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Nicotine stimulates urokinase-type plasminogen activator receptor expression and cell invasiveness through mitogen-activated protein kinase and reactive oxygen species signaling in ECV304 endothelial cells

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ABSTRACT

Urokinase-type plasminogen activator receptor (uPAR) expression is elevated during inflammation, tissue remodeling and in many human cancers. This study investigated the effect of nicotine, a major alkaloid in tobacco, on uPAR expression and cell invasiveness in ECV304 endothelial cells. Nicotine stimulated uPAR expression in a dose-dependent manner and activated extracellular signal-regulated kinases-1/2 (Erk-1/2), c-Jun amino-terminal kinase (JNK) and p38 mitogen activated protein kinase (MAPK). Specific inhibitors of MEK-1 (PD98059) and JNK (SP600125) inhibited the nicotine-induced uPAR expression, while the p38 MAPK inhibitor SB203580 did not. Expression vectors encoding dominant negative MEK-1 (pMCL-K97M) and JNK (TAM67) also prevented nicotine-induced uPAR promoter activity. The intracellular hydrogen peroxide (H_2O_2) content was increased by nicotine treatment. The antioxidant N-acetylcysteine prevented nicotine-activated production of reactive oxygen species (ROS) and uPAR expression. Furthermore, exogenous H_2O_2 increased uPAR mRNA expression. Deleted and site-directed mutagenesis demonstrated the involvement of the binding sites of transcription factor nuclear factor-kappaB (NF-κB) and activator protein (AP)-1 in the nicotine-induced uPAR expression. Studies with expression vectors encoding mutated NF-κB signaling molecules and AP-1 decoy confirmed that NF-κB and AP-1 were essential for the nicotine-stimulated uPAR expression. MAPK (Erk-1/2 and JNK) and ROS functioned as upstream signaling molecules in the activation of AP-1 and NF-κB, respectively. In addition, ECV304 endothelial cells treated with nicotine displayed markedly enhanced invasiveness, which was partially abrogated by uPAR neutralizing antibodies. The data indicate that nicotine induces uPAR expression via the MAPK/AP-1 and ROS/NF-κB signaling pathways and, in turn, stimulates invasiveness in human ECV304 endothelial cells.

Introduction

Cigarette smoke is a complex chemical mixture containing thousands of different compounds, of which 100 are known to be carcinogens, cocarcinogens, mutagens, and/or tumor promoters that have been shown to induce neoplastic lesions in humans and animals (Hoffmann et al., 2001; Sasazuki et al., 2002). The exact mechanism(s) for the carcinogenic action of cigarette smoke remains unclear. Nicotine is the major psychoactive compound in cigarette smoke and has been suggested to play a role in carcinogenesis (Tsurutani et al., 2005). Studies have highlighted that nicotine is a key compound acting as a central regulator of a complex network that governs growth (Lee et al., 2010) and angiogenesis (Cooke, 2007).

Urokinase-type plasminogen activator (uPA) and uPA receptor (uPAR) form a complex proteolytic system that has been implicated in cancer invasion and metastasis. uPA is a serine protease with the ability to convert plasminogen to active plasmin (Petersen et al., 1988). In addition, the uPA–uPAR binding interaction can independently affect cell motility, integrin function, and gene expression (Waltz et al., 2000). uPAR regulates proteolysis by binding the extracellular protease uPA and also activates many intracellular signaling pathways, which frequently indicates poor prognosis (Smith and Marshall, 2010). Overexpression of uPA and uPAR has been detected in different tumors, and the correlation between uPA and uPAR concentration is consistent with the idea that uPAR-bound uPA at the surface of cancer cells is responsible for the invasiveness of cancer cells and metastasis (Andreasen et al., 1997). Moreover, antibodies that prevent binding of uPA to uPAR inhibit uPA-induced cell invasion (Kim et al., 2008) and inhibition of uPA and uPAR expression drastically reduces cell growth and migration. Inhibition of cancer metastasis by a 3′ end antisense urokinase receptor mRNA in a nude mouse model has also been reported (Wang et al., 2001). Besides, the overexpression of uPAR increases the ability of cells to penetrate the barrier of the reconstituted basement membrane. In contrast, blockade of uPAR through the expression of a catalytically inactive enzyme or an antisense uPAR complementary DNA markedly decreases cell invasiveness. In cancers, increased levels

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of uPAR are essential for maintaining the invasive and metastatic phenotypes, and these increases are considered prognostically significant (Cho et al., 1997). The level of uPAR synthesis is increased by a diverse set of agents including epidermal growth factor, vascular endothelial growth factor, hepatocyte growth factor, fibroblast growth factor, transforming growth factor, okadaic acid, and phorbol ester in a number of different cell types (Wang et al., 1995).

Nicotine suppresses apoptosis and enhances the growth of endothelial cells, which could provide evidence for nicotine upregulated angiogenesis. In addition, nicotine-stimulated changes in the expression of various genes in endothelial cells, including endothelial nitric oxide synthase, angiotensin I-converting enzyme, tissue-type plasminogen activator, platelet-derived growth factor, and basic fibroblast growth factor have been described (Conklin et al., 2001). Endothelial cells activated by growth factors lead to disruption of cell–extracellular matrix contacts. The initial disruption of the endothelial cell–extracellular matrix contact requires degradation of the extracellular matrix, which is facilitated by a variety of proteases including uPA and matrix metalloproteinases (MMPs) (Stoppelli et al., 1985; Vassalli et al., 1985). However, the direct role of nicotine on the uPAR in endothelial cells remains unknown.

In this study, it was found that nicotine induces the expression of uPAR via mitogen activated protein kinase (MAPK)/activator protein-1 (AP-1) and reactive oxygen species (ROS)/nuclear factor-kappa B (NF-κB) signaling pathways in endothelial cells, thereby enhancing the invasion of the cells essential for tumor growth and metastasis.

Materials and methods

Cell culture and reagents. Human endothelial ECV304 cells obtained from the American Type Culture Collection (Manassas, VA, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin at 37 °C in an atmosphere containing 5% CO_2. Nicotine, the antioxidant compound N-acetyl cysteine (NAC), and the AP-1 inhibitor curcumin were obtained from Sigma-Aldrich (St. Louis, MO, USA) and the MAPK/Erk kinase (MEK) inhibitor PD98059, c-jun-N-terminal kinase (JNK) inhibitor SP600125, p38 MAPK inhibitor SB203580, and NF-κB inhibitor BAY11-7082 were from Calbiochem (La Jolla, CA, USA).

Cell viability. Cells (5×10^3) were incubated in a 96-well plate with low serum (1% FBS) containing 0–500 μg/ml nicotine for 24 h, and cell respiration was determined by an established 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich) assay. After the incubation, 10 μl of 5 mg/ml MTT was added to each well of the 96-well plates and incubated at 37 °C for 2 h. The formazan granules obtained were dissolved in 100% dimethyl sulfoxide, and absorbance 562 nm was detected with a 96-well ELISA reader.

Reverse transcription-polymerase chain reaction (RT-PCR). ECV304 cells were incubated overnight in the medium containing 1% FBS and then treated with 0–500 μg/ml nicotine for 5 h. After the incubation, the total cellular RNA was isolated from the cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). One microgram of total RNA was used for first-strand complementary DNA synthesis using random primers and Superscript reverse transcriptase (Invitrogen). The complementary DNA was subjected to PCR amplification with the primer sets for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and uPAR. The specific primer sequences were GAPDH sense, 5′-TTG TTG CCA TCA ATG ACC CC-3′; GAPDH antisense, 5′-TGA CAA AGT GGT CGT TGA GG-3′ (836 bp); uPAR sense, 5′-CAC GAT CGT GCG CTT GTG GG-3′, and uPAR antisense, 5′-TGT TCT TCA GGG CTG CGG CA-3′ (285 bp). The PCR conditions were denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s, and extension at 72 °C for 45 s. The products were electrophoresed in 1.5% agarose gel containing ethidium bromide.

Northern blot analysis. Total RNA extraction and Northern blot hybridization were performed as described previously (Kim et al., 2008). Each complementary DNA probe was radiolabeled with [α-^{32}P] deoxyribonucleoside triphosphate using the random priming technique with the Rediprime labeling system (Amersham, Piscataway, NJ, USA). The probed nylon membranes were exposed to radiographic film (Life Technologies, Grand Island, NY, USA).

Western blot analysis. Cells pretreated with 0–500 μg/ml nicotine for various periods were washed in phosphate buffered saline (PBS), detached using Trypsin-EDTA buffer, and stored at −70 °C until needed. The protein was extracted with RIPA buffer [1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS)] and protease inhibitors (aprotinin, leupeptin, phenylmethanesulfonylfluoride (PMSF), benzamidine, trypsin inhibitor, sodium orthovanadate). Fifty micrograms of the proteins was then separated by 10% SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes. The membranes were blocked in a PBS solution containing 5% nonfat dry milk, incubated with primary antibody in a blocking solution overnight at 4 °C, and washed three times with 0.1% Tween-20 in Tris buffered saline (TBST) at 10 min intervals. Horseradish peroxidase-conjugated secondary antibody (Amersham, Arlington Heights, IL, USA) was used to detect the immunoreactive proteins by chemiluminescence. The following antibodies were used: anti-uPAR (American Diagnostica, Greenwich, CT, USA), anti-IκBα (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-phospho-p44/42 MAPK (Erk-1/2) (Cell Signaling Technology, Danvers, MA, USA), anti-phospho-JNK (Cell Signaling Technology), and anti-phospho-p38MAPK (Cell Signaling Technology). The total protein levels were assayed by washing the blotted membrane with a stripping solution composed of 100 mM 2-mercaptoethanol, 2% SDS, and 62.5 mM Tris–HCl (pH 6.7) for 30 min at 50 °C, and the membrane was then reprobed with either the anti-β-actin (Sigma-Aldrich) and anti-Erk-1/2 (Cell Signaling Technology), anti-JNK-2 (Cell Signaling Technology), or anti-p38MAPK (Cell Signaling Technology) monoclonal antibody.

Measurement of uPAR promoter activity. The transcriptional regulation of uPAR was examined by the transient transfection of an uPAR promoter luciferase reporter construct (pGL3-uPAR). The plasmid pGL3-uPAR promoter (Wang et al., 1995) was kindly provided by Dr. Y. Wang (Australian National University). ECV304 cells (5×10^5) were seeded and grown until they reached 60–70% confluence, then pRL-TK (an internal control plasmid containing the Herpes simplex thymidine kinase promoter linked to the constitutively active Renilla luciferase reporter gene) and pGL3-uPAR were cotransfected into the cells using FuGENE (Boehringer-Mannheim, Mannheim, Germany) according to the manufacturer's protocol. pRL-TK was transfected as an internal control. Cells were incubated in the transfection medium for 20 h and treated with 0–500 μg/ml nicotine for 5 h. The effects of signaling inhibitors on uPAR promoter activity were determined by pretreating cells with the inhibitors for 1 h prior to the addition of nicotine. The cotransfection studies were performed in the presence or absence of the AP-1 decoy oligodeoxynucleotides (ODNs) or MEK-1 (pMCL-K97M), c-Jun (TAM67), I-κBα, I-κBβ, or NF-κB-inducting kinase (NIK). The phosphorothioate double-stranded ODNs with the sequences against the AP-1 binding site (5′-CAC TCA GAA GTC ACT TC-3′ and 3′-GAA GTG ACT TCT GAG CTG-5′) were prepared (Genotech, St Louis, MO, USA) and annealed (AP-1 decoy ODNs). The expression vector encoding the inactive MEK-1 (pMCL-K97M; Emrick et al., 2001) and c-Jun (TAM67; Brown et al., 1994) was a gift from Dr. N.G. Ahn (University of Colorado, Boulder) and Dr. M.J. Birrer (University of Helsinki), respectively. The dominant negative mutants of I-κBα and I-κBβ (McKinsey et al., 1996) and NIK (Geleziunas et al., 1998) were kindly provided by Dr. D.W. Ballard (Vanderbilt University, Nashville) and Dr. W.C. Greene (University of California), respectively. The importance of NF-κB and AP-1 during the induction of uPAR by nicotine was examined by