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N-Formylated humanin activates both formyl peptide receptor-like 1 and 2☆

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Abstract

We have discovered that humanin (HN) acts as a ligand for formyl peptide receptor-like 1 (FPRL1) and 2 (FPRL2). This discovery was based on our finding that HN suppressed forskolin-induced cAMP production in Chinese hamster ovary (CHO) cells expressing human FPRL1 (CHO-hFPRL1) or human FPRL2 (CHO-hFPRL2). In addition, we found that N-formylated HN (fHN) performed more potently as a ligand for FPRL1 than HN: in CHO-hFPRL1 cells, the effective concentration for the half-maximal response (EC_{50}) value of HN was 3.5 nM, while that of fHN was 0.012 nM. We demonstrated by binding experiments using [^{125}I]-W peptide that HN and fHN directly interacted with hFPRL1 on the membrane. In addition, we found that HN and fHN showed strong chemotactic activity for CHO-hFPRL1 and CHO-hFPRL2 cells. HN is known to have a protective effect against neuronal cell death. Our findings contribute to the understanding of the mechanism behind HN's function.

Keywords: Formyl peptide receptor-like 1; Formyl peptide receptor-like 2; Humanin; Mitochondria; Chemotaxis; Alzheimer's disease

G-protein-coupled receptors (GPCRs) are currently among the most successful targets for the drug market. Thus, we consider GPCRs whose ligands are not yet known, that is, 'orphan' GPCRs, to be good target sources for discovering new drugs. However, to investigate orphan GPCRs as potential drug targets, it is indispensable to identify their ligands. Previously [1,2], we established a widely applicable strategy to identify orphan GPCR ligands, which involves searching for ligands by monitoring signal transductions, such as changes in cAMP or Ca^{2+} mobilization, in cells expressing orphan GPCRs [3]. Applying this approach, we have succeeded in identifying various orphan GPCR ligands [4–11]. In the present study, we expressed green fluorescence protein (GFP)-fused orphan GPCRs in Chinese hamster ovary (CHO) cells—thus ensuring the expression of orphan GPCRs by selecting only GFP-positive cells—and subsequently screened our ligand libraries by measuring changes in cAMP production in these cells. Through this screening, we found that humanin (HN) and its related peptides specifically suppressed cAMP production in CHO cells expressing GFP-fused formyl peptide receptor-like 1 (FPRL1). Human FPRL1 (hFPRL1) and 2 (hFPRL2) share 69% and 57% amino-acid identity with human formyl peptide receptor 1

☆ *Abbreviations:* HN, humanin; FPRL1, formyl peptide receptor-like 1; FPRL2, formyl peptide receptor-like 2; FPR1, formyl peptide receptor 1; CHO, Chinese hamster ovary; fHN, N-formylated humanin; EC_{50}, half-maximal response; GPCRs, G-protein-coupled receptors; GFP, green fluorescence protein; AD, Alzheimer's disease; Aβ, β-amyloid peptide; IC_{50}, 50% inhibition of specific binding; DMEM, Dulbecco's modified minimum essential medium; FAM, 6-carboxyfluorescein; TAMRA, 6-carboxy-tetramethylrhodamine; HNG, [Gly^{14}]-humanin; PMNs, polymorphonuclear leukocytes.

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(hFPR1), respectively, and are mainly expressed in phagocytic cells [12,13]. In addition, hFPRL1 mRNA has reportedly been detected in and around the senile plaque in the brain tissue of Alzheimer's disease (AD) patients [14]. A variety of substances, including lipoxin A4 [15], serum amyloid A [16], T21/DP107 [17], the V4-C4 region of HIV-1 envelope gp120 [18], W peptide [19], cathelicidin [20], prion peptide (PrP(106–126)) [21], β-amyloid peptide (Aβ) [14], chenodeoxycholic acid [22], *Helicobacter pylori* Hp(2–20) [23], MMK-1 [24], and CKβ8-1 [25], have been reported to function as ligands for FPRL1. Despite their unrelated structure, these ligands bind to FPRL1 [25,26]. On the other hand, ligands for FPRL2 have not yet been reported, and so this receptor has remained an orphan GPCR. FPRL2 is mainly expressed in monocytes, but not in neutrophils [13].

HN cDNA was originally identified through a functional expression screening on the basis of its ability to suppress neuronal cell death induced by familial AD genes (K595M/N596L-APP) [27]. HN cDNA encodes a 24-amino-acid residue polypeptide, MAPRGFSCLLLL TSEIDLPVKRRA, which inhibits neuronal cell death caused by both familial AD genes (i.e., APP mutants, PS1 mutants, and PS2 mutants) and β-amyloid peptides [27–29]. However, the mechanism behind HN's function has not been clarified.

Here we report that HN and its related peptides function as ligands for both FPRL1 and FPRL2. In addition, we show that N-formylation of HN remarkably increases its agonistic activity for FPRL1.

Experimental procedures

Peptides. All peptides were purchased from Peptide Institute (Osaka, Japan).

Preparation of CHO cells expressing GPCRs. The entire coding regions of hFPR1, hFPRL1 or hFPRL2 cDNAs were inserted downstream of the SRα promoter in an expression vector pAKKO-111H [30]. To express the fusion protein of hFPRL1 and GFP, a vector plasmid was constructed by inserting a fused DNA, in which hFPRL1- and GFP-coding regions were tandemly connected in-frame, into pAKKO-111H. These expression plasmids were transfected into *dhfr*$^-$ CHO cells, and the resultant transformed *dhfr*$^+$ CHO cells expressing hFPRL1, hFPRL2 or hFPR1 (CHO-hFPRL1, CHO-hFPRL2, and CHO-hFPR1 cells, respectively) were selected as described elsewhere [30].

Internalization of hFPRL1. CHO cells stably expressing hFPRL1-GFP were seeded onto chambered coverglasses (Nalge Nunc International, NY, USA) and cultured overnight. Intracellular localization of the fusion protein before and after treatment with 2 μM [Gly^{14}]-HN (HNG) for 120 min was observed under a confocal fluorescence microscope.

cAMP production-inhibitory activities of HN and its related peptides. cAMP production-inhibitory assays using CHO cells in the presence of forskolin were performed according to our method previously described [31]. Intracellular cAMP concentrations were measured by an enzyme immunoassay (cAMP-Screen [Applied Biosystems, CA, USA]).

Binding of HN and its related peptides to hFPRL1. Receptor binding assays were conducted essentially as described previously [32]. Briefly, in the competitive binding assays, membrane fractions prepared from CHO-hFPRL1 cells were suspended in the assay buffer (25 mM Tris–HCl at pH 7.5 containing 0.1% bovine serum albumin, 1 mM EDTA, 0.03% NaN_3, 0.25 mM phenylmethylsulfonyl fluoride, 20 μg/ml leupeptin, 10 μg/ml phosphoramidon, and 1 μg/ml pepstatin), and then HN or one of its related peptides was incubated together with [^{125}I]-W peptide (Perkin–Elmer Life and Analytical Sciences, MA, USA). Non-specific binding was determined in the presence of 1 μM unlabeled W peptide. After incubation at 25 °C for 60 min, [^{125}I]-W peptide unbound to the membrane fraction was removed by filtration using polyethyleneimine-treated UniFilter GF/C plates (Perkin–Elmer Life and Analytical Sciences, MA, USA) which had been presoaked with 50 mM Tris–HCl at pH 7.5 and washed five times with the assay buffer. [^{125}I]-W peptide bound to the membrane fraction was detected with a TopCounts Microplate Scintillation and Luminescence Counter (Perkin–Elmer Life and Analytical Sciences, MA, USA). The test sample concentrations causing 50% inhibition of specific binding (IC_{50}) were calculated by fitting the binding data into a pseudo-Hill equation: $\log[\%SB/(100-\%SB)] = n[\log(C) - \log(IC_{50})]$, where SB is specific binding, n is the pseudo-Hill constant, and C is the test sample concentration.

Chemotactic activities of HN and its related peptides. Chemotactic assays were performed with 96-well microchemotaxis chambers (ChemoTx-96 No. 106-5; NeuroProbe, MD, USA). The test samples were diluted with Dulbecco's modified minimum essential medium supplemented with 0.1% BSA (DMEM/BSA), and then 30 μl of each solution was added to the lower chamber. A framed chemotaxis filter with 5-μm pores was used after precoating with 10 μg/ml bovine fibronectin (Yagai Research Center, Yamagata, Japan). The CHO cells were suspended in DMEM/BSA and each cell suspension (2.5×10^5cells/50μl/well) was added into the upper chamber forming a hemispherical drop on the surface of the filter. The cells were then incubated at 37 °C for 4 h in 5% CO_2 in air. After scraping off cells which did not migrate from the upper surface of the filter, those migrating to the lower surface were fixed and stained with Diff-Quick (International Reagent, Hyogo, Japan). The stained cells were then detected with absorbance at 540 nm. Data were expressed as the percentage of maximum absorbance corresponding to the number of migrating cells.

Quantitative reverse transcription-polymerase chain reaction analyses for FPRL1 and FPRL2 mRNAs. Poly(A)$^+$RNA fractions from human tissues were purchased from Clontech. Poly(A)$^+$RNA fractions were prepared from the tissues of 8- to 12-week-old Wistar rats. Random-primed cDNAs were synthesized as described previously [33], and subjected to quantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis using an ABI Prism 7700 sequence detector (Applied Biosystems, CA, USA), with primers and fluorescent-labeled probes (5′-TTGTTGCCATCTGCTATGG-3′, 5′-GGACGGCTGGATTTAATCAT-3′, and 5′[FAM]-CATTGCAGC CAAGATCCACAAAAAGG-[TAMRA]3′ for human FPRL1; 5′-GATGACACGCACAGTCAACA-3′, 5′-GGATGGCACTGAAA GAGAAGT-3′, and 5′[FAM]-CATCTGTTACCTGAACCTGGCC CTAGCT-[TAMRA]3′ for human FPRL2).

Results

Identification of HN as a ligand for hFPRL1

We prepared CHO-hFPRL1-GFP cells to screen for ligands of hFPRL1. GFP was used as a marker to ascertain the expression of hFPRL1. We screened more than 1000 compounds by measuring the suppression of forskolin-induced cAMP production in CHO-hFPRL1-GFP, and detected a specific response to HN and HNG at 0.4 μM (data not shown). In the absence of a

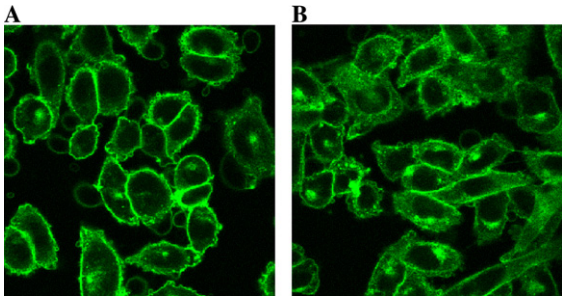

Fig. 1. Internalization of hFPRL1-GFP induced by HNG. (A) CHO cells expressing hFPRL1-GFP without treatment. (B) CHO cells expressing hFPRL1-GFP after treatment with HNG (2 μM) at 37 °C for 120 min.

ligand, the hFPRL1-GFP was typically localized at the plasma membrane (Fig. 1A). In contrast, in the presence of HNG, the hFPRL1-GFP was internalized into the cytoplasm (Fig. 1B).

cAMP production-inhibitory activities of HN and its related peptides

To confirm the receptor–ligand relationship between HN and FPRL1 family members, we prepared CHO-hFPRL1, CHO-hFPRL2, and CHO-hFPR1 cells, and examined changes in the inhibition of forskolin-stimulated cAMP production of these cells in response to HN and its related peptides (Fig. 2). We found that HN as well as HNG inhibited cAMP production in both CHO-hFPRL1 and CHO-hFPRL2 cells. The EC_{50} values of HN in CHO-hFPRL1, CHO-hFPRL2, and CHO-hFPR1 cells were 3.5, 2.6, and >1000 nM, respectively (Table 1). The potencies of HN and HNG on both CHO-hFPRL1 and CHO-hFPRL2 cells were comparable, indicating that the substitution of Ser14 with Gly in HN does not affect its interaction with hFPRL1 or 2. In the CHO-hFPR1 cells, HN and its related peptides were much less potent than fMLF and W peptide. Our results indicate that HN and HNG act as specific ligands for not only FPRL1 but also FPRL2.

HN is encoded in human mitochondrial 16S rRNA gene and it is known that the N-termini of proteins encoded in the mitochondrial genome are usually N-formylated [34]. We therefore synthesized N-formylated HN (fHN) and determined its potency in CHO-hFPRL1, CHO-hFPRL2, and CHO-hFPR1 cells. Based on the EC_{50} value of fHN in CHO-hFPRL1 (i.e., 0.012 nM), fHN was found to be about 300 times more potent than HN. On the other hand, the EC_{50} value of fHN in CHO-hFPRL2 cells was almost the same as those of HN and its related peptides (Table 1). In addition, fHN showed weak cAMP-production-inhibitory activity on CHO-hFPR1 cells. These results indicate that N-formylation confers remarkable changes to the functional properties of HN, although it remains to be determined whether fHN could actually be produced under physiological conditions.

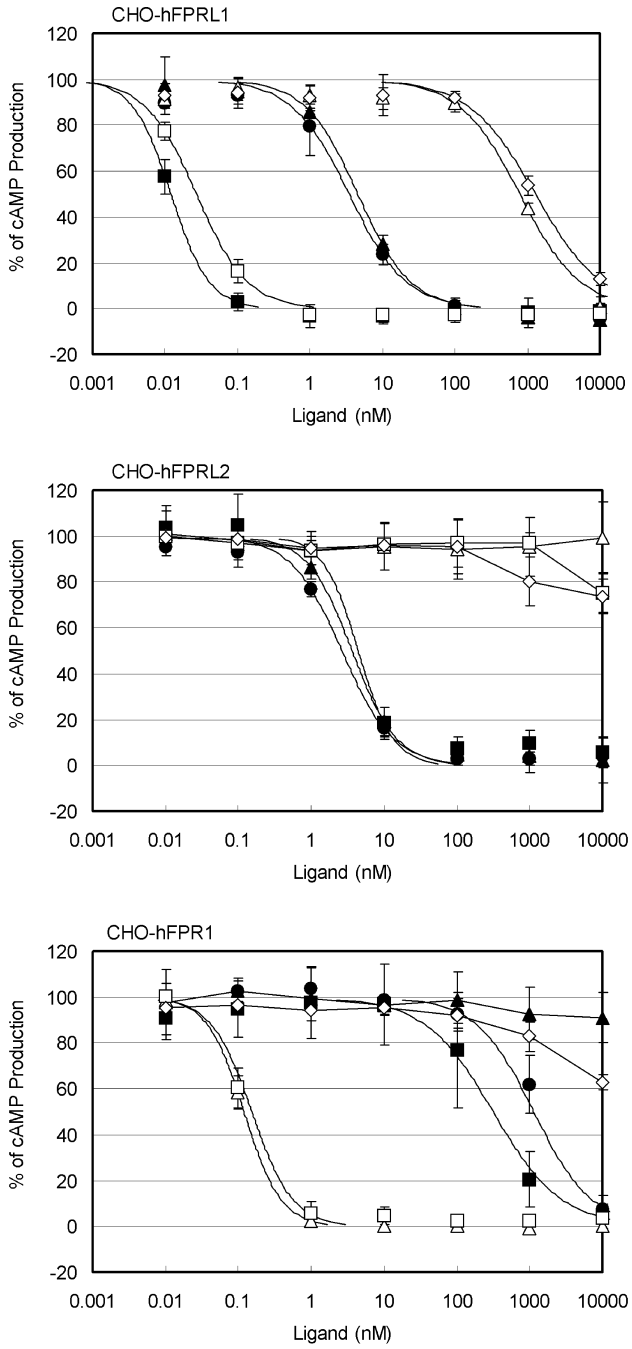

Fig. 2. Inhibition of cAMP production in CHO-hFPRL1, CHO-hFPRL2, and CHO-hFPR1 cells. After culturing overnight in 96-well microplates, CHO-hFPRL1, CHO-hFPRL2, and CHO-hFPR1 cells were incubated for 30 min with 1 μM forskolin and HN (●), HNG (▲), fHN (■), fMLF (△), W peptide (□), or Aβ(1–42) (◊). Intracellular cAMP amounts were measured with cAMP-Screen. Data are expressed as means ± SD in triplicate assays.

Binding of HN and its related peptides to hFPRL1

To characterize the binding properties of HN and its related peptides to hFPRL1, we prepared membrane

Table 1
EC_{50} and IC_{50} values of HN and its related peptides in various functional assays

Ligand	cAMP EC_{50} (nM)			Binding IC_{50} (nM)
	hFPRL1	hFPRL2	hFPR1	hFPRL1
HN	3.5	2.6	>1000	68
HNG	4.6	3.7	>1000	70
fHN	0.012	4.4	310	1.8
fMLF	790	>1000	0.12	>1000
W peptide	0.027	>1000	0.16	0.14
Aβ(1–42)	1300	>1000	>1000	>1000

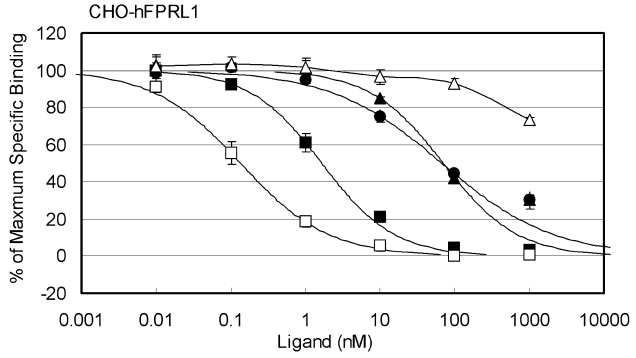

Fig. 3. Displacement of [^{125}I]-W peptide by HN and its related peptides. Binding of [^{125}I]-W peptide to CHO- hFPRL1 membrane fractions was examined in the presence of HN (●), HNG (▲), fHN (■), fMLF (△), or W peptide (□). Results are presented as a percentage of specific [^{125}I]-W peptide binding, and data represent means ± SD in triplicate assays.

fractions of CHO-hFPRL1 cells and examined the displacement of [^{125}I]-W peptide, which is known to act as a ligand for FPRL1 and FPR1. The IC_{50} values of fHN, HN, and HNG were 1.8, 68, and 70 nM, respectively (Fig. 3 and Table 1). The binding affinities of HN and HNG were 30 times weaker than that of fHN. These results demonstrate that HN and its related peptides directly bound to FPRL1 on the membrane.

Chemotactic activities of HN and its related peptides

To characterize function, we examined the chemotactic activity of HN and its related peptides for CHO-hFPR1, CHO-hFPRL1, and CHO-hFPRL2 cells (Fig. 4). fHN showed strong activity for CHO-hFPRL1 cells, with its dose–response forming a typical bell-shaped curve. HN also showed chemotactic activity for CHO-hFPRL1 cells, however, it was less potent than fHN. The maximal response of fHN was detected at 10^{-8} M, whereas that of HN was at 10^{-6} M. fMLF, a ligand for FPR1, did not show significant chemotactic activity for CHO-hFPRL1 cells. HN and fHN induced chemotaxis in CHO-hFPRL2 cells. Their chemotactic activities were weaker for CHO-hFPRL2 cells than for CHO-hFPRL1 cells. The maximal

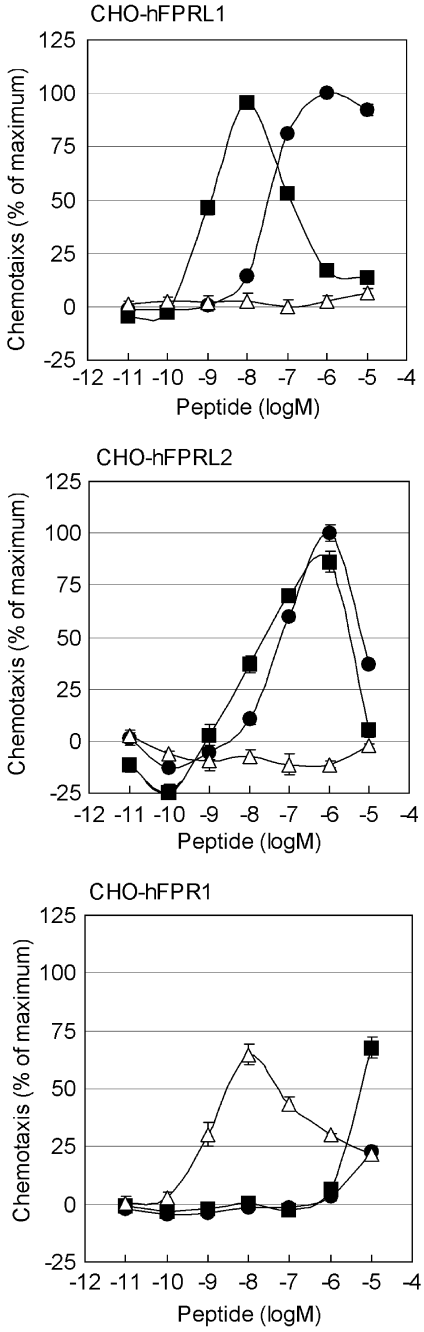

Fig. 4. Chemotactic activities of HN and its related peptides for CHO-hFPRL1, CHO-hFPRL2, and CHO-hFPR1 cells. CHO-hFPRL1, CHO-hFPRL2, and CHO-hFPR1 cells were incubated on chemotaxis filters with the indicated concentrations of HN (●), fHN (■), or fMLF (△) in 96-well chemotaxis chambers. After fixing, the cells were stained with Diff-Quick and the number of migrating cells was estimated by absorbance at 540 nm.

responses of both fHN and HN were detected at 10^{-6} M. However, fMLF did not induce chemotaxis in CHO-hFPRL2 cells. In contrast, fMLF showed more potent chemotactic activity for CHO-hFPR1 cells than HN or fHN. The chemotactic activities of these peptides seemed to correlate well with their potency as determined in the

cAMP-production-inhibitory and receptor binding assays. However, these peptides did not show chemotactic activity for mock-transfected CHO cells (data not shown).

Tissue distribution of hFPRL1 and hFPRL2 mRNAs

We analyzed the tissue distribution of hFPR1, hFPRL1, and hFPRL2 mRNAs by RT-PCR. High levels of hFPR1 and hFPRL1 mRNA expression were mainly detected in the bone marrow, while moderate levels were detected in the lung and spleen (Fig. 5). In contrast, hFPRL2 mRNA expression was detected in various tissues including the spleen and lymph nodes, with its highest expression in the lung and low expression in the bone marrow (Fig. 5).

Discussion

In this paper, we have demonstrated that HN and its related peptides act as specific ligands for FPRL1 and 2. Although it has been recently reported that HN uses FPRL1 as a functional receptor [35], we believe this is the first report demonstrating that HN acts as a ligand for not only FPRL1 but also FPRL2. Furthermore, until now there has been no report demonstrating direct interaction between HN and FPRL1. Through our binding assays using [^{125}I]-W peptide, we have shown here the direct interaction of HN and FPRL1.

Since HN is encoded in human mitochondrial 16S rRNA gene and the N-termini of proteins encoded in the mitochondrial genome are usually N-formylated [34], we synthesized fHN and found that its affinity for

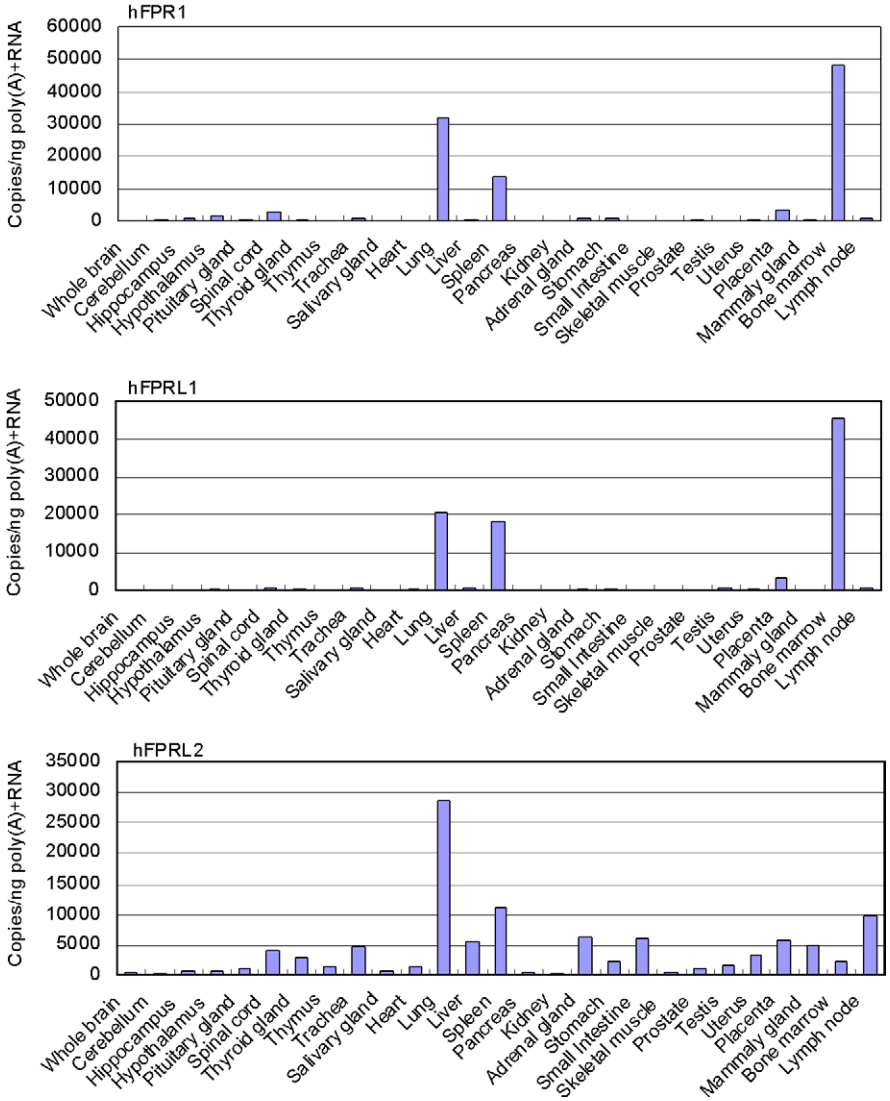

Fig. 5. Tissue distribution of hFPR1, hFPRL1,and hFPRL2 mRNAs. Poly(A)$^+$ RNA preparations were subjected to quantitative RT-PCR using an ABI Prism 7700 sequence detector. Each column represents the mean value in duplicate determinations.

hFPRL1 was much higher than that of HN. fHN also showed strong chemotactic activity for CHO-hFPRL1 cells. N-formylated mitochondrial proteins have been reported to show chemotactic activity for polymorphonuclear leukocytes (PMNs) [34]. Thus, fHN would be expected to show chemotactic activity for PMNs, although future studies are necessary to confirm this. The neuroprotective function of HNG in primary neuronal cells against Aβ(1–43) peptides has been found to be 1000 times greater than that of HN [27]. However, we found the agonistic activities of HN and HNG for hFPRL1 to be almost the same. Therefore, the direct neuroprotective function of HN and HNG appears to have no relation to their agonistic activities for hFPRL1. However, a previous report has suggested that FPRL1 is implicated in HN's neuroprotective activity mechanism [35]. Future studies must be conducted to resolve this discrepancy.

Since Aβ(1–42) is known to be a ligand for hFPRL1, it has been suggested that FPRL1 has some relationship to the proinflammatory aspects of AD [14]. Abundant expression of hFPRL1 mRNA has been detected in $CD11b^{+}$ mononuclear phagocytes that surround or infiltrate the plaque in the brains of AD patients [36]. hFPRL1 has thus been thought to mediate the cellular uptake of Aβ(1–42) by rapidly internalizing Aβ(1–42)-hFPRL1 complexes, resulting in the formation of Congo-red positive fibrils in mononuclear phagocytes. On the other hand, HN inhibits neuronal cell death caused by familial AD genes and β-amyloid peptides [27–29]. Considering this together, we speculate that HN affects AD through FPRL1.

In addition, we have demonstrated that HN and its related peptides also activate FPRL2. Although there have been no reports suggesting any relationship between AD and FPRL2, FPRL2 mRNA is widely expressed in various tissues, including those expressing FPRL1 mRNA. Not only FPRL1 but also FPLR2 may be involved in AD. We hope that our findings will contribute to understanding the mechanisms behind the progress of AD and provide opportunities to develop anti-AD drugs.

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