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Nicotinic acetylcholine receptor mediates nicotine-induced actin cytoskeletal remodeling and extracellular matrix degradation by vascular smooth muscle cells

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ABSTRACT

Cigarette smoking is a significant risk factor for atherosclerosis, which involves the invasion of vascular smooth muscle cells (VSMCs) from the media to intima. A hallmark of many invasive cells is actin cytoskeletal remodeling in the form of podosomes, accompanied by extracellular matrix (ECM) degradation. A7r5 VSMCs form podosomes in response to PKC activation. In this study, we found that cigarette smoke extract, nicotine, and the cholinergic agonist, carbachol, were similarly effective in inducing the formation of podosome rosettes in A7r5 VSMCs. α-Bungarotoxin and atropine experiments confirmed the involvement of nicotinic acetylcholine receptors (nAChRs). Western blotting and immunofluorescence experiments revealed the aggregation of nAChRs at podosome rosettes. Cycloheximide experiments and media exchange experiments suggested that autocrine factor(s) and intracellular phenotypic modulation are putative mechanisms. In situ zymography experiments indicated that, in response to PKC activation, nicotine-treated cells degraded ECM near podosome rosettes, and possibly endocytose ECM fragments to intracellular compartments. Invasion assay of human aortic smooth muscle cells indicated that nicotine and PKC activation individually and synergistically enhanced cell invasion through ECM. Results from this study suggest that nicotine enhances the ability of VSMCs to degrade and invade ECM. nAChR activation, actin cytoskeletal remodeling and phenotypic modulation are possible mechanisms.

1. Introduction

Cigarette smoking is a significant risk factor for atherosclerosis, which involves the invasion of vascular smooth muscle cells from the media to intima (Balakumar and Kaur, 2009; Doran et al., 2008; Erhardt, 2009; Newby, 2005). Nicotine and nicotinic acetylcholine receptors are known to mediate smoking addition, and their roles in stimulating angiogenesis have been studied extensively, as reviewed by Cooke and Ghebremariam (2008) and Egleton et al. (2009). For example, using mouse models of lung cancer and atherosclerosis, Heeschen et al. (2001) were the first to show that nicotine stimulates angiogenesis and promotes tumor growth and atherosclerosis by activating nicotinic acetylcholine receptors in endothelial cells. Other studies together showed that all components for synthesizing and degrading acetylcholine are present in endothelial cells, suggesting that endogenous acetylcholine functions as autocrine and/or paracrine in endothelial cells. Ng et al. (2007) established the physiological significance of nicotinic acetylcholine receptors in endothelial cell migration by showing that activation of nicotinic acetylcholine receptor is required for growth factor-induced endothelial cell migration. Thus, there is consensus among endothelial biologists that nicotine stimulates angiogenesis by activating nicotinic acetylcholine receptors in endothelial cells. In contrast, the effect of nicotine and function of nicotinic acetylcholine receptor in vascular smooth muscle invasion are not fully understood.

A hallmark of many invasive cells is actin cytoskeletal remodeling in the form of podosomes, accompanied by extracellular matrix degradation (Gimona and Buccione, 2006; Linder, 2006). We and others have reported the formation of podosomes by A7r5 vascular smooth muscle cells in response to PKC activation (Burgstaller and Gimona, 2005; Dorfleutner et al., 2008; Eves et al., 2006; Gu et al., 2007; Hai et al., 2002; Wang et al., 2010). In this study, we tested the hypothesis that nicotinic acetylcholine receptor mediates cigarette smoke extract and nicotine-induced actin cytoskeletal remodeling and extracellular matrix degradation by A7r5 vascular smooth muscle cells in response to PKC activation. In addition, we investigated the individual and interactive effects of nicotine and PKC activation on invasion of human aortic smooth muscle cells.

We identified nicotinic acetylcholine receptors and podosome-associated proteins in A7r5 cells by Western blotting and immunofluorescence microscopy. We differentiated the functions of nicotinic and muscarinic acetylcholine receptors in mediating nicotine-induced

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actin cytoskeletal remodeling using the nicotinic and muscarinic acetylcholine receptor antagonists, α-bungarotoxin and atropine, respectively. We investigated the involvement of phenotypic modulation in nicotine-induced actin cytoskeletal remodeling by performing cycloheximide and media exchange experiments. We studied nicotine-induced extracellular matrix degradation by A7r5 cells by performing in situ zymography experiments using cross-linked Alexa Fluor 488-conjugated gelatin and DQ-gelatin. Finally, we investigated the individual and interactive effects of nicotine and PKC activation on invasion of human aortic smooth muscle cells using matrigel-coated transwell assay. Results from this study suggest that long-term exposure to nicotine enhances the ability of VSMCs to degrade and invade extracellular matrix. Nicotinic acetylcholine receptor activation, actin cytoskeletal remodeling and phenotypic modulation are possible mechanisms.

2. Materials and methods

2.1. Cell culture

The procedure was the same as described previously (Gu et al., 2007). A7r5 rat vascular smooth muscle cells were purchased from ATCC (Manassas, VA), and grown in low-glucose (1000 mg/ml) DMEM without phenol red, supplemented with 10% fetal bovine serum, GlutaMAX™-I Supplement, and penicillin/streptomycin at 37 °C and 5% CO_2. Human aortic smooth muscle cells were purchased from Invitrogen (Grand Island, NY) and cultured in medium 231, supplemented with smooth muscle differentiation supplement, according to the vendor's instructions. All cell culture reagents were purchased from Invitrogen (Grand Island, NY). Podosome formation in cultured A7r5 cells was induced by 1 μM phorbol-12,13-dibutyrate (PDBu; Sigma) in serum-free medium.

2.2. Cigarette smoke extract experiments

The procedure was similar to that described by Su et al. (1998). Briefly, cigarette smoke extract was prepared by bubbling cigarette smoke through 30 ml sterile phosphate-buffered saline (PBS) of the following composition: 138 mM NaCl, 26 mM KCl, 84 mM Na_2HPO_4, pH 7.4 (37 °C) using a vacuum pump. Each cigarette was "smoked" for 5 min, and six cigarettes were used per 30 ml PBS to generate a cigarette smoke extract. The [nicotine] in this cigarette smoke extract should be approximately 15 μM (Lee et al., 2001). Since the average plasma [nicotine] in chronic smokers was approximately 1 μM (Moreyra et al., 1992), we studied the effect of 5% cigarette smoke extract on A7r5 cells.

2.3. Confocal immunofluorescence microscopy

The procedure was the same as described previously (Gu et al., 2007). For immunofluorescence microscopy studies, cells cultured on 15-mm glass coverslips were washed three times in PBS, fixed in 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) in PBS for 15 min, and extracted with 0.3% Triton X-100 in PBS for 10 min. Coverslips containing triton-extracted cells were labeled with primary antibodies for 1 hr, washed three times in PBS, incubated with secondary antibodies, washed three times in PBS, and then mounted on glass slides with FluorSave™ Reagent (Calbiochem, San Diego, CA). Immunofluorecence images were captured using a Leica TCS SP2 AOBS confocal microscope equipped with a 63×1.4 Plan Aprochromatic oil-immersion objective and a digital camera controlled by Leica Confocal Software package (version 2.5; Leica Microsystems, Exton, PA).

2.4. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis

The procedure was the same as described previously (Gu et al., 2007). For SDS-PAGE, A7r5 cells were lysed and scraped with an ice-cold cell lysis buffer of the following composition: 1% Triton X-100, 50 mM HEPES, 150 mM NaCl, 5 mM EDTA, 5 mM EGTA, 20 mM NaF, 20 mM sodium pyrophosphate, 1 mM PMSF, 1 mM Na_2VO4; pH 7.4. DC protein assays (Bio-Rad Laboratories, Hercules, CA) were performed on cell lysate samples. Equal amount of protein from each sample was run on each lane of 7.5% SDS-PAGE gels. After gel electrophoresis, proteins were transferred to nitrocellulose membranes for Western blot analysis. Proteins on the membranes were labeled with primary antibodies overnight at 4 °C, then labeled by peroxidase-conjugated secondary antibodies, and visualized by Enhanced Chemiluminescence detection reagents (Amersham Biosciences, Piscataway, NJ).

2.5. In situ zymography of extracellular matrix degradation

We studied nicotine-induced extracellular matrix degradation by A7r5 cells by performing in situ zymography experiments using two different substrates — cross-linked Alexa Fluor 488-conjugated gelatin and DQ-gelatin. The cross-linked Alexa Fluor 488-conjugated gelatin method was similar to that described by Bowden et al. (2001). Briefly, glass coverslips were covered by 0.5 mg/ml crosslinker SulfoSANPHA (G-Biosciences) in PBS, followed by ultraviolet irradiation for 10 min to cross-link one end of the SulfoSANPHA molecule to glass. After ultraviolet irradiation, coverslips were washed three times with PBS, and then incubated with 0.1 mg/ml Alexa Fluor 488-conjugated gelatin (Invitrogen) for 4 hrs to cross-link Alexa Fluor 488-conjugated gelatin to the free end of SulfoSANPHA. A7r5 cells were seeded onto cross-linked Alexa Fluor 488-conjugated gelatin-coated coverslips in 10% serum-containing media for 2 hrs to allow cell attachment. The serum-containing media was then replaced with serum-free media to minimize the release of metalloproteinase by cells during overnight incubation. Cells were cultured overnight in serum-free media with or without 2 μM nicotine, depending on the experiment, and then stimulated by 1 μM PDBu for 1 hr.

The DQ-gelatin method was similar to that described by Busco et al. (2010) and Cortesi et al. (1998). Briefly, a PBS solution containing 5% bovine skin gelatin (Sigma-Aldrich; St. Louis, MO) and 2.5% sucrose was prepared by heating the solution to 40 °C. DQ-gelatin (Invitrogen) was then added to the solution at a final concentration of 30 μg/ml. Glass coverslips were coated with the sucrose-gelatin/DQ-gelatin mix and stored in the refrigerator. DQ-gelatin-coated coverslips were equilibrated in a cell culture incubator for 1 hr, incubated in culture media for 1 hr, plated with A7r5 cells, and then incubated overnight before treatment with nicotine and/or PDBu. At the end of an experiment, A7r5 cells were fixed with 4% paraformaldehyde, labeled with the nuclear stain DAPI, and then imaged using a Nikon Diaphot 300 fluorescence microscope equipped with a 40× Plan Aprochromatic oil-immersion objective and a digital camera (Zeiss Axiocam) controlled by the Zeiss Axiovision software.

2.6. Transwell invasion assay

The method for studying invasion of human aortic smooth muscle cells through extracellular matrix using a matrigel-coated transwell system (BD Biosciences, San Jose, CA) was similar to that described by Maqbool et al. (2012). Briefly, human aortic smooth muscle cells (5×10^4; Invitrogen, Grand Island, NY) were loaded onto the matrigel-coated membrane in the upper chamber of the transwell system, and allowed to invade through the matrigel to the bottom side of the membrane for 24 hrs. The upper chamber contained serum-free 231 medium (Invitrogen, Grand Island, NY), whereas the