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Eosinophil peroxidase induces expression of cholinergic genes via cell surface neural interactions

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ABSTRACT

Eosinophils localize to and release their granule proteins in close association with nerves in patients with asthma and rhinitis. These conditions are associated with increased neural function. In this study the effect of the individual granule proteins on cholinergic neurotransmitter expression was investigated. Eosinophil peroxidase (EPO) upregulated choline acetyltransferase (ChAT) and vesicular acetylcholine transporter (VAChT) gene expression. Fluorescently labeled EPO was seen to bind to the IMR-32 cell surface. Both Poly-L-Glutamate (PLG) and Heparinase-1 reversed the up-regulatory effect of EPO on ChAT and VAChT expression and prevented EPO adhesion to the cell surface. Poly-L-arginine (PLA) had no effect on expression of either gene, suggesting that charge is necessary but insufficient to alter gene expression. EPO induced its effects via the activation of NF-κB. MEK inhibition led to reversal of all up-regulatory effects of EPO. These data indicate a preferential role of EPO signaling via a specific surface receptor that leads to neural plasticity.

1. Introduction

The presence of eosinophils and their degranulation products in tissues are the hallmark of a variety of diseases, including the allergic conditions asthma and rhinitis, as well as inflammatory bowel disease and eosinophilic gastroenteritis (Jacobsen et al., 2007; Hogan et al., 2008; Rothenberg et al., 2001; Rothenberg, 2004; Gleich, 2000; Aceves and Ackerman, 2009). In asthma, eosinophils have been shown to localize to airway nerves (Costello et al., 1997; Jacoby et al., 2001). This interaction occurs via nerve-eosinophil adhesion molecules (Kingham et al., 2002) and results in intracellular signaling within nerve cells (Walsh et al., 2004). These intracellular cascades lead to changes in nerve cell morphology, changes in production of neurotransmitters, and protection from apoptosis (Kingham et al., 2003; Morgan et al., 2004; Sawatzky et al., 2003; Durcan et al., 2006). Adhesion also leads to effects on eosinophils, including eosinophil degranulation (Sawatzky et al., 2002) and the release of mediators such as major basic protein (MBP), eosinophil peroxidase (EPO), eosinophil-derived neurotoxin (EDN), and eosinophil cationic protein (ECP). These cationic proteins have anti-parasitic activity in vitro (Klion and Nutman, 2004) and they may be important in the defence against certain viral infections. Eosinophil cationic proteins also play a role in diseases such as asthma and rhinitis because they are cytotoxic to mammalian cells (Walsh, 2001; Bystrom et al., 2012). At non-cytotoxic concentrations, eosinophil cationic proteins have been shown to induce nerve cell signaling pathways by phosphorylation of the MAP kinases ERK 1/2, p38 and AKT and subsequent activation of the nuclear transcription factor NFκB (Morgan et al., 2005). Each of the eosinophil proteins have different effects, for example MBP protects nerve cells from apoptosis by Bfl-2 upregulation (Morgan et al., 2005), while MBP and EPO upregulate muscarinic M2 receptor expression (Durcan et al., 2006).

Abbreviations: Ach, acetylcholine; AKT, protein kinase B; AP-1, activator protein 1; ATP, adenosine triphosphate; BSA, bovine serum albumin; CD 11/18, β2 integrin; cDNA, copy DNA; ChAT, choline acetyltransferase; DC, dendritic cell; DMEM, Dulbecco's modified eagle medium; DTT, dithiothreitol; ECP, eosinophil cationic protein; EDN, eosinophil derived neurotoxin; EDTA, ethylenediaminetetraacetic acid; EMSA, electrophoretic mobility shift assay; EPO, eosinophil peroxidase; ERK, extracellular signal-regulated kinase; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; FPRL-1, formyl peptide-like receptor 1; GAG, glycosaminoglycan; h, hour(s); HEP1, heparinase 1; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HER-2, epidermal growth factor-2; hPR, human pancreatic RNase; HRP, horseradish peroxidase; ICAM1, intracellular adhesion molecule 1; IκB, inhibitory factor κB; KCl, potassium chloride; MAPK, mitogen activated protein kinase; MBP, major basic protein; MEK, mitogen-activated protein kinase kinase; $MgCl_2$, magnesium chloride; min, minute(s); mRNA, messenger RNA; NaCl, sodium chloride; NFκB, nuclear factor κB; NGF, nerve growth factor; PBS, phosphate buffered saline; PLA, poly-L-arginine; PLG, Poly-L-Glutamate; PMSF, phenylmethylsulfonyl fluoride; RIPA buffer, radioimmunoprecipitation assay buffer; RT-PCR, real-time polymerase chain reaction; SDS-PAGE, sodium dodecyl-sulphate polyacrylamide gel electrophoresis; SDW, sterile distilled water; SEM, standard error of mean; VAChT, vesicular acetylcholine transporter; VCAM-1, vascular adhesion molecule-1; VLA4, very late antigen 4; vol, volume; wt, weight.

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The dominant innervation of airways is by vagal parasympathetic nerves (Costello et al., 1999). Stimulation of these nerves causes release of ACh onto M_3 muscarinic receptors resulting in bronchoconstriction, production of mucus, and dilation of the bronchiolar vasculature (Nadel and Barnes, 1984). The regulation of the production of ACh in nerves is co-ordinated through a series of distinct enzymatic processes, including the enzyme choline acetyltransferase (ChAT) and the transport protein vesicular acetylcholine transporter (VAChT), the genes both of these proteins are encoded in a "cholinergic locus" (Kitamoto et al., 1998). The coordinated expression of ChAT and VAChT is complex and is the basis of the rationale of studying the dynamic effects on these two genes specifically. This is discussed further in Section 4.

Once released the effects of ACh are limited by protective neuronal M_2 muscarinic receptors present on the parasympathetic cholinergic terminals (Fryer and Maclagan, 1984). In animal models, eosinophil MBP is associated with the development of vagally mediated hyperreactivity of cholinergic nerves (Lefort et al., 1996) by allosteric inhibition of protective M_2 muscarinic receptors (Jacoby et al., 1993; Fryer and Jacoby, 1992; Evans et al., 1997). Hence, cholinergic nerve function is carefully regulated both at the level of ACh production and its effects once it has been released.

Because eosinophils exert a pathogenic role via their action on nerves we sought to examine the effects of eosinophil granule proteins on cholinergic gene expression in IMR32 nerve cells. We tested the hypothesis that following cell surface interactions, eosinophil granule proteins may promote intracellular signaling leading to changes in cholinergic gene expression.

2. Materials and methods

2.1. Cell culture

Fibroblast-depleted human cholinergic neuroblastoma IMR-32 cells were maintained in culture in proliferation medium [DMEM Plus Glutamax, 5% FCS, 100 U/ml penicillin/streptomycin (GIBCO® Invitrogen, Paisley, UK)] at 37 °C in an atmosphere of 5% CO_2, as described previously (Walsh et al., 2004). Cells were plated at a density of 5×10^5/well in 6-well cell culture dishes and cultured in differentiation medium [DMEM Plus Glutamax, 2% FCS, 2 mM sodium butyrate (Sigma, Poole, UK), 100 U/ml penicillin/streptomycin] for 6 days.

2.2. Eosinophil cationic granule proteins

EPO, MBP and EDN were kindly donated by Dr. Gerald Gleich, Department of Dermatology, School of Medicine, University of Utah, Salt Lake City. The concentration of 1 μg/ml for all three proteins was established as optimal and non cytotoxic in our previous work (Durcan et al., 2006; Morgan et al., 2005).

2.3. RNA and cDNA synthesis and RT-PCR

To assess the effects of EPO, MBP and EDN on cholinergic ChAT and VAChT gene expression, differentiated IMR32 cells were incubated with 1 μg of purified granule protein per 1 ml of medium for varying time points. Two negative controls were utilized for all experiments with IMR32 cells incubated in differentiation medium alone. Three experiments were conducted and each included two replicates.

In some experiments, the synthetic cationic polypeptide PLA (1 μg/ml) (Sigma) was used as a cationic charge mimetic in place of EPO. In other experiments, the dependence of EPO gene regulatory effects on direct interaction with the cell membrane was studied using cell adhesion inhibitors including the polyanionic molecule PLG (1 μg/ml) (Sigma) or the glycosaminoglycan inhibitor Heparinase 1 (5 sigma units) (Sigma). The intracellular pathway resulting in genetic effects in the nucleus were studied by inhibiting ERK1 using the MEK inhibitor PD98059 (50 μM) (Sigma).

Table 1
Primer sequences used in this study.

Primer	Sequence[a]	T_m (°C)[b]
β-Actin F	5′-GGA CTT CGA GCA AGA GAT GG-3′	59.4
β-Actin R	5′-AGG AAG GAA GGC TGG AAG AG-3′	59.4
ChAT F	5′-CTA CAG GCT CCA CCG AAG AC-3′	61.4
ChAT R	5′-GTC AGT CAC GGC TCT CAC AA-3′	59.4
VAChT F	5′-ACT ATG CGG CCT CTG TTT TG-3′	59.4
VAChT R	5′-CGC TGC CAT AGA CTG AGA CA-3′	59.4

[a] Forward and reverse sequences are shown for each primer used in RT-PCR.
[b] Melting temperature shown for each primer.

Total RNA was isolated from the cells with TRI reagent™ (Sigma), according to the manufacturer's instructions. For all PCR, 1 μg of RNA was reverse transcribed using the QuantiTect Reverse Transcription kit cDNA synthesis kit (Qiagen, Hilden, Germany). Quantitative RT-PCR analysis was carried out on the LightCycler™ 1.0 (Roche, West Sussex, UK) using fast start Taq DNA polymerase containing the double-stranded DNA binding dye SYBR Green 1 from the QuantiTect SYBR Green PCR kit (Qiagen), according to the manufacturer's instructions. Primers specific to ChAT, VAChT or β-actin as a normalizing gene were manufactured by Eurofins (Ebersberg, Germany); primer sequences are shown in Table 1. The samples were denatured at 95 °C for 15 min followed by 35-40 cycles of denaturation, annealing and extension at 95 °C for 15 s, 55 °C for 25 s, and 72 °C for 11 s (ChAT, VAChT), or 95 °C for 15 s, 55 °C for 20 s, and 72 °C for 20 s (β-actin). Characteristic melting curves were obtained at the end of amplification by cooling the samples to 65 °C for 15 s followed by further cooling to 40 °C for 30 s. Serial 10-fold dilutions were prepared from individual PCR products purified using the Wizard® PCR Preps DNA Purification System was obtained from Promega (Madison, WI, USA), which were then used as standards to plot against the unknown samples. Quantification of data was analyzed using the LightCycler™ analysis software, and values were normalized to the level of β-actin expression for each sample on the same template cDNA.

2.4. Protein preparation

Whole cell protein lysates were extracted in RIPA buffer. Nuclear and cytoplasmic extracts were isolated, as described previously (Walsh et al., 2004). Protein concentration was established by the Bradford method (Bradford, 1976).

2.5. Western blotting

Cytoplasmic or total protein extracts (10 μg) were heated to 95 °C in sample buffer (1 M Tris pH 6.8, 10% (wt/vol) SDS, 0.1% (wt/vol) bromophenol blue, 20% (vol/vol) glycerol, 10% (vol/vol) β-mercaptoethanol) and separated by SDS-PAGE on 10% polyacrylamide separating gel overlaid with 4% stacking gel at 500 V for 1 h. The separated proteins were transferred on to nitrocellulose membranes in transfer buffer (20 mM Tris, 150 mM glycine, 10% (wt/vol) SDS, 20% (vol/vol) methanol) at 500 V overnight. For immunodetection rabbit anti-human/rat/mouse ChAT (H-95) antibody, rabbit anti-human/rat/mouse VAChT (H-160) antibody, rabbit anti-rat ERK2 (C-14) antibody, mouse anti- human IκB-α (H-4) antibody were used. Membranes were incubated in blocking buffer (Dulbecco's PBS containing 0.2% (wt/vol) I-block and 0.1% (vol/vol) Tween-20) for 1 h at room temperature then incubated for overnight in blocking buffer containing the individual respective primary antibody (1:1000 for ChAT, VAChT, and ERK2