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TOXICOLOGICAL SCIENCES **108(2)**, 290–300 (2009)
doi:10.1093/toxsci/kfp035
Advance Access publication February 16, 2009

Molecular Signatures of N-nitroso Compounds in Caco-2 Cells: Implications for Colon Carcinogenesis

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Received January 8, 2009; accepted February 10, 2009

N-nitroso compounds (NOC) are genotoxic, carcinogenic to animals, and may play a role in human cancer development. Because the gastro-intestinal tract is an important route of exposure through endogenous nitrosation, we hypothesize that NOC exposure targets genetic processes relevant in colon carcinogenesis. To investigate these genomic responses, we analyzed the transcriptomic effects of genotoxic concentrations of two nitrosamides, N-methyl-N′-nitro-N-nitrosoguanidine (MNNG, 1μM) and N-methyl-N-nitrosurea (MNU, 1mM), and four nitrosamines, N-nitrosodiethylamine (NDEA, 50mM), N-nitrosodimethylamine (NDMA, 100mM), N-nitrosopiperidine (NPIP, 40mM), and N-nitrosopyrrolidine (NPYR, 100mM), in the human colon carcinoma cell line Caco-2. Gene Ontology gene group, consensus motif gene group and biological pathway analysis revealed that nitrosamides had little effect on gene expression after 24 h of exposure, whereas nitrosamines had a strong impact on the transcriptomic profile. Analyses showed modifications of cell cycle regulation and apoptosis pathways for nitrosamines which was supported by flow cytometric analysis. We found additional modifications in gene groups and pathways of oxidative stress and inflammation, which suggest an increase in oxidative stress and proinflammatory immune response upon nitrosamine exposure, although less distinct for NDMA. Furthermore, NDEA, NPIP, and NPYR most strongly affected several developmental motif gene groups and pathways, which may influence developmental processes. Many of these pathways and gene groups are implicated in the carcinogenic process and their modulation by nitrosamine exposure may therefore influence the development of colon cancer. In summary, our study has identified pathway modifications in human colon cells which may be associated with cancer risk of nitrosamine exposure in the human colon.

Key Words: **gene expression profiling; N-nitroso compounds; nitrosamides; nitrosamines; Caco-2; colon carcinogenesis.**

More than 5 decades ago it was discovered that N-nitroso compounds (NOC) were carcinogenic in test animals (Druckrey *et al*., 1967), raising the question whether exposure to this class of compounds might also be relevant for the development of human cancer. Additional research demonstrated the occurrence of NOC in the environment and certain types of food, such as beer, fish, and nitrite-preserved meat (Tricker and Preussmann, 1991). Furthermore, multiple bacterial mutagenicity studies and animal experiments resulted in dozens of NOC being classified by the National Toxicology Program as "reasonably anticipated to be a human carcinogen" (U.S. Department of Health and Human Services, 2005). NOC 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). As a consequence, measures have been taken to reduce dietary and environmental exposure to NOC (Frommberger, 1989).

NOC can be divided into two main subclasses, nitrosamines and nitrosamides (Preussmann and Eisenbrand, 1984), which can both induce alkylating DNA damage by formation of a highly reactive diazonium ion (Mirvish, 1995; Tricker and Preussmann, 1991). DNA bases are alkylated by diazonium ion species at the N^7 and O^6 positions of guanine and the O^4 position of thymine. O^6-alkylguanine adducts have been identified as the main premutagenic lesion and cause GC-AT transition mutations (Mirvish, 1995). O^4-alkylthymine results in TA-GC transitions, whereas N^7-alkylguanine adducts have not been shown to be mutagenic (Saffhill *et al*., 1985).

There are, however, several indications for differences in modes-of-action between nitrosamines and nitrosamides. Cyclic nitrosamines can also form similarly reactive cyclic oxonium ions (Young-Sciame *et al*., 1995). Further, although nitrosamides can spontaneously decompose to form the diazonium ion, nitrosamines require metabolic activation through α-hydroxylation by several P450 enzymes (Mirvish, 1995). Additionally, nitrosamines may release nitric oxide radicals (NO·) under certain conditions and generate formaldehyde during metabolism, which may ultimately lead to deamination and hydroxymethyl adduct formation (Cheng *et al*., 2008; Hiramoto *et al*., 2002).

Evidence of NOC involvement in human cancer development is mostly based on epidemiological studies and data on endogenous nitrosation. However, further studies are required to establish whether or not NOC are true human carcinogens

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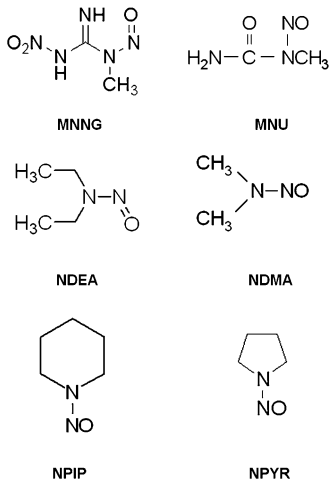

FIG. 1. Molecular structures of the six NOC used in this study.

(Mensinga *et al*., 2003; Mirvish, 1995). Although the genotoxic and carcinogenic effects of NOC are usually attributed to their DNA damaging properties, little is known about other molecular events caused by these compounds which may also be relevant in the carcinogenic process in humans.

Microarray-based gene expression analysis provides a valuable tool for identifying different modes-of-action and molecular responses to NOC exposure. Although there are a few animal studies investigating gene expression modulation in NOC-induced tumor or preneoplastic tissues, little is known on gene expression modifications after NOC exposure in human cells or tissues (Osada *et al*., 2006; Yao *et al*., 2007). Therefore, the analysis of such genomic responses may be indicative of the role of NOC in human carcinogenesis. Because the gastro-intestinal tract presents the first target of dietary or endogenously formed NOC and the colon has been identified as an important site for endogenous nitrosation it is of relevance to investigate gene expression modifications in colon cells after NOC exposure (Kuhnle *et al*., 2007). Within this respect, previous animal studies have implicated nitrosamides in the development of colon cancer (Lijinsky, 1992), whereas nitrosamines are known to be genotoxic in intestinal cell lines (Robichova *et al*., 2004).

We therefore hypothesize that analysis of molecular pathways modified by NOC will help to determine the role of these compounds in human colon carcinogenesis. To investigate this, we analyzed genome-wide gene expression in the human colon adenocarcinoma cell line Caco-2 after exposure to different NOC. Caco-2 cells were exposed to six NOC known to be relevant for human exposure (Fig. 1): the nitrosamines N-nitrosodiethylamine (NDEA), N-nitrosodimethylamine (NDMA), N-nitrosopiperidine (NPIP), and N-nitrosopyrrolidine (NPYR) and the nitrosamides N-methyl-N′-nitro-N-nitrosoguanidine (MNNG), and N-methyl-N-nitrosurea (MNU) (Tricker and Preussmann, 1991). By comet assay analysis, we selected noncytotxic, equally genotoxic concentrations of these NOC to identify corresponding modifications of transcriptomic levels which may reveal genetic processes relevant in the carcinogenic process and also may discriminate between different NOC. Various data analysis techniques were used to identify NOC-induced modulations of gene groups and pathways. Individual gene expression data were also linked with levels of apoptosis and cell cycle distribution as phenotypic markers of effect to functionally anchor gene sets differentially modified by NOC exposure.

MATERIAL 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.

Treatment and isolation of cells. Caco-2 cells were treated with increasing concentrations of MNNG, MNU, NDEA, NDMA, NPIP, and NPYR or the solvent control (MilliQ or dimethyl sulfoxide [DMSO], final concentration 0.1%) in six-wells plates or 25-cm^2 culture flasks for 24 h. All NOC 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%]). Experiments were carried out in quadruplicate. Cells were isolated by trypsinization and suspended in phosphate-buffered saline and subsequently placed on ice (for comet assay) or fixed in methanol and stored at −20°C (for flow cytometry). Cells for microarray experiments were isolated with TRIzol Reagent (Invitrogen, Breda, The Netherlands), which stabilizes RNA, and stored at −20°C. Viability of cells was determined with the MTT test as described by Mosmann (1983) with minor modifications.

Immunocytochemistry and flow cytometric analysis. Methanol-fixed cells were indirectly stained for cleaved cytokeratin 18 (an early sign of apoptosis) using the primary antibody M30 CytoDeath (Roche, Penzberg, Germany) and subsequently analyzed using a FACSort (Becton Dickinson, Sunnyvale, CA) equipped with an Argon ion laser and a diode laser as described by Schutte *et al*. (2006). Data analysis was performed using CellQuest software (version 3.1, Becton Dickinson, San Jose, CA). Data were gated on pulse-processed PI signals to exclude doublets and larger aggregates from analysis. M30 CytoDeath positive and negative signals were sorted in the gated population and displayed as percentage of total cells. In addition, cell cycle distribution was determined using ModFIT LT (version 2.0, Verity Software House, Topsham, ME).

Alkaline single-cell gel electrophoresis (comet assay). The alkaline comet assay allows for the detection of single and double strand breaks as well as abasic sites and sites where DNA repair is taking place, which are all expected to be induced by NOC exposure. The assay was performed as described by Singh *et al*. (1988) with minor modifications. Comets were visualized using a Zeiss Axioskop fluorescence (Carl Zeiss MicroImaging GmbH, Göttingen, Germany) microscope (at ×200 magnification). Randomly, 50 cells were analyzed using the Comet assay III software (Perceptive Instruments, Haverhill, UK). DNA damage was expressed as tail moment (TM, the product of tail DNA content and mean tail migration distance).

RNA isolation and purification of microarray samples. Test concentrations for microarray experiments were selected based on the induction of

TABLE 1
Main target organs, Viability, and Genotoxicity (Mean ± SD) of Caco-2 Cells after 24 h of Exposure to Concentrations Used for Microarray Testing

NOC	Main target organs[a]	Concentration	Viability (%)[b]	Genotoxicity (TM)[c]
MNNG	Stomach	1μM	106	0.89 ± 0.52
MNU	Stomach, nervous system	1mM	107	1.92 ± 0.98
NDEA	Liver, esophagus, nasal	50mM	105	0.70 ± 0.43
NDMA	Liver, kidney, lung	100mM	100	3.03 ± 1.17
NPIP	Esophagus, nasal, liver	40mM	103	2.96 ± 2.08
NPYR	Liver, lung	100mM	98	1.34 ± 0.58

[a]From Lijinsky (1992).

[b]Viability as compared with control. Viability at NOC concentrations shown did not significantly deviate from control levels.

[c]Genotoxicity for blank and DMSO control were 0.05 ± 0.05 and 0.06 ± 0.04, respectively. Genotoxicity at NOC concentrations shown were all significantly increased versus control levels (p value < 0.05).

genotoxicity as assessed by comet assay, as well as on the absence of cytotoxicity as evaluated by means of MTT tests (Table 1). The lowest concentrations resulting in a significant increase in DNA damage (two-tailed Student's t-test, p value < 0.05) in the comet assay, and having a TM between 0.5 and 3.5 (i.e., moderate DNA damage levels) and a viability > 90%, were selected. These concentrations were MNNG (1μM), MNU (1mM), NDEA (50mM), NDMA (100mM), NPIP (40mM), and NPYR (100mM). RNA was isolated from TRIzol suspended cells according to the manufacturer's protocol with minor modifications, followed by a clean up, using an RNeasy Mini Kit (Qiagen, Venlo, The Netherlands) with DNase treatment. RNA quantity and purity were determined spectrophotometrically. RNA quality was assessed by automated gel electrophoresis on an Agilent 2100 Bioanalyzer (Agilent Technologies, Amstelveen, The Netherlands). All samples were pure and free of RNA degradation.

Target preparation, hybridization, and scanning procedure. The Two-Color Microarray-Based Gene Expression Analysis kit from Agilent Technologies (Amstelveen, The Netherlands) was used to generate Cyanine (Cy) labeled cRNA according to the manufacturer's protocol. Two of the quadruplicate test and reference samples were labeled with Cy3 and Cy5 (PerkinElmer Corp., Norwalk, CT), whereas the remaining two test and reference samples were dye-swapped. Hybridization was carried out on Agilent 4 x 44K Whole Human Genome microarrays. Dye incorporation rates were used to put appropriate amounts of Cy3- and Cy5-labeled test and reference samples together (i.e., 10 pmol Cy3 vs. 10 pmol Cy5). After hybridization and washing slides were dried applying a controlled N_2 gas flow before scanning.

Slides were scanned on a GenePix 4000B Microarray Scanner (Molecular Devices, Sunnyvale, CA). Cy3 and Cy5 were excited at wavelengths of 532 and 635 nm, respectively. Laser power was set to 100%. The photo multiplier tube gain was set to a saturation tolerance of 0.02% to minimize background and saturated spots. The images obtained (resolution 5μM, 16-bit tiff image) were processed with Imagene 8.0.1 software (BioDiscovery Inc., El Segundo, CA) to measure mean signal intensities for spots and local backgrounds followed by a quality control in Microsoft Excel (Microsoft Corporation, Redmond, WA). Poor spots were flagged manually and automatically.

Data preparation and normalization. Data were further processed in GeneSight 4.1 (Biodiscovery). Flagged spots were excluded from analysis. For each spot, mean local background intensity was subtracted from mean signal intensity, and spots with a mean net signal of < 10.0 were omitted from analysis. Background corrected mean intensities were $\log_2$ transformed. Normalization of expression differences was performed using LOWESS and the replicates of each gene were combined to a mean expression difference with exclusion of outliers (beyond two standard deviations). 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.

Data analysis. T-profiler analyses were performed to identify responsive gene groups within the complete gene expression profiles, without the need for selection of significantly modulated genes. T-profiler uses the unpaired t-test to score changes in average transcriptomic activity of predefined groups of genes within a single expression profile and expresses the significance of the change as a t value (Boorsma *et al*., 2005; van Leeuwen *et al*., 2008). Adjusted p values (E values) are calculated and gene groups with an E value of ≤ 0.05 are considered to be significantly regulated. T-profiler analyses were performed with $\log_2$ ratio data to identify gene groups based on Gene Ontology (GO) and consensus motif/transcription factor (TF) categorization. GO categories with over 1000 genes were excluded because they provide little relevant information.

For further pathway analysis of significantly modulated genes (two-tailed Student's t-test, p value < 0.05, unadjusted p values) two repositories of pathways were used. MetaCore (GeneGo, San Diego, CA) 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. Pathways with a p value < 0.05 were considered significantly modulated. The second repository of pathways was PathVisio version 1.1 beta (van Iersel *et al*., 2008). PathVisio calculates a Z score, based on a hypergeometric distribution, for each biological pathway term. Pathways with a Z score > 2 were considered significantly modulated. The reason for combining MetaCore and PathVisio is that, because both repositories contain different pathways, more information may be retrieved from the database.

The online software suite GenePattern version 3.1 (http://www.broad.mit.edu/cancer/software/genepattern/) was used for hierarchical clustering analysis (HCA) and principal component analysis (PCA) of the tested compounds. HCA was performed using the unweighted average as a clustering method and Pearson correlation as a similarity measure. PCA plots were generated in GenePattern by reducing the data to two dimensions and visualized in Microsoft Excel.

Spearman's rank correlations between gene expression and levels of apoptosis or cell cycle distribution were calculated using the online Gene Expression Profile Analysis Suite (http://gepas.bioinfo.cipf.es/). Ratios of apoptosis or cell cycle phase percentages 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. These transformed values were then correlated with the gene expression data.

RESULTS

Transcriptomic Response at Selected Genotoxic NOC Concentrations

For microarray experiments a suitable concentration of each NOC was first determined by testing a doses range of 0.1μM–100mM in several assays. The goal was to select a genotoxic concentration, which did not result in a cytotoxic effect in the MTT test. The lowest concentrations resulting in a significant increase in DNA damage in the comet assay, and having a TM between 0.5 and 3.5 (i.e., moderate DNA damage levels) and a viability > 90%, were selected. Final concentrations were

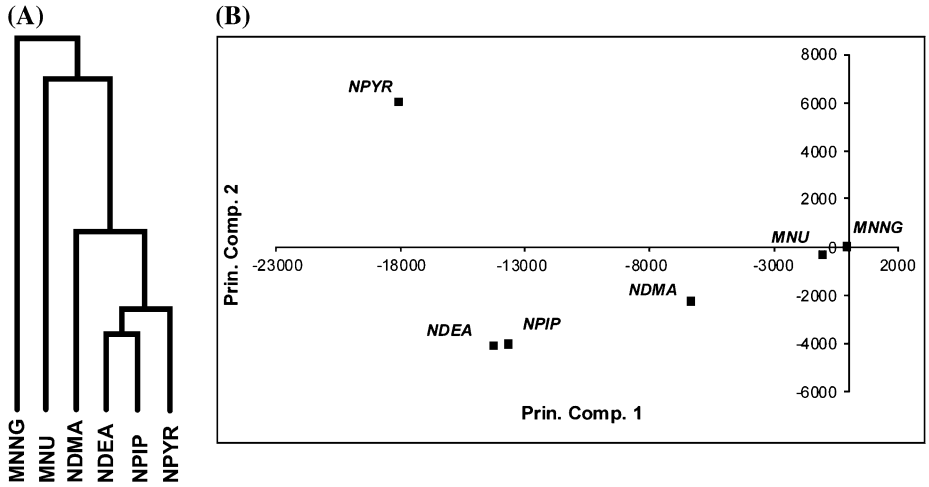

FIG. 2. Dendogram of Pearson correlation HCA (A) and PCA plot (B) for genes significantly modulated for at least one of the six NOC. The percentage variation for principal components 1 and 2 were 81.22 and 6.87%.

1μM MNNG, 1mM MNU, 50mM NDEA, 100mM NDMA, 40mM NPIP, and 100mM NPYR. Corresponding viability and genotoxicity levels are presented in Table 1. Main target organs for carcinogenicity are also reported for each NOC.

Modulation of gene expression by the selected genotoxic NOC concentrations was subsequently analyzed on microarrays to elucidate the underlying modes-of-action of these compounds and to detect and identify differential gene expression profiles. The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (GEO) (Edgar *et al*., 2002) and are accessible through GEO Series accession number GSE14284 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc = GSE14284). An initial analysis of differentially expressed genes revealed that a much smaller number of genes were significantly modulated by the nitrosamides MNNG and MNU (357 and 744 resp.) compared with the nitrosamines NDEA, NDMA, NPIP, and NPYR (10271, 5440, 9847, and, 12609 resp.). In fact, the number of genes modulated by these nitrosamides is smaller than the number of false positives that could be expected at a 5% significance level (2200 genes on a 44K array).

To visualize the differences in gene expression profiles between NOC we first performed a Pearson correlation HCA on genes significantly modulated (p value < 0.05) for at least one of the six NOC. The clustering dendogram (Fig. 2A) shows a distinction between the four nitrosamines and two nitrosamides. MNNG and MNU cluster separately from the nitrosamines and each other. Although the NDMA and NPYR expression patterns cluster within the nitrosamine group, the corresponding PCA plot (Fig. 2B) shows that both compounds dissociate from the other two nitrosamines, a pattern that is analogous to the number of significantly modulated genes. The PCA also shows the nitrosamides grouping together near the origin, indicating little effect on gene expression levels.

Gene Group and Pathway Identification

T-profiler, MetaCore, and PathVisio were used to identify significantly modulated gene groups and pathways. Here only a selection of results is presented. Complete gene group and pathway lists and additional data can be found in the Supplementary Data Excel file.

For a functional interpretation of all expressed genes, without pre-selection, we first performed a T-profiler analysis on gene groups. GO categories, significantly modulated in T-profiler for at least one of the six NOC and with less than 100 genes per group, are listed in Table 2A. The nitrosamides (MNNG and MNU) failed to modulate any GO gene groups, whereas nitrosamines affected a wide variety of categories. All nitrosamines caused strong up regulations of genes in the cadmium and copper ion binding groups. NPIP also shows strong up regulation of genes involved in protein folding. Genes were mostly down regulated by all nitrosamines in two mitochondrion related gene groups, which mainly contain ribosomal proteins and NADH dehydrogenases and some apoptosis related genes. Effects on protein synthesis were also evident by modulation of the gene groups Nucleolar part, Ribosome biogenesis and assembly, rRNA metabolic process, rRNA processing and tRNA processing. Oxidoreductase activity is modified by NDEA and NPIP, whereas alkyl/aryl transferase activity is only modified by NPYR. NPYR also uniquely modified genes associated with FAD binding, biosynthesis of glutamine family amino acids, and glutathione transferase activity.

GO gene groups significantly regulated (p value < 0.05) for at least one of the six NOC, generated by T-profiler, were subsequently used for Pearson correlation HCA. As shown in Figure 3A the gene groups again show a discrimination between nitrosamides and nitrosamines, but in comparison to Figure 2A nitrosamines cluster better. The accompanying PCA

plot (Fig. 3B) also shows the nitrosamines grouping together indicating comparable effects on a functional level, although NDMA is located more distinctly. MNU and MNNG clearly form a separate group.

T-profiler was also used to perform an analysis on motif/TF associated gene groups, which are defined as genes with a match to a particular consensus motif within 600 base pairs upstream of the open reading frame (ORF) allowing no overlap with neighboring ORFs (Boorsma *et al.*, 2005). Most commonly affected motif gene groups, sorted by similarity in biological function, are shown in Table 2B. Most motif gene groups were considered up regulated and the genes in these groups contained motifs specific to TFs that are involved in many different biological functions. The Forkhead box TFs (FOX), for example, are involved in the regulation of cell cycle, apoptosis, oxidative stress and developmental processes, including embryogenesis. Other TFs, such as CREB1 and the POU family, are implicated in neuronal development, whereas the ATF family is associated with several aspects of the stress response, including oxidative stress. There were also eight gene groups with binding motifs associated with homeobox containing genes, involved in development. Down regulation of genes with TF motifs for CDC5L, the E2F family, and MYC/MAX mainly involved regulation of the G1/S transition, apoptosis and DNA repair. Nitrosamines were again most strongly involved in gene expression differences, whereas MNNG only affected two gene groups associated with development and homeobox gene expression. MNU was not involved in any gene group modifications. The complete list of modified GO and motif gene groups can be found in the Supplementary Data (worksheet 1 and 2).

We subsequently performed additional pathway analyses using MetaCore and PathVisio to further elucidate modifications of the underlying pathways and their theoretical effects on cellular processes. Theoretical effects were determined by individually assessing the effects of up and down regulated genes in each pathway. The nitrosamides were excluded from this analysis because the number of significant genes was beneath the false positive threshold. A complete list of modulated pathways can be found in the Supplementary Data (worksheet 3 and 4).

Cellular processes most strongly affected by modified pathways and their theoretical effects are presented in Table 3. Both tools identified a large number of pathways involved in apoptosis, cell cycle regulation, and DNA repair after exposure to nitrosamines. Cell cycle progression is inhibited by modifications in the G1/S phase transition, DNA replication, and metaphase checkpoints, whereas DNA damage recognition and repair pathways, involved in double strand breaks among others, are activated. All four nitrosamines showed modifications of apoptosis response and cell cycle regulation pathways. DNA damage response pathways were modified by NDEA, NPIP, and NPYR, but not by NDMA.

Pathways related to oxidative stress appear to be activated by nitrosamine exposure. Glutathione metabolism and ASK1-mediated oxidative stress induced apoptosis are both stimulated. Immune response pathways were strongly activated by NDEA, NPIP, and NPYR, but again not by NDMA, resulting in a stimulation of proinflammatory genes. Changes in nucleotide metabolism were induced by all four nitrosamines and are indicative of a DNA replication block and modifications of the nucleotide turnover. Lipid metabolism was also affected, resulting in inhibition of fatty acid synthesis and beta oxidation.

MetaCore and PathVisio analysis also showed significant modulations of several pathways involved in development, such as the Notch and EGFR1 signaling pathways (Supplementary Data worksheet 3 and 4), for NDEA, NPIP, and NPYR. These pathways were absent for NDMA exposure.

Phenotypic Markers of Effect: Apoptosis and Cell Cycle Distribution

Because the gene group and pathway analysis tools all identified cell cycle regulation and apoptosis pathways as significantly modulated for the nitrosamines we performed a flow cytometry analysis of the cell cycle distribution and apoptosis levels to see whether these effects could also be found on a phenotypic level. Effects on apoptosis and cell cycle distribution induced by all six NOC are displayed in Figures 3A and 3B, respectively. The nitrosamides MNNG and MNU do not show any increase in apoptosis as compared with control levels, whereas treatment with the nitrosamines results in a significant increase in the number of apoptotic cells ranging from 8 to 15% (p value < 0.01), which is in agreement with the gene group and pathway analyses. Caco-2 cells exposed to MNNG and MNU at these concentrations also do not show effects on cell cycle distribution (Fig. 4B). For NDMA there seems to be an accumulation of cells in the S and G2 phases, whereas the other nitrosamines display a decrease in S phase cells and a G2 accumulation, although the G2 accumulation for NPIP and NPYR was not significant.

Correlation of Genes with Phenotypic Markers of Effect

In order to link the gene expression changes with the phenotypic effects on apoptosis and cell cycle distribution following NOC exposure, we performed Spearman's rank correlation tests between gene $\log_2$ ratios and $\log_2$ transformed apoptosis and cell cycle distribution data. Significantly correlating genes (p value < 0.05) were subsequently used for a pathway analysis in MetaCore. In the Supplementary Data (worksheet 5) the significantly modulated pathways that were related to apoptosis and the S and G2 phases of the cell cycle are shown. Although the G1 phase did correlate with a number of genes, these genes did not return any significant pathways. Genes correlating with apoptosis did not return any apoptosis pathways, but there were several cell cycle, cytoskeleton remodeling, and cell adhesion pathways associated as well as a number of immune

TABLE 2
T-Profiler Analysis of (A) GO gene groups with < 100 Genes; (B) Most Common Motif Gene Groups and their Associated TFs and Biological Functions

A.

GO gene groups	NOC	*t* values	No. of genes
Cadmium ion binding	3/4/5/6	13/5.9/5.25/12.12	6
Copper ion binding	3/6	6.08/6.45	57
Response to protein stimulus/unfolded protein	5	6.1	44
FAD binding	6	−4.58	58
Glutamine family amino acid biosynthetic process	6	−4.88	16
Glutathione transferase activity	6	−4.4	19
Glycolysis	5	−4.43	44
Intramolecular oxidoreductase activity	3/5	−4.46/−4.38	38
Mitochondrial lumen/matrix	3/5/6	−4.58/−4.34/−6.05	87
Mitochondrial membrane part	3/4/5/6	−5.09/−4.4/−5.24/−5.05	91
Nucleolar part	3	−4.42	26
Oxidoreductase activity, acting on the aldehyde or oxo group of donors	5	−5.09	28
Ribosome biogenesis and assembly	3/4/6	−5.24/−4.93/−4.39	65
rRNA metabolic process	3/4	−5.07/−4.91	56
rRNA processing	3/4/6	−5.17/−4.82/−4.46	53
Transferase activity, transferring alkyl or aryl (other than methyl) groups	6	−5.08	45
tRNA processing	6	−5.28	57

B.

Motif gene groups	NOC	*t* values	TF	Biological functions[a]
TTGTTT_V$FOXO4_01	3/4/5/6	5.45/4.57/5.05/8.07	FOXO4	Cell cycle, cell death, cell metabolism, oxidative stress, embryonic development
RTAAACA_V$FREAC2_01	5/6	4.55/5.44	FOXF2	
V$FOXO3_01	6	4.3	FOXO3A	
V$FOXO1_01	6	4.03	FOXO1A	
V$CREB_Q2_01	3/5	4.38/5	CREB1	Neuronal development, stress response
V$CREB_Q4_01	3/5	4.3/5.8	CREB1	
V$CREB_Q2	3/5	4.19/4.76	CREB1	
V$CREB_01	3/5	4.18/5.09	CREB1	
V$ATF3_Q6	3/5/6	5.09/6.46/4.04	ATF3	Stress response
TGAYRTCA_V$ATF3_Q6	3/5/6	4.94/5.64/5.11	ATF3	
V$CREBP1CJUN_01	5	4.39	JUN/ATF2	
TGACGTCA_V$ATF3_Q6	5	4.38	ATF3	
V$CREBP1_01	6	4.53	ATF2	
V$OCT1_06	6	4.24	POU2F1	Neuronal development, hormone production
V$POU3F2_02	6	4.63	POU3F2	
V$OCT1_03	6	5.19	POU2F1	
V$POU1F1_Q6	1	4.14	POU1F1	
TGACAGNY_V$MEIS1_01	3/6	4.73/6.17	MEIS1	Modulation of homeodomain responsive genes involved in development
TAATTA_V$CHX10_01	3/6	4.62/6.24	VSX1	
CGTSACG_V$PAX3_B	3/6	4.21/4.01	PAX3	
V$TGIF_01	3	4.03	TGIF	
YTAATTAA_V$LHX3_01	6	4.02	LHX3	
V$LHX3_01	6	4.18	LHX3	
V$NKX25_02	6	4.66	NKX2–5	
V$EN1_01	1	4.12	EN1	

TABLE 2—*Continued*

B.

Motif gene groups	NOC	*t* values	TF	Biological functions[a]
V$CDC5_01	3/6	4.23/5.81	CDC5L	Cell cycle regulation (G1/S transition),
V$E2F1_Q6	4	−4.02	E2F1	apoptosis, DNA repair
V$E2F1_Q6_01	4	−4.17	E2F1	
V$E2F_Q4_01	4	−4.59	E2F/TFDP1	
V$E2F_Q6_01	3	−4.25	E2F/TFDP1	
SGCGSSAAA_V$E2F1DP2_01	3/4/5	−4.32/−4.46/−4.39	E2F1/TFDP1/RB1	
V$MYCMAX_01	3/4	−4.62/−4.1	MYC/MAX	

Note. Significant results (E value ≤ 0.05) are reported with t value > 4.32 or < −4.32 for GO gene groups and t value > 4 or < −4 for motif gene groups. Gene groups with positive and negative t values can, on average, be considered up regulated and down regulated, respectively.

[a]According to GO from http://www.ncbi.nlm.nih.gov/sites/entrez?db=gene and http://www.genecards.org/. NOC are indicated with 1 (MNNG), 2 (MNU), 3 (NDEA), 4 (NDMA), 5 (NPIP), 6 (NPYR).

response pathways. The S phase and G2 phase correlating genes also returned several cell cycle related pathways.

DISCUSSION

In this study, we identified the gene expression modulating effects of six NOC in the human colon adenocarcinoma cell line Caco-2, in order to gain more insight in their respective molecular mechanisms at genotoxic doses. The noncytotoxic concentrations we selected for microarray analysis resulted in a comparable level of genotoxicity as measured by comet assay (Table 1). It was quite surprising to find very little effect of the two nitrosamides as compared with the nitrosamines. The number of genes significantly modulated by both nitrosamides was smaller than the number of expected false positives. An HCA of genes significantly modulated by at least one of the NOC shows a clear separation of nitrosamides and nitrosamines (Fig. 2A). The corresponding PCA (Fig. 2B) suggests that this discrimination is mainly based on the relatively small transcriptomic effects induced by MNU and MNNG. This was confirmed by functional data on apoptosis and cell cycling which also indicated little if no effects of nitrosamides (Figs. 3A and 3B).

To study these changes in gene expression in more detail we performed T-profiler gene group and MetaCore/PathVisio pathway analyses. Because the transcriptomic response of the nitrosamides was beneath the false positive threshold, these data were excluded from the pathway analysis.

Gene group and pathway analyses revealed a considerable number of processes modified by nitrosamine exposure (Tables 1, 2 and Supplementary Data worksheet 1–4). Pathway analyses indicated an increase in apoptosis and inhibition of cell cycle progression following nitrosamine exposure. In addition, a number of nucleotide metabolism pathways were modulated which may cause a DNA replication block, whereas the inhibition of lipid metabolism pathways is associated with apoptosis (Table 3) (Zhou *et al*., 2003). The GO gene group

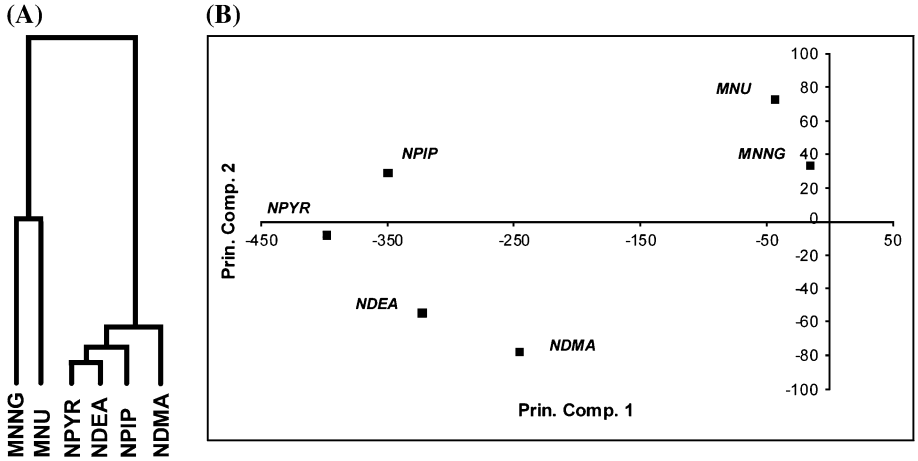

FIG. 3. Dendogram of Pearson correlation HCA (A) and PCA plot (B) for T-profiler GO gene groups significantly regulated for at least one of the six NOC. The percentage variation for principal components 1 and 2 were 91.59 and 4.39%.

TABLE 3
Theoretical Effects of Pathways Significantly Modulated by Nitrosamines as Found in MetaCore and PathVisio Analyses for a Selection of Strongly Affected Cellular Processes

Cellular process (theoretical effect)	Modulated pathways	NOC	Genes in pathway[a]	
			Significant	Total
Apoptosis (stimulation of apoptotic process)	Anti-apoptotic TNFs/NF-kB/Bcl-2 pathway	3/5/6	17/21/21	41
	Caspase cascade	3	17	31
	FAS signaling cascades	3	24	41
	NGF activation of NF-Kb	5	15	26
	p53-dependent apoptosis	3/5/6	17/16/18	29
	Role of IAP-proteins in apoptosis	3/5	15/10	25
	TNFR1 signaling pathway	3/5/6	23/18/23	38
	Apoptosis mechanisms[b]	4	22	84
	Proteasome degradation[b]	4	19	68
	TGF-beta receptor signaling pathway[b]	5/6	54/65	153
Cell cycle regulation (cell cycle block in S and G2 phase)	ATM/ATR regulation of G1/S checkpoint	5/6	17/20	32
	Brca1 as a transcription regulator	5	16	30
	ESR1 regulation of G1/S transition	5	17	30
	Initiation of mitosis	5	15	25
	Nucleocytoplasmic transport of CDK/Cyclins	3	12	14
	Regulation of G1/S transition	3/5/6	25/21/28	40
	Role of SCF complex in cell cycle regulation	5	15	28
	Start of DNA replication in early S phase	3/4/5/6	18/15/21/22	31
	The metaphase checkpoint	3/6	3/16/19	36
	Transition and termination of DNA replication	3/4/5	17/12/15	26
	Cell cycle[b]	3/5/6	34/36/44	87
	DNA replication[b]	3/4/5/6	22/15/22/21	42
	G1 to S cell cycle control[b]	3/4/5/6	32/17/34/34	68
DNA damage recognition/repair (stimulation of DNA repair)	Role of Brca1 and Brca2 in DNA repair	3	14	30
	NHEJ mechanisms of DSBs repair	3/6	11/10	18
	ATM/ATR regulation of G1/S checkpoint	5/6	17/20	32
	Brca1 as a transcription regulator	5	16	30
Response to oxidative stress (stimulation of antioxidant genes and inhibition of glutathione metabolism)	Glutathione metabolism[b]	4/6	9/25	36
	Role of ASK1 under oxidative stress	3/5/6	10/7/11	15
Immune response (stimulation of proinflammatory genes)	MIF-mediated glucocorticoid regulation	5/6	10/12	21
	Signaling pathway mediated by IL-6 and IL-1	3/5/6	8/11/16	26
	Bacterial infections in normal airways	5	19	39
	IL-1 signaling pathway[b]	3/5/6	16/20/23	33
	MIF in innate immunity response	3/5/6	13/16/18	24
	MIF-JAB1 signaling	5/6	11/10	17
	Toll-like receptor (TLR) ligands and common TLR signaling pathway leading to cell proinflammatory response	5	20	37
Nucleotide metabolism (DNA replication block)	ATP/ITP metabolism	3/4/5/6	46/22/36/44	74
	dATP/dITP metabolism	3/4/5/6	29/20/26/29	53
	dCTP/dUTP metabolism	3/4/5	26/17/25	46
	dGTP metabolism	3/4/5	24/14/22	42
	GTP-XTP metabolism	3/6	34/33	59
	TTP metabolism	3/4/5/6	23/14/22/22	38
Lipid metabolism (inhibition of fatty acid |synthesis and oxidation)	Cholesterol biosynthesis	4[c]	2	14
	PDGF activation of prostacyclin synthesis	5	8	11
	Regulation of fatty acid synthase activity in hepatocytes	3	10	16
	Fatty acid beta oxidation[b]	3/6	5/6	8
	Fatty acid biosynthesis[b]	3/4/6	15/7/14	22
	Triacylglyceride synthesis[b]	5	15	26

Note. NOC are indicated with: 3 (NDEA), 4 (NDMA), 5 (NPIP), 6 (NPYR).
[a]Significantly modulated genes (p value < 0.05 or Z score > 2) versus total number of genes in pathway.
[b]PathVisio pathways (sometimes overlapping with MetaCore pathways).
[c]Theoretical effect unclear due to low number of significant genes compared with total.

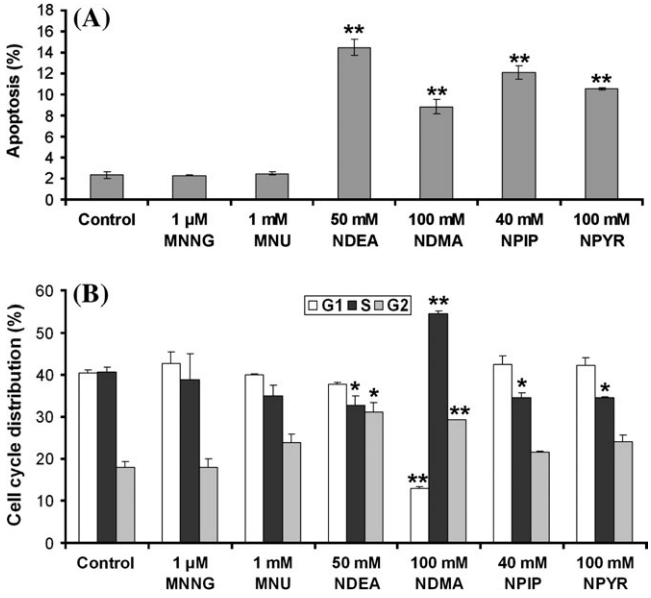

FIG. 4. Levels of apoptosis (A) and cell cycle distribution (B) in Caco-2 cells exposed to genotoxic concentrations of six NOC for 24 h as measured by flow cytometry analysis. DMSO control is not shown but was identical to normal control. Error bars indicate standard deviation. *p value < 0.05; **p value < 0.01.

analysis revealed gene groups likely to play a role in apoptosis and cell cycle regulation after nitrosamine exposure, such as the ubiquitine cycle, several mitochondria related GO categories, oxidoreductase activity, and electron transport chain gene groups (Table 2A and Supplementary Data worksheet 1). Though less explicit, also DNA repair pathways were modified by NDEA, NPIP, and NPYR (Table 2A). NPYR also affected an alkyl/aryl transferase activity gene group that may be involved in alkyl/aryl adduct repair. Motif gene group analysis revealed multiple gene groups that are associated with TFs known to play an important role in these processes (Table 2B), including several Forkhead box TFs and E2F1 (Burgering and Kops, 2002; Stevens and La Thangue, 2004). Apoptosis, regulation of cell cycle progression and DNA repair are all direct consequences of DNA damage, which activates and regulates these downstream cellular processes through the activation of the p53 tumor suppressor protein (Levine, 1997).

Interestingly, gene groups and pathways associated with oxidative stress were found for all nitrosamine exposures. The cadmium and copper ion binding GO gene groups (Table 2A), for example, contain many metallothioneins which are implicated in free radical scavenging (Sato and Bremner, 1993). Furthermore, regulation of oxidative stress pathways, resulting in stimulation of antioxidant genes, was identified by MetaCore, whereas motif gene groups responsive to the FOX and CREB/ATF TFs, were also modulated. These TFs are known to play an important role in the cellular oxidative stress response (Fawcett *et al*., 1999; Sedding, 2008). These results indicate an increase in radical formation following nitrosamine exposure, which may be related to the release of NO· from nitrosamines or radical formation during nitrosamine metabolism as reported by others (Bartsch *et al*., 1989; Heur *et al*., 1989; Hiramoto *et al*., 2002; Yamada *et al*., 2006). This suggests an important role for reactive oxygen species (ROS) in the induction of DNA damage by nitrosamines, and this may play a significant part in the carcinogenic process.

NDEA, NPIP, and NPYR also strongly affected cellular immune response pathways (Table 3). All three compounds influenced pathways involved in the stimulation of proinflammatory cytokines, including the IL-1 and IL-6 signaling pathways. Immune response GO gene groups were also modified after NPIP and NPYR exposures (Supplementary Data worksheet 1). These gene groups contain many proinflammatory genes, including a number of interleukins and tumor necrosis factor genes and their corresponding receptors. Intestinal epithelial cells, including the Caco-2 cell line, are known to excrete proinflammatory cytokines in response to different stimuli and also express cytokine receptors (Varilek *et al*., 1994; Vitkus *et al*., 1998). Such stimuli include oxidative stress which has been shown to increase cytokine production in Caco-2 cells (Nemeth *et al*., 2007; Yamamoto *et al*., 2003). Cytokine production, especially IL-6, plays an important role in the induction of the acute phase response and stimulation of the intestinal inflammatory response (Akira *et al*., 1993). It could be hypothesized that a high intestinal exposure to these specific nitrosamines, originating either directly from the diet or formed endogenously, incites intestinal inflammation, possibly triggered by increased oxidative stress levels. Such an increase in inflammation could contribute to the carcinogenic process and play a part in NOC exposure related cancers (Aggarwal *et al*., 2006).

Another interesting result from the motif gene group analysis was the identification of several TF associated gene groups containing homeodomain responsive genes which are implicated in essential developmental processes, including embryogenesis (Table 2B). These gene groups were mainly identified for NDEA, NPIP, and NPYR. Deregulated expression of homeobox genes has also been found in many types of cancer, including colon cancer, and they seem to play a pivotal role in the carcinogenic process (Abate-Shen, 2002). Pathway analyses also showed significant modulations of several pathways involved in development, such as the Notch and EGFR1 signaling pathways, after NDEA, NPIP, and NPYR exposure (Supplementary Data worksheet 3 and 4).

NDEA, NPIP, and NPYR thus seem to pose a higher cancer risk than NDMA. NDMA had a smaller effect on gene expression modifications compared with the other nitrosamines and NDMA was absent in pathways involved in the immune response and developmental TF associated gene groups, both of which can be linked to an increased carcinogenic potential. Although the HCA and PCA analyses (Figs. 3C and 3D) show a rather close grouping of the nitrosamines, suggesting similar effects on a functional level, NDMA does dissociate from the other nitrosamines. NDMA uniquely affected steroid

metabolism pathways which are involved in cholesterol, androstenedione and testosterone synthesis and metabolism (Supplementary Data worksheet 3 and 4): the implications of this, however, are unclear. NDMA also uniquely modified three E2F family related motif gene groups involved in cell cycle regulation among others (Table 2B). Regarding the other three nitrosamines, NPIP and NPYR modulated more pathways than NDEA. This difference is mainly attributable to the larger number of pathways affected by NPIP and NPYR that are involved in the immune response and development (Supplementary Data worksheet 1, 3, and 4). Exposure to NPIP or NPYR may therefore also imply a higher carcinogenic risk than exposure to NDEA or NDMA. The differences in response between the nitrosamines may be related to the type of adducts generated by each individual compound. NDMA will result in methyl adducts and NDEA in ethyl adducts, whereas NPIP and NPYR are expected to generate a heterogeneous group of alkyl and aryl adducts (Young-Sciame *et al*., 1995).

Animal studies with exogenous exposure to NOC have not identified the colon as one of the main target organs for NOC (Table 1). We, however, hypothesize that the colon is a relevant target tissue, predominantly as a result of high exposures following the endogenous formation of NOC which is known to occur in the colon (Kuhnle *et al*., 2007). Although our results are based on an intestinal adenocarcinoma cell line, and extrapolation of these results to normal colonic tissue should be done with restraint, Caco-2 cells are generally considered a good model for the human colon (Sambuy *et al*., 2005). Our findings can therefore be considered as indicative of mechanistic effects following colonic NOC exposure.

Measurements of cell cycle distribution and the level of apoptosis following NOC exposure demonstrated an increased number of apoptotic cells and a shift in cell cycle distribution (Figs. 4A and 4B). The S phase accumulation after NDMA treatment indicates a delay during the replication process, possibly due to the presence of DNA adducts. The decrease in S phase and increase in G2 phase cells following NDEA, NPIP and NPYR exposure, may indicate that DNA adducts are largely repaired and cells are resuming the cell cycle. The difference in NDMA response with regards to cell cycle distribution may be reflected by the three uniquely modified E2F family motif gene groups identified by T-profiler (Table 2B).

Although apoptosis, as shown by flow cytometry, did not correlate with apoptosis pathways it was related with several cytoskeleton remodeling, cell adhesion and cell cycle regulation pathways (Supplementary Data worksheet 5), which is to be expected because apoptosis is associated with such processes. The S and G2 phases were also associated with many of these pathways, in addition to being linked with apoptosis. Pathways involved in the immune response and signaling were also abundantly linked to apoptosis and cell cycle regulation, which may suggest a role for the immune response in these responses.

The absence of strong gene modulating effects by MNU and MNNG may be related to the direct acting properties of nitrosamides. Because nitrosamides do not require metabolic activation to become genotoxic, this may result in rapid transcriptomic responses that also subside faster. This requires further study where in view of their genomics responses in human Caco-2 cells *in vitro* it is suggested that nitrosamines rather than nitrosamides play a significant role in colon cancer development in humans. This is in contrast to animal studies which implicate nitrosamides in colon cancer development, rather than nitrosamines (Lijinsky, 1992).

The data obtained in this study provide new insights into possible modes-of-action of nitrosamines and their gene expression effects at genotoxic levels in the human intestinal epithelial cell line Caco-2 and its implications for colon carcinogenesis. We have confirmed the possible relevance of ROS production after NOC exposure and identified modulation of the inflammatory response and developmental pathways as potential NOC targets using different approaches. Where it has to be considered that the gene expression responses *in vitro* have been sampled at rather high incubation concentrations of nitrosamines, identified processes may be of mechanistic importance in assessing carcinogenic risk after NOC exposure in the human colon.

SUPPLEMENTARY DATA

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

FUNDING

This study was funded by both the European Network of Excellence (FOOD-CT-2005-513943) on Environmental Cancer Risk, Nutrition, and Individual Susceptibility, and by Maastricht University.

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