Toxicology and Applied Pharmacology 233 (2008) 254–261

Contents lists available at ScienceDirect

Toxicology and Applied Pharmacology

journal homepage: www.elsevier.com/locate/ytaap

Nicotine and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone induce cyclooxygenase-2 activity in human gastric cancer cells: Involvement of nicotinic acetylcholine receptor (nAChR) and β-adrenergic receptor signaling pathways

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ARTICLE INFO

Article history:
Received 30 April 2008
Revised 6 August 2008
Accepted 13 August 2008
Available online 6 September 2008

Keywords:
Nicotine
NNK
Nicotinic acetylcholine receptor
β-adrenergic receptor
COX-2

ABSTRACT

Induction of cyclooxygenase-2 (COX-2) associates with cigarette smoke exposure in many malignancies. Nicotine and its derivative, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), are the two important components in cigarette smoke that contributes to cancer development. However, the molecular mechanism (s) by which nicotine or NNK promotes gastric carcinogenesis remains largely unknown. We found that nicotine and NNK significantly enhanced cell proliferation in AGS cells that expressed both alpha7 nicotinic acetylcholine receptor (α7 nAChR) and β-adrenergic receptors. Treatment of cells with α-bungarotoxin (α-BTX, α7nAChR antagonist) or propranolol (β-adrenergic receptor antagonist) blocked NNK-induced COX-2/PGE_2 and cell proliferation, while nicotine-mediated cell growth and COX-2/PGE_2 induction can only be suppressed by propranolol, but not α-BTX. Moreover, in contrast to the dependence of growth promoting effect of nicotine on Erk activation, inhibitor of p38 mitogen-activated protein kinase (MAPK) repressed NNK-induced COX-2 upregulation and resulted in suppression of cell growth. In addition, nicotine and NNK mediated COX-2 induction via different receptors to modulate several G1/S transition regulatory proteins and promote gastric cancer cell growth. Selective COX-2 inhibitor (SC-236) caused G1 arrest and abrogated nicotine/NNK-induced cell proliferation. Aberrant expression of cyclin D1 and other G1 regulatory proteins are reversed by blockade of COX-2. These results pointed to the importance of adrenergic and nicotinic receptors in gastric tumor growth through MAPK/COX-2 activation, which may perhaps provide a chemoprevention strategy for cigarette smoke-related gastric carcinogenesis.

Introduction

Gastric cancer is the second leading cause of cancer mortality worldwide (Kelley and Duggan, 2003). Several factors are strongly correlated with increase risk of gastric cancer, including dietary, genetic factors, environmental factors, infectious agents and pathological condition in the stomach (Tsugane and Sasazuki, 2007). Tobacco smoking is a risk factor for gastric cancer (Ladeiras-Lopes et al., 2008). Cigarette smoke consist various groups of carcinogens, such as phenolic compounds, polycyclic aromatic (PAH), inorganic, *N*-nitrosamines, and trace amount of metal sources (Hecht, 1999). Among them, the nicotine-derived nitrosamines, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) is activated within the body to form a potent alkylating agent causing DNA mutations that lead to cancer development. This may explain why smokers have twice the risk of getting gastric cancer than non-smokers (Unakami et al., 1989).

Cigarette smoke elicited a typical malignancy response similar to *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) (Tayler and Piper, 1977), implicating that cigarette smoke exerted some carcinogenic effects on the stomach. However, the underlying mechanism(s) of how cigarette smoke promoted gastric carcinogenesis remain unresolved. Abnormalities of the cell cycle regulatory proteins are frequent in gastric cancer and are associated with prognosis. Overexpression of cyclin D1 has been shown to act in several cancers including breast and GI cancers (Motokura and Arnold, 1993). Cyclin-dependent kinase inhibitors, the CIP/KIP family, p21 and p27 would bind to cyclin-CDK complexes to cause cell cycle arrest in G1 phase. It has been demonstrated that TGFβ caused upregulation of p21 that blocked the association of cyclin–CDK complex formation, causing growth arrest in G1 phase in gastric cancer cells (Yoo et al., 1999). Regulation

Abbreviations: COX-2, Cyclooxygenase-2; PGE_2, prostaglandin E_2; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; nAChR, nicotinic acetylcholine receptor; α-BTX, α-bungarotoxin; Erk, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; CDK, cyclin-dependent kinase.

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doi:10.1016/j.taap.2008.08.012

of the cell cycle is important in cancer, due to its contribution to tumor growth, apoptosis and therapeutic resistance. Thus, the role played by cell cycle regulatory proteins in cigarette smoke-induced gastric cancer cell proliferation might provide a basis for treating cancer patients in smokers.

It has been shown that nicotine stimulated tumor growth and angiogenesis (Heeschen et al., 2001; Shin et al., 2005). Nicotine itself does not cause neoplastic transformation but induces cancer cell proliferation, angiogenesis and inhibit apoptosis through nicotinic acetylcholine receptor (nAChR) and/or β-adrenergic receptor (Arredondo et al., 2006; Shin et al., 2007). The development of NNK-induced lung cancer was shown to modulate through β-adrenergic receptor in vivo (Schuller et al., 2000). nAChR is the receptor for nicotine, and it is critical in regulating non-neuronal cells (Lindstrom, 1997; Grando et al., 2003). Other than nicotine, its derived nitrosamine, like NNK, mediated tumor growth in small-cell lung carcinoma cells (Cattaneo et al., 1997; Razani-Boroujerdi and Sopori, 2007). Increasing evidence showed that α7 nAChR plays an important role in cigarette smoke-related lung carcinogenesis (Lam et al., 2007; Razani-Boroujerdi and Sopori, 2007).

Earlier studies demonstrated that exposure of nicotine or NNK increases the carcinogenic action in gastric and colon cancers (Shin et al., 2004; Wu et al., 2005). A complete understanding of the mechanisms underlying cigarette smoke-induced carcinogenesis is important for the development of new therapeutic drugs in smokers. Thus, it is of great interest to examine whether nicotine and NNK are the active compounds that exhibited the tumorigenic effect in gastric cancer cells. Moreover, we investigated the involvement of α7nAChR and β-adrenergic receptors in nicotine/NNK-induced gastric carcinogenesis.

Materials and methods

Reagents and drugs. 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) was obtained from ChemSyn Laboratories (Lenexa, KS). 4-[5-(4-Chlorophenyl)-3-(trifluoromethyl)-^{1}H-pyrazol-1-yl] benzenesulfonamide (SC-236) was purchased from Pharmacia (Peapack, NJ). SB203580 (specific p38 inhibitor) was obtained from Calbiochem (La Jolla, CA). U0126 (specific MEK-1/2 inhibitor) was purchased from Cell Signaling Technology (Beverly, MA). p38 and p-p38 antibodies were from BioLabs (Beverly, MA). Antibodies to cyclin D1, CDK4, CDK6, Rb, p-Rb, p53, p21 and p27 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). PGE_2 enzyme immunoassay kit was bought from R&D Systems (Minneapolis, MN). COX-2 activity assay kit was purchased from Cayman Chemical Company (Ann Arbor, MI). Other chemicals and reagents were from Sigma (St Louis, MO) unless otherwise specified.

Cell culture and drug treatment. Human gastric adenocarcinoma cells (AGS) were purchased from the American Type Culture Collection (CRL-1739, ATCC, USA). Cells were cultured in RPMI 1640 (GibcoBRL, Grand Island, USA) containing 10% fetal bovine serum (GibcoBRL, 100 U/ml penicillin G, 100 μg/ml streptomycin, and maintained at 37 °C, 95% humidity and 5% carbon dioxide. AGS cells were treated with various concentrations of nicotine (20, 100, 200 or 400 μM) or NNK (1, 10, 50 or 100 nM) for 5 h. The concentration of nicotine used in the present study mimicked the daily intake of cigarettes in smokers. The concentration of nicotine (100 μM) is equivalent to concentration in the saliva in smoker who intake 25 cigarettes/day (Cheng and Tsai, 1999). Prokopczyk et al. reported that the concentration of NNK in saliva is 0.96–1.61 μg of NNK/g of tobacco (Prokopczyk et al., 1992).

Real-time RT-PCR. Total RNA was extracted from cells with Tri-Reagent according to the protocol provided. Gene expressions were screened by RT^2 Profiler™ PCR JAK/STAT Array (SuperArray Bioscience Corporation, Frederick, MD). Extracted RNA was purified by genomic DNA elimination mixture, and reversed-transcribed into cDNA. RT^2 qPCR was set under the condition at 95 °C for 10 min, 95 °C for 15 s and 60 °C for 1 min (40 cycles). Fold change in expression of each gene is calculated by a comparative threshold cycle (C_t) method using the formula: $2^{-[\Delta Ct\ (experiment) - \Delta Ct\ (control)]}$. For validation, real-time RT-PCR was performed using Power SYBR-Green real-time RT-PCR system and ABI PRISM 7500 detection system (Applied Biosystems, Foster City, CA). The primers for cyclin D1 were as follows: (forward), 5′-CAAATGTGTGCAGAAGGAGG-3′; (reverse), 5′-AGGCCACGAACAT-GCAAGTG-3′. The reaction conditions include 50 °C for 5 min, 95 °C for 10 min, 95 °C for 15 s and 60 °C for 1 min (40 cycles). The screening with RT^2 Profiler™ PCR JAK/STAT Array, were carried out twice with RNAs from two independent experiments. For the validation, real-time RT-PCR was done three times independently.

Prostaglandin E_2 assay. Cell lysates were homogenized in enzyme immunoassay buffer [containing 50 mM Tris–HCl at pH 7.4, 100 mM NaCl, 1 mM $CaCl_2$, 1 mg/ml D-glucose and 28 μM indomethacin] for 30 s on ice. Samples were then centrifuged for 15 min at 10,000 ×*g* at 4 °C. The supernatant was used for the determination of PGE_2 using the immunoassay kit (R&D Systems Inc., Minneapolis, USA) according to the manufacturer's instructions.

Cyclooxygenase-2 (COX-2) activity assay. After treatment, cells were collected using a scraper and centrifuged at 1500 ×*g* for 10 min at 4 °C. Cell lysates were sonicated in iced-cold buffer containing 0.1 M Tris-HCl (pH 7.8), 1 mM EDTA, 250 mM mannitol and 0.3 mM diethyldithiocarbamic acid, and centrifuged at 10,000 ×*g* for 15 min at 4 °C. The supernatant was used to determine the peroxidase activity of COX using the COX activity assay kit (Cayman Chemical Company, MI). The peroxidase activity is measured spectrophotometrically the amount of oxidized *N*, *N*, *N*, *N*′-tetramethyl-*p*-phenylenediamine (TMPD) at 590 nm according to the manufacturer's instructions.

[^{3}H]-thymidine incorporation assay. A modified [^{3}H]-thymidine incorporation assay was used to determine the amount of DNA synthesis (Tones et al., 1988). After confluent, cells were incubated in the presence or absence of nicotine or NNK for 5 h, and then incubated with 0.5 μCi/ml [^{3}H]-thymidine (Amersham Corporation, Arlington Heights, IL, USA) for another 4 h and washed with iced-cold 0.15 M NaCl, followed by 10% trichloroacetic acid and incubated with 15 min at room temperature. After several washings, 1% sodium dodecyl sulfate (SDS) was added and incubated for another 15 min at 37 °C. Finally, hydrophilic scintillation fluid was added into the vial and the amount of DNA synthesized was measured using a liquid scintillation spectrometry on a beta-counter (Beckman Instruments, Fullerton, CA, USA).

Western blot analysis. Cells were harvested in radioimmunoprecipitation buffer containing protease inhibitor. Protein was quantified using a protein assay kit (Bio-Rad Laboratories, Hercules, CA). Equal amount of protein (70 μg per lane) were resolved by SDS-PAGE gel and transferred to Hybond C nitrocellulose membranes (Amersham Biosciences Corporation, Arlington Heights, IL). The membrane was blocked with 5% non-fat milk probed with the antibodies overnight at 4 °C and the signal was amplified with secondary peroxidase-conjugated antibodies. They were developed with an enhanced chemiluminescence system (Amersham Corporation) and exposed to an X-ray film (Fuji Photo Film Co., Ltd, Tokyo, Japan). Quantitation was carried out with a video densitometer (Scan Maker III, Microtek, Carson, CA).

Immunoprecipitation. Immunoprecipitation was performed as described (Pérez-Roger et al., 1999). Cell lysates were suspended in lysis buffer [50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 1% Nonidet P-40] for 30 min, and centrifuged at 10,000 ×*g* for 15 min at 4 °C. Protein A-agarose beads (50 μl) (Oncogene Research Products, Boston, MA) were added to