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Oxidized lipids and lysophosphatidylcholine induce the chemotaxis and intracellular calcium influx in natural killer cells

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ABSTRACT

We previously reported that human NK cells express G2A and they respond to LPC. Here, we report that oxidized lipids such as 9-R-HODE, 9-S-HODE and 13-R-HODE, as well as LPC induced the *in vitro* chemotaxis of human NK cells, although with variable efficacies. The chemotactic effects of these lipids were inhibited by prior treatment of NK cells with pertussis toxin (PTX). 9-S-HODE, 9-R-HODE and LPC optimally induced the influx of intracellular Ca^{2+} in NK cells. Addition of 9-S-HODE prior to the addition of LPC inhibited more than 50% of the effect of LPC, whereas addition of LPC prior to the addition of 9-S-HODE completely inhibited the effect of the latter lipid. Also, there was a complete reciprocal desensitization among 9-R-HODE and LPC on the influx of intracellular Ca^{2+}. Further analysis showed that the four lipids did not affect NK cell lysis of tumor target cells. 9-R-HODE but not any other lipid increased the percentages of NK cells producing IFN-γ and is the only lipid that enhanced the release of this cytokine by these cells. In conclusion, we provide novel evidence showing that oxidized lipids and LPC exert important functions for cells of innate immune system.

Introduction

Oxidized lipids significantly contribute to various diseases which include cancer, atherosclerosis, among many others. These lipids undergo modification, for example, insertion of oxygen into the fatty acids producing molecules such as HPETE (Soberman et al. 1985). Other oxidized lipids particularly those derived from the hydrolysis of linoleic acid including hydroxyoctadecadienoic acid (HODE) have been described (Fang et al. 1999). Several isomers of HODE, 9-HODE and 13-HODE are formed in either enzymatic process by 15-lipooxygenase or cyclooxygenase, or by no enzymatic process upon lipid peroxidation (Soberman et al. 1985; Fang et al. 1999). A mixture of the R- and S-enantiomers of 13-HODE and the isomer 9-HODE are formed upon oxidation of linoleic acid by cyclooxygenase. It was previously reported that 13-HODE is taken up by endothelial cells which is incorporated into phosphatidylcholine "PC" (Fang et al. 1999). It was also reported that the vasoactive effect of HODE may be related to the ability of 13-HODE and to a lesser extent 9-HODE to induce the mobilization of intracellular calcium from cultured porcine aortic and pulmonary artery smooth muscle cells (Stoll et al. 1994). Another lipid containing free hydroxyl group, i.e. lysophosphatidylcholine (LPC) also robustly increased the intracellular calcium in these cells (Stoll et al. 1994). Further, most of the PC is converted into LPC by phospholipase A (PLA) activity during oxidation.

LPC is a pleotropic lipid with multiple activities. It increases mRNA for MCP-1 in rat vascular smooth muscle cells (Rong et al. 2002), induces the release of inflammatory cytokines such as IFN-γ and TNF-α in peripheral mononuclear cells (Huang et al. 1999), induces the release of intracellular Ca^{2+} in Jurkat T cell line (Légrádi et al. 2004), and upregulates the expression of CXCR4 in human $CD4^+$ cells (Han et al. 2004), among many other functions. Due to its various activities, it was used to treat mice with experimental sepsis (Yan et al. 2004). The receptor for LPC has been a matter of controversy. It was originally reported that G2 accumulation "G2A" is the receptor for this lipid (Kabarowski et al. 2001), but this report was retracted due to the absence of binding data (Witte et al. 2005). G2A is a GPCR that may reorganize the cytoskeleton of lymphocytes through the activation of $G_{\alpha 13}$ and RhoA (Kabarowski et al. 2000), and was originally suggested to be a tumor suppressor due to its ability to arrest cell cycle progression through the G2 checkpoint (Weng et al. 1998). Although the association among G2A and LPC activity has been doubted, several findings support that such an association might exist. Yang et al. (2005) reported that macrophages are chemoattracted towards LPC which was dependent on the expression of G2A in these cells. Further, G2A-expressing neutrophils respond by fluxing calcium when LPC was used as a stimulus (Frasch et al. 2007). G2A is also involved in atherosclerosis and it was reported that macrophages from $G2A^{-/-}$ deficient animals had increased proinflammatory

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state (Bolick et al. 2007). Parks et al. (2009) reported that G2A modulates hepatic high density lipoprotein (HDL) metabolism which might contribute to apolipoprotein E-dependent pathogenesis, rather than this receptor promotes atherosclerosis in low density lipoprotein receptor deficient mice ($LDLR^{-/-}$) mice via attraction of monocytes

Despite these convincing evidences, it was reported that G2A may function as a proton sensing GPCR (Murakami et al. 2004). However, this was doubted by others (Im 2005), because in contrast to other GPCRs in this family (for example OGR1, GRP4 and TDGA), G2A is less sensitive to the fluctuations in pH due to the absence of the histidine residues important for pH sensing from G2A, whereas it is conserved in OGR1, GRP4 and TDGA8 (Radu et al. 2005). A third proposal suggests that G2A functions as a sensor for oxidative stress that induces the production of ligands for G2A including oxidized fatty acids. For example, the products of linoleic acid, 9-HODE activates G2A resulting in the mobilization of intracellular calcium, incorporation of [^{35}S]GTPγS, activation of MAPK and inhibition of cAMP in G2A transfected cell lines (Obinata et al. 2005). This was confirmed in keratinocytes where 9-HODE induced calcium mobilization, cytokine production and inhibition of proliferation, activities that were enhanced by overexpression of G2A and reduced by its ablation (Hattori et al. 2008).

We previously reported that the anti-tumor/anti-viral effector natural killer (NK) cells express G2A and they respond chemotactically to LPC (Jin et al. 2005). NK cells are effector cells of the innate immune system that perform several important functions; among them is the regulation of the adaptive immune response by secreting cytokines such as IFN-γ, shaping the innate immune system by interacting with dendritic cells, defending against viral infection, and lysing and destroying tumor cells. The fact that activated NK cells highly express G2A makes them an excellent model to examine the activities of various ligands reported to bind this receptor in a physiological system. For these reasons, we initiated this study to investigate the response to oxidized lipids in cells of innate immunity.

Materials and methods

Reagent

Pertussis toxin (PTX) and ionomycin were purchased from Sigma–Aldrich (Oslo, Norway). 9-S-HODE, 9-R-HODE, 13R-HODE and (±) 13-HODE were obtained from Cayman Chemicals (Ann Arbor, MI, USA). Sphingosine 1-phosphate (S1P) and lysophosphatidic acid (L-oleolyl LPA) were purchased from Avanti Polar Lipids, Inc. (Alabaster, Alabama, USA). PE-conjugated mouse anti-IFN-γ and mouse PE-conjugated IgG were all from R&D Systems (R&D Systems Europe Ltd., Abingdon, UK).

Cell preparation

Buffy coats of healthy volunteers were obtained from the blood bank (Ullevål Hospital, Oslo, Norway). After histopaque (Sigma–Aldrich) separation, the cells were purified using RosetteSep negative selection human NK cell enrichment cocktail (StemCell Technologies SARL, Grenoble, France), which removes CD3, CD4, CD19, CD36, CD66b, and glycophorin A positive cells, leaving NK cells intact. These preparations were almost devoid of $CD3^+$ (T), or $CD19^+$ (B) cells but they express CD56, as well as the NK cell cytotoxicity receptors NKp44, NKp46 and NKG2D (Fig. 1). The cells were incubated at 1×10^6 cells/mL with 200 U/mL IL-2 at 37 °C in a 5% CO_2 incubator for 5–7 days.

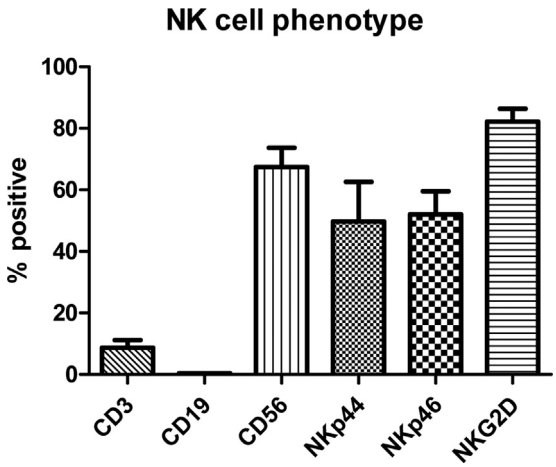

Fig. 1. Phenotypic expression of IL-2-activated NK cells utilized in this study. The expression of CD3 (T cells), CD19 (B cells), as well as CD56, NKp44, NKp46 and NKG2D (NK cells) was determined on the surface of IL-2-activated NK cells. Mean ± SEM of results generated from three different donors.

NK cell cytotoxicity assay

The human myeloid leukemia K562 cells were used as targets. Target cells were incubated at 1×10^6 cells/mL with 5 μg/mL calcein-AM (Sigma–Aldrich) for 1 h at 37 °C, washed and plated at 10,000 cells/well onto 96-well plates. To obtain total killing, target cells were incubated with 0.5% Triton-X, whereas total viability was obtained by incubating the cells with media only. The plates were spun down at 500 rpm for 5 min and incubated for 4 h at 37 °C. After incubation, the cells were centrifuged, supernatants removed and PBS added to each well. The Fluorescence intensity of the calcein AM-loaded cells was measured in a BioTek FLX TBI plate reader (FLx 800, Bio-Tek Instruments, Inc., Winooski, VM, USA), using 485/528 nm fluorescence filters. The percentage of cytotoxicity was calculated according to the following formula: % viability = fluorescence units (FU) of targets incubated with NK cells (experimental), minus FU of targets incubated with Triton-X (total lysis), divided by FU of targets incubated in media only (total viability), minus FU of targets incubated with Triton-X (total lysis). Percent cytotoxicity was then calculated as 100% minus % viability as previously described (Damaj et al. 2007). In the figures, the 10:1 effector: target (E:T) cell ratio is shown, however, similar results were obtained using other E:T cell ratios (2.5:1 and 5:1).

Calcium mobilization

NK cells (1×10^7/mL) were suspended in a buffer containing 0.25% BSA, 145 mM NaCl, 5 mM KCl, 10 mM Na/MOPS, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, 10 mM Hepes (all from Sigma–Aldrich), pH 7.4, and incubated with 2 μM Fura-2-AM (Molecular Probes, Eugene, OR), for 40 min at room temperature. The cells were washed once, resuspended in buffer containing 0.25% BSA, and kept at room temperature. Just before use, aliquots of the cells (4×10^5) were washed and resuspended in 2 mL buffer containing 0.05% BSA in a stirred cuvette at 37 °C. Measurement of $[Ca^{2+}]_i$ was performed as previously described (Maghazachi et al. 1997).

In vitro chemotaxis assay

Nucleopore blind well chemotaxis chambers with a lower well volume of 200 μL were used. A maximum volume of 200 μL