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Inhibition of superoxide anion production by extracellular acidification in neutrophils

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ABSTRACT

Extracellular acidification inhibited formyl-Met-Leu-Phe- or C5a-induced superoxide anion (O_2^-) production in differentiated HL-60 neutrophil-like cells and human neutrophils. A cAMP-increasing agonist, prostaglandin E_1, also inhibited the formyl peptide-induced O_2^- production. The inhibitory action on the O_2^- production by extracellular acidic pH was associated with cAMP accumulation and partly attenuated by H89, a protein kinase A inhibitor. A significant amount of mRNAs for T-cell death-associated gene 8 (TDAG8) and other proton-sensing ovarian cancer G-protein-coupled receptor 1 (OGR1)-family receptors is expressed in these cells. These results suggest that cAMP/protein kinase A, possibly through proton-sensing G-protein-coupled receptors, may be involved in extracellular acidic pH-induced inhibition of O_2^- production.

1. Introduction

Continuous ambulatory peritoneal dialysis (CAPD) has been used as an effective therapy for renal failure. Conventional peritoneal dialysis fluid contains high concentrations of glucose and lactate in acidic pH solutions, leading to the production of high amounts of harmful glucose degradation products. This unphysiological composition adversely affects peritoneal host defense and may partly contribute to the development of peritoneal dialysis-related peritonitis [1]. Indeed, the conventional lactate and glucose-based dialysis solutions with low pH of 5.2–5.3 have been shown to have detrimental effects on the capacity of peritoneal macrophages and neutrophils to kill bacteria, including phagocytosis and superoxide (O_2^-) anion production [2–4]. The composition and pH of peritoneal dialysis fluid are now improved [5,6]; however, the molecular mechanisms of the detrimental effects of the conventional peritoneal dialysis fluid have not been fully characterized. Extracellular acidification occurs not only under such an artificial circumstance of treatment for therapies but also under pathological processes, including inflammation, where extracellular acidification is achieved by the facilitation of lactate production and proton efflux to maintain physiological intracellular pH in the inflammatory cells [7].

In the present study, we found that extracellular acidification and a cAMP-increasing agent, prostaglandin (PG) E_1, inhibited formyl-Met-Leu-Phe (fMLP)-induced O_2^- production in differentiated HL-60 neutrophil-like cells and human neutrophils. The inhibitory action on O_2^- production was associated with cAMP accumulation and partly reversed by the protein kinase A (PKA) inhibitor. Moreover, T-cell death-associated gene 8 (TDAG8) and other ovarian cancer G-protein-coupled receptor 1 (OGR1) family receptors, which have previously been reported as receptors for lysolipids but now recognized as proton-sensing G-protein-coupled receptors (GPCRs) [8–10], were expressed in these cells. These results imply that proton-sensing GPCRs may play a role in the extracellular acidic pH-induced attenuation of neutrophil functions.

2. Materials and methods

2.1. Materials

C5a, fMLP, PGE_1, A23187, phorbol 12-myristate 13-acetate (PMA), 4-(3-butoxy-4-methoxybenzyl)-imidazolidin-2-one (Ro20-1724), and *N*-(2-[*p*-bromocinnamylamino]ethyl)-5-isoquinolinesulfonamide (H89) were purchased from Sigma–Aldrich (St. Louis, MO). The human G2A (Hs00203431)-, OGR1 (Hs00268858)-, TDAG8 (Hs00269247)-, GPR4 (Hs00270999)-, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Hs99999905)-specific probes for real-time PCR were obtained from TaqMan gene expression assays (Applied Biosystems, Foster City, CA). The sources of all other reagents were described previously [11–14].

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2.2. Cell culture

HL-60 cells were routinely cultured in a RPMI 1640 medium (Sigma) supplemented with 10% fetal calf serum (Life Technologies, Inc.) and maintained in a humidified atmosphere of 95% air and 5% CO_2. The cells are differentiated into neutrophil-like cells by culturing for 4 days in a medium containing 0.5 mM dibutyryl cyclic AMP (dbcAMP) as described previously [12]. The differentiated HL-60 cells are harvested and washed twice with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes)-buffered medium which consisted of 20 mM Hepes (pH 7.6), 134 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 2 mM $CaCl_2$, 2.5 mM Na_2HCO_3, 5 mM glucose, and 0.1% (w/v) bovine serum albumin (BSA) (fraction V) before experiments.

2.3. Preparation of human neutrophils

Venous blood samples were obtained from healthy volunteers, using syringes containing heparin (final concentration 2 units/ml). All protocols were in strict compliance with Gunma University Ethics Committee guidelines. Neutrophils were isolated using a standard method of dextran sedimentation, followed by centrifugation in a Ficoll–Paque PLUS (GE Healthcare) gradient and the hypotonic lysis of erythrocytes. Purified neutrophils that contained >98% viable cells, as determined by the trypan blue exclusion method, were resuspended in Ca^{2+}-free Hepes-buffered medium as described above and maintained at 4 °C before use.

2.4. O_2^- production

O_2^- production was measured by colorimetric method based on the cytochrome *c* reduction by O_2^- as previously described [11]. In brief, the cells (1×10^6 cells in 0.35 ml) were first incubated for 10 min at 37 °C in the presence of cytochrome *c* (1 mg/ml), cytochalasin B (5 μg/ml) and adenosine deaminase (1 unit/ml) with or without superoxide dismutase (120 units/ml) in the regular Hepes-buffered medium. Adenosine deaminase was included to rule out the possible involvement of released adenosine during incubation in O_2^- production [15,16]. The cell suspension was then further incubated with test agents under the appropriate extracellular pH, which was adjusted with HCl, for 15 min at a final volume of 0.4 ml. The reaction was quenched by the addition of *N*-ethylmaleimide (1.5 mM), and change in absorbance of cytochrome *c* at 550 nm was measured.

2.5. cAMP accumulation

In the experiments for measurement of both cAMP and O_2^- production (Fig. 4), 0.1 mM Ro20-1724, was also included in the assay medium. Other experimental conditions are the same as those for measurement of O_2^- production. After the 15-min incubation, the reaction was terminated by adding 40 μl of 1 N HCl. Cyclic AMP in the acid extract was measured by radioimmunoassay as described previously [17].

2.6. Quantitative real-time PCR using real-time TaqMan technology

Total RNA was isolated using TRI REAGENT (Sigma–Aldrich, St. Louis, MO) according to the instructions from the manufacturer. After DNase I (Promega, Madison, UI) treatment to remove possible traces of genomic DNA contaminating in the RNA preparations, 5 μg of the total RNA was reverse-transcribed using random priming and Multiscribe reverse transcriptase according to the instructions from the manufacturer (Applied Biosystems, Foster City, CA). To evaluate the expression level of the OGR1, TDAG8, G2A, and GPR4 mRNAs, quantitative real-time PCR was performed using real-time TaqMan technology with a Sequence Detection System model 7700 (Applied Biosystems, Foster City, CA). Other experimental conditions were described previously [12,13]. The expression level of the target mRNA was normalized to the relative ratio to the expression of GAPDH mRNA.

2.7. Data presentation

All experiments were performed in duplicate or triplicate. The results of multiple observations are presented as means ± SEM of six determinations of separate experiments or means ± SD of three determinations of a representative result of more than two separate experiments. Statistical significance was assessed by the Student's *t*-test; values were considered significant at $^*p < 0.05$.

3. Results

3.1. Extracellular acidification and PGE_1 inhibit fMLP-induced O_2^- production in human neutrophils

Consistently with previous results [18,19], the chemoattractant fMLP-induced O_2^- production was remarkably inhibited by extra-

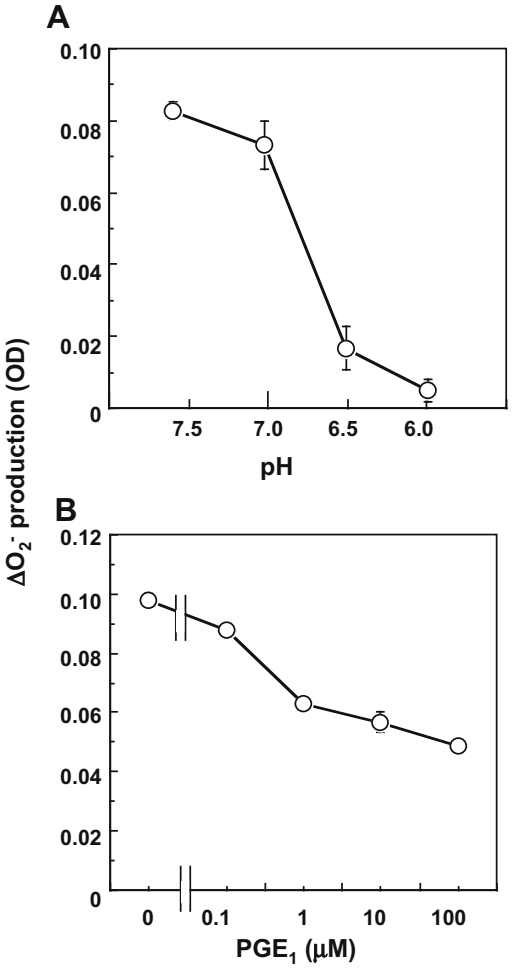

Fig. 1. Extracellular acidification and PGE_1 inhibit O_2^- production in human neutrophils. Human neutrophils prepared by a Ficoll–Paque PLUS gradient method were incubated with 1 μM fMLP under the indicated pH (A) or in the presence of the indicated concentrations of PGE_1 (B) for 15 min to measure O_2^- production. Results are expressed as increased absorbance (OD) over the control absorbance without any stimulant. Data are the means ± SD of three determinations of a representative result. Other two experiments gave similar results.