 1989; Wade *et al*., 1987; Yang *et al*., 1994). Indeed, formation of CCRs from nitrosamines was observed following incubation of nitrosamines with rat liver microsomes, indicating a CYP enzyme–catalyzed process being responsible (Floyd *et al*., 1978). Moreover, UV light–induced release of $^{\bullet}$NO (Grover *et al*., 1987), which shares similarity with the denitrosation pathway (Fig. 6), results in the formation of CCRs with similar spectra. We now show that this CCR is also formed upon cellular exposure to nitrosamines. The α-nitrosamino radical can subsequently either dealkylate or denitrosate. The latter metabolic route is usually considered a nongenotoxic alternative (Keefer *et al*., 1987). Because all four nitrosamines used in this study are metabolized via hydroxylation of the α-carbon, the α-nitrosamino radical DMPO adducts we measured gave similar HFSCs, which explains the analogous spectra we found for the nitrosamines.

The observation that higher levels of both ROS and compound-specific CCRs are formed in the presence of Caco-2 cells (Fig. 4, Table 1), strongly suggests the involvement of CYP metabolism, known to be present in this cell line (Borlak and Zwadlo, 2003). In the absence of cells, spontaneous formation of CCRs by nitrosamines was also observed, albeit at significantly lower levels (Fig. 2A, Table 1). Nitrosamines have been shown to spontaneously denitrosate at low levels, which is similar to UV light–induced denitrosation (Grover *et al*., 1987; Hiramoto *et al*., 2002; Wink and Desrosiers, 1991). UV light–induced formation of CCRs, as described by Grover *et al*. (1987), cannot be completely ruled out, although precautions were taken to minimize light exposure. Trace amounts of iron or other transition metals could also have resulted in a Fenton-induced degradation of nitrosamines (Heur *et al*., 1989; Wink and Desrosiers, 1991). Cells lacking strong CYP activity could therefore also be exposed to the CCR, which could either denitrosate or dealkylate after reacting with a hydroxyl radical.

At a lower concentration of 1mM, no ROS formation above control levels was observed for nitrosamines and CCRs were entirely absent from the spectra (Fig. 5A), clearly indicating that radical formation is nitrosamine concentration dependent and detected only at concentrations that are genotoxic in the comet assay.

The results we found after nitrosamide exposure were quite different from the nitrosamines. At comparably genotoxic doses, both MNU and MNNG produced a small amount of ROS above control levels in Caco-2 cells, although this was only significant

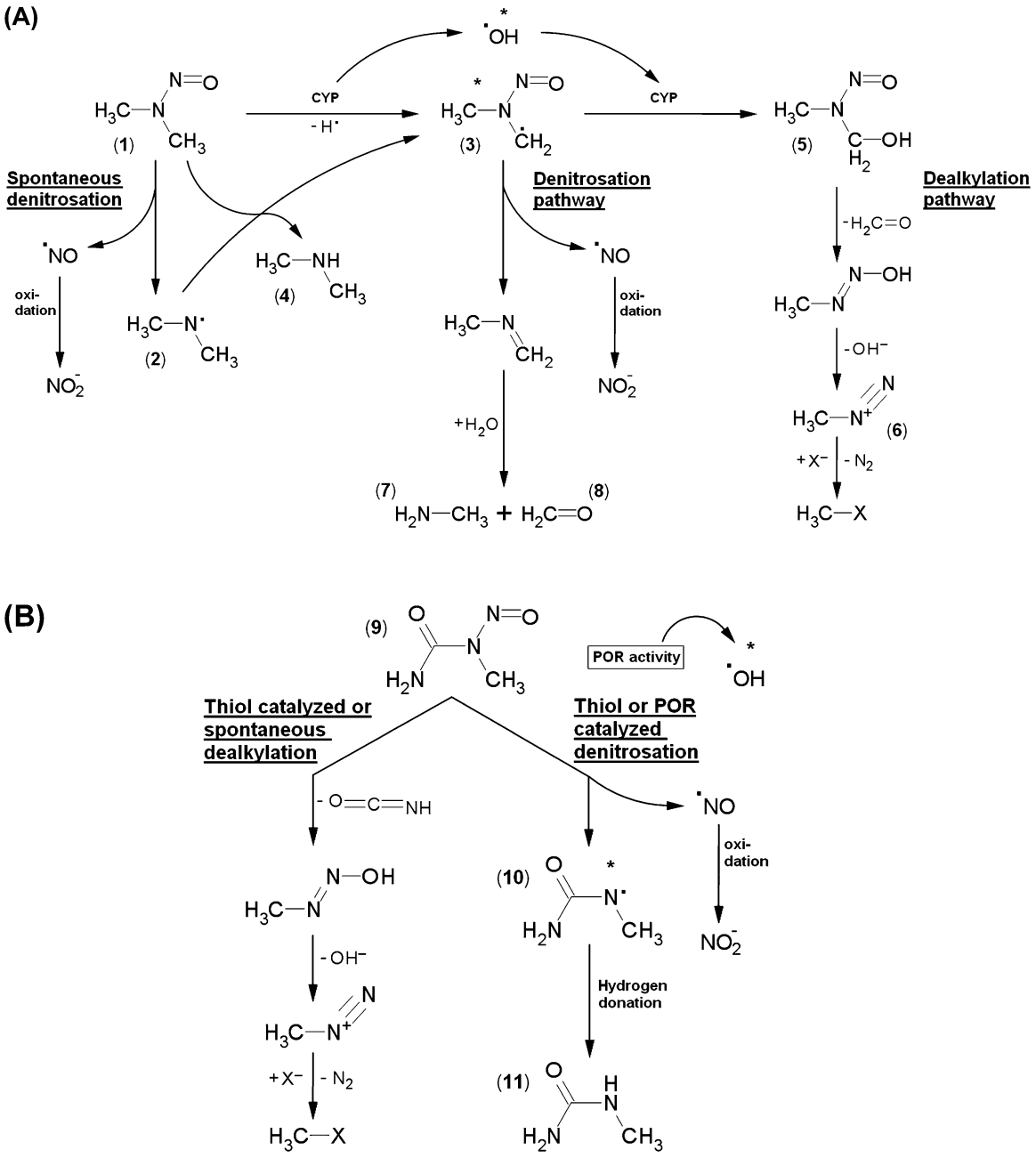

FIG. 6. (A) Metabolism of nitrosamines (shown for NDMA (1) as an example). Spontaneous denitrosation gives rise to the alkylaminyl radical (2), which can react with the parent compound, resulting in the formation of the α-nitrosamino CCR (**3**) and the corresponding amine (4). During CYP metabolism, hydroxyl radicals ($^{\bullet}$OH) and CCRs are formed, which can recombine in the dealkylation pathway and form the α-hydroxynitrosamine (5) which decomposes into the highly reactive diazonium species (6) that can damage nucleophilic compounds (X^{-}), such as DNA. Alternatively, denitrosation can occur, which gives rise to $^{\bullet}$NO, methylamine (7) and formaldehyde (8). (B) Metabolism of nitrosamides (shown for MNU (9) as an example). Thiol- or CYP oxidoreductase–catalyzed denitrosation results in the formation of an NCR (10), which subsequently produces the corresponding amide (11). Alternatively, dealkylation can occur (thiol catalyzed or spontaneous) similar to the nitrosamine dealkylation pathway. Asterisk indicates radicals trapped by DMPO in this study.

for MNU (Fig. 4). At a higher dose of 1mM (equimolar to MNU), MNNG produced an ROS signal in cells that was significantly higher than control levels and also higher than MNU-induced ROS levels (Fig. 5B and Table 2). MNU and MNNG are both known to cause oxidative stress in animal models and cellular systems (Bai *et al.*, 2007; Mikuni and Tatsuta, 2002; Prater *et al.*, 2007), and it appears that this ROS formation is dependent on cellular components such as glutathione and the hypoxanthine-xanthine-oxidase system, which may explain the ROS levels seen in the Caco-2 cell incubations.

Incubation of cells with MNU at a genotoxic concentration also generated a compound-specific NOC radical (Fig. 3C). We propose that this could be an NCR (the amidyl radical, Fig. 6B), which was also found after UV irradiation and the concomitant denitrosation of MNU (Grover *et al.*, 1987). Although absent at a genotoxic concentration of 1μM, at 1mM, MNNG also generates such a radical (Fig. 5B). The formation of these radicals has also been suggested by others (Nagata *et al.*, 1972), and simulations of DMPO-trapped amidyl radicals and DMPO-OH indeed resulted in a composite spectrum that exactly fits our detected spectra (Figs. 3D and 5C). The observation that cells enhance the formation of this NCR (Table 1) suggests a stimulating effect of cells for nitrosamide exposure as well. Although nitrosamides do not require metabolic activation by CYP enzymes to become genotoxic, nitrosamides are known to react with thiols, such as glutathione, which can result either in denitrosation or dealkylation (Fig. 6B). The latter is similar to the spontaneous decomposition of nitrosamides. The presence of cellular thiols could therefore catalyze the formation of amidyl radicals. The biological relevance of these radicals is questionable, because they would normally recombine with the hydrogen residue of glutathione to form a harmless amide. Moreover, Romert *et al.* (1991) have shown that under physiological conditions, the thiol-mediated denitrosation pathway plays a relatively small role and metabolism is mostly directed toward dealkylation. Additionally, thiol-catalyzed denitrosation appears only relevant for MNNG, not MNU (Jensen, 1983). Another explanation for the cellular stimulation of NCR formation from nitrosamides is via microsome-dependent denitrosation, which may occur as a result of CYP oxidoreductase activity and is augmented by CYP (Jensen *et al.*, 1990; Potter and Reed, 1983). Activity of these enzymes could also contribute to the DMPO-OH signal observed following cellular nitrosamide exposure. Denitrosation of nitrosamides is considered as a nongenotoxic metabolic route and is unlikely to result in significant damage to cellular components, although the concomitant ROS formed could lead to oxidative damage. Because the genotoxicity of nitrosamide and nitrosamine concentrations in our study was similar, and the denitrosation route for nitrosamides appears less important for DNA damage formation, the genotoxic effect of nitrosamides is most likely related to DNA alkylation. It is interesting that MNNG induces genotoxicity at a much lower concentration than MNU (Lee *et al.*, 1977; Swenberg *et al.*, 1976; Walker and Ewart, 1973) and this could be related to a higher reactivity of MNNG with thiols or a higher rate of spontaneous dealkylation.

The fact that at comparably genotoxic doses, a higher ROS formation is observed for nitrosamines (Fig. 4), whereas alkylating damage appears to be more relevant in the genotoxicity of MNNG and MNU (Romert *et al.*, 1991) does not necessarily mean that nitrosamine exposure–related alkylating damage is less important. CCR levels clearly indicate the formation of α-nitrosamino radicals that are likely to further react to form alkylating diazonium ion species. The fact that nitrosamine exposure does not show radical formation at a concentration of 1mM, indicates that at equal dose, nitrosamides are likely to be more genotoxic than nitrosamines, as also demonstrated by the higher concentrations needed to induce a similar level of DNA damage in the comet assay. Although the comet assay may be more sensitive to ROS-induced DNA breaks, thereby detecting ROS-related effects more readily, the alkaline comet assay is also known to respond to alkylating damage as a result of DNA breaks induced by alkyl adduct repair mechanisms and alkali labile sites (Rojas *et al.*, 1999). Because MNNG and MNU exposure also resulted in a significant level of DNA damage, whereas ESR analysis showed (almost) no ROS formation above background at these genotoxic concentrations, the DNA damage level cannot be attributed to ROS and can only be ascribed to alkylating damage. Consequently, the DNA damage level induced by nitrosamines at genotoxic concentrations also has to be (partly) attributed to alkylating damage. Alkylating damage can be expected after exposure to 1mM nitrosamides (Stephanou *et al.*, 1996) and the formation of DMPO-OH (Table 2) suggests oxidative damage is also a likely outcome, whereas the absence of nitrosamine-induced ROS and CCR signals at this concentration suggests that these types of damage are not likely to occur with nitrosamines in Caco-2 cells. Of course, this depends on the metabolic CYP activity of cells, which in the Caco-2 cell line is lower than that in, e.g., liver tissue (Borlak and Zwadlo, 2003). A higher metabolic rate could result in a higher nitrosamine-related radical production and genotoxicity compared with nitrosamides. A higher level of genotoxicity is, however, not automatically associated with a greater carcinogenicity as shown by Brambilla *et al.* (1981). In view of the complex relationship between radical formation, genotoxicity and human carcinogenicity as well as the multiple mechanisms underlying the carcinogenic process, it is difficult to assess, based on our data, which compounds would pose a higher carcinogenic risk in the human colon, using Caco-2 cells as an *in vitro* model.

To establish the link between ROS formation and the transcriptomic response, we identified gene expression changes that significantly correlated with ROS levels (Table 3). Although we identified a number of pathways that were affected after 1 h of exposure, the relatively low number of significantly affected genes in each pathway makes it difficult to assess the direction of the biological effect of these modified pathways. The correlation between ROS formation and gene expression data after 24 h, however, shows much more pronounced effects on the same cellular processes, including apoptosis, cell cycle regulation, DNA repair, and the oxidative stress response. In view of the residual amount of NDMA after 24 h, radicals should be formed continuously during the exposure. This indicates that the gene expression modifications associated with ROS formation become more enhanced over time. The difference in the number of significantly modulated

genes and molecular pathways between nitrosamines and nitrosamides shows a similarity, with the ROS-induced DMPO-OH signal being much higher following nitrosamine exposure. The lower ROS level of NDMA compared with the other three nitrosamines is also reflected by the absence of a number of cellular processes following NDMA exposure after 24 h, again suggesting that the level of ROS has a determinant effect on the extent of gene expression changes. Oxidative stress pathways are most strongly associated with exposure to NDEA, NPIP, and NPYR, which also display the highest levels of ROS formation. The correlations that were found also indicate that ROS may be partially responsible for the cell cycle blocks and increased apoptosis levels we previously observed (Hebels *et al*., 2009), in addition to the well-known effect of alkyl adducts on cell cycle blockage and apoptosis (Roos and Kaina, 2006). This is likely to be a direct consequence of the DNA-damaging properties of ROS, because DNA damage repair is also correlated with ROS levels (Table 3). Other processes like cytoskeleton remodeling and nucleotide metabolism are likely related to cytoskeletal changes associated with apoptosis and cell cycle regulation and changes in the nucleotide pool as a result of the DNA replication blockage during S phase. A number of significantly modulated pathways involved in the immune response were also found. As nitric oxide has important immunoregulatory functions and oxidative stress is known to stimulate cytokine production in Caco-2 cells (Bogdan, 2001; Nemeth *et al*., 2007; Yamamoto *et al*., 2003), these modulated pathways could be related to $^{\bullet}$NO formed after denitrosation of nitrosamines or oxidative stress caused by nitrosamine-induced ROS formation. In the Supplementary data, a complete overview of modulated pathways can be found, which in addition to the cellular processes already mentioned also shows a number of significantly modulated pathways involved in G-protein signaling and cellular development. Modulations in G-protein signaling and developmental pathways, many of which are involved in crucial parts of cell differentiation, could influence the cancer risk and contribute to the genotoxic aspects of ROS in the carcinogenic process, in addition to NOC-induced alkylating damage and its associated gene expression changes. Although correlation analyses could not be performed with the NOC radicals, it is likely they contribute to these ROS-associated gene expression changes because metabolism of NOC results in a concomitant production of both ROS and NOC radicals.

In summary, we have demonstrated for the first time NOC-induced ROS formation and compound-specific NOC radicals in colon cells. By analyzing both nitrosamides and nitrosamines, differences in radical generation mechanisms were found, which are helpful in explaining the observed difference in genotoxicity in the Caco-2 cell line between these two classes of potentially carcinogenic compounds. The data indicate that the levels of ROS generated by NOC may contribute to the genotoxic effects of alkylation and that they strongly influence pivotal gene expression pathways, which increase in effect over time. Therefore, at comparably genotoxic concentrations, nitrosamines not only display a stronger concomitant radical production than nitrosamides but also affect several molecular pathways much stronger than nitrosamides. In addition to the carcinogenic risk associated with NOC-induced DNA alkylation, the identified ROS-related mechanisms may also contribute to the development of human colon cancer. Overall, it is concluded that radical formation by NOC, i.e., both CCRs and ROS, may play an important role in health effects associated with colonic NOC exposure and that nitrosamides and nitrosamines induce distinct molecular responses.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

REFERENCES

Aiub, C. A., Pinto, L. F., and Felzenszwalb, I. (2009). DNA-repair genes and vitamin E in the prevention of N-nitrosodiethylamine mutagenicity. *Cell Biol. Toxicol.* **25,** 393–402.

Arranz, N., Haza, A. I., Garcia, A., Moller, L., Rafter, J., and Morales, P. (2006). Protective effects of isothiocyanates towards N-nitrosamine-induced DNA damage in the single-cell gel electrophoresis (SCGE)/HepG2 assay. *J. Appl. Toxicol.* **26,** 466–473.

Arranz, N., Haza, A. I., Garcia, A., Rafter, J., and Morales, P. (2007). Protective effect of vitamin C towards N-nitrosamine-induced DNA damage in the single-cell gel electrophoresis (SCGE)/HepG2 assay. *Toxicol. In Vitro* **21,** 1311–1317.

Bai, P., Hegedus, C., Erdelyi, K., Szabo, E., Bakondi, E., Gergely, S., Szabo, C., and Virag, L. (2007). Protein tyrosine nitration and poly(ADP-ribose) polymerase activation in N-methyl-N-nitro-N-nitrosoguanidine-treated thymocytes: Implication for cytotoxicity. *Toxicol. Lett.* **170,** 203–213.

Bartsch, H., Hietanen, E., and Malaveille, C. (1989). Carcinogenic nitrosamines: Free radical aspects of their action. *Free Radic. Biol. Med.* **7,** 637–644.

Bogdan, C. (2001). Nitric oxide and the immune response. *Nat. Immunol.* **2,** 907–916.

Borlak, J., and Zwadlo, C. (2003). Expression of drug-metabolizing enzymes, nuclear transcription factors and ABC transporters in Caco-2 cells. *Xenobiotica* **33,** 927–943.

Brambilla, G., Cavanna, M., Pino, A., and Robbiano, L. (1981). Quantitative correlation among DNA damaging potency of six N-nitroso compounds and their potency in inducing tumor growth and bacterial mutations. *Carcinogenesis* **2,** 425–429.

Floyd, R. A., Soong, L. M., Stuart, M. A., and Reigh, D. L. (1978). Spin trapping of free radicals produced from nitrosoamine carcinogens. *Photochem. Photobiol.* **28,** 857–862.

Grover, T. A., Ramseyer, J. A., and Piette, L. H. (1987). Photolysis of nitrosamines and nitrosamides at neutral pH: A spin-trap study. *Free Radic. Biol. Med.* **3,** 27–32.

Hebels, D. G., Jennen, D. G., Kleinjans, J. C., and de Kok, T. M. (2009). Molecular signatures of N-nitroso compounds in Caco-2 cells: Implications for colon carcinogenesis. *Toxicol. Sci.* **108,** 290–300.

Heur, Y. H., Streeter, A. J., Nims, R. W., and Keefer, L. K. (1989). The Fenton degradation as a nonenzymatic model for microsomal denitrosation of N-nitrosodimethylamine. *Chem. Res. Toxicol.* **2,** 247–253.

Hiramoto, K., Ryuno, Y., and Kikugawa, K. (2002). Decomposition of N-nitrosamines, and concomitant release of nitric oxide by Fenton reagent under physiological conditions. *Mutat. Res.* **520,** 103–111.

Jensen, D. E. (1983). Denitrosation as a determinant of nitrosocimetidine in vivo activity. *Cancer Res.* **43,** 5258–5267.

Jensen, D. E., Stelman, G. J., and Williams, A. W. (1990). Microsomally-mediated denitrosation of nitrosoguanidinium compounds. *Carcinogenesis* **11,** 1075–1082.

Keefer, L. K., Anjo, T., Wade, D., Wang, T., and Yang, C. S. (1987). Concurrent generation of methylamine and nitrite during denitrosation of N-nitrosodimethylamine by rat liver microsomes. *Cancer Res.* **47,** 447–452.

Knekt, P., Jarvinen, R., Dich, J., and Hakulinen, T. (1999). Risk of colorectal and other gastro-intestinal cancers after exposure to nitrate, nitrite and N-nitroso compounds: A follow-up study. *Int. J. Cancer* **80,** 852–856.

Kuhnle, G. G., Story, G. W., Reda, T., Mani, A. R., Moore, K. P., Lunn, J. C., and Bingham, S. A. (2007). Diet-induced endogenous formation of nitroso compounds in the GI tract. *Free Radic. Biol. Med.* **43,** 1040–1047.

Kumaraguruparan, R., Chandra Mohan, K. V., Abraham, S. K., and Nagini, S. (2005). Attenuation of N-methyl-N′-nitro-N-nitrosoguanidine induced genotoxicity and oxidative stress by tomato and garlic combination. *Life Sci.* **76,** 2247–2255.

Kushida, H., Fujita, K., Suzuki, A., Yamada, M., Endo, T., Nohmi, T., and Kamataki, T. (2000). Metabolic activation of N-alkylnitrosamines in genetically engineered Salmonella typhimurium expressing CYP2E1 or CYP2A6 together with human NADPH-cytochrome P450 reductase. *Carcinogenesis* **21,** 1227–1232.

Lee, K., Gold, B., and Mirvish, S. S. (1977). Mutagenicity of 22 N-nitrosamides and related compounds for Salmonella typhimurium TA1535. *Mutat. Res.* **48,** 131–138.

Lewis, D. F. V. (2002). Oxidative stress: The role of cytochromes P450 in oxygen activation. *J. Chem. Technol. Biotechnol.* **77,** 1095–1100.

Lijinsky, W. (1992). *Chemistry and Biology of N-Nitroso Compounds*. Cambridge University Press, Cambridge, UK.

Mabrouk, G. M., Moselhy, S. S., Zohny, S. F., Ali, E. M., Helal, T. E., Amin, A. A., and Khalifa, A. A. (2002). Inhibition of methylnitrosourea (MNU) induced oxidative stress and carcinogenesis by orally administered bee honey and Nigella grains in Sprague Dawley rats. *J. Exp. Clin. Cancer Res.* **21,** 341–346.

Mikuni, T., and Tatsuta, M. (2002). Production of hydroxyl free radical in the xanthine oxidase system with addition of 1-methyl-3-nitro-1-nitrosoguanidine. *Free Radic. Res.* **36,** 641–647.

Mirvish, S. S. (1995). Role of N-nitroso compounds (NOC) and N-nitrosation in etiology of gastric, esophageal, nasopharyngeal and bladder cancer and contribution to cancer of known exposures to NOC. *Cancer Lett.* **93,** 17–48.

Nagata, C., Nakadate, M., Ioki, Y., and Imamura, A. (1972). Electron spin resonance study on the free radical production from N-methyl-N′-nitro-N-nitrosoguanidine. *Gann* **63,** 471–481.

Nemeth, E., Halasz, A., Barath, A., Domokos, M., and Galfi, P. (2007). Effect of hydrogen peroxide on interleukin-8 synthesis and death of Caco-2 cells. *Immunopharmacol. Immunotoxicol.* **29,** 297–310.

Paraskeva, C., Corfield, A. P., Harper, S., Hague, A., Audcent, K., and Williams, A. C. (1990). Colorectal carcinogenesis: Sequential steps in the in vitro immortalization and transformation of human colonic epithelial cells (review). *Anticancer Res.* **10,** 1189–1200.

Potter, D. W., and Reed, D. J. (1983). Involvement of FMN and phenobarbital cytochrome P-450 in stimulating a one-electron reductive denitrosation of 1-(2-chloroethyl)-3-(cyclohexyl)-1-nitrosourea catalyzed by NADPH-cytochrome P-450 reductase. *J. Biol. Chem.* **258,** 6906–6911.

Prater, M. R., Laudermilch, C. L., and Holladay, S. D. (2007). Does immune stimulation or antioxidant therapy reduce MNU-induced placental damage via activation of Jak-STAT and NFkappaB signaling pathways? *Placenta* **28,** 566–570.

Preussmann, R., and Eisenbrand, G. (1984). N-nitroso carcinogens in the environment. In: *Chemical Carcinogens*. (C. E. Searle, Ed.), 2nd ed., pp. 829–868. American Chemical Society, Washington DC.

Rojas, E., Lopez, M. C., and Valverde, M. (1999). Single cell gel electrophoresis assay: Methodology and applications. *J. Chromatogr. B Biomed. Sci. Appl.* **722,** 225–254.

Romert, L., Swedmark, S., and Jenssen, D. (1991). Thiol-enhanced decomposition of MNNG, ENNG, and nitrosocimetidine: Relationship to mutagenicity in V79 Chinese hamster cells. *Carcinogenesis* **12,** 847–853.

Roos, W. P., and Kaina, B. (2006). DNA damage-induced cell death by apoptosis. *Trends Mol. Med.* **12,** 440–450.

Rosen, H., and Klebanoff, S. J. (1979). Hydroxyl radical generation by polymorphonuclear leukocytes measured by electron spin resonance spectroscopy. *J. Clin. Invest.* **64,** 1725–1729.

Sen, N. P., Seaman, S. W., Burgess, C., Baddoo, P. A., and Weber, D. (2000). Investigation on the possible formation of N-nitroso-N-methylurea by nitrosation of creatinine in model systems and in cured meats at gastric pH. *J. Agric. Food Chem.* **48,** 5088–5096.

Stephanou, G., Vlastos, D., Vlachodimitropoulos, D., and Demopoulos, N. A. (1996). A comparative study on the effect of MNU on human lymphocyte cultures in vitro evaluated by O^6-mdG formation, micronuclei and sister chromatid exchanges induction. *Cancer Lett.* **109,** 109–114.

Swenberg, J. A., Petzold, G. L., and Harbach, P. R. (1976). In vitro DNA damage/alkaline elution assay for predicting carcinogenic potential. *Biochem. Biophys. Res. Commun.* **72,** 732–738.

Tricker, A. R., and Preussmann, R. (1991). Carcinogenic N-nitrosamines in the diet: Occurrence, formation, mechanisms and carcinogenic potential. *Mutat. Res.* **259,** 277–289.

U.S. Department of Health and Human Services, P.H.S., National Toxicology Program. (2005). *Report on Carcinogens*, 11th ed. U.S. Department of Health and Human Services, Washington, DC. Available at: http://ntp.niehs.nih.gov/index.cfm?objectid=32BA9724-F1F6-975E-7FCE50709CB4C932.

Wade, D., Yang, C. S., Metral, C. J., Roman, J. M., Hrabie, J. A., Riggs, C. W., Anjo, T., Keefer, L. K., and Mico, B. A. (1987). Deuterium isotope effect on denitrosation and demethylation of N-nitrosodimethylamine by rat liver microsomes. *Cancer Res.* **47,** 3373–3377.

Walker, I. G., and Ewart, D. F. (1973). The nature of single-strand breaks in DNA following treatment of L-cells with methylating agents. *Mutat. Res.* **19,** 331–341.

Wink, D. A., and Desrosiers, M. F. (1991). Unusual spin-trap chemistry for the reaction of hydroxyl radical with the carcinogen N-nitrosodimethylamine. *Radiat. Phys. Chem.* **38,** 467–472.

Yamamoto, K., Kushima, R., Kisaki, O., Fujiyama, Y., and Okabe, H. (2003). Combined effect of hydrogen peroxide induced oxidative stress and IL-1 alpha on IL-8 production in CaCo-2 cells (a human colon carcinoma cell line) and normal intestinal epithelial cells. *Inflammation* **27,** 123–128.

Yang, C. S., Smith, T. J., Hong, J. Y., and Zhou, S. (1994). Kinetics and enzymes involved in the metabolism of nitrosamines. In: *Chemistry and Biochemistry of Nitrosamines and Related N-Nitroso Compounds*. (R. N. Loeppky and C. J. Michejda, Eds.), pp. 169–178. ACS, Washington, DC.

Young-Sciame, R., Wang, M., Chung, F. L., and Hecht, S. S. (1995). Reactions of alpha-acetoxy-N-nitrosopyrrolidine and alpha-acetoxy-N-nitrosopiperidine with deoxyguanosine: Formation of N2-tetrahydrofuranyl and N2-tetrahydropyranyl adducts. *Chem. Res. Toxicol.* **8,** 607–616.