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Quantitative Proteomic Analysis Revealed 4-(methylnitrosamino)-1-(3-pyridinyl)-1-butanone-induced Up-regulation of 20S Proteasome in Cultured Human Fibroblast Cells

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Abstract

The tobacco-specific N-nitrosamine, 4-(methylnitrosamino)-1-(3-pyridinyl)-1-butanone (NNK), is a well-known carcinogen. Although the ability of the metabolically activated form of NNK to generate DNA adducts is well established, little is known about the cellular pathways perturbed by NNK in its native state. In this study, we utilized stable isotope labeling by amino acid in cell culture (SILAC), together with mass spectrometry, to assess the perturbation of protein expression in GM00637 human skin fibroblast cells upon NNK exposure. With this approach, we were able to quantify 1412 proteins and 137 of them were with significantly altered expression following NNK exposure, including the up-regulation of all subunits of the 20S proteasome core complex. The up-regulation of the 20S core complex was also reflected by a significant increase in 20S proteasome activities in GM00637, IMR90 and MCF-7 cells upon NNK treatment. Furthermore, the β -adrenergic receptor (β -AR) antagonist propranolol could attenuate significantly the NNK-induced increase in proteasome activity in all the three cell lines, suggesting that up-regulation of the 20S proteasome may be mediated through the β -AR. Additionally, we found that NNK treatment altered the expression levels of other important proteins including mitochondrial proteins, cytoskeleton-associated proteins, and proteins involved in glycolysis and gluconeogenesis. Results from the present study provided novel insights into the cellular mechanisms targeted by NNK.

Keywords

Tobacco-specific N-nitrosamines (TSNAs); NNK; mass spectrometry; protein quantitation; 20S proteasome; β -adrenergic receptor

Introduction

Tobacco use is the leading cause of preventable death and is estimated to kill more than 5 million people worldwide in each year. The adverse health effects associated with tobacco use are well-known and have been widely reported. More than 4,000 chemicals have been identified in tobacco smoke, among which 250 and 50 are known to be harmful and cause cancer, respectively¹. The toxic constituents of tobacco include polycyclic aromatic hydrocarbons, nitrosamines and heavy metals; exposure to these chemicals have long been known to be associated with the development of various human diseases^{2–4}.

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Supporting Information Available: Tables for protein identification and quantification results. This material is available free of charge via the Internet at <http://pubs.acs.org>.

Tobacco-specific N-nitrosamines (TSNAs) are considered one of the most important classes of carcinogens found in tobacco products and numerous studies have demonstrated that TSNAs are present in substantial quantities in unburned tobacco and its smoke^{5, 6}. In addition, TSNAs can be formed in tobacco smoke residues via surface-mediated reactions between nicotine and ambient nitrous acid⁷. One of the most important TSNAs found at significant amounts in tobacco and its smoke is 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK). NNK has been widely studied due to its carcinogenic activity and it is classified as a human carcinogen by the International Agency for Research on Cancer⁸. Exposure to NNK has been shown to result in DNA adduct formation and induce lung tumors in all species tested⁶. NNK exposure can also lead to the development of tumors in the pancreas, nasal mucosa, and liver of laboratory animals^{5, 6}. DNA adduct formation is considered the central step in the process of NNK carcinogenesis, which requires cytochrome P450-mediated activation of NNK to DNA-reactive metabolites. These metabolites can induce the methylation, pyridyloxobutylation and pyridylhydroxybutylation of nucleobases in DNA⁶. However, apart from DNA adduct formation, there is a great deal of evidence indicating that other pathways may also contribute to TSNA carcinogenesis⁵. For instance, NNK has been shown to bind and activate nicotinic and β -adrenergic receptors, leading to the activation of downstream cell signaling pathways^{9, 10}. These aspects of NNK toxicity at the protein level have not been adequately examined and the potential importance of these molecular mechanisms underlying NNK-induced carcinogenic effect requires further investigation.

The developing field of proteomics has delivered a variety of techniques that can be used to analyze the expression levels of proteins in biological systems following xenobiotic exposure¹¹. Quantitative proteomics has become a valuable tool for this type of study since it can provide information about the toxic effects of xenobiotics at the global proteome scale, which may allow for the discovery of novel cellular pathways that are altered upon NNK exposure. In this study, we employed a quantitative proteomic technique, based on stable isotope labeling by amino acid in cell culture (SILAC) together with LC-MS/MS, to assess the perturbation of protein expression in GM00637 human skin fibroblast cells induced by NNK exposure. SILAC is a metabolic labeling method and it facilitates the in-vitro incorporation of stable isotope-labeled amino acids into proteins for mass spectrometry-based protein quantification in the whole proteome¹². SILAC relies on the incorporation of a given 'light' or 'heavy' form of essential amino acids into proteins, allowing for the generation of two unique cell populations that can be differentiated by LC-MS/MS (Figure 1A). The method facilitates the relative quantification of small changes in protein abundance in cells with and without xenobiotic exposure¹². Using this technique, we were able to quantify a total of 1412 unique proteins in all three sets of SILAC measurements (Figure 1B), among which 137 were significantly altered upon NNK treatment, including the increased expression of 14 subunits of the 20S proteasome core complex. The up-regulation of the 20S proteasome core complex following NNK treatment resulted in a corresponding increase in the chymotrypsin-like, trypsin-like, and peptidylglutamyl-peptide hydrolyzing (PGPH) activities of the 20S proteasome in human skin and lung fibroblast cells. Additionally, we observed the altered expression of mitochondrial proteins, cytoskeleton-associated proteins, and proteins involved in glycolysis and gluconeogenesis. The identification of proteins perturbed by NNK exposure helped elucidate multiple cellular pathways that are altered and unveiled novel cellular mechanisms targeted by this important tobacco carcinogen.

Materials and Methods

Materials

Heavy lysine and arginine ($[^{13}\text{C}_6, ^{15}\text{N}_2]$ -L-lysine and $[^{13}\text{C}_6]$ -L-arginine) were purchased from Cambridge Isotope Laboratories (Andover, MA). NNK was obtained from Toronto Research Chemicals Inc. (North York, Ontario, Canada). Propranolol and all other chemicals/reagents unless otherwise noted were purchased from Sigma-Aldrich (St. Louis, MO).

Cell culture

GM00637 human skin fibroblast cells, obtained from the Coriell Institute for Medical Research (Camden, NJ), were cultured in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% fetal bovine serum (FBS, Invitrogen, Carlsbad, CA) and penicillin/streptomycin (100 IU/mL). Cells were maintained in a humidified atmosphere with 5% CO_2 at 37°C, and the culture medium was changed at every 2–3 days as necessary. For SILAC experiments, custom IMDM medium was prepared without L-lysine or L-arginine according to the American Type Culture Collection (ATCC, Manassas, VA) formulation. The complete light and heavy IMDM media were prepared by the addition of light or heavy lysine and arginine, along with 10% dialyzed FBS. The GM00637 cells were cultured in the heavy IMDM medium for at least 10 days or 5 cell doublings to achieve complete heavy isotope incorporation.

IMR-90 human lung fibroblast and MCF-7 human breast adenocarcinoma cells were obtained from ATCC, and were cultured in Eagles minimum essentials medium (EMEM). All culture media were supplemented with 10% FBS and cells were maintained in humidified atmosphere with 5% CO_2 at 37°C with medium renewal in every 2 to 3 days depending on cell density.

NNK treatment and cell lysate preparation for SILAC experiments

GM00637 cells were cultured to a density of approximately 7.5×10^5 cells/mL. The cells were washed twice with ice-cold phosphate-buffered saline (PBS) to remove the residual FBS, and replaced with FBS-free heavy or light media containing 5 μM NNK or vehicle control (DMSO, final concentration < 0.0025%). In forward SILAC experiments, the cells cultured in light medium were treated with 5 μM NNK for 24 hr, whereas the cells cultured in heavy medium were untreated and used as control. For reverse SILAC experiments, cells cultured in the heavy medium were treated with NNK and light cells were used as the untreated control (Figure 1A). After 24 hr, the light and heavy cells were collected by centrifugation at 3,000 g at 4°C, and washed three times with ice-cold PBS. The cell pellets were re-suspended in the CellLytic™ M cell lysis buffer containing a protease inhibitor cocktail and placed on ice for 30 min with vortexing at 10-min intervals. Cell lysates were centrifuged at 12,000 g at 4°C for 30 min, and the resulting supernatants were collected. The protein concentrations of the cell lysates were determined by using Quick Start Bradford Protein Assay kit (Bio-Rad, Hercules, CA).

LC-MS/MS for protein identification and quantification

Samples were automatically injected and separated by online liquid chromatography on an EASY-nLC, and analyzed by an LTQ Orbitrap Velos mass spectrometer equipped with a nanoelectrospray ionization source (Thermo, San Jose, CA). The separation was conducted by using a home-made trapping column (150 $\mu\text{m} \times 50$ mm) and a separation column (75 $\mu\text{m} \times 120$ mm), packed with ReproSil-Pur C18-AQ resin (5 μm in particle size, Dr. Maisch HPLC GmbH, Germany). Peptide samples were initially loaded onto the trapping column with a solvent mixture of 0.1% formic acid in $\text{CH}_3\text{CN}/\text{H}_2\text{O}$ (2:98, v/v) at a flow rate of 4.0

$\mu\text{L}/\text{min}$. The peptides were then separated with a 120-min linear gradient of 2–40% acetonitrile in 0.1% formic acid and at a flow rate of 300 nL/min. The LTQ-Orbitrap Velos mass spectrometer was operated in the positive-ion mode, and the spray voltage was 1.8 kV. The data were collected in data-dependent scan mode where one full-scan MS was followed with twenty MS/MS scans. The full-scan mass spectra (from m/z 350 to 2,000) were acquired with a resolution of 60,000 at m/z 400 after accumulation to a target value of 500,000. The twenty most abundant ions found in MS at a threshold above 500 counts were selected for fragmentation by collision-induced dissociation at a normalized collision energy of 35%.

Data processing

MaxQuant software (Version B.01.03) was used to extract the MS and MS/MS data¹³. Database search was performed in MaxQuant against the human International Protein Index version 3.68 (87,083 entries) to which contaminants and reverse sequences were added. The search was performed with an initial precursor mass tolerance of 25 ppm and MS/MS tolerance of 0.6 Da. We included cysteine carbamidomethylation as a fixed modification and methionine oxidation as a variable modification. SILAC quantification setting was adjusted to doublets, with lysine (+8 Da) and arginine (+6 Da) being selected as heavy labels. Only proteins with at least two peptides being identified by MS/MS were considered as reliably identified. The minimal peptide length was six amino acids, and the maximum number of miss-cleavages for trypsin was set at two per peptide. For peptide and protein identifications, the false discovery rates were set to 0.01 at both peptide and protein levels¹³. Proteins with significant changes in SILAC experiments were determined by a combination of ratio and ratio significance calculated by MaxQuant. The p-value for the significance of enrichment was set to be <0.01 in both forward and reverse SILAC labeling experiments. The quantification was based on three independent SILAC and LC-MS/MS experiments, which included two forward and one reverse SILAC labelings, and the significantly changed proteins discussed below could be quantified in all three sets of SILAC experiments (Figure 1B).

Measurements of 20S proteasome activity

The chymotrypsin-like, trypsin-like, and peptidylglutamyl-peptide hydrolyzing (PGPH) activities were assessed using a 20S Proteasome Activity Assay kit (Millipore, Billerica, MA), where we measured the fluorescence of free 7-amino-4-methylcoumarin (AMC, excitation, 360 nm; emission, 460 nm) released from succinyl-Leu-Leu-Val-Tyr-AMC, Boc-Leu-Arg-Arg-AMC (Boston Biochem, Inc., Cambridge, MA), and Z-Leu-Leu-Glu-AMC, respectively. All cell lines were cultured as described above. After reaching confluence, the cells were treated with NNK, alone or in combination with 10 μM propranolol, at the indicated concentrations for 24, 48, or 72 hr in appropriate serum-free media. Crude lysates containing 20S proteasome were prepared following a protocol supplied with the kit and as described previously with minor modifications¹⁴. Along this line, cells were collected in PBS by scraping from culture flasks and centrifuged at 3,000 g for 10 min. The resultant cell pellet was lysed in 0.3 mL lysis buffer [50 mM HEPES (pH 7.5), 5 mM EDTA, 150 mM NaCl and 1% Triton X-100] for 30 min on ice with vortexing at 10-min intervals. The cell lysates were subsequently centrifuged at 15,000 g for 20 min at 4°C, and the supernatant was collected and used for assessing the 20S proteasome activity. To assure that the measured fluorescence was due to 20S proteasome activity, duplicate samples for each treatment group were pretreated for 20 min with 2 μM MG-132, a 20S proteasome inhibitor, and fluorescence readings from these samples were subtracted out of final reading.

Results

Protein identification and quantification

To investigate the cellular pathways targeted by NNK, we employed a quantitative proteomic approach utilizing SILAC coupled with LC-MS/MS to assess the NNK-induced perturbation of protein expression in GM00637 cells. GM00637 cells were exposed to 5 μ M NNK for 24 hr, which was found to induce a less than 5% cell death as determined by MTT assay. It is of note that we employed serum-free medium for all NNK treatment experiments to avoid the potential interactions between NNK and proteins in the FBS. To ensure that the observed changes in protein expression were a result of NNK treatment, we conducted the SILAC experiments in triplicate, incorporating both forward and reverse SILAC labeling (Figure 1A, see also Materials and Methods). Figure 2 displays the quantification of the peptide ATAGAYIASQTVK from the β 5 subunit of the 20S proteasome, the subunit responsible for the chymotrypsin-like activity¹⁵, which clearly reveals the up-regulation of this protein in both forward and reverse SILAC experiments (Figure 2A and 2B). The sequence for both the light- and heavy-labeled peptide was confirmed by MS/MS analysis (Figure 2C and 2D).

Our proteomic analysis allowed for the identification and quantification of 2394 and 2075 proteins, respectively. A total of 1412 proteins could be quantified in all three SILAC labeling experiments (Figure 1B), and the detailed quantification results for these proteins are summarized in Table S1. Among the quantified proteins, 137 exhibit significant changes upon NNK treatment (the ratio of treated/untreated was greater than 1.5 or less than 0.67), with 39 and 98 being up- and down-regulated, respectively. The quantification results for all the proteins with significant changes are summarized in Table S2.

Pathways perturbed by NNK treatment, as identified by GenMapp

Analysis of the protein quantification results using GenMAPP/MAPPFinder revealed the perturbation of several cellular pathways in GM00637 cells following NNK treatment^{16, 17}. Proteins with greater than a 1.5-fold change in expression following NNK treatment were included for the analysis, and those pathways with z-scores > 2.0 were considered significant. The altered pathways included the proteasome core complex (GO, z-score = 7.2), glycolysis and gluconeogenesis (GO, z-score = 3.0), and voltage gated ion channel porin activity (GO, z-score = 3.0).

20S proteasome subunits are up-regulated upon NNK treatment

An overarching feature of our quantitative proteomic results was the up-regulation of almost all subunits of the 20S proteasome core complex (Table 1). The 20S proteasome harbors multiple catalytic sites and is a proteinase complex responsible for the degradation of most intracellular proteins, including proteins crucial for cell cycle regulation and apoptosis^{18, 19}. The proteasome contains a highly ordered ring-shaped 20S catalytic core structure consisting of 4 rings of 28 non-identical subunits; each of the 2 outer rings are composed of 7 α subunits and each of the 2 inner rings are comprised of 7 β subunits¹⁹. Our quantitative proteomic results revealed that NNK treatment induced a significant increase in expression of 14 different subunits of the 20S proteasome core complex (Table 1), suggesting that NNK treatment induced an up-regulation of the 20S proteasome in GM00637 human skin fibroblast cells.

NNK treatment led to elevated activity of the 20S proteasome

The 20S proteasome is a multi-catalytic proteinase with chymotrypsin-, trypsin-, and PGPH-like activities which are provided by the β 5, β 2, and β 1 subunits, respectively¹⁵. The results from our quantitative proteomic analysis indicated that NNK treatment of GM00637 cells

resulted in significant increases in expression levels of all the three subunits. We reason that the increased expression of the 20S proteasome should confer a rise in proteasomal activity. To test this, we examined the effects of NNK treatment on chymotrypsin-, trypsin- and PGPH-like activities by monitoring the release of free AMC from the fluorogenic peptides Suc-Leu-Leu-Val-Tyr-AMC, Boc-Leu-Arg-Arg-AMC and Z-Leu-Leu-Glu-AMC, respectively²⁰. We found that all three types of proteasomal activities were significantly elevated in GM00637 cells at 24, 48, and 72 hr following 5 μ M NNK treatment, with the increases being most pronounced at 72 hr after treatment (Figure 3A). To examine whether this finding is general, we next assessed whether NNK treatment can also result in the alteration of proteasomal activities in two other cells lines, i.e., IMR-90 human lung fibroblast and MCF-7 breast cancer cells. For IMR-90 cells, we found that both the chymotrypsin- and trypsin-like activities were significantly increased at 24, 48, and 72 hr; however, PGPH activity was only modestly elevated by 1.3 fold at 72 hr of exposure (Figure 3B). In contrast, we found only slight rises (by 1.2 fold) in chymotrypsin- and trypsin-like activities in MCF-7 cells at 72 hr after treatment with 5 μ M NNK, whereas the PGPH-like activity was significantly increased at all the time points tested (Figure 3C).

NNK was reported to act as an agonist for the β -adrenergic receptor^{10, 21} and stimulation of the β -adrenergic receptor with its agonist, i.e., isoproterenol, results in a rise in proteasome subunits in cardiac proteasomes²². Furthermore, the proteasome system may help modulate the abundance of β -adrenergic receptors and several key elements of the β -adrenergic pathway through their degradation²³. To explore the mechanisms involved in the NNK-induced increase in proteasome activity, we assessed whether the up-regulation of 20S proteasome is mediated through the β -adrenergic receptor. It turned out that co-exposure with 5 μ M NNK and 10 μ M propranolol, which is a β -adrenergic receptor antagonist²⁴, for 72 hr diminished markedly the NNK-induced increase in all three types of 20S proteasome activities in both GM00637 and IMR-90 cell lines (Figure 3B). In this regard, the chymotrypsin-, trypsin- and PGPH-like activities dropped from 2.9, 2.1 and 2.2 fold in GM00637 cells treated with NNK alone to 1.2, 1.3, and 1.0 fold, respectively, upon exposure with both NNK and propranolol (Figure 3A). Similarly, co-treatment of IMR-90 cells with propranolol rendered these three types of activities dropping from 2.3, 2.1, and 1.3 fold to 1.2, 1.2, and 1.0 fold, respectively (Figure 3B). Likewise, the addition of propranolol reduced the PGPH-like activity from 1.8 to 1.3 fold in NNK-treated MCF-7 cells at the 72 hr time point. These results support that the NNK-stimulated activation of β -adrenergic receptor constitutes an important upstream event contributing to the up-regulated proteasome activity in human fibroblast cells.

Up-regulation of casein kinase II

Apart from the observed up-regulation of the 20S proteasome core complex, our quantitative proteomic analysis showed that NNK treatment also resulted in the perturbation of other proteins which play important roles in a variety of cellular pathways and processes. Along this line, NNK treatment of GM00637 cells led to the up-regulation casein kinase II (protein kinase CK2), whose α 1 and β subunits were stimulated by 3.2 and 4.3 folds, respectively. Casein kinase II is a serine/threonine protein kinase that catalyzes the phosphorylation of acidic proteins and is thought to have possibly thousands of protein substrates²⁵. Casein kinase II is ubiquitously expressed in mammalian cells, where it functions in a variety of cellular processes, including cell cycle progression, apoptosis, and transcription²⁶. Furthermore, the expression of casein kinase II was shown to be significantly elevated in many types of tumor cells, which is associated with increased cellular proliferation²⁷. Thus, the observed up-regulation of casein kinase II may also play an important role in NNK-induced cellular proliferation. Additionally, a previous report showed that casein kinase II

can be co-purified with the 20S proteasome from cells ²⁸, suggesting a possible interaction between the two.

Up-regulation of proteins involved in glycolysis and gluconeogenesis

Our proteomic analysis also demonstrated that NNK treatment resulted in the increased expression of several proteins involved in cellular energy homeostasis and metabolic pathways, including glycolysis and gluconeogenesis. We found that NNK treatment resulted in an up-regulation of 11 proteins involved in the glycolysis and gluconeogenesis pathways (Table 1), among which the glycolytic enzymes α -enolase and aldolase A were up-regulated by 1.64 and 1.85 fold, respectively. Pyruvate kinase isozymes M1/M2, which catalyze the last and rate-limiting step of glycolysis ²⁹, were up-regulated by 2.2 fold, suggesting cellular energy homeostasis may be perturbed following NNK exposure. Furthermore, enhanced glycolysis is known to be associated with proliferating cells ³⁰. Therefore, the observed increase in glycolytic enzymes may also contribute to NNK-induced cellular proliferation.

NNK induced decrease in voltage-dependent anion channels 1 and 2

Previous reports have suggested that mitochondria may represent a potential target of NNK ^{31, 32}. We found that NNK treatment resulted in the altered expression of several mitochondrial proteins (Table 1) including the decreased expression of two isoforms of the voltage-dependent anion channel (VDAC), i.e. VDAC 1 and VDAC 2, by 1.9 and 3.2 fold, respectively. VDAC is an ion channel located in the mitochondrial outer membrane that plays a central role in regulating energy metabolism in cells by maintaining cellular ATP levels and regulating calcium homeostasis ^{33, 34}. Decreased VDAC expression has been shown to result in decreased ATP synthesis and diminished levels of ADP and ATP in the cytosol ³³. In addition, previous experiments using VDAC-knockout mice demonstrated that each VDAC isoform appears to have specialized functions. The knockout of VDAC1 and VDAC2 results in a reduction of mitochondrial respiratory capacity ³⁵, demonstrating the importance of these proteins in maintaining cellular energy levels.

NNK Treatment resulted in altered expression of the chaperonin-containing TCP1 complex

Several types of molecular chaperones also displayed elevated expression following NNK treatment, including three subunits of the chaperonin-containing TCP1 complex (CCT), subunit-1 α , subunit 7 (η) and subunit 8 (θ), which were increased by ~ 2.5, 2.3, and 2.3 fold, respectively. The chaperonin CCT is involved in the folding of cytoskeletal proteins such as tubulin and actin³⁶, and it is also an important factor in a variety of processes including cell viability and proliferation ³⁶.

Discussion

Quantitative proteomic analysis revealed significantly altered expression of 137 proteins in human skin fibroblast cells following NNK exposure. NNK has been widely studied and is considered one of the most potent carcinogens found in tobacco and its products. Exposure to NNK has been shown to induce lung tumors in all species tested ⁶. After metabolic activation, NNK can induce the generation of an array of DNA adducts, which may contribute to the carcinogenic effect of this important TSNA ⁶. However, we found that exposure to the un-metabolized form of NNK leads to the altered expression of many proteins associated with several important cellular pathways.

One of the main findings made from our proteomic analysis was the up-regulation of the 20S proteasome core complex as represented by the marked up-regulation of nearly all subunits of the 20S proteasome in GM00637 human skin fibroblast cells. We further confirmed the up-regulation of the 20S proteasome core complex in GM00637 cells and

IMR-90 human lung fibroblast cells by measuring the 20S proteasomal activity, where we observed a significant increase in the chymotrypsin- and trypsin-like activity of the 20S proteasome following 24, 48, and 72 hr of NNK exposure. Additionally, NNK treatment resulted in a significant rise in PGPH-like activity in GM00637 cells following 24, 48, and 72 hr of exposure, though only a modest increase was seen at 72 hr for IMR-90 cells. In contrast, NNK treatment failed to induce an elevation in chymotrypsin- and trypsin-like activities MCF-7 cells at 24 or 48 hr of exposure; however, modest increases of these two types of activities were found at 72 hr and PGPH activity was significantly increased at all time points tested. Further studies are needed for uncovering the molecular events underlying the differences in the types of proteasomal activities that are activated in different cell lines. In addition, we found that the co-exposure with a β -adrenergic receptor antagonist, propranolol, could abolish the NNK-induced increase in chymotrypsin-, trypsin-, and PGPH-like activities in both GM00637 and IMR-90 cells and PGPH-like activity in MCF-7 cells. These results, along with previous observations that NNK can bind to and activate β -adrenergic receptor^{9, 10}, revealed that the NNK-induced up-regulation of the 20S proteasome may be mediated, at least in part, through the binding of NNK to the β -adrenergic receptor. Altered proteasome activity has been observed in a variety of diseases including cancer^{18, 22, 37}, and proteasome inhibitors have been found to be effective drugs for cancer treatment and prevention^{38, 39}. The elevated proteasomal activity may contribute significantly to the enhanced cellular proliferation associated with NNK exposure⁴⁰.

Aside from an increase in the 20S proteasome core complex, we found that NNK treatment induced considerable changes in the expression levels of other important proteins involved in a variety of cellular functions (Supplementary Table S2), including molecular chaperones, cytoskeleton-associated proteins, etc. Notably, increases in casein kinase II and glycolytic proteins are known to be associated with increased cellular proliferation and may also contribute to NNK-induced proliferation^{27, 30}. Together, results from this study offered novel insights into the mechanisms of toxicity of NNK.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

TSNAs	Tobacco specific N-nitrosamines
NNK	4-(methylnitrosamino)-1-(3-pyridinyl)-1-butanone
SILAC	stable isotope labeling by amino acid in cell culture
IMDM	Iscove's modified Dulbecco's medium
EMEM	Eagles minimum essentials medium
DMEM	Dulbecco's modified essentials medium
FBS	fetal bovine serum
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
AMC	7-amino-4-methylcoumarin

VDAC	voltage-dependent anion channel
PGPH	peptidylglutamyl-peptide hydrolyzing

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Figure 1. SILAC-based quantitative proteomics. (A) Flowchart of reverse SILAC coupled with LC-MS/MS for the comparative analysis of protein expression in GM00637 cells following NNK treatment. In forward SILAC experiments, light Lys- and Arg-labeled cells were treated with 5 µM NNK, whereas the heavy Lys- and Arg-labeled cells were used as vehicle control. (B) A Venn diagram summarizing the number of proteins quantified from three independent SILAC (two forward and one reverse) experiments.

Figure 2.

Representative LC-MS/MS data revealing the NNK-induced up-regulation of the peptide ATAGAYIASQTVK from the $\beta 5$ subunit of human 20S proteasome. Shown are the mass spectra for the $[M + 2H]^{2+}$ ions of the $\beta 5$ subunit peptide ATAGAYIASQTVK from the forward (A) and reverse (B) SILAC samples. The sequence for both the light- and heavy-labeled peptide was confirmed by MS/MS analysis, as depicted in (C) and (D), respectively.

Figure 3.

Chymotrypsin-, trypsin-, and PGPH-like activities of 20S proteasome in lysates of GM00637 (A), IMR90 (B) and MCF-7 (C) cells upon treatment with 5 μ M NNK for 24, 48, or 72 hr or together with 10 μ M propranolol for 72 hr. The activity of 20S proteasome was assessed by fluorescence of free AMC (excitation, 360 nm; emission, 460 nm) released from AMC-bearing fluorogenic peptides (See Materials and Methods), and results are presented as % of vehicle control. Data represent the mean $\pm$ standard deviation from three independent treatment experiments. ‘*’, $P < 0.05$, ‘**’, $P < 0.01$, ‘***’, $P < 0.001$, calculated by ANOVA followed by Tukey’s post hoc test ($n = 3$). All P values were calculated relative to the vehicle control except the results obtained from dual treatment with NNK and propranolol, the P values of which were calculated by comparing with the data for cells treated with NNK alone for 72-hr.

Table 1

A partial list of proteins quantified with more than 1.5 fold changes, with IPI numbers, protein names, average ratios and standard deviations (based on three independent sets of SILAC experiments) listed. A complete list of all proteins with >1.5 fold changes is shown in Table S2.

International Protein Index	Protein Name	Average Ratio (Treated/ Untreated)
	<i>A. Proteasome Associated Proteins</i>	
IPI00025019	Proteasome subunit beta type-1	2.29±0.51
IPI00028006	Proteasome subunit beta type-2	1.80±0.11
IPI00555956	Proteasome subunit beta type-4	2.33±0.40
IPI00479306	Proteasome subunit beta type-5	1.86±0.25
IPI00000811	Proteasome subunit beta type-6	1.91±0.16
IPI00003217	Proteasome subunit beta type-7	1.80±0.15
IPI00000783	Proteasome subunit beta type-8	1.61±0.20
IPI00472442	Proteasome subunit alpha type-1	2.83±1.40
IPI00219622	Proteasome subunit alpha type-2	2.53±1.10
IPI00419249	Proteasome subunit alpha type-3	3.47±2.06
IPI00299155	Proteasome subunit alpha type-4	1.77±0.12
IPI00291922	Proteasome subunit alpha type-5	3.97±2.76
IPI00029623	Proteasome subunit alpha type-6	2.54±0.84
IPI00024175	Proteasome subunit alpha type-7	2.86±1.03
	<i>B. Casein Kinase</i>	
IPI00016613	Casein kinase II alpha 1 subunit isoform a	3.16±0.60
IPI00640088	Casein kinase 2 beta polypeptide	4.30±2.75
	<i>C. Glycolysis and Gluconeogenesis</i>	
IPI00796333	Fructose-bisphosphate aldolase A	1.85±0.71
IPI00169383	Phosphoglycerate kinase 1	2.96±1.62
IPI00027497	Glucose-6-phosphate isomerase	3.11±1.08
IPI00479186	Pyruvate kinase isozymes M1/M2	2.18±1.24
IPI00465248	Alpha-enolase	1.64±0.62
IPI00947127	L-lactate dehydrogenase	2.04±0.52
IPI00219217	L-lactate dehydrogenase B chain	1.68±0.17
IPI00291006	Malate dehydrogenase, mitochondrial	1.78±0.18
IPI00018206	Aspartate aminotransferase, mitochondrial	3.62±2.07
IPI00008215	Malic enzyme 1	2.38±0.68
	<i>D. Voltage-dependent anion channel</i>	
IPI00216308	Voltage-dependent anion-selective channel 1	0.52±0.37
IPI00902560	Voltage-dependent anion-selective channel 2	0.31±0.31
	<i>E. Molecular Chaperone Proteins</i>	
IPI00018465	Chaperonin containing TCP1, subunit 7 (Eta) variant	2.27±1.21
IPI00784090	Chaperonin containing TCP1, subunit 8 (Theta), isoform CRA_c	2.33±0.10

International Protein Index	Protein Name	Average Ratio (Treated/ Untreated)
IPI00290566	T-complex protein 1 subunit alpha	2.50±0.82