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Auto/paracrine control of inflammatory cytokines by acetylcholine in macrophage-like U937 cells through nicotinic receptors

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ABSTRACT

Although acetylcholine (ACh) is well known for its neurotransmitter function, recent studies have indicated that it also functions as an immune cytokine that prevents macrophage activation through a 'cholinergic (nicotinic) anti-inflammatory pathway'. In this study, we used the macrophage-like U937 cells to elucidate the mechanisms of the physiologic control of cytokine production by auto/paracrine ACh through the nicotinic class of ACh receptors (nAChRs) expressed in these cells. Stimulation of cells with lipopolysaccharide up-regulated expression of α1, α4, α5, α7, α10, β1 and β3 subunits, down-regulated α6 and β2 subunits, and did not alter the relative quantity of α9 and β4 mRNAs. Distinct nAChR subtypes showed differential regulation of the production of pro- and anti-inflammatory cytokines. While inhibition of the expression of the TNF-α gene was mediated predominantly by the α-bungarotoxin sensitive nAChRs, that of the IL-6 and IL-18 genes—by the mecamylamine-sensitive nAChRs. Both the Mec- and αBtx-sensitive nAChRs regulated expression of the IL-1β gene equally efficiently. Upregulation of IL-10 production by auto/paracrine ACh was mediated predominantly through α7 nAChR. These findings offer a new insight on how nicotinic agonists control inflammation, thus laying a groundwork for the development of novel immunomodulatory therapies based on the nAChR subtype selectivity of nicotinic agonists.

1. Introduction

Although acetylcholine (ACh) is well known for its neurotransmitter function, recent studies have indicated that it also functions as an immune cytokine that prevents macrophage activation through a 'cholinergic (nicotinic) anti-inflammatory pathway' [1,2]. The nicotinic ACh receptor (nAChR) agonists have been shown to prevent or treat experimentally induced endotoxemic shock [3–5], sepsis [5–7], hemorrhagic shock [8,9], ischemia-reperfusion [10], subcutaneous inflammation [11], postoperative ileus [12], pancreatitis [13], allergic lung inflammation [14,15], and acute lung injury [16]. The agonist of nAChRs nicotine has been used in clinical trials, but its clinical potential is limited by its collateral toxicity [17]. Appreciations of an important role of α7 nAChR in regulation of the immune inflammation urged a search for selective nicotinic agonists that avoid the undesired side effects of nicotine [2]. Further elucidation of the nAChR-mediated regulation of inflammation should help develop novel treatments allowing to regulate specific types of immune reactions by selectively activating or blocking particular nAChR subtypes expressed in monocytes/macrophages.

Various immune cells possess diverse repertoires of nAChRs and, therefore, respond differently to the nicotinic agonists that exhibit varying affinities to distinct nAChR subtypes. The pharmacologic subtype of the ACh-gated ion channel is determined by a specific combination of the nAChR subunits forming the channel. The "muscle"-type nAChRs can be comprised by α1, β1, γ, δ, and ε subunits, and the "neuronal"-type nAChRs—by α2–α10 and β2–β4 subunits [18–21]. The α7, and α9 subunits can form homomeric nAChR channels sensitive to α-bungarotoxin (αBtx). The heteromeric channels can be composed of α2, α3, α4, α5, α6, β2, β3 and β4 subunits, e.g., α3(β2/β4) ± α5, and α9 can also form a heteromeric channel with α10 [21]. The signal transduction pathways downstream of different nAChRs may be activated by both ionic events, such as Ca^{2+} influx, and changes of the stoichiometry of a multiprotein complex formed by the nAChR subunit(s) [22,23]. Therefore, a net biologic effect of ACh in a particular type of immune cell depends on the subunit composition of the major nAChR subtypes expressed by the cell at a given stage of its development and activation.

The presence of nAChRs in human monocyte/macrophages was suggested by the inhibitory effect of αBtx on monocyte activation [24] and nicotine binding to the human monocytic THP-1 cell line [25]. By

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now, it has been documented that human, murine and monkey macrophages express classic nAChR subunits [4,26,27]. Expression of α1, α7, and α10 mRNAs has been detected in human macrophages [4], whereas both bone marrow-derived dendritic cells and macrophages from C57BL/6J mice possess mRNAs encoding the nAChR subunits α2, α5, α6, α7, α10 and β2 [28]. Macrophages also express the muscarinic class of ACh receptors [29,30] that can modify the cell response to auto/paracrine ACh.

The human monoblastoid tumor cell line U937 [31] that can be differentiated into macrophage-like cells by treatment with phorbol-12-myristate 13-acetate (PMA) exhibits ACh synthesizing activity of choline acetyltransferase and contains approximately 0.02 pmol/10^6 of ACh [32]. Although, to the best of our knowledge, the subunit composition of nAChRs expressed in U937 cells have not been established, it has been reported that these cells respond to nicotine [33,34]. Therefore, U937 cells provide a useful model for studying basic mechanisms of macrophage regulation by auto/paracrine ACh through nAChRs.

In this study, we characterized the profile of nAChR subunits expressed in the macrophage-like differentiated U937 cells and demonstrated how the receptor repertoire changes upon cell activation with lipopolysaccharide (LPS). We also established relative contributions of α7- and non-α7 nAChR subtypes expressed in these cells to regulation of the pro- and anti-inflammatory cytokine production. The obtained results indicated that the macrophage nAChR subtypes are differentially coupled to regulation of production of distinct cytokines by auto/paracrine ACh. These findings offer a new insight on how nicotinic agonists control inflammation, thus laying a groundwork for the development of novel immunomodulatory therapies based on the nAChR subtype selectivity of nicotinic agonists.

2. Materials and methods

2.1. Cells and reagents

The human monoblastoid tumor cell line U937 was purchased from ATCC (Catalog #CRL-2367; Manassas, VA) and grown in the ATCC complete growth medium (Catalog #30-2001) at 37 °C in a humid, 5% CO_2 incubator. To differentiate into macrophages, the U937 cells were treated with 200 nM PMA (Sigma-Aldrich Corporation, St Louis, MO) and allowed to adhere to tissue culture plate for 3 days [35]. The nicotinic ligands epibatidine (Epi), mecamylamine (Mec), methyllycaconitine (MLA) and αBtx, the inhibitor of ACh synthesis hemicholinium-3 (HC-3), and LPS were from Sigma-Aldrich Corporation. AR-R17779 was a gift from AstraZeneca Pharmaceuticals (Wilmington, DE). Particular doses of all drugs were selected based on the pilot dose–response experiments.

2.2. Characterization of nAChRs expressed in U937 cells

The profile of nAChR subunits expressed in differentiated, macrophage-like U937 cells was determined in a standard reverse-transcription PCR (RT-PCR) assay using the published primer sets for human α1–α7, α9, α10, β1–β4, γ, δ and ε nAChR subunits (Operon, Alameda, CA) and the amplification conditions shown in Table 1. All primers were tested using normal human muscle and brain PCR ready first strand cDNAs purchased from BioChain Institute Inc. (Hayward, CA) [36]. To control for contamination of DNase-treated samples with residual genomic DNA, the reverse-transcription step was omitted.

2.3. Real-time quantitative polymerases chain reaction (qPCR) experiments

Total RNA was extracted from U937 cells at the end of exposure experiments with the RNeasy Mini Kit (Qiagen, Valencia, CA) and used in the qPCR assay detailed elsewhere [37]. All qPCR primers were designed with the assistance of the Primer Express software version 2.0 computer program (Applied Biosystems, Foster City, CA) and the service Assays-on-Demand provided by Applied Biosystems. The qPCR reactions were performed using an ABI Prism 7500 Sequence Detection System (Applied Biosystems) and the TaqMan Universal Master Mix reagent (Applied Biosystems) in accordance to the manufacturer's protocol, as described by us in detail elsewhere [38]. To correct for minor variations in mRNA extraction and reverse-transcription, the gene expression values were normalized using the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase. The data were analyzed with a sequence detector software (Applied Biosystems) and expressed as mean ± standard deviation of mRNA in question relative to that of control.

2.4. In-cell Western (ICW) assay

The ICW assay was performed as described by us in detail elsewhere [39], using the reagents and equipment from LI-COR Biotechnology (Lincoln, NE). After incubation of 3×10^4 U937 cells/well of a 96-well plate in the growth medium with or without test agents for 16 h, the experimental and control U937 cells were fixed *in situ*, washed, permeabilized with Triton solution, incubated with the LI-COR Odyssey Blocking Buffer for 1.5 h and then treated overnight at 4 °C with a primary mouse antibody to human IL-1β, IL-6, IL-10 or IL-18, TNF-α (R&D Systems, Minneapolis, MN). After that, the cells were washed, and stained for 1 h at room temperature with a secondary LI-COR IRDye

Table 1
Primers used for RT-PCR analysis of nAChRs in differentiated U937 cells.[a]

Target mRNA	Forward/reverse primers	Product size, bp	Anneal temperature	Sources
α1	CGT CTG GTG GCA AAG CT CCG CTC TCC ATG AAG TT	580	55	[86]
α2	GGA GCT CTG CCA CCC CCT AC AAC ATA CTT CCA GTC CTC	327	64	[87]
α3	CTG GTG AAG GTG GAT GAA GT CTC GCA GCA GTT GTA CTT GA	464	58	[86]
α4	GGA TGA GAA GAA CCA GAT GA CTC GTA CTT CCT GGT GTT GT	444	58	[86]
α5	TCA ACA CAT AAT GCC ATG GC CCT CAC GGA CAT CAT TTT CC	219	64	[87]
α6	GTG GCC TCT GGA CAA GAC AA AAT TAT AAA TAC CCA AAG A	372	58	[86]
α7	CTT CAC CAT CAT CTG CAC CAT C GGT ACG GAT GTG CCA AGG ATA T	308	55	[87]
α9	GTC CAG GGT CTT GTT TGT ATC CGC TCT TGC TAT GAT	403	58	[87]
α10	CTC TCA AGC TGT TCC GTG ACC AAG GCT GCT ACA TCC ACG C	394	64	[88]
β1	TGT ACC TGC GTC TAA AAA GG GCA GGT TGA GAA CCA CGA CA	455	60	[87]
β2	CAG CTC ATC AGT GTG CA GTG CGG TCG TAG GTC CA	347	58	[89]
β3	AGA GGC TCT TTC TGC AGA GCC ACA TCT TCA AAG CAG	354	60	[89]
β4	GTG AAT GAG CGA GAG CAG AT GGG ATG ATG AGG TTG ATG GT	524	58	[86]
δ	CAG ATC TCC TAC TCC TGC AA CCA CTG ATG TCT TCT CAC CA	471	58	[86]
γ	CGC CTG CTC TAT CTC AGT CA GGA GAC ATT GAG CAC AAC CA	546	56	[86]
ε	GTA ACC CTG ACG AAT CTC AT GTC GAT GTC GAT CTT GTT GA	432	55	[86]

[a] All products were sequenced by the designers of the PCR primer used in this study.