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Differential production of leukotriene B4 or prostaglandin E_2 by WKYMVm or serum amyloid A via formyl peptide receptor-like 1

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Abbreviations:
AA, arachidonic acid
LTB4, leukotriene B4
PGE_2, prostaglandin E_2
GPCR, G-protein coupled receptor
PLA_2, phospholipase A_2
$cPLA_2$, cytosolic PLA_2
$iPLA_2$, calcium independent PLA_2
$sPLA_2$, secretory PLA_2
5-LO, 5-lipoxygenase
COX, cyclooxygenase
FPRL1,, formyl peptide receptor-like 1
SAA, serum amyloid A
WKYMVm,
Trp-Lys-Tyr-Met-Val-D-Met
BAPTA/AM,

ABSTRACT

Serum amyloid A (SAA) and Trp-Lys-Tyr-Met-Val-D-Met (WKYMVm) have been reported as formyl peptide receptor-like 1 (FPRL1) ligands. WKYMVm but not SAA stimulated superoxide generation by human neutrophils. In terms of the downstream signalings triggered by WKYMVm and SAA, both agonists stimulated cytosolic phospholipase A_2-mediated arachidonic acid release, a precursor of leukotriene B4 (LTB4) and prostaglandin E_2 (PGE_2). WKYMVm also strongly stimulated LTB4 production in human neutrophils without affecting PGE_2 production, whereas SAA strongly stimulates cyclooxygenase-2 (COX-2) expression and PGE_2 production but not LTB4 production. In terms of the receptors responsible for the differential actions of these two agonists, we found that FPRL1 is involved in the production of LTB4 by WKYMVm and PGE_2 production by SAA. This study demonstrates that the chemoattractant receptor, FPRL1, can be differentially regulated by distinct ligands to generate different lipid mediators, and thus, different immune responses.

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1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetoxymethyl ester
MAFP, methyl arachidonylfluorophosphonate
BEL, bromoenol lactone
AA-861, 2-(12-hydroxydodeca-5,10-diynyl)-3,5,6-trimethyl-1,4-benzoquinone
NDGA, nodihydroguaiaretic acid
$[Ca^{2+}]_i$, intracellular calcium concentration
PTX, pertussis toxin

1. Introduction

Arachidonic acid (AA) is a precursor of several essential lipid mediators, such as leukotriene B4 (LTB4) and prostaglandin E_2 (PGE_2) [1,2]. Previous reports have demonstrated that many extracellular ligands that bind to cell surface bound G-protein coupled receptor (GPCR) elicit AA release in several cell types [3–5], and that this AA release is induced via the activation of phospholipase A_2 (PLA_2). Three major groups of PLA_2 isoforms have been identified to date; cytosolic PLA_2 ($cPLA_2$), calcium independent PLA_2 ($iPLA_2$), and secretory PLA_2 ($sPLA_2$) [6]. Upon neutrophil activation, the 5-lipoxygenase (5-LO) is translocated to the perinuclear membrane where it converts AA into LTB4 [7]. LTB4 has been reported to be an important host defense component that stimulates leukocyte chemotaxis, degranulation, phagocytosis, and superoxide generation [8]. AA can also be converted into PGE_2 via the activation of cyclooxygenase (COX), which exists as two isoforms, COX-1 and COX-2. COX-1 is responsible for the basal and constitutive synthesis of PGE_2, whereas COX-2 is an inducible enzyme and plays an important role during various inflammatory conditions [8]. PGE_2 also modulate various cellular responses, e.g., it causes vasodilation and promotes edema induced by bradykinin or histamine [8]. Moreover, PGE_2 may act as a proinflammatory or as an anti-inflammatory mediator, depending on the biomolecular environment [8].

Formyl peptide receptor-like 1 (FPRL1) has been reported to play an important role in the regulation of immune responses against pathogen infections. FPRL1 is a chemoattractant receptor, and various FPRL1 agonists, both natural and synthetic, have been identified [9–12]. Among these, several agonists such as serum amyloid A (SAA) and Trp-Lys-Tyr-Met-Val-D-Met (WKYMVm) have been reported to act as chemoattractants [9,13]. In terms of the signaling downstream of FPRL1, it was previously demonstrated that FPRL1 activation by several ligands induces phospholipase C-mediated intracellular calcium increase, and the activation of PLA_2 and phospholipase D [14]. Activated FPRL1 also elicits the activation of mitogen-activated protein kinase in phagocytic cells, like neutrophils [14], and more recently, we demonstrated that different FPRL1 ligands can induce differential signaling and cellular responses [14,15]. Moreover, Trp-Lys-Tyr-Met-Val-Met and lipoxin A4 differentially stimulate human neutrophils, i.e., superoxide anion is generated by Trp-Lys-Tyr-Met-Val-Met but not by lipoxin A4 [14]. In addition, we also demonstrated that synthetic peptides, His-Arg-Tyr-Leu-Pro-Met and His-Glu-Tyr-Leu-Pro-Met, differentially stimulate FPRL1 [15]. However, the effects of FPRL1 on the generation of lipid mediators like LTB4 and PGE_2, and on the expression of COX-2 have not been investigated.

In this study we undertook to determine whether FPRL1 can affect the generation of lipid mediators (LTB4 and PGE_2) and to identify their modes of regulation. We also compared the effects of different FPRL1 ligands to determine whether they differentially affect lipid mediator generation.

2. Materials and methods

2.1. Reagents

WKYMVm was obtained from A&Pep Inc. (Yeongi, Korea). Recombinant human SAA was purchased from Peprotech (Rocky Hill, NJ). Cytochrome *c* was purchased from Sigma (St. Louis, MO). 1,2-Bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetoxymethyl ester (BAPTA/AM) was purchased from Molecular Probes (Eugene, OR). RPMI 1640 medium, DMEM medium, and fetal bovine serum (FBS) were obtained from Invitrogen Corp. (Carlsbad, CA). Methyl arachidonylfluorophosphonate (MAFP), bromoenol lactone (BEL), 2-(12-hydroxydodeca-5,10-diynyl)-3,5,6-trimethyl-1,4-benzoquinone (AA-861), MK-886, and nodihydroguaiaretic acid (NDGA) were from Biomol (Plymouth Meeting, PA). *p*-Bromophenacyl bromide (BPB) was purchased from Fluka (St. Louis, MO). [5,6,8,9,11,12,14,15-^{3}H(N)]-AA was from PerkinElmer (Boston, MA). Goat polyclonal COX-2 antibodies were purchased from Cayman Chemical (Ann Arbor, MI), and horseradish peroxidase-conjugated antibodies to rabbit IgG were purchased from Kirkegaard & Perry Inc. (Gaithersburg, MD).

2.2. Isolation of human neutrophils

Peripheral blood was collected from healthy donors in sodium citrate solution (3.8%). Donors had not taken anti-inflammatory drugs for at least 3 weeks before sampling and were free of systemic illnesses, such as asthma or allergic rhinitis. Human

neutrophils were isolated by dextran sedimentation, hypotonic lysis of erythrocytes, and by using a lymphocyte separation medium gradient as described previously [15]. Briefly, the cellular portion was mixed with a solution of 3% dextran in 0.9% NaCl solution and kept at 25 °C for 45 min. The neutrophil-rich upper layer of the suspension was then collected and centrifuged (250 × *g*, 10 min, 4 °C). Residual erythrocytes were removed by hypotonic lysis and the pellet obtained was suspended in ice-cold PBS. The suspension was centrifuged (250 × *g*, 45 min) on Histopaque solution at 4 °C. Isolated neutrophils were maintained in RPMI 1640 medium supplemented 10% FBS at 37 °C. Cell viability was determined by trypan blue dye exclusion assay and >98% of neutrophils were viable. Isolated human neutrophils were used promptly.

2.3. Cell culture

RBL-2H3 cells or FPRL1-expressing RBL-2H3 cells were a kind gift from Dr. Richard D. Ye (University of Illinois). The cells were maintained at 37 °C in a humidified atmosphere containing 5% CO_2 in high glucose DMEM supplemented with 20% (v/v) heat-inactivated FBS and G418 (500 μg/ml), as described previously [15]. The cells were sub-cultured every 3 days.

2.4. Measurement of superoxide anion generation

Superoxide anion generation was determined by measuring cytochrome *c* reduction using a microtiter 96-well plate ELISA reader (Bio-Tek instruments, EL312e, Winooski, VT) as previously described [15]. Human neutrophils (2×10^6 cells in RPMI 1640 medium) were preincubated with 5 μM of cytochalasin B at 37 °C for 5 min and then incubated with each peptide in the presence of 50 μM of cytochrome *c*. Superoxide generation was determined by measuring light absorption changes at 550 nm over 10 min at 1 min intervals.

2.5. Measurement of PLA_2 activity in cells

Isolated human neutrophils (10^7 cells/ml) were prelabeled with 0.5 μCi/ml of [^{3}H]-AA in RPMI 1640 medium containing 10% FBS for 2 h at 37 °C in a humidified atmosphere containing 5% CO_2, as described before [16]. The labeled cells were then washed twice with serum-free RPMI 1640 medium and incubated in RPMI 1640 medium containing 0.1% fatty acid-free BSA for 15 min at 37 °C. After discarding the medium, the cells were stimulated with various concentrations of SAA or WKYMVm for several lengths of time. Radioactivity in the medium was measured using a liquid scintillation counter.

2.6. LTB4 measurement

LTB4 measurement was performed as previously described [17]. Neutrophils (3×10^6 cells/0.3 ml of RPMI 1640 medium) were stimulated with vehicle alone, indicated concentrations of WKYMVm or SAA for several lengths of time at 37 °C. After stimulation, cell-free supernatants were collected, centrifuged, and measured for LTB4 by enzyme-linked immunosorbent assay (Pierce Biotechnology Inc., Rockford, IL) according to the instruction of the vender.

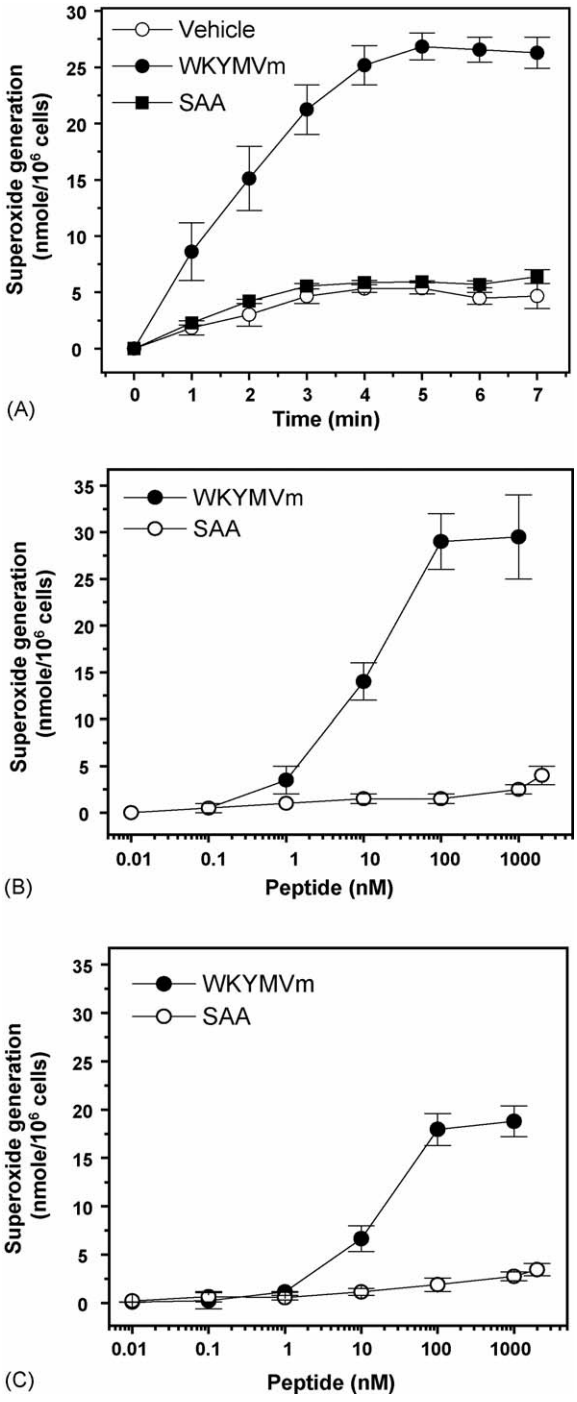

Fig. 1 – Effects of WKYMVm and SAA on superoxide generation in human neutrophils. Isolated human neutrophils (2×10^6 cells/100 μl/assay) were preincubated for 5 min at 37 °C with 5 μM of cytochalasin B before being stimulated with WKYMVm (100 nM) or SAA (2 μM) for several lengths of time (A). Human neutrophils were stimulated with different concentrations of WKYMVm or SAA for 5 min in the presence (B) or absence (C) of 5 μM of cytochalasin B. Cytochrome c reductions were monitored as changes in absorption at 550 nm at 1 min intervals, as described in Section 2. Superoxide anion generation is expressed as mean ± S.E. of three independent experiments performed in duplicate (A–C).

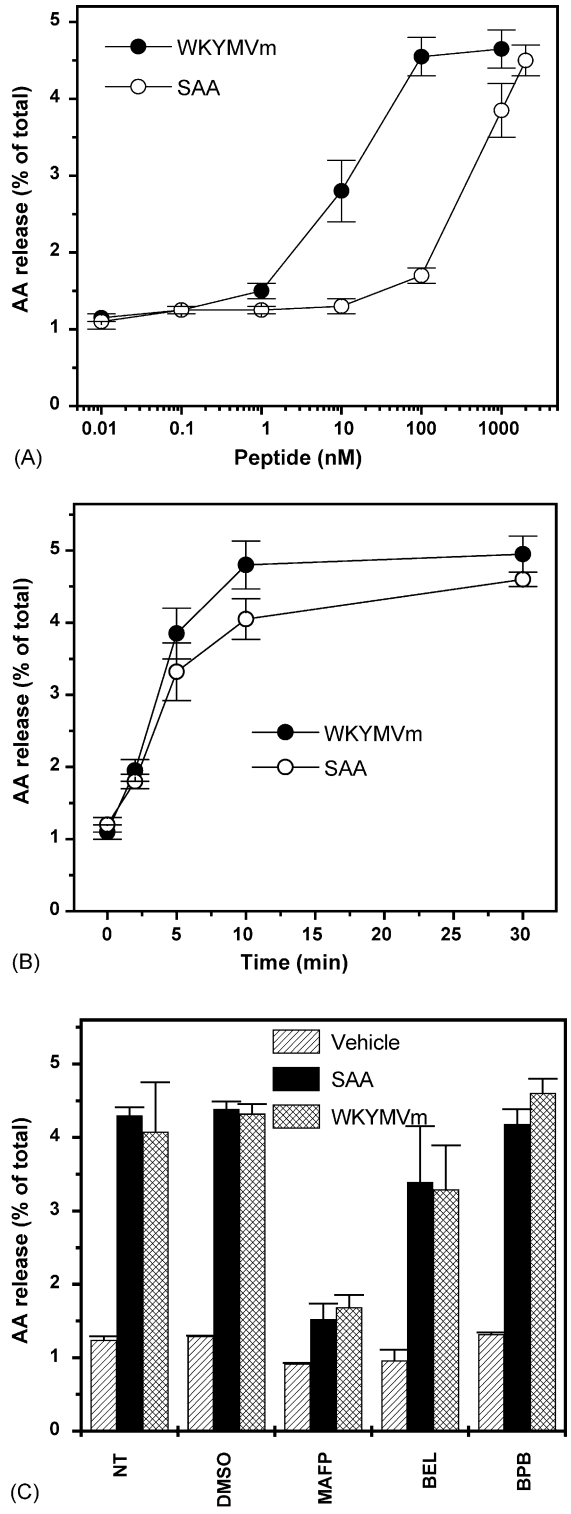

Fig. 2 – Effects of WKYMVm and SAA on AA release in neutrophils. Human neutrophils labeled with [^{3}H] AA were stimulated with different concentrations of WKYMVm or SAA for 30 min (A) and for different time with 100 nM WKYMVm or 2 μM SAA (B) in the presence of 0.1% fatty acid-free BSA at 37 °C. Neutrophils were suspended in RPMI 1640 medium containing 0.1% fatty acid-free BSA, and incubated with DMSO, MAFP (20 μM, 15 min), BEL (20 μM, 15 min), or BPB (20 μM, 15 min) at 37 °C, and then stimulated with 100 nM WKYMVm or 2 μM of SAA for 30 min (C). [^{3}H] AA release into medium was determined using a liquid scintillation counter. Results are expressed as percentages of total cellular radioactivity, and are presented as mean ± S.E. of three independent experiments performed in duplicate.

2.7. PGE_2 measurement

Neutrophils (3×10^6 cells/0.3 ml of RPMI 1640 medium) were stimulated with vehicle alone, indicated concentrations of WKYMVm or SAA for several lengths of time at 37 °C. After stimulation, cell-free supernatants were collected, centrifuged, and measured for PGE_2 levels using an enzyme immunoassay kit (GE healthcare, Uppsala, Sweden), according to the manufacturer's instructions [18].

2.8. Stimulation of cells with peptides

Cultured RBL-2H3 cells, FPRL1-expressing RBL-2H3 cells, or freshly isolated human neutrophils (2×10^6) were stimulated with 2 μM of SAA or 100 nM of WKYMVm for several lengths of time. After stimulation, the cells were washed with serum free RPMI 1640 medium and lysed in lysis buffer (20 mM HEPES, pH 7.2, 10% glycerol, 150 mM NaCl, 1% Triton X-100, 50 mM NaF, 1 mM Na_3VO_4, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride). Detergent insoluble materials were pelleted by centrifugation (12,000 × *g*, 15 min, at 4 °C), and the soluble supernatant fraction was removed and stored at either −80 °C or used immediately. Protein concentrations in the lysates were determined by Bradford protein assay.

2.9. Electrophoresis and immunoblot analysis

Protein samples were analyzed by electrophoresis on a 10% SDS-polyacrylamide gel using the buffer system described previously [15]. Following the electrophoresis, the proteins were transferred onto nitrocellulose membrane, which was blocked by incubating with TBST (Tris-buffered saline, 0.05% Tween-20) containing 5% non-fat dried milk. The membranes were then incubated with anti-COX-2 antibody or anti-actin antibody and washed with TBST. Antigen–antibody complexes were visualized after incubating the membrane with 1:5000 diluted donkey anti-goat IgG antibody coupled to horseradish peroxidase by enhanced chemiluminescence.

2.10. Statistics

The results are expressed as mean ± S.E. of the number of determinations indicated. Statistical significance of differences was determined by ANOVA. Significance was accepted when $p < 0.05$.

3. Results

3.1. Effects of WKYMVm and SAA on superoxide generation in human neutrophils

We examined the effects of WKYMVm and SAA on superoxide generation by measuring cytochrome *c* reduction, as described

previously [15]. Stimulation of human neutrophils with 100 nM WKYMVm in the presence of cytochalasin B was found to generate superoxide in a time-dependent manner (Fig. 1A), but 2 μM SAA did not affect superoxide generation (Fig. 1A). We also examined the effect of various concentrations of WKYMVm or SAA on this superoxide generation. WKYMVm was found to generate superoxide generation in a concentration-dependent manner, with a maximal effect at 100 nM (Fig. 1B), but SAA did not affect superoxide generation in the concentration range of 10 nM–2 μM (Fig. 1B). We also examined the effect of WKYMVm and SAA on superoxide anion generation in the absence of cytochalasin B. As shown in Fig. 1C, WKYMVm stimulated superoxide anion generation in the absence of cytochalasin B. The superoxide anion generation by WKYMVm was found to be weakly induced in the absence of cytochalasin B. However, SAA did not stimulate superoxide anion generation in the absence of cytochalasin B (Fig. 1C). The higher concentrations of SAA (5, 10, and 25 μM) did not stimulate superoxide anion generation in the absence or presence of cytochalasin B (data not shown). To check whether we failed to observe superoxide anion generation by SAA because SAA can scavenge superoxide anion, we examined its effect on N-formyl-Met-Leu-Phe-induced superoxide production. Addition of SAA did not affect on N-formyl-Met-Leu-Phe-induced superoxide production (data not shown), ruling out the possibility of SAA as a scavenger of superoxide anion.

3.2. *WKYMVm and SAA stimulate AA release by activating cPLA$_2$*

Unsaturated fatty acids, especially AA generated by the action of PLA$_2$, are known to play an essential role in superoxide production as catalyzed by the NADPH oxidase complex [19]. In this context, we investigated PLA$_2$ activity as a downstream effector of WKYMVm or SAA signaling. When stimulated with various concentrations of WKYMVm, human neutrophils responded with a concentration-dependent increase in AA release with a maximal effect at a peptide concentration around 100 nM (Fig. 2A). At 100 nM, WKYMVm caused a rapid release of AA from human neutrophils, which peaked after 10 min (Fig. 2B). Moreover, the stimulation of neutrophils with various concentrations of SAA also induced a dramatic AA release in a concentration-dependent manner, with maximal activity at 2 μM (Fig. 2A). To identify the isoform of PLA$_2$ responsible for WKYMVm- or SAA-induced AA release, we pretreated cells with different PLA$_2$ isoform-specific inhibitors. Pretreatment with the cPLA$_2$-specific inhibitor, MAFP, blocked the WKYMVm- and SAA-induced liberations of AA in a concentration-dependent manner (Fig. 2C). Moreover, at a MAFP concentration of 10 μM WKYMVm-induced AA release was almost completely inhibited. Other PLA$_2$ inhibitors, i.e., BEL (a specific inhibitor of iPLA$_2$) and BPB (specific for sPLA$_2$), did not interfere with AA release by either peptide (Fig. 2C). Both WKYMVm- and SAA-stimulated AA releases were also inhibited by chelating intracellular Ca^{2+} with BAPTA/AM, which also supports the notion of cPLA$_2$ activation (data not shown). These results indicate that both WKYMVm and SAA evoke AA release by

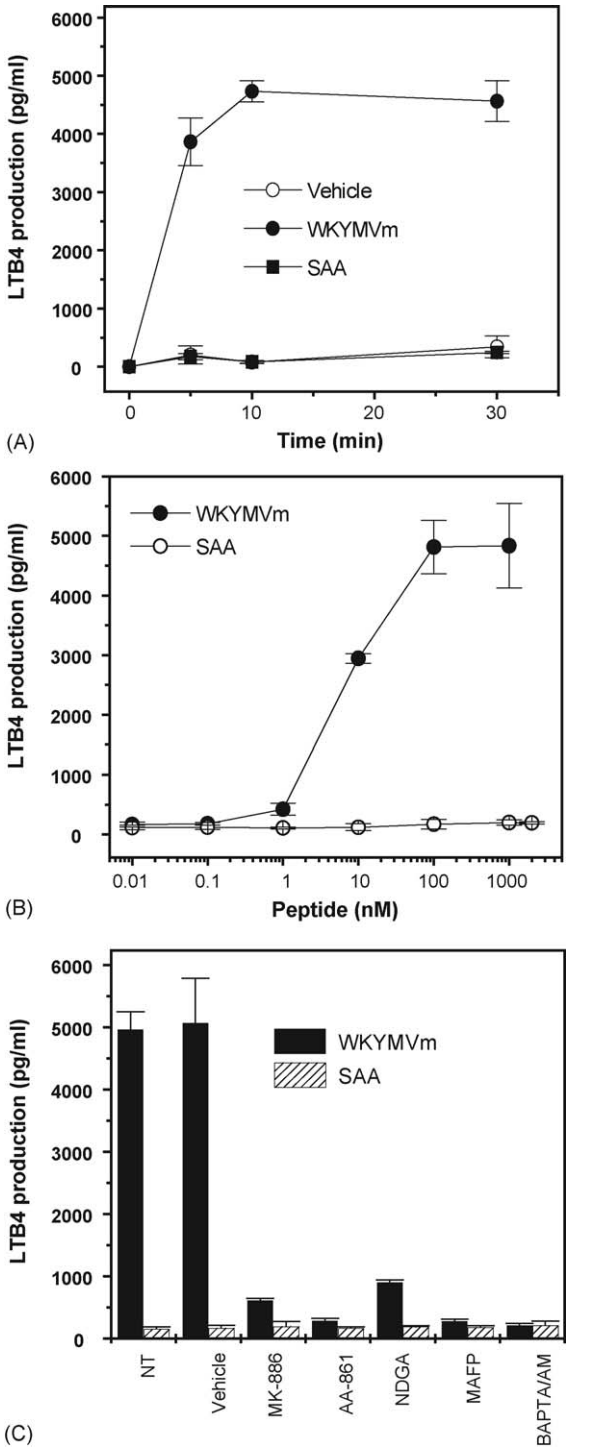

Fig. 3 – Effects of WKYMVm and SAA on LTB4 production in neutrophils. Freshly isolated human neutrophils were stimulated with 100 nM of WKYMVm or 2 μM of SAA for different lengths of time (A) and at different concentrations for 30 min (B). Freshly isolated human neutrophils were preincubated with; vehicle alone (DMSO), MK-886 (1 μM), AA-861 (10 μM), NDGA (10 μM), MAFP (10 μM) for 15 min, or BAPTA/AM for 60 min, and then stimulated with 100 nM WKYMVm or 2 μM SAA for 30 min. Levels of LTB4 were measured by ELISA. Data represent the mean ± S.E. of three independent experiments performed in duplicate (A–C).

stimulating $cPLA_2$, and not by stimulating $iPLA_2$ or $sPLA_2$ in human neutrophils.

3.3. WKYMVm but not SAA stimulates LTB4 production in human neutrophils

It is well established that LTB4 is the major metabolite of AA in human neutrophils [1,8]. We next investigated whether WKYMVm or SAA stimulate LTB4 production in these cells. Freshly isolated human neutrophils were stimulated with 100 nM WKYMVm or 2 μM SAA up to 30 min. As shown in Fig. 3A, stimulation with 100 nM WKYMVm induced LTB4 production in a time-dependent manner; LTB4 production was apparent after 5 min and increased until 30 min (Fig. 3A). We also investigated the concentration-dependency of WKYMVm-induced LTB4 production. LTB4 production became apparent at 10 nM and peaked at 100 nM (Fig. 3B). In contrast, SAA did not stimulate LTB4 production in human neutrophils (Fig. 3B). To examine the mode of regulation of WKYMVm-induced LTB4 formation, we examined the effects of three different 5-LO-selective inhibitors (MK-886, AA-861, and NDGA). Fig. 3C shows that human neutrophils preincubated with MK-886 (1 μM), AA-861 (10 μM), or NDGA (5 μM) prior to adding 100 nM WKYMVm almost completely inhibited WKYMVm-induced LTB4 production. WKYMVm-stimulated LTB4 formation was also inhibited by MAFP and by the chelation of intracellular Ca^{2+} with BAPTA/AM (Fig. 3C). These observations indicate that the $cPLA_2$ and 5-LO pathways are involved in WKYMVm-stimulated LTB4 formation in human neutrophils.

3.4. SAA but not WKYMVm stimulates PGE_2 synthesis in human neutrophils

Previous reports have demonstrated that AA is also a precursor of PGE_2, and that AA release is associated with PGE_2 synthesis in several cell types [1,2,8]. Thus, we investigated whether WKYMVm or SAA stimulates PGE_2 production in human neutrophils. Freshly isolated neutrophils were stimulated with 100 nM WKYMVm or 2 μM SAA for different lengths of time. As shown in Fig. 4A, stimulation with 2 μM SAA induced PGE_2 production in a time-dependent manner; PGE_2 production was apparent after 3 h of stimulation and increased for up to 6–12 h (Fig. 4A). The concentration-dependency of SAA-induced PGE_2 production was investigated in a similar manner; PGE_2 production became apparent at 100 nM and peaked at 2 μM (Fig. 4B). However, WKYMVm did not stimulate PGE_2 production in human neutrophils (Fig. 4A and B). It has been reported that COX-2 is essential for the synthesis of PGE_2 from AA in various cells [27]. Thus, we examined the effects of WKYMVm and SAA on COX-2 expression by Western blotting with anti-COX-2 antibody. As expected, SAA strongly stimulated COX-2 expression in a concentration-dependent manner, COX-2 expression was apparent after 3 h of stimulation and increased up to 6–12 h (Fig. 4C). This correlates well with the kinetics of PGE_2 synthesis by SAA, and thus, suggests a relationship between COX-2 expression and PGE_2 synthesis (Fig. 4C). However, neutrophil stimulation with WKYMVm had no affect on COX-2 expression (Fig. 4C).

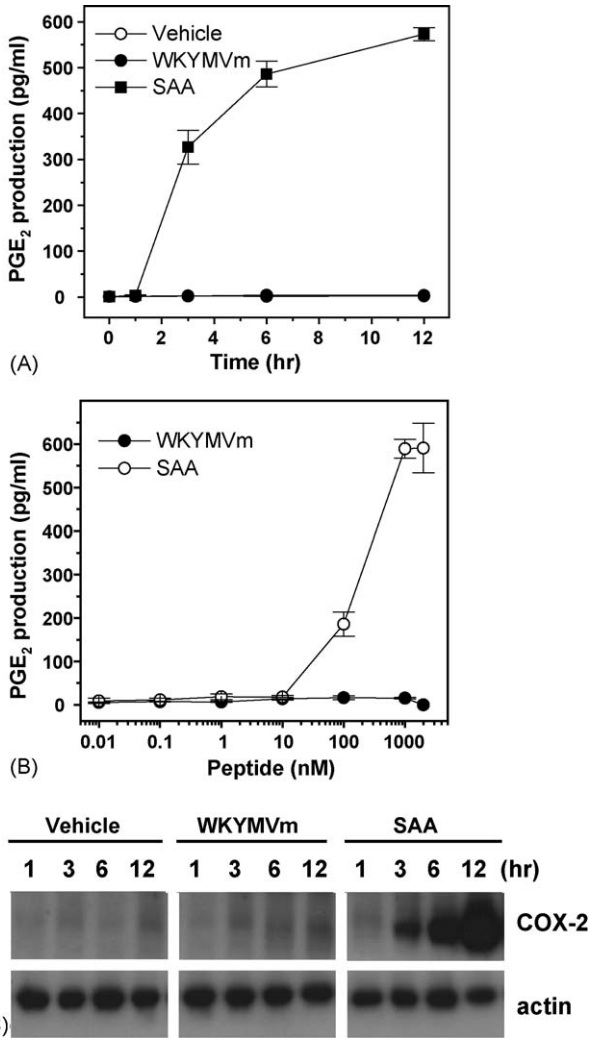

Fig. 4 – Effects of WKYMVm and SAA on PGE_2 production and COX-2 expression in neutrophils. Freshly isolated human neutrophils were stimulated with 100 nM of WKYMVm or 2 μM of SAA for different lengths of time (A) and at different concentrations for 12 h (B). Levels of PGE_2 were measured by ELISA. Data represent the mean ± S.E. of three independent experiments performed in duplicate (A and B). Freshly isolated human neutrophils were stimulated with 100 nM of WKYMVm or 2 μM of SAA for different lengths of time (C). Samples (30 μg of protein) were subjected to 10% SDS-PAGE, and the expression levels of COX-2 were determined by immunoblot analysis using anti-COX-2 antibody (C). The results shown are representative of three independent experiments (C).

3.5. WKYMVm and SAA induce different signals via FPRL1

To further support the notion that WKYMVm and SAA differentially stimulate human neutrophils via FPRL1 activation, we investigated the effects of WKYMVm and SAA on LTB4 production and PGE_2 synthesis in FPRL1-expressing RBL-2H3 cells. Stimulation of FPRL1-expressing RBL-2H3 cells with 100 nM WKYMVm caused a dramatic time-dependent

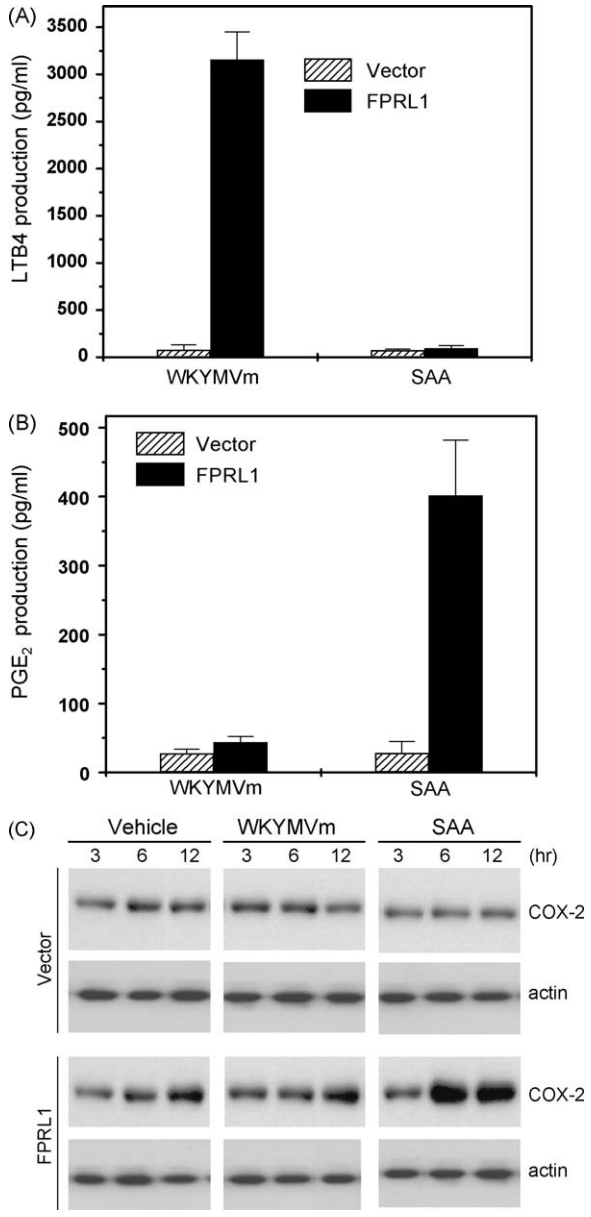

Fig. 5 – The role of FPRL1 on the differential production of LTB4 or PGE_2 by WKYMVm or SAA. Vector- or FPRL1-expressing RBL-2H3 cells were stimulated with 100 nM of WKYMVm or 2 μM of SAA for 30 min or for 12 h (for PGE_2). Levels of LTB4 and PGE_2 were measured by ELISA. Data represent the mean ± S.E. of three independent experiments performed in duplicate (A and B). Vector- or FPRL1-expressing RBL-2H3 cells were stimulated with 100 nM of WKYMVm or 2 μM of SAA for different lengths of time (C). Samples (30 μg of protein) were subjected to 10% SDS-PAGE, and COX-2 expression levels were determined by immunoblotting using anti-COX-2 antibody (C). The results shown are representative of three independent experiments (C).

increase in LTB4 (Fig. 5A). However, when we stimulated FPRL1-expressing RBL-2H3 cells with 2 μM SAA, no significant enhancement of LTB4 production was observed (Fig. 5A). To verify that the observed WKYMVm-induced LTB4 production was mediated by the activation of FPRL1, we investigated the effect of WKYMVm on LTB4 production in vector-expressing RBL-2H3 cells. As shown in Fig. 5A, WKYMVm did not stimulate LTB4 production in these cells. We also examined the effect of SAA on PGE_2 synthesis in FPRL1-expressing RBL-2H3 cells and in vector-expressing RBL-2H3 cells. As shown in Fig. 5B, SAA stimulated PGE_2 synthesis in FPRL1-expressing RBL-2H3 cells but not in vector-expressing RBL-2H3 cells, suggesting the involvement of FPRL1 in SAA-induced PGE_2 synthesis. SAA also stimulated COX-2 expression in FPRL1-expressing RBL-2H3 cells but not in vector-expressing RBL-2H3 cells (Fig. 5C), suggesting the involvement of FPRL1 in SAA-induced COX-2 expression. Taken together, these results demonstrate that while WKYMVm stimulates FPRL1 resulting in LTB4 production, the stimulation of FPRL1 by SAA causes COX-2 expression and PGE_2 synthesis.

4. Discussion

The present study, demonstrates that the chemoattractant receptor FPRL1 is modulated by distinct peptide ligands, and that these modulations lead to differential lipid mediator production in human neutrophils. As shown in Figs. 3 and 4, whilst WKYMVm stimulated LTB4 production, SAA stimulated PGE_2 synthesis and COX-2 expression in human neutrophils.

SAA is regarded as a proinflammatory mediator, and is highly elevated in the circulation and locally in tissues during various pathologic conditions, like sepsis. SAA stimulates TNF-α secretion from human T lymphocytes by forming an SAA-extracellular matrix complex [20], and has also been reported to induce IL-8 in human neutrophils by activating its membrane-bound specific receptor, FPRL1 [21]. Moreover, SAA-induced IL-8 production was found to be mediated by the activation of NF-κB in human neutrophils [21]. Taken together, previous reports suggest that SAA has a proinflammatory effect, and that FPRL1 participates in the inflammatory process. The present study shows that SAA, an endogenous ligand of FPRL1, stimulates COX-2 expression, which results in the generation of PGE_2 in human neutrophils. Since PGE_2 and its specific cell surface receptor EP2 system have been suggested to contribute to the local production of G-CSF during acute inflammation [22], the production of PGE_2 by SAA may further contribute to inflammation. The SAA used in the present study was a recombinant SAA produced in *E. coli*. Although the endotoxin content of SAA preparation was negligible (0.1 ng/μg), we further examined the possible contribution of lipopolysaccharide (LPS) to SAA-induced COX-2 expression and PGE_2 production using polymyxin B (a potent inhibitor of LPS). Preincubating human neutrophils with polymyxin B (10 μg/ml) prior to adding LPS completely inhibited COX-2 expression by LPS, but SAA-induced COX-2 expression and PGE_2 production were unaffected (data not shown). From these results, we can rule out the possibility of LPS contribution to SAA-induced COX-2 expression and PEG_2 production, thus supporting our notion that SAA directly stimulates COX-2 expression and PGE_2 production via FPRL1. Our neutrophil data, did not exclude the possibility that another receptor is involved in the differential signaling initiated by WKYMVM or SAA in human neutrophils. However,

we subsequently found that the differential signals from FPRL1, in terms of LTB4 and PGE_2 production, were induced by WKYMVm or SAA in FPRL1-expressing RBL-2H3 cells, but not in vector-transfected RBL-2H3 cells (Fig. 5). Furthermore, we also found that both of WKYMVm and SAA stimulated AA release in FPRL1-transfected RBL-2H3 cells but not in vector-transfected RBL-2H3 cells (data not shown). The results strongly suggest that AA releases induced by WKYMVm and SAA are the outcome of FPRL1 activation. Taken together, it appears reasonable to assume that FPRL1 can mediate the production of different lipid mediators (LTB4 and PGE_2) by ligating different agonists in human neutrophils.

The present study demonstrates that WKYMVm and SAA differentially stimulate the production of two distinct lipid mediators, LTB4 and PGE_2 (Figs. 3 and 4). Previous reports have shown that LTB4 and PGE_2 have various physiological activities in different cell types [23,24]. For example, LTB4 has been reported to enhance the levels of an important chemokine, MCP-1, in monocytes and macrophages, which has a crucial role in the induction of atherosclerosis [23]. On the other hand, PGE_2 has been reported to modulate inflammation during atherogenesis and other inflammatory diseases by suppressing macrophage-derived MCP-1 production [24]. Since WKYMVm and SAA stimulated LTB4 or PGE_2 production, respectively, we suggest that they may modulate differential immune responses via the generation of different lipid mediators. In terms of the regulation of MCP-1 expression, FPRL1 can play a dual role by generating LTB4 or PGE_2. Moreover, since WKYMVm is a synthetic FPRL1 ligand, it will be interesting to identify a natural ligand that selectively induces LTB4 production without affecting PGE_2 synthesis in human neutrophils.

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