4-(Methylnitrosamino)-1-(3-Pyridyl)-1-Butanone Enhances the Expression of Apolipoprotein A-I and Clara Cell 17-kDa Protein in the Lung Proteomes of Rats Fed a Corn Oil Diet but not a Fish Oil Diet

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Abstract

The nicotine-derived nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butane (NNK) is one of the most potent lung carcinogens in rodents. Several epidemiologic studies indicated that the development of lung cancer in smokers is influenced by the type and amount of dietary polyunsaturated fatty acids. A high corn oil diet has been shown to increase lung tumor volume and to decrease tumor latency in rats treated with NNK. In this study, we investigated the effect of dietary polyunsaturated fatty acids in the form of corn oil or fish oil on lung proteomes in F344 rats treated with or without NNK. The fish oil diet contained 17% fish oil and 3% corn oil, and the corn oil diet contained 20% corn oil. Rats were sacrificed after 3 months, and lungs were excised. Whole lung tissue proteins were separated by two-dimensional liquid chromatography, and differentially expressed proteins were identified by trypsin digestion and tandem mass spectrometry. Apolipoprotein A-I and Clara cell 17-kDa protein may be involved in the development of NNK-induced lung cancer in rats fed a high corn oil diet. Therefore, we propose that both proteins may serve as potential biomarkers in future molecular epidemiologic and clinical chemoprevention intervention studies. (Cancer Epidemiol Biomarkers Prev 2007;16(2):228–35)

Introduction

Over 4,000 chemicals have been identified in tobacco smoke, and among those, at least 20 are known to induce lung cancer in laboratory animals (1). Specifically, the nicotine-derived nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butane (NNK) is known to be one of the most potent lung carcinogens in rodents (2). NNK induces adenocarcinoma in the lung of mice, rats, and Syrian golden hamsters, independent of the route of administration (3). NNK is metabolically activated to intermediates that damage DNA in rodents, and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol, the predominant metabolite of NNK, has been detected in smokers and in people exposed to environmental tobacco smoke (2, 4, 5). Recently, the IARC has classified NNK as a class I carcinogen, which is carcinogenic to humans (6).

Some but not all epidemiologic studies have indicated that the type and amount of daily dietary fat are important factors in the development of lung cancer (7-9). Previous epidemiologic studies have reported an effect of fatty acid consumption on smoking-related lung diseases, including lung cancer. Intake of ω-3 polyunsaturated fatty acids (PUFA), abundant in fish oil, protects cigarette smokers against chronic obstructive pulmonary disease (10, 11) and the deterioration of lung function (12). Zhang et al. (13) suggested that the consumption of dietary ω-3 PUFA is associated with a reduced risk of lung cancer mortality in males in countries with high levels of cigarette smoking and high animal fat consumption. Data from several animal studies suggest that dietary PUFA may be an important modulator in the development of certain cancers. Consumption of ω-6 PUFA has been shown to increase the incidence of colon, mammary, and pancreatic cancers, whereas consumption of ω-3 PUFA has been shown to inhibit carcinogenesis (14). In rodents, only a few studies have explored the influence of dietary PUFA in tobacco carcinogenesis (15-17). For example, Hoffmann et al. (15) showed that in NNK-treated rats, the increase of dietary corn oil from 5% to 23.5% resulted in an increase in lung tumor volume and a decrease in tumor latency.

To provide insights into the mechanisms responsible for the effects of dietary PUFA on lung cancer, in this study, we sought to investigate early changes in lung proteomes of Fisher rats in response to type of fat (corn oil or fish oil) and to NNK treatment.

Materials and Methods

Bioassay and Tissue Collection. The protocol used in this study is similar to that reported by Hoffmann et al. (15). In this pilot study, we determined whether the 3-month exposure to NNK and/or dietary PUFA is sufficient to induce biochemical and molecular changes in rats that may account for the increase in tumor volume and the decrease in tumor latency as reported by Hoffmann et al. in a 2-year bioassay (15). Briefly, male F344 rats (Charles River Breeding Laboratories, Kingston, NY) were divided into four groups and housed in solid-bottomed...
Polycarbonate cages under standard conditions [20 ± 2°C (SD), 50 ± 10% relative humidity, 12-h light and dark cycle]. Beginning at the age of 8 weeks, the animals were maintained on the following diets: groups 1 and 3, 20% corn oil; groups 2 and 4, 17% fish oil and 3% corn oil. The diets are based on the modified AIN-76 diet (18), and the composition of diets is given in Table 1. The 17% fish oil diet was supplemented with 3% corn oil to prevent any potential deficiency in essential fatty acids. Fresh diets were prepared weekly and stored at 4°C in a container purged with nitrogen. Lipid peroxidation of the oils does not occur under the conditions employed for diet preparation. All ingredients of experimental diets were obtained from Dyets, Inc. (Bethlehem, PA), except casein, which was purchased from BioServ (Frenchtown, NJ). The fish oil (menhaden) was generously donated by the Menhaden Oil Refinery of Zapata Protein, Inc. (Reedville, VA). Animals in groups 1 and 3 were given a 2-ppm solution of NNK in tap water, whereas animals in groups 2 and 4 received tap water without NNK. A 2-ppm solution of NNK in tap water was prepared once weekly and stored in amber bottle at 4°C before use. Body weights of rats were determined once weekly throughout the duration of the bioassay (3 months). To investigate early changes in the lung proteome, six animals from each group were sacrificed after 3 months, and the lungs were removed, snap frozen in liquid nitrogen, and stored at −80°C.

Preparation and Separation of Rat Lung Proteins. The ProteomeLab PF2D (Beckman Coulter, Fullerton, CA) employs two-dimensional high-performance liquid chromatography to separate proteins. A schematic presentation of sample preparation and analysis of rat lung protein is shown in Fig. 1. Briefly, tissues were first lysed, and the resulting solution was desalted. The first-dimension high-performance liquid chromatography step in protein separation, based on isoelectric point (pI), was accomplished using a pH gradient. Each pI-dependent fraction collected from this step was then further separated by reverse-phase high-performance liquid chromatography in the second dimension. Chromatographic data obtained from the PF2D separation were analyzed using ProteoVue and DeltaVue software packages. Individual two-dimensional proteome maps were generated with ProteoVue, and then comparisons among two-dimensional maps and detailed peak analysis were made by using DeltaVue. Differentially expressed proteins were subjected to trypsin digestion and analyzed by matrix-assisted laser desorption/ ionization time-of-flight tandem mass spectrometry (MALDI-TOF-MS/MS).

In this study, samples of rat lung tissues (~100 mg) were homogenized and lysed in 2 mL of an optimized lysis buffer [5 mol/L urea, 2 mol/L thiourea, 10% glycerol, 2.5% (w/v) N-decyl-N,N-dimethyl-3-ammonio-1-propane sulfonate, 50 mmol/L Tris, 2% n-octylglucoside, 5 mmol/L Tris (2-carboxyethyl)-phosphino-HCl, 1 mmol/L protease inhibitor]. The lysates were cleared by centrifugation at 80,000 × g for 60 min at 18°C. The supernatant was removed and applied to a PD-10 desalting column (Amersham Pharmacia Biotech, Piscataway, NJ) which was equilibrated with 25 mL of the PF2D Start Buffer (pH 8.5 ± 0.1). Proteins were eluted with the PF2D start buffer; the first 3.5 mL were collected; and the total protein concentration of this fraction was then determined by using the BCA protein assay kit (Pierce, Rockland, IL). Five milligrams of total protein from each sample were removed and diluted in the PF2D start buffer to bring the volume for each sample to 1.5 mL. For the PF2D separation, the standard PF2D default running method provided by the manufacturer was used. Briefly, samples were injected onto the chromatofocusing column in the first-dimension compartment, and protein separation was done at room temperature with a constant flow rate of 0.2 mL/min. The absorbance of the column effluent was monitored at 280 nm. Fractions (~ 0.6 to 1.0 mL) were collected every 0.3 pH units for proteins within the pH range of 4 to 8. The reversed-phase high-performance liquid chromatography column in the second-dimension compartment was pre-equilibrated with 0.1% trifluoroacetic acid in water and 200 μL of each first-dimension fraction was then injected onto the reverse-phase column. The second-dimension separation was done at 50 ± 1°C, with a flow rate of 0.75 mL/min, and absorbance of the column effluent was monitored at 214 nm. The column was eluted with a gradient of 0% to 100% of acetonitrile containing 0.08% trifluoroacetic acid over 30 min, and fractions were collected every 15 s and stored at −20°C before MALDI-TOF-MS/MS analysis.

MALDI-TOF-MS/MS Analysis. Differentially expressed proteins as determined by the PF2D analysis were further analyzed by MALDI-TOF-MS/MS. After the samples of

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Table 1. Percentage composition of experimental diets

<table>
<thead>
<tr>
<th>Diet ingredients</th>
<th>Corn oil diet (%)</th>
<th>Fish oil diet (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein</td>
<td>23.50</td>
<td>23.5</td>
</tr>
<tr>
<td>DL-methionine</td>
<td>0.35</td>
<td>0.35</td>
</tr>
<tr>
<td>Cornstarch</td>
<td>35.70</td>
<td>35.7</td>
</tr>
<tr>
<td>Dextrose</td>
<td>9.02</td>
<td>9.02</td>
</tr>
<tr>
<td>Alginic acid</td>
<td>5.00</td>
<td>5.00</td>
</tr>
<tr>
<td>Corn oil</td>
<td>20.00</td>
<td>3.00</td>
</tr>
<tr>
<td>Fish oil</td>
<td>—</td>
<td>17.0</td>
</tr>
<tr>
<td>Mineral mix</td>
<td>4.11</td>
<td>4.11</td>
</tr>
<tr>
<td>Vitamin mix</td>
<td>1.18</td>
<td>1.18</td>
</tr>
<tr>
<td>Choline bitartrate</td>
<td>0.24</td>
<td>0.24</td>
</tr>
</tbody>
</table>

NOTE: All diets were formulated on the basis of the AIN standard reference diet with the modification of varying sources of carbohydrate (18).

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Dr. Bandaru Reddy, Rutgers, The State University of New Jersey, personal communication.

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**Figure 1.** Schematic representation of experimental methods used in the preparation, separation, and identification of rat lung tissue proteins.
interest were concentrated to a volume of ~10 µL, DTT and NH₄HCO₃ were added to give final concentrations of 2.5 and 50 mmol/L, respectively. Following incubation at 50°C for 30 min, the samples were cooled to room temperature; iodoacetamide was added to give a final concentration of 10 mmol/L; and the solution was then incubated in the dark at 37°C for 30 min. After alkylation, the samples were brought to a volume of 100 µL with a solution containing 50 mmol/L NH₄HCO₃ (pH 8), 10% v/v acetonitrile, and 0.15 µg Promega Gold Trypsin (Promega, Madison, WI). The samples were incubated at 37°C for 16 to 18 h and then evaporated to dryness. To completely remove the bicarbonate, the protein digests were resuspended thrice in 200 µL water, evaporated to dryness, then resuspended a fourth time in water and evaporated to ~10 µL instead of complete dryness. After the addition of 1 µL of a 1% trifluoroacetic acid solution in water, peptides were desalted and concentrated using ZipTip SCX tips (Millipore, Billerica, MA) and eluted directly onto stainless steel target plates (Applied Biosystems, Framingham, MA). After drying, the sample spots were overlaid with 0.7 µL of a 1% cyano-4-hydroxycinnamic acid (5 mg/mL in 50% acetonitrile, 2 mg/mL NH₄HPO₄) and then dried at room temperature. The peptide mixtures were analyzed using an Applied Biosystems 4700 Proteomics Analyzer (MALDI-TOF-TOF). The MS spectra were calibrated using a plate calibration derived from six external calibration spots on each MALDI plate, resulting in a precursor mass accuracy of better than ±50 ppm. Protein identification was done using the Mascot algorithm version 1.9 (Matrix Science, London, United Kingdom) as implemented in the GPS Explorer analysis program (version 3.5, Applied Biosystems) against nonredundant protein database and Swissprot (version 2005.0315). Peptide mass fingerprints and MS/MS spectra with a confidence interval of >95% were used for protein identification.

**Western Blot Analysis.** Lung tissue was homogenized in 1 mL of lysis buffer containing 20 mmol/L Tris buffer (pH 7.5), 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1% Triton X-100, 1 mmol/L phenylmethylsulfonyl fluoride, and 10 µg/mL leupeptin. The lysates were clarified by centrifugation (22,000 x g) at 4°C for 40 min, and protein concentration was determined using the BCA protein assay. A volume equivalent to 50 µg protein was removed from each sample, diluted 2-fold with 2/3 Laemmli buffer (Bio-Rad, Hercules, CA) containing β-mercaptoethanol (at a final concentration of 5%) and bromophenol blue, and then placed in boiling water for 5 min. The samples were run a 10% SDS-PAGE gel. The proteins were electrotransferred to nitrocellulose membranes. Blots with equal protein loading were probed with a goat polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) for apolipoprotein A-I (apo A-I) in 1% fat-free dry milk. Because a specific antibody for Clara cell 17-kDa (CC17) protein was not commercially available, a rabbit polyclonal Clara cell 10-kDa (CC10) protein antibody (Santa Cruz Biotechnology) was used to probe CC17. The incubations with primary antibodies were done overnight at 4°C. The blots were then incubated with horseradish peroxidase-conjugated antibody (donkey anti-goat IgG-HRP for apo A-I and donkey anti-rabbit IgG-HRP for CC17; Santa Cruz Biotechnology) for 1 h at room temperature. The Western blots were developed using the ECL chemiluminescence system according the manufacturer’s instructions (Amersham Pharmacia, Little Chalfont, United Kingdom).

**Statistical Analyses.** Statistical evaluation for chromatographic data obtained from the PF2D analysis was done by using a two-way ANOVA with NNK (absent versus present) and oil (corn oil versus fish oil) as between-animal factors followed by post hoc contrasts using PROC MIXED in SAS version 9.1. All tests are two sided and considered significant at P < 0.05.
Results and Discussion

Body weights of rats were determined once per week throughout the duration of the bioassay (3 months); no significant differences in the mean body weights of rats were observed among the four groups (data not shown).

Two-dimensional proteome maps for proteins isolated from lung tissue of F344 rats fed high fat diet containing either corn oil or fish oil with or without NNK are shown in Fig. 2. The maps are displayed as UV/pI profiles, where the pI fractions are shown in horizontal lanes 1 to 14, and the UV chromatograms obtained in the second dimension are shown in vertical position. The DeltaVue software revealed ~500 peaks in the chromatograms of protein fractions within the pI range of 4 to 8. A comparison between samples with different treatments was done lane by lane, and local alignment was visually optimized using the DeltaVue’s peak pattern match feature and lane adjustment capabilities. Proteome maps among samples with different treatments showed similarity in the overall fingerprints of lung tissue proteins within the pI range.

Figure 3. Two-dimensional protein maps of the lung tissue protein of F344 rats fed either high-fat corn oil diet or fish oil diet with or without NNK. Each lane represents a fraction with a pI range of 5.4 to 5.7 separated by the first-dimension chromatofocusing column, and to the outside of each map are the high-performance liquid chromatograms for corresponding lanes.
of 4 to 8. However, measurable differences of protein signatures were seen in the fraction of pI 5.4 to 5.7. The PF2D profiles of these fractions displayed in DeltaVue are shown as chromatograms (Fig. 3). Each lane represents the protein fraction, and the UV chromatograms for the second-dimension separation for corresponding lanes are shown to the outside of each map. The center portion of Fig. 3 shows the qualitative and quantitative differences between samples, where the intensity of the color represents the abundance of the proteins associated with the peaks displayed. A total of 38 peaks were detected in the protein fractions within the pI range of 5.4 to 5.7.

Examination of the two-dimensional profiles showed a protein peak (P1) with high intensity at 19.1 min in the fraction from rats fed corn oil with or without NNK, compared with those fed fish oil with or without NNK (Fig. 3). Another protein peak (P3) with high intensity was observed at the retention time 21.1 min in the same set of samples. The intensity of a protein peak (P2) detected at the retention time 20.5 min varied from sample to sample. Table 3 shows relative peak areas of the selected proteins in the pI fraction of 5.4 to 5.7. Quantitative assessment of protein levels was determined using the peak integration feature of the DeltaVue software for data collected from three replicate runs (n = 3 per group). The levels of P1 and P3 increased in the lungs of rats fed corn oil diet, compared with those fed fish oil diet; however, P2 was not identified by the PF2D and MALDI-TOF-MS/MS analyses. Further studies are needed to explain why CC10 is not detectable by the PF2D and MALDI-TOF-MS/MS analyses. It is possible that loss of some proteins, including CC10, may occur during the chromatofocusing step of the first-dimension separation of proteins in the PF2D analysis.

Table 3. Proteins identified by MALDI-TOF-MS/MS

<table>
<thead>
<tr>
<th>Spot no.</th>
<th>Protein identity</th>
<th>MASCOT molecular weight</th>
<th>Protein score</th>
<th>Total ion score</th>
<th>Sequence coverage (%)</th>
<th>Accession no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>Apo A-I</td>
<td>30,100</td>
<td>125</td>
<td>56</td>
<td>27.8</td>
<td>P04639</td>
</tr>
<tr>
<td>P2</td>
<td>Apo A-IV</td>
<td>44,428</td>
<td>98</td>
<td>50</td>
<td>19.4</td>
<td>P02651</td>
</tr>
<tr>
<td>P3</td>
<td>CC17</td>
<td>10,670</td>
<td>110</td>
<td>50</td>
<td>31.2</td>
<td>pdb:1CCCD</td>
</tr>
</tbody>
</table>

The selected proteins were successfully identified by trypsin digestion and MALDI-TOF-MS/MS analysis. Mascot search for peptides based on Swissprot database led to the identification of apo A-I, apo A-IV, and CC17 proteins for P1, P2, and P3, respectively (Table 3). Typical mass spectra of the trypsin digest of these proteins are shown in Fig. 4. The MALDI-TOF-MS/MS confirmation of these proteins in the same elution fractions from samples isolated from different lung tissues showed that the chromatographic data collected using the PF2D were highly reproducible. The results of the present study revealed for the first time that apo A-I and CC17 responded differently to corn oil or fish oil, implying that the type of PUFA influences their expression levels in the lungs of rats; NNK exposure enhanced the effects of corn oil on all these proteins. The identification of apo A-I and CC17 using the PF2D and MALDI-TOF-MS/MS is consistent with the results obtained by Western blot analysis (Fig. 5). Whole tissue proteins were subjected to Western blot analysis, and as shown in Fig. 5, apo A-I (30 kDa) and CC17 (17 kDa) were detected in the lungs of corn oil–treated rats with or without NNK administration. We also showed that CC10 protein is overexpressed in the lungs of rats treated with NNK and fed corn oil diet; however, CC10 was not identified by the PF2D and MALDI-TOF-MS/MS analyses. Further studies are needed to explain why CC10 is not detectable by the PF2D and MALDI-TOF-MS/MS analyses. It is possible that loss of some proteins, including CC10, may occur during the chromatofocusing step of the first-dimension separation of proteins in the PF2D analysis.

Apo A-I is a major protein component of high-density lipoprotein cholesterol and is synthesized principally in the small intestine and liver in mammal. It is an important mediator of reverse cholesterol transport, a process by which free cholesterol is removed from the peripheral tissues of the body and transferred back to the liver (19). Previous studies suggested that apo A-I protein plays a potential role as an antioxidant and an anti-inflammatory factor to reduce cell or tissue damage (20, 21). Apo A-I has been shown to inhibit the production of interleukin-1β and tumor necrosis factor-α at both protein and mRNA levels by binding to T lymphocytes and thus blocking contact-mediated activation of monocytes (20). The expression of apo A-I has also been associated with breast cancer and colon cancer progression in humans (22, 23).

Clara cell proteins (CC10 or CC16) is one of the major proteins secreted by Clara cells and is a steroid-inducible and multifunctional polypeptide that accounts for ~7% of the total protein in bronchiolar lavage fluid (24). Clara cell proteins have been cited interchangeably in the literature as CC10, CC16, and CC17, depending on the organ and species. Although their exact physiologic functions are not fully understood, literature data suggest that Clara cell proteins play a protective role against pulmonary inflammatory response. CC10 is known to inhibit the activity of phospholipase A2, a key enzyme in the production of prostaglandins and leukotrienes, as well as the production and activity of tumor necrosis factor-α and interleukin-1β (25). In this study using the PF2D combined with MALDI-TOF-MS/MS, we identified CC17 as overexpressed in the lung of rats after...
Figure 4. MALDI-TOF-MS spectra of the tryptic digest of the protein fraction P1, P2, and P3 from the two-dimensional chromatographic separation of rat lung proteins; arrows label peaks that correspond to proteins identified by peptide mass fingerprinting, and asterisks indicate peaks in which the identities of these proteins were confirmed by MS/MS analysis.
3-month exposure to corn oil; its overexpression was further enhanced by NNK treatment.

Enhanced expression of CC17 is considered an early response of cells providing protection against inflammation or oxidative stress during the initial stage of lung cancer induced by NNK in combination with high-fat corn oil diet (15). Although the levels of CC17 expression at various time points during the progress of the bioassay need to be determined, they are likely to be decreased in rat lungs over time in response to corn oil and also in combination with NNK. Previous studies suggest that the reduced levels of CC10 protein are associated with tobacco smoking (26-28) and lung tumorigenesis (29). Longer exposure of hamsters and mice to NNK has been shown to be associated with reduced CC10 expression (26, 29). Broeckaert et al. (28) showed that the serum levels of Clara cell protein (CC16 or CC10) are decreased in subjects with chronic lung damage caused by tobacco smoke and other air pollutants as a consequence of the destruction of Clara cells. A recent study showed that plasma CC10 levels are significantly lower in current smokers compared with former smokers, indicating that sustained smoking cessation is associated with up-regulation of plasma CC10 levels (27).

Corn oil is rich in ω-6 PUFA (~12% linoleic acid), whereas fish oil (menhaden) is high in ω-3 PUFA (~14% docosahexaenoic acid and ~30% eicosapentaenoic acid). Although the mechanism underlying the enhanced expression of apo A-I and CC17 in the lungs of NNK-treated rats fed ω-6 PUFA from corn oil are not clear, both apo A-I and CC17 proteins may play a protective role against oxidative stress induced by lipid peroxidation and/or by NNK at an early stage of NNK-induced carcinogenesis possibly because of alterations in prostaglandin synthesis mediated by cyclooxygenase-2 (30).

To our knowledge, there are no published data on the influence of dietary fat and/or NNK on the expression of these proteins in a well-defined animal model system. We reasoned that the enhanced expression of apo A-I and CC17 proteins in rats fed corn oil diet and treated with NNK represents an early antioxidant or anti-inflammatory response to the oxidative stress induced by corn oil or in combination with NNK. In a future long-term bioassay, we intend to determine which this increased expression would persist, or whether it is simply an early response to oxidative stress induced by corn oil or NNK, which may vary over time. Furthermore, in future studies, we will also explore the effects of corn oil and fish oil diet with and without NNK in F344 rats on the same proteins in pancreatic tissue, a relevant target organ of NNK-induced carcinogenesis (15). In addition, similar studies will also be conducted using either fish oil or NNK alone.

The present study suggests that the enhanced expression of apo A-I and CC17 may contribute in the development of NNK-induced lung cancer in rats fed corn oil diet, and both proteins may serve as potential biomarkers in future molecular epidemiologic (28) and clinical chemoprevention intervention trials. However, the mechanisms by which corn oil alone or in combination with NNK modulate the expression of the two proteins require further investigation.

Acknowledgments

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References

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