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Mitocryptide-2, a neutrophil-activating cryptide, is a specific endogenous agonist for formyl-peptide receptor-like 1

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ABSTRACT

Peptides simultaneously produced during maturation and degradation of peptidergic hormones and functional proteins have recently become a great interest because they display unpredictably different biological roles than the parent proteins. Namely, we discovered two novel functional cryptic peptides, mitocryptide-1 (MCT-1) and mitocryptide-2 (MCT-2), hidden in mitochondrial cytochrome *c* oxidase and cytochrome *b*, that efficiently induced neutrophilic migration and activation at nanomolar concentrations. We named these functional "cryptic" peptides hidden in protein structures as "cryptides." In this study, we investigated the receptor molecules and cellular signaling mechanisms for neutrophil-activating *N*-formylated cryptide MCT-2. In order to identify the receptor molecules, we established HEK-293 cells stably expressing either formyl-peptide receptor (FPR) or its homologue FPR-like 1 (FPRL1), because neutrophilic cells express these receptor molecules which recognize *N*-formylated peptides. We observed that MCT-2 directly bound to FPRL1 and promoted an increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), and neither interacted with nor activated FPR, demonstrating that MCT-2 is a specific agonist for FPRL1. Moreover, MCT-2 induced not only $[Ca^{2+}]_i$ increase and phosphorylation of extracellular signal-regulated protein kinases 1 and 2, but also β-hexosaminidase release in neutrophilic/granulocytic cells differentiated from HL-60 cells. Such signaling events were diminished by pretreatment with pertussis toxin, indicating that MCT-2-promoted neutrophilic function is a consequence of G_i- or G_o-type G protein-dependent intracellular signaling events via FPRL1 activation. These findings suggest that MCT-2, a cryptide derived from mitochondrial cytochrome *b*, is a specific endogenous agonist for FPRL1 which is proposed to play key roles in inflammatory responses but whose physiological agonists are equivocal.

1. Introduction

Recently, we isolated and purified a novel class of neutrophil-activating peptides mitocryptide-1 (MCT-1, Table 1) and mitocryptide-2 (MCT-2, Table 1), which were derived from mitochondrial cytochrome *c* oxidase and cytochrome *b*, from porcine heart [1,2]. These peptides efficiently promoted neutrophil migration and activation at nanomolar concentrations. We also found many neutrophil-activating peptides derived from mitochondrial proteins other than MCT-1 and MCT-2 [1–3]. These findings suggest that some of the peptides simultaneously produced during maturation and degradation of mitochondrial proteins are involved in innate defense mechanisms against tissue damages. Moreover, peptides produced from not only mitochondrial but also other proteins such as hemoglobin and aminoacyl-tRNA synthetase were shown to regulate a variety of biological functions including cell proliferation and cytokine release [3–7]. These lines of evidence suggest that such "cryptic" peptides within various proteins may play critical roles including triggering inflammatory responses and healing of damaged tissues. We therefore named these functional "cryptic" peptides that are hidden in protein structures as "cryptides" [1–3,8,9].

The identification of novel cryptides and their regulatory mechanisms including receptor molecules is now intensively being studied [1,2,5,6,8,9]. In particular, MCT-2 was found to efficiently induce migration and activation of peripheral neutrophils as well as neutrophilic/granulocytic cells differentiated from HL-60 cells (differentiated HL-60 cells) [1,2]. However, MCT-2-induced cellular signaling in neutrophilic cells and its biological functions on various cells other than neutrophils have not been elucidated yet. In order to study the functions of MCT-2, identification of receptor molecules for MCT-2 is crucial.

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Table 1
Amino acid sequences of mitocryptides and other neutrophil-activating agonists.

Peptide name	Amino acid sequence
Mytocryptide-1	LSFLIPAGWVLSHLDHYKRSSAA
Mytocryptide-2	formyl-MTNIRKSHPLMKIIN
fMLF	formyl-MLF
WKYMVm	W K Y M V m

Uppercase or lowercase letter indicates L- or D-amino acid, respectively.

MCT-2 is a pentadecapeptide produced from cytochrome *b* whose *N*-terminus is formylated [2]. Thirteen proteins, including cytochrome *b*, encoded by mitochondrial DNA are known to be translated as *N*-formylated forms in mammals [10]. Formyl-peptide receptor (FPR), which recognizes *N*-formylated peptides and proteins, as well as its homologue FPR-like1 (FPRL1) are expressed in neutrophils [11,12]. However, there is a slight possibility that *N*-formylated mitochondrial proteins induce FPR or FPRL1 activation, because most of such mitochondrial proteins are insoluble membrane proteins and may not serve as extracellular signaling messengers. The existence of MCT-2, a soluble *N*-formylated mitochondrial-derived peptide as an efficient neutrophil-activating factor described above, however, proposes that mitochondrial derived *N*-formylated peptides including MCT-2 may be recognized by FPR and/or FPRL1, and may be involved in inflammation caused by tissue damage.

FPR plays a crucial role in the host defense mechanisms of neutrophils against infectious microorganisms by recognizing bacterial *N*-formylated peptides and proteins such as *N*-formyl-Met-Leu-Phe (fMLF, Table 1) [11,12]. Although FPRL1 is proposed to play key roles in acute inflammation and anti-inflammatory responses, its physiological agonists, however, are equivocal. The involvement of FPRL1 in monitoring bacterial *N*-formylated peptides is still in debate, because FPRL1 interacts with the peptides at much lower affinity than does FPR [11,13]. A lipid metabolite lipoxin A_4, which interacts with FPRL1, promotes chemotaxis but does not induce production of reactive oxygen species (ROS) and cytokine release [14,15]. From a screening of random peptide libraries, artificial peptides such as Trp-Lys-Tyr-Met-Val-D-Met-NH_2 (WKYMVm, Table 1) were identified as potent agonists for the formyl-peptide receptor family which consists of not only FPRL1 but also its homologues FPR and FPR-like 2 (FPRL2) [11,12,16]. The fact that such artificial peptides efficiently activate FPRL1 suggests the existence of endogenous peptidergic FPRL1 agonists. After many attempts at identifying physiological peptidergic agonists for the receptor, several peptides such as serum amyloid A and amyloid β_{1-42} were found to promote chemotaxis, ROS production and cytokine releases [15,17–19]. However, all activate FPRL1 at considerably high concentrations that are not likely found in tissues. Thus, the existence of endogenous peptidergic agonists for FPRL1 still remains unknown.

In this study, we investigated which formyl-peptide receptor subtypes expressed in neutrophilic cells are activated by mammalian *N*-formylated cryptide MCT-2. We demonstrated that MCT-2 specifically binds and activates FPRL1 and not FPR, suggesting that MCT-2 is a specific endogenous agonist for FPRL1 which is proposed to play key roles in inflammatory responses but whose physiological agonists have been elusive.

2. Materials and methods

2.1. Peptides

MCT-2 was chemically synthesized by solid-phase peptide synthesis using the *t*-butyloxycarbonyl method described previously [20–22]. Synthetic MCT-2 was more than 95% pure by analytical RP-HPLC using a C18 column (Develosil ODS-HG5, 4.6 × 150 mm) (Nomura Chemicals, Aichi, Japan). fMLF and WKYMVm were purchased from Sigma–Aldrich (St. Louis, MO) and TOCRIS (Avonmouth, Bristol, UK), respectively.

2.2. Cell preparation

HEK-293 cells (ATCC, Manassas, VA) were maintained in DMEM (Sigma–Aldrich) containing 10% FBS (Equitech-bio, Kerrville, TX) and the cells stably expressing FPR or FPRL1 were established as described in the Supplementary content. HL-60 cells (Riken Cell Bank, Ibaraki, Japan) were maintained in RPMI-1640 medium (Sigma–Aldrich) containing 10% FBS. HL-60 cells were treated with dibutyryl cyclic adenosine monophosphate (500 μM, Sigma–Aldrich) for 72 h in RPMI-1640 containing 15% FBS to differentiate into neutrophilic/granulocytic cells as previously described [1,2]. To examine the inhibitory effects by pertussis toxin (PTX) which renders G_i- and G_o-types of G proteins insensitive to the regulation of receptors, cells were treated with PTX (50 ng/mL, List Biological Laboratories, Campbell, CA) for 18 h as described [1,2].

2.3. Binding assay

The membrane fraction from FPR- or FPRL1-expressing cells was prepared as described [23] and was suspended in binding assay buffer [BAB: 5.8 mM NaCl, 5 mM KCl, 0.75 mM $CaCl_2$, 2 mM

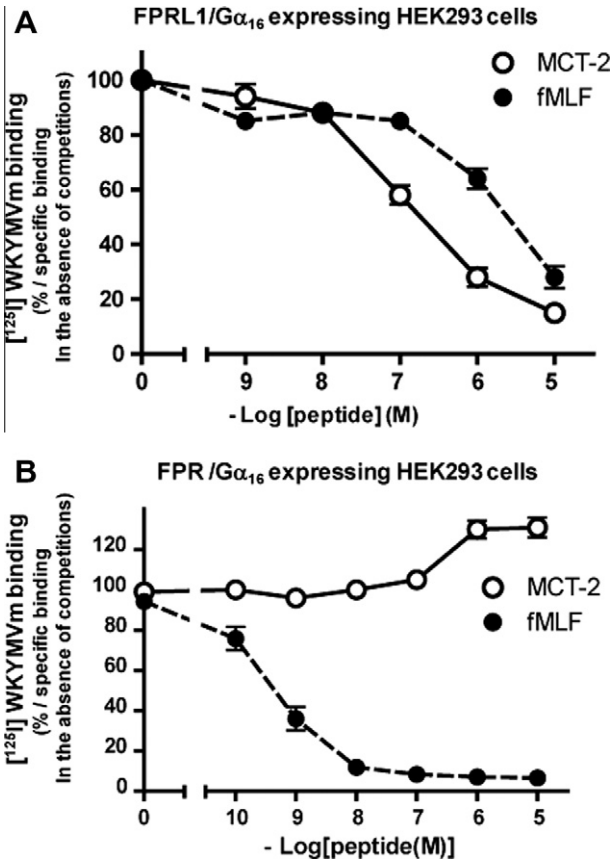

Fig. 1. MCT-2 specifically binds to FPRL1 (A) and not FPR (B). The binding of MCT-2 (open circle) or fMLF (closed circle) to FPRL1 or FPR was evaluated by the competition of these peptides at various concentrations against [^{125}I]-WKYMVm binding in membrane fraction prepared from FPRL1- or FPR-expressing HEK-293 cells. Each value was expressed as percentage to maximum [^{125}I]-WKYMVm binding without competitors. Data are expressed as mean ± SEM of six independent experiments.

$MgCl_2$, 320 mM sucrose, 0.2% BSA, 10 mM HEPES at pH 7.4, containing protease inhibitor cocktails (Nakarai, Kyoto, Japan)]. The suspended membrane fraction (10 μg protein in 90 μL of BAB) was added to tubes containing [^{125}I]-WKYMVm (10 μL, final concentration, 10 nM, PerkinElmer, Waltham, MA) with or without peptides at various concentrations. The suspension was incubated for 2 h at room temperature, and unbound [^{125}I]-WKYMVm was removed by filtration using a presoaked UniFilter GF/C plate (PerkinElmer). The plate was washed with ice-cold wash buffer (5.4 mM KCl, 10 mM HEPES, 0.25% BSA, at pH 7.4) and the radioactivity in each well was counted using a microplate scintillation and luminescence counter (PerkinElmer). Specific binding was estimated by subtracting the value obtained in the presence of excess of nonlabeled WKYMVm (10 μM).

2.4. Measurement of $[Ca^{2+}]_i$ increase

The increase in $[Ca^{2+}]_i$ stimulated by peptides or ionomycin was assessed as described previously [24–26] with the following modifications. Fluo-4/AM (final concentration, 2 μM, Dojindo, Kumamoto, Japan) was loaded into HEK-293 cells stably expressing FPR or FPRL1, or differentiated HL-60 cells in calcium assay buffer (CAB; 137 mM NaCl, 5.4 mM KCl, 0.4 mM KH_2PO_4, 3.4 mM Na_2HPO_4, 0.8 mM $MgSO_4$, 1 mM $CaCl_2$, 4.2 mM $NaHCO_3$, 5.6 mM glucose, and 10 mM HEPES at pH 7.4) for 1 h at room temperature. Loaded cells were washed four times with CAB, and cell suspension in CAB (180 μL) was placed in wells of 96-well plate. Cells were then stimulated at various concentrations of peptides or ionomycin (20 μL of the solution), and fluorescence changes at 520 nm excited at 488 nm were recorded using a fluorescent imaging plate reader (Molecular Devices, Sunnyvale, CA). In Fig. 2, the data were expressed relative to the maximum fluorescent changes induced at various concentrations of MCT-2 or fMLF against those induced by 1 μM WKYMVm. Traces of fluorescent changes induced by MCT-2 or fMLF (100 nM) in pretreated cells with or without PTX, were expressed relative to the maximum response induced by 1 μM ionomycin (Fig. 3A).

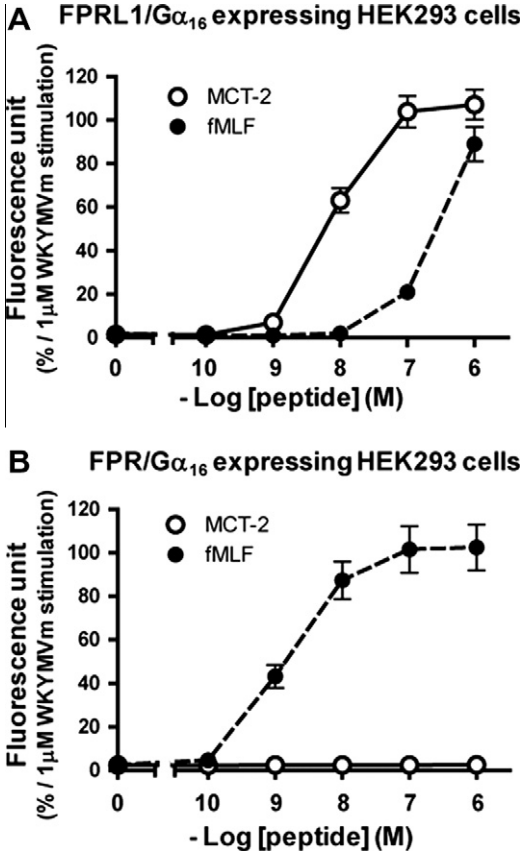

Fig. 2. MCT-2 specifically induces $[Ca^{2+}]_i$ increase in HEK-293 cells stably expressing FPRL1 (A) and not in HEK-293 cells expressing FPR (B). $[Ca^{2+}]_i$ increase induced by MCT-2 (open circle) or fMLF (closed circle) at various concentrations was investigated in Fluo-4-loaded HEK-293 cells stably expressing FPRL1 (A) or FPR (B). Data were normalized to maximum response of 1 μM WKYMVm. Data are expressed as mean ± SEM of six independent experiments.

2.5. β-Hexosaminidase (β-HA) release assay

β-HA release from differentiated HL-60 cells was assessed as described previously [1,2] with the following modifications. Cells (3.5×10^4 cells) in Tyrode's solution (20 μL: 10 mM HEPES, 135 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5.6 mM glucose containing 0.1% BSA at pH 7.4) was stimulated with a peptide solution (5 μL) at various concentrations for 30 min at 37 °C. To initiate an enzymatic reaction, cells were incubated with a substrate solution (25 μL) containing 4-metylumbelliferyl-2-acetamide-2-deoxyl-β-D-glucopyranoside (final concentration, 1.2 mM, Wako Chemical) at 37 °C for 20 min. The fluorescence in each well from the resulting methylumbelliferone was measured at 460 nm excited at 355 nm using a fluorescence microplate reader (BMG Labtech, Offenburg, Germany). The enzyme activity released from the cells was expressed as a percentage of the total enzyme activity, which was estimated by disruption of the cells with 0.05% Triton X-100.

2.6. Phosphorylation of extracellular signal-regulated protein kinases 1 and 2 (ERK1/2)

Peptide-induced phosphorylated and endogenous ERK1/2 protein levels in differentiated HL-60 cells were quantified by Western blot analysis using antibodies against phospho-ERK1/2 (Thr^{202}/Tyr^{204}) and ERK1/2 (Cell Signaling Technology, Danvers, MA). Cells that were stimulated by the peptides for 5 min at 37 °C were resuspended in lysis buffer (50 mM Tris–HCl, 150 mM NaCl, 1% TritonX-100, 1% SDS, 0.5% sodium deoxycholate, 10% glycerol, at pH 7.4 with a protease and phosphatase inhibitor cocktail) and were briefly sonicated at 4 °C. The obtained proteins (10 μg) were separated by 4–20% SDS–PAGE, transferred to polyvinylidene fluoride membranes (Millipore, Billerica, MA), and immunoblotted with the antibodies. Antibody-bound ERK1/2 or phosphorylated ERK1/2 were visualized by the ECL method using anti-rabbit IgG conjugated with horseradish peroxidase (GE Healthcare, Little Chalfont, Buckinghamshire, UK).

3. Results

3.1. MCT-2 specifically activates FPRL1 and not FPR

In order to identify receptor molecules for mammalian *N*-formylated peptide MCT-2, we examined whether MCT-2 binds and activates FPR and/or FPRL1 that are expressed in neutrophilic cells. For this purpose, we established HEK-293 cells stably expressing either FPR or FPRL1. The activity of ligand binding to FPR or FPRL1 was evaluated by competition between [^{125}I]-WKYMVm and MCT-2 or fMLF for a membrane fraction prepared from either FPR- or FPRL1-expressing cells. [^{125}I]-WKYMVm is a promiscuous agonist for the formyl-peptide receptor family. The activation of the receptor molecule was determined by an increase in $[Ca^{2+}]_i$ in these cells stably co-expressing $G\alpha_{16}$-type G-protein.

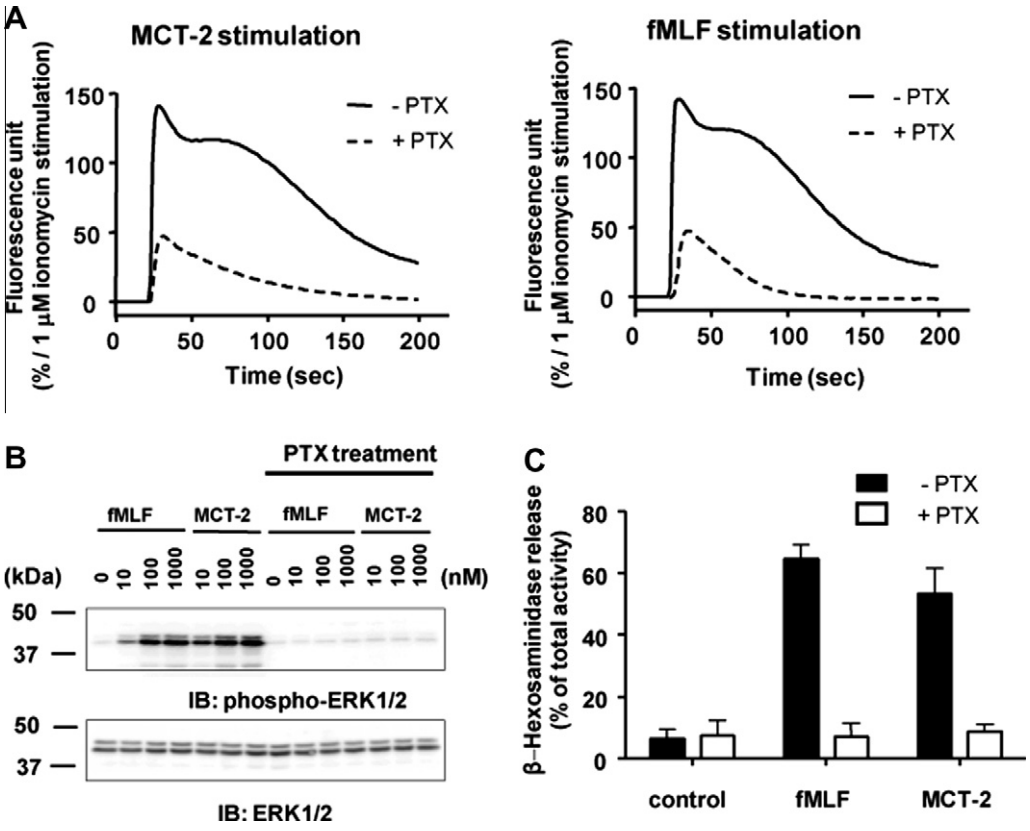

Fig. 3. Intracellular signaling effects and β-HA release induced by MCT-2 and the inhibition of its stimulation by PTX. Neutrophilic/granulocytic cells differentiated from HL-60 cells were treated with or without PTX, and $[Ca^{2+}]_i$ mobilization (A), phosphorylation of ERK1/2 (B), and β-HA release (C) promoted by MCT-2 or fMLF were investigated. (A) $[Ca^{2+}]_i$ traces stimulated by MCT-2 (100 nM) and fMLF (100 nM) were normalized to the response induced by 1 μM ionomycin. The experiment was confirmed three times independently. (B) Total ERK1/2 and phosphorylated ERK1/2 proteins in cells were detected by western blot analysis. The experiment was confirmed three times independently. (C) Cells were stimulated by a vehicle, MCT-2 (100 nM), or fMLF (100 nM) and induction of β-HA release was evaluated. Each value was expressed as mean ± SEM of three independent experiments.

The $G\alpha_{16}$ protein promiscuously interacts with a variety of G-protein coupled receptors and effectively induces agonist-promoted $[Ca^{2+}]_i$ increase via activation of phospholipase C [27].

Expression of FPR or FPRL1 receptor proteins on the plasma membrane in each respective cell line was confirmed by flow cytometric analysis using antibodies against each receptor subtype (Fig. S1). We observed that bacterial *N*-formylated peptide fMLF inhibited the binding of [^{125}I]-WKYMVm to the membrane fraction prepared from both FPR- and FPRL1-expressing cells, although the binding affinity of fMLF for the membrane fraction prepared from FPRL1-expressing cells was more than 100 times lower than in that from FPR-expressing cells (Fig. 1A and B). fMLF also caused $[Ca^{2+}]_i$ increase in HEK-293 cells expressing either FPR or FPRL1 (EC_{50} ~240 nM for FPRL1-expressing cells; ~1.4 nM for FPR-expressing cells, Fig. 2A and B). These results demonstrated that fMLF binds and activates both FPR and FPRL1 to promote $[Ca^{2+}]_i$ increase, in agreement with previous reports [13,16].

In the case of MCT-2, [^{125}I]-WKYMVm binding to the membrane fraction prepared from FPRL1-expressing cells was blocked by MCT-2 in a dose-dependent manner, and the binding was completely diminished at the presence of higher concentrations of MCT-2 (Fig. 1A). In contrast to fMLF, MCT-2 did not compete with [^{125}I]-WKYMVm for the binding of the membrane fraction prepared from FPR-expressing cells (Fig. 1B). Moreover, MCT-2 promoted $[Ca^{2+}]_i$ increase in a concentration-dependent manner in HEK-293 cells stably expressing FPRL1 (EC_{50} ~6.9 nM, Fig. 2A). However, cells stably expressing FPR did not increase $[Ca^{2+}]_i$ even after stimulation at 1 μM MCT-2 (Fig. 2B). These findings indicate that MCT-2 directly binds to FPRL1 to induce $[Ca^{2+}]_i$ increase, whereas MCT-2 neither interacts nor activates FPR, demonstrating that MCT-2 is a specific agonist for FPRL1.

3.2. MCT-2-induced cellular signaling

Once MCT-2 was demonstrated as a specific agonist for FPRL1, we examined the cellular signaling mechanisms induced by MCT-2 in neutrophilic/granulocytic cells differentiated from HL-60 cells. MCT-2 promoted not only an increase in $[Ca^{2+}]_i$ but also phosphorylation of ERK1/2 in a concentration-dependent manner (Fig. 3A *left* and B). These signaling events were largely prevented by cells pretreated with PTX, which ADP-ribosylates G_i- and G_o-types G proteins and renders them insensitive to receptor regulation [28]. Moreover, MCT-2 induced a neutrophilic function of β-HA release, which is a glycosidase that is released from neutrophilic cells upon stimulation of their activating factors such as fMLF [1,2]. The enzyme release promoted by MCT-2 was completely inhibited by PTX treatment (Fig. 3C). Similarly, fMLF induced not only $[Ca^{2+}]_i$ increase and ERK1/2 phosphorylation, but also β-HA release (Fig. 3A *right*, B, and C). Again, these events were diminished by PTX pretreatment. These results indicate that G_i- or G_o-type G proteins are involved in MCT-2-promoted and fMLF-promoted intracellular signaling events that induce β-HA release, a neutrophilic function.

4. Discussion

For a long time, most peptides produced from the degradation of functional proteins as well as peptidergic hormones and

neurotransmitters were considered as molecules lacking physiological roles. However, we recently discovered that MCT-1 and MCT-2, which are produced from mitochondrial proteins, efficiently promote neutrophilic migration and activation at nanomolar concentrations [1,2,29,30]. We named such functional cryptic peptides that are hidden in protein structures, "cryptides." Following our findings, various lines of experimental evidence that point to the physiological roles for cryptides are amassing, and such cryptides have attracted considerable interests [3,5,6].

In this study, we identified which formyl-peptide receptor subtypes expressing in neutrophilic cells are activated by mammalian *N*-formylated cryptide MCT-2. We found that MCT-2 specifically binds and activates FPRL1, and neither interacts nor activates FPR, demonstrating that MCT-2 is a specific agonist for FPRL1 (Figs. 1 and 2). Moreover, we indicated that MCT-2 induced the neutrophilic function of β-HA release as a result of G_i- or G_o-protein-dependent intracellular signaling events including $[Ca^{2+}]_i$ increase and ERK1/2 phosphorylation via FPRL1 activation (Fig. 3). The formyl-peptide receptor family consists of not only FPR and FPRL1, but also their homologue FPRL2 which is not expressed in neutrophils. However, MCT-2 did not activate FPRL2 (unpublished observation). These findings suggest that MCT-2, a cryptide derived from mitochondrial cytochrome *b*, is a specific endogenous agonist for FPRL1 which is proposed to play key roles in inflammatory responses but whose physiological agonists are equivocal. Because FPRL1 is known to be expressed in various cells including monocytes, macrophages, microglia and dendritic cells [11,31], the discovery of MCT-2 as a specific agonist for FPRL1 promises to explore the physiological roles of FPRL1 in various cells expressing FPRL1. MCT-2 is also an expected useful tool to investigate the pathophysiological mechanisms of diseases involving neutrophils or FPRL1, such as Alzheimer's disease, rheumatoid arthritis, Prion disease, and myocardial reperfusion injury [18,32–34].

Here we have indicated that MCT-2 singly activates FPRL1 and not FPR to promote neutrophilic function (Figs. 1–3). Our finding proposes a functional sharing between FPR and FPRL1 in early immune responses involving neutrophils (Fig. 4). Namely, the bacterial *N*-formylated peptide fMLF prefers to interact with FPR rather than FPRL1 (Figs. 1 and 2), thus suggesting that FPR mainly monitors for bacterial infection by recognizing exogenous *N*-formylated proteins and peptides. In contrast, the mitochondrial-derived peptide MCT-2 selectively binds and activates FPRL1 (Figs. 1 and 2), thus suggesting that FPRL1 monitors for injury-associated inflammation by recognizing endogenous *N*-formylated peptide MCT-2 which is released from mitochondria during tissue damage. The notion of functional sharing between FPR and FPRL1 is also supported by our previous observations that fMLF causes neutrophilic migration at lower concentrations and promotes β-HA release at higher concentrations with neutrophilic migration; while MCT-2 initiates chemotaxis at lower concentrations, and stimulation for β-HA release is promoted at higher concentrations, at which neutrophilic migration is desensitized [2]. These findings suggest that MCT-2 may be involved in the clearance of endogenous toxic debris without much undesirable damage to healthy tissues.

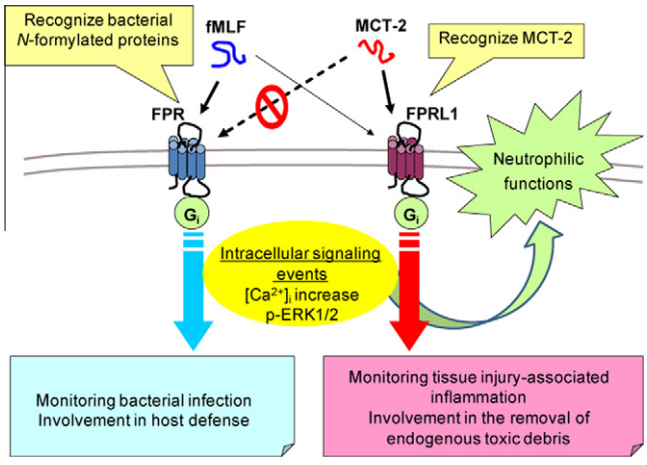

Fig. 4. Proposed functional sharing between FPR and FPRL1 in neutrophilic cells. Endogenous mitochondrial-derived peptide MCT-2 exclusively activates FPRL1, whereas bacterial-derived peptide fMLF selectively activates FPR rather than FPRL1 to induce neutrophilic functions.

Since the early 1990's, we began our attempt to purify neutrophil-activating factors from healthy organs because it has not been known for a long time which factors promote immediate neutrophil migration to early stage inflammatory sites caused by tissue damage. In 1999, we discovered novel neutrophil-activating peptides MCT-1 and MCT-2, and also found the existence of a number of activating peptides derived from mitochondrial proteins [1–3,29,30]. Based on these lines of evidence, we proposed a regulatory mechanism in acute inflammation involving mitochondrial-derived peptides and neutrophils [1,29,30]. Very recently, our proposed mechanism was supported by Zhang et al., from a pathological perspective [35]. Namely, damage-associated molecular patterns (DAMPs) that contain mitochondrial DNAs and proteineous mitochondrial-derived molecules are released from mitochondria in injured tissues of traumatic patients and cause systemic inflammation by promoting neutrophil activation. Although Zhang et al. did not identify the proteineous molecules, DAMPs may contain MCT-1 and MCT-2, because we indicated the existence of mitochondrial protein-derived peptides as neutrophil-activating factors in mammalian tissues [1–3]. The present finding that MCT-2 activates FPRL1 suggests that the FPRL1 receptor also contributes to damage-associated immune responses involving neutrophils.

In conclusion, we demonstrated that MCT-2, a mitochondrial cytochrome *b* derived cryptide, directly interacts and activates FPRL1 among formyl-peptide receptor subtypes expressing in neutrophilic cells, suggesting that MCT-2 specifically activates FPRL1 to induce cellular signaling for induction of neutrophilic functions. Moreover, the remarkable fact that receptor molecule FPRL1 specifically and efficiently recognizes cryptide MCT-2 suggests that many peptides produced by the degradation of functional proteins may be associated with unidentified physiological regulations. Thus, the discovery of novel cryptides and the elucidation of their regulatory mechanisms are crucial to understand various biological functions.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2010.12.007.

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