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Nicotinic receptor involvement in regulation of functions of mouse neutrophils from inflammatory site

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ABSTRACT

Participation of nicotinic acetylcholine receptors (nAChRs) in functioning of polymorphonuclear neutrophils (PMNs) isolated from inflammatory site of mice and expression of different nAChR subunits were studied. Nicotine and acetylcholine (ACh) modified respiratory burst induced by a chemotactic peptide N-formyl-MLF in neutrophils of male (but not female) mice. Antagonists of nAChRs α-cobratoxin (αCTX), α-conotoxins MII and [A10L]PnIA at concentrations of 0.01–5 μM, 0.2 μM and 1 μM, respectively, eliminated nAChR agonist effects. ACh also affected adhesion of PMNs, this effect was also prevented by αCTX (100 nM) and MII (1 nM). Neutrophils of female mice after chronic nicotine consumption acquired sensitivity to nAChR agonists. Changes of free intracellular Ca^{2+} concentration in neutrophils under the action of nAChR ligands were analyzed. In cells with no Ca^{2+} oscillations and relatively low resting level of intracellular Ca^{2+}, nicotine triggered Ca^{2+}-spikes, the lag of the response shortened with increasing nicotine concentration. A nicotinic antagonist caramiphen strongly decreased the effect of nicotine. RT-PCR analysis revealed mRNAs of α2, α3, α4, α5, α6, α7, α9, β2, β3, and β4 nAChR subunits. Specific binding of [^{125}I]-α-bungarotoxin was demonstrated. Thus in view of the effects and binding characteristics the results obtained suggest a regulatory role of α7, α3β2 or α6* nAChR types in specific functions of PMNs.

1. Introduction

PMNs participate in innate and adaptive immunity (Ishikawa and Miyazaki, 2005; for review see Jaillon et al., 2013; Scapini and Cassatella, 2014; Takashima and Yao, 2015). They are potent in microbicidal action, production of cytokines and pattern recognition molecules. PMNs, developed in bone marrow and released into circulation, express numerous proteolytic enzymes and cytotoxic proteins, as well as a set of membrane and cytoplasmic subunits of NADPH oxidase to produce superoxide, a predecessor of other reactive oxygen species (ROS). PMNs are the quickest and the most powerful effectors of the acute stage of inflammation (Jaillon et al., 2013; Rigby and DeLeo, 2012; Segal, 2005). Inadequate activity of PMNs may lead to uncontrolled inflammatory reaction. A proper intensity of neutrophil immune reactions is regulated by different membrane receptors: G-protein-coupled, Fc-, adhesion, cytokine and innate immune receptors (for review, see Futosi et al., 2013). nAChRs have been also revealed in PMNs. High affinity binding of their specific agonists [^{3}H]-nicotine and [^{3}H]-epibatidine was observed in human neutrophils (Benhammou et al., 2000; Lebargy et al., 1996), the latter was shown for smokers (Cormier et al., 2004). α-Bungarotoxin (α-Bgt) binding sites were found in PMNs (Cormier et al., 2004) that indicated expression of α7 or α1 nAChR subunits. mRNAs coding α4, α3, β2 and β4 subunits and corresponding proteins were disclosed in human PMNs suggesting the existence of α4β2 and α3β4 nAChR types (Benhammou et al., 2000). α7 nAChRs

Abbreviations: ACh, acetylcholine; BSA, bovine serum albumin; αBgt, α-bungarotoxin; $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration; αCTX, α-cobratoxin; fMLF, *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine; HBSS, Hanks' balanced salt solution; IL-8, interleukin-8; LPS, lipopolysaccharide; MIP-2, macrophage inhibiting protein-2; MMP-9, matrix metallopeptidase-9; nAChR, nicotinic acetylcholine receptor; NF-kB, nuclear factor-kB; PKC, protein kinase C; PMSF, phenylmethylsulfonyl fluoride; PMN, polymorphonuclear neutrophilic granulocyte; ROS, reactive oxygen species; RT-PCR, Real-Time Reverse-Transcription Polymerase Chain Reaction; TNF-α, tumor-necrosis factor-α.

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appear on the surface of the neutrophil in the period of its maturation from promyelocyte to segmented phenotype (Xu et al., 2008). mRNAs encoding a full-length (ordinary) α7 nAChR and a shorter (specific human) variant were revealed in human non-fractionated leukocyte population from blood; overexpression of one of those variants led to cell phenotype change and enhancement of cell to cell adhesion (Costantini et al., 2015).

α7 nAChRs are of a peculiar interest, especially due to their unique role in "cholinergic anti-inflammatory pathway" (Tracey, 2009). The central nervous system is considered to guard an organism from excessive inflammation by regulating activity of innate immune cells through α7 nAChRs (Olofsson et al., 2012; Pavlov et al., 2007; Wu et al., 2014). Stimulation of α7 nAChRs protected an organism against sepsis by inhibiting Toll-like receptors via phosphoinositide 3-kinase activation, as it was shown using the mouse models (polymicrobial sepsis and endotoxemia), and LPS-treated RAW264.7 macrophage culture cells (Kim et al., 2014). Production of TNF-α and MIP-2, as well as alveolar transmigration, was significantly lower in mouse neutrophils after the activation of α7 nAChRs as shown by *E. coli* pneumonia and LPS-induced acute lung injury models (Su, 2010). Adhesion of PMNs and their transmigration across the blood-brain barrier were diminished in α7 nAChR-deficient mice as compared with the wild phenotype; this resulted in decreasing neuronal injury in α7-/- mice with meningitis (Chi, 2011) and in neuronal inflammation (Yu et al., 2015). Nicotine-enhanced *E. coli* K1 invasion and transmigration of PMNs across the blood-brain barrier were blocked by methyllycaconitine and memantin, α7 nAChR antagonists (Yu et al., 2015). Effects of nicotine, epibatidine and anatoxin-a on IL-8 production associated with NF-kB translocation and ROS generation were demonstrated in human neutrophils; whereas mecamylamine inhibited it (Iho et al., 2003). The concentrations of nAChR ligands used in that study were very high, and although the authors suggested a role of α3β2 nAChR in the action of agonists, additional nAChR subtypes might be involved. Thus, the information on nAChR subtypes expressed in PMNs and on their role in defense reactions is not sufficient and additional experiments are required.

We tested the capability of several nAChR agonists and antagonists to modify specific neutrophil reactions: namely, the respiratory burst induced by a chemotactic tripeptide N-formyl-MLF (fMLF) and adhesion. Nicotine and ACh changed production of ROS by neutrophils, whereas nAChR antagonists eliminated their action. Influence of nicotinic ligands on dynamics of intracellular free Ca^{2+} was studied. Besides, expression of diverse nAChR subunits in mouse neutrophils was shown at both mRNA and protein levels. The results obtained suggest a modifying role of nAChRs (including α7 and α3β2 or α6* types) in specific neutrophil functions.

2. Materials and methods

2.1. Materials

Acetylcholine iodide and nicotine hydrogen tartrate were purchased from Sigma (St-Louis, MO, USA). α-CTX was purified from crude venom of *Naja kaouthia* as described previously (Kukhtina et al., 2000). α-Conotoxins MII and [A10L]PnIA were synthesized as described (Surin et al., 2012; Kasheverov et al., 2006; respectively). Monoiodinated (3-[^{125}I]iodotyrosyl^{54})-α-Bgt was from Amersham Biosciences (Little Chalfont, UK). Caramiphen (diethylaminoethyl ester of diphenyl cyclopentan carbonic acid), gangleron (3-diethylamino-1,2-dimethylpropyl-p-isobutoxybenzoat), and hexonium were kindly provided by Prof. M.J. Michelson (Sechenov Institute of Evolutionary Physiology and Biochemistry, Russian Academy of Sciences, St-Petersburg, Russia). Tubarine (d-tubocurarine chloride) was purchased from Burroughs Wellcome & Co (London, UK). Anti-Gr-1 antibody (PE-anti-mouse Ly6G/Ly6C) was purchased in BioLegend (San-Diego, CA, USA). Hanks' balanced salt solution (HBSS), phosphate buffered saline (PBS), N-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLF), trypan blue, luminol (5-Amino-2,3-dihydro-1,4-phthalazinedione), zymosan, leupeptin, pepstatin, bestatin all were purchased from Sigma-Aldrich (St-Louis, MO, USA). Fura-2AM was from Molecular probes (Eugene, OR, USA). Bisbenzimide H 33258 (2-[2-(4-Hydroxyphenyl)-6-benzimidazoyl]-6-(1-methyl-4-piperazyl)-benzimidazol) was obtained from Merck/Millipore (Darmstadt, Germany). Romanovsky-Himsa dye was purchased from Minimed-R (Suponevo, Bryansk region, Russia), ethyl alcohol was from Donskoi' (Tula region, Epifan', Russia), 2-propanol from Component-Reactive (Moscow, Russia). BSA was from MP Biomedicals (Illkirch, France). PMSF was obtained from Helicon (Moscow, Russia). Polyethylenimine was from Fluka (Switzerland). Glass microfibre paper GF/F was from Whatman (Maidstone, England).

2.2. Animals

6-7 week old mice of outbred strains C57Bl/6 and BALB/c (23–27 g weight) and of inbred strain NMRI (30–32 g weight) were purchased from the Animals Breeding Centre (Branch of Shemyakin and Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences, Pushchino, Russia). All experiments were performed under protocols of the Animal Care and Use Commission No. 12306 (2006) of the Institute of Cell Biophysics, Russian Academy of Sciences (Pushchino, Russia).

2.3. Mouse model of nicotine chronic consumption

Four week-old female NMRI mice were divided into 2 groups: 10 mice (control) and 20 mice (experimental group). Both groups received *accesso libero* drinking water containing 2.5% sucrose without (control) or with nicotine hydrogen tartrate (4.2 μg/kg/day) during 3 weeks. Fresh water with sucrose (control group) or with sucrose + nicotine (experimental group) was given daily in the morning, and drinking in each group was measured.

2.4. Preparation of peritoneal neutrophils

Inflammation in mice of three strains was induced by intraperitoneal injection of zymosan suspension (5 mg/ml, 150 μl per mouse). 5 h later peritoneal cavity was washed with 3 ml Ca^{2+}-free Hanks' balanced salt solution (HBSS, pH 7.4, 4 °C), wash-out was centrifuged at 600 g for 5 min at 4 °C. Purity of PMN population exceeded 95% as estimated by luminescent microscopy (Leica DM6500, ×40) with anti-Gr-1 antibody (PE-anti-mouse Ly6G/Ly6C) and Bisbenzimide H 33258. Survival of cells was 97–99% as determined by trypan blue staining. Isolated cells were kept in HBSS without Ca^{2+} and phenol red for 1 h at 4 °C before use.

2.5. Chemiluminescent analysis of ROS generation

ROS generation by PMNs was determined by luminol-dependent chemiluminescence technique as was described earlier (Filina et al., 2014). Chemiluminometre device CHEMLUM-12 (Institute of Cell Biophysics, Russian Academy of Sciences, Pushchino, Russia) was used. The influence of preliminary treatment with nAChR ligands on fMLF-induced ROS production was studied. In brief, in each independent experiment 12 minidishes with samples (200 μl; 10^6 cells/ml) were used. Samples of cells, prepared in triplet, were: (i) intact – control; (ii) treated with an agonist; (iii) incubated with an antagonist; (iv) treated with