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Modulation of immune functions in polymorphonuclear neutrophils induced by physostigmine, but not neostigmine, independent of cholinergic neurons

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ABSTRACT

Background: Cholinesterase inhibitors (Ch-I) improve survival in experimental sepsis consistent with activation of the cholinergic-anti-inflammatory-pathway. So far, less is known about whether Ch-I have a direct immunomodulatory effect on immune cells (polymorphonuclear neutrophils, PMN) in the absence of cholinergic neurons. We investigated the concentration–response-effects of physostigmine and neostigmine on the oxidative burst activity (human and rat PMN) and the expression of adhesion molecules on the surface of human PMN under *in vitro* conditions.
Methods: PMN from 10 healthy humans or 10 rats were pretreated with 2, 10, 24, 97 μM physostigmine or 3, 15, 30, 150 μM neostigmine, primed with tumor-necrosis-factor-alpha (TNF-alpha) followed by stimulation with n-formyl-methionyl-leucylphenylalanine (fMLP) or stimulated with phorbol-12-myristate-13-acetate (PMA). Human and rat samples were assessed by flow cytometry for the generation of oxidative free radicals. Stimulated human PMN were additionally incubated with antibodies against Mac-1 (CD11b) or L-selectin (CD62l).
Results: Physostigmine and neostigmine did not alter oxidative burst activity or the expression of adhesion molecules of PMN induced by receptor-dependent activators like fMLP or TNF-alpha/fMLP (rat and human PMN, p = n.s.). Physostigmine, but not neostigmine, inhibited the protein-kinase-C-mediated oxidative burst activity by PMA in a dose-dependent manner (rat and human PMN, $p < 0.05$). Physostigmine, in the concentration range tested, suppressed the expression of CD11b following stimulation with PMA not significantly (human PMN: control: 63.1 ± 10.7 vs. 97 μM physostigmine: 49.9 ± 12.8 MESF, p = n.s.).
Conclusion: While neostigmine has no effect on functional and phenotypic changes of PMN, the lipid soluble Ch-I physostigmine causes a dose dependent reduction in PMA-induced oxidative burst, independent of neuronal released acetylcholine.

Introduction

The cholinergic-anti-inflammatory-pathway (CAP), which has been described as a mechanism for the neuronal control of inflammation via the efferent fiber of the vagus nerve, is important in modulating the inflammatory response in local and systemic diseases, including sepsis, hemorrhagic shock, pancreatitis and other inflammatory syndromes (Pavlov and Tracey 2006). Studies describing the CAP show that acetylcholine, the primary neurotransmitter released by the vagus nerve, and nicotinic acetylcholine receptor agonists (nAChR) inhibit the synthesis of proinflammatory cytokines via the alpha7-nAChR on immune cells and protect against septic shock in experimental bacteremia (Tracey 2007).

This hard-wired connection between the nervous and immune system, considered a critical regulator of inflammation, can also be activated by cholinesterase inhibitors (Ch-I). Peter et al. show that the number of rolling leukocytes is significantly reduced by the application of physostigmine during experimental endotoxemia (Peter et al. 2010). Hofer and colleagues demonstrate that Ch-I improve survival in murine experimental sepsis consistent with activation of the CAP. Animals treated with neostigmine show no difference compared with physostigmine-treated animals (Hofer et al. 2008).

Abbreviations: CAP, cholinergic-anti-inflammatory-pathway; CD11b, Mac-1; CD62l, L-selectin; Ch-I, cholinesterase inhibitors; DHR, dihydrorhodamine; FITC, fluoresceinisothiocyanate; fMLP, n-formyl-methionyl-leucylphenylalanine; nAChR, nicotinic acetylcholine receptor; PI, propidium iodide; PKC, protein-kinase-C; PMA, phorbol-12-myristate-13-acetate; PMN, polymorphonuclear neutrophils; ROS, reactive oxygen species; SNARF1/AM, carboxy-seminaphthorhodafluor-L-acetoxymethylester; TNF-alpha, tumor-necrosis-factor-alpha.

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These data are quite different from previous studies by Akinci et al., which failed to demonstrate protective effects of neostigmine in an animal model of endotoxin-induced septic shock (Akinci et al. 2005). The cause for the variation in results with similar experimental setups between two studies is still unknown.

In the last years it has been shown that the cholinergic system is not confined to the nervous system, but is ubiquitous, leading to the concept of "non-neuronal-cholinergic-system" (Grando et al. 2007). Acetylcholine, as well as its receptors, can all be found in a wide variety of cellular systems such as epithelial cells, blood cells, and vascular endothelial cells (Fujii and Kawashima 2001). Although its function is not yet clarified, the "non-neuronal-cholinergic-system" appears to be involved in the regulation of several biological functions, such as cell–cell-contact or immune functions (Wessler et al. 2003).

So far, less is known about whether Ch-I have a direct immunomodulatory effect on immune cells under *in vitro* conditions, *i.e.* in the absence of cholinergic neurons. Polymorphonuclear neutrophils (PMN) are multifunctional cells that play a pivotal role in the innate immune system and appear to be a central component in the inflammatory injury of sepsis (Brown et al. 2006).

Reactive oxygen species (ROS) are generated by the NADPH-oxidase during the PMN oxidative burst. Bacterial peptides, such as n-fomyl-methionyl-leucyl-phanylalanin (fMLP), trigger activation of the PMN-oxidase directly. FMLP exerts its effects through a specific G-protein-coupled receptor of the PMN. Tumor-necrosis-factor-alpha (TNF-alpha) is an incomplete agonist, which only primes the PMN-oxidase for subsequent activation. In contrast, phorbol-12-myristate-13-acetate (PMA) stimulates PMN by a direct activation of protein-kinase-C (PKC) bypassing post-receptor signaling (Fröhlich et al. 1997).

Regardless of its ability to generate ROS, PMN migration is a central component during inflammation. PMN-rolling and the adherence of PMN to activated endothelium are critical steps for transendothelial migration. L-selectin (CD62l) is an adhesion molecule on the surface of PMN which promotes rolling. Firm adherence and diapedesis are mediated by Mac-1 (CD11b/CD18) (Trabold et al., 2007a,b).

To elucidate differential functional and phenotypic changes in response to clinical relevant Ch-I, we investigated in this comparative study the concentration–response-effects of physostigmine and neostigmine on typical immune functions of PMN like adhesion and the generation of the oxidative burst under *in vitro* conditions.

Materials and methods

Preparation of stock solutions

Physostigminesalicylate (Köhler Chemie, Bensheim, Germany) and physostigminehemisulfate (Bioszol Diagnostica, Eching, Germany) were diluted with PBS (Sigma Aldrich Chemie GmbH, Steinheim, Germany) to equimolar final concentrations of 2, 10, 24 or 97 μM. Neostigminemethylsulfate (Rotexmedica, Trittau, Germany) was diluted with PBS to final concentrations of 3, 15, 30 or 150 μM. We used physostigmine and neostigmine in doses, which are similar to the doses already used in literature (Hartvig et al. 1986) and made a dilution series for each drug to determine a concentration–response relation.

The 1 mM stock solutions of fMLP (437.6 μg/mL) and PMA (667 μg/ml; Sigma Aldrich Chemie GmbH, Steinheim, Germany) are prepared with DMF and stored at −20 °C. Stock solutions of Dihydrorhodamine (DHR, Cayman Chemical Company, MI, USA), Carboxy-seminaphthorhodafluor-L-acetoxymethylester (SNARF1/AM, Invitrogen Corporation, Grand Island, NY, USA), fMLP, PMA and propidium iodide (PI, Electrophoresis GmbH, Heidelberg, Germany) were diluted with PBS to working solutions with concentrations of 100 μM for DHR, fMLP and PMA, 10 μM for SNARF1/AM and 1.5 mM for PI. The stock solutions of DHR, SNARF1/AM and PI were also stored at −20 °C.

Ammonium chloride buffer, made of 4.15 g of ammonium chloride, 0.84 g of sodium bicarbonate and 1 mL of 0.5 M EDTA ad 500 mL water, was used as a red blood cell lysis buffer (Li and Chung 2003).

Experimental design

The study was arranged in three steps: To elucidate differential functional and phenotypic changes in response to Ch-I, we first examined the changes in the generation of ROS by rat PMN in the presence of physostigminesalicylate and neostigminemethylsulfate in a dose dependent manner. Oxidative burst was induced in diluted rat whole blood by either the bacterial peptide fMLP, priming with TNF-alpha followed by fMLP or the unspecific non-receptor PKC-activator PMA.

In a second step, the concentration–response-relation of physostigminehemisulfate and neostigminemethylsulfate on the fMLP, TNF-alpha/fMLP or PMA-induced oxidative burst has been determined in separated human PMN. This was done to examine whether there are any species differences, and to ensure that the effects are independent of other cell- or blood plasma-influences like protein binding. Furthermore we used physostigminehemisulfate to ensure that potential effects are independent of the salt ligated to the Ch-I.

Step three comprised the evaluation of the effects of physostigminesalicylate on the expression of CD62l and CD11b on the surface of human PMN.

Blood collection and cell preparation

Blood collection, cell preparation and oxidative burst of rat PMN

Animal experiments have been conducted in accordance to the German laws regulating animal care, the European Communities Council Directive and institutional guidelines. Ten male Wistar rats weighing 250–350 g were included into the study. Blood was drawn by cardiac puncture during deep inhalational anesthesia (sevoflurane/oxygen) into lithium heparin containing commercial containers (Sarstedt, Nümbrecht, Germany).

The cell preparation and stimulation assays have been performed as described previously (Bitzinger et al. 2008): Twenty microliters of heparinised rat whole blood were suspended 1:50 (20 μL + 980 μL PBS). All incubation steps were performed at 37 °C if not stated otherwise. First, all cell suspensions were loaded with 10 μL of the fluorogenic substrates DHR and SNARF1/AM. The final concentrations were 1 μM for DHR and 0.1 μM for SNARF1/AM. Thereafter, physostigminesalicylate (final concentrations: 2, 10, 24 or 97 μM) or neostigminemethylsulfate (3, 15, 30 or 150 μM) were added. Following incubation for 10 min, the cells were primed by TNF-alpha (final concentration: 10 ng/mL, Pepro Tech GmbH, Hamburg, Germany) prior fMLP stimulation and incubated for further 5 min. Afterwards the oxidative burst was induced by 1 μM fMLP or 1 μM PMA, so that we received three stimulated sample groups (TNF-alpha/fMLP, fMLP and PMA). Samples without any stimulus were used as a negative control. Finally after further incubation for 15 min the reaction was stopped at 0 °C. Dead cells were labeled with 10 μL PI at a final concentration of 15 μM. The samples were immediately washed once with 2 mL of 4 °C PBS (400 g, 3 min) to eliminate extracellular chemicals. Then 2 mL of 4 °C red blood cell lysis buffer were added prior to incubation at 4 °C in the dark until the suspension of cells lost its turbidity (10–15 min). After a final washing step, the cell pellets were resuspended in 200 μL PBS and stored on ice until flow-cytometric analysis.