Neurochem Res
DOI 10.1007/s11064-010-0301-5

ORIGINAL PAPER

Expression and Signaling of Formyl-Peptide Receptors in the Brain

Fabio Cattaneo · Germano Guerra · Rosario Ammendola

Accepted: 14 October 2010

Abstract The human formyl-peptide receptor (FPR) and its variants FPRL1 and FPRL2 belong to the G-protein coupled seven transmembrane receptor (GPCR) family sensitive to pertussis toxin. FPR and FPRL1 were first detected in phagocytic leukocytes, and FPRL2 was found in monocytes and in dendritic cells. The three receptors were subsequently identified in other cell types or tissues, including neuronal cells and brain, where FPR and FPRL1 play a key role in angiogenesis, cell proliferation, protection against and cell death, as well as in neuroendocrine functions. Binding of different agonists to FPRs triggers several signaling pathways, activates NFkB and STAT3 transcriptional factors and induces the accumulation of the CDK inhibitors $p21^{waf1/cip1}$, $p16^{INK4}$ and $p27^{kip1}$. Signaling molecules, such as ERKs, JNK, PKC, p38MAPK, PLC and PLD are involved in these intracellular cascades. In this article we briefly review FPRs expression and signaling in neuronal cells.

Keywords Formyl-peptides receptors · Signal transduction · Cell cycle · Neuronal cells · Inhibitors of cyclins/CDK complexes

Special issue article in honor of Prof. Abel Lajtha.

F. Cattaneo · R. Ammendola (✉)
Dipartimento di Biochimica e Biotecnologie Mediche, Università degli Studi di Napoli Federico II, Via S. Pansini 5, 80131 Naples, Italy
e-mail: rosario.ammendola@unina.it

G. Guerra
Dipartimento di Scienze per la Salute, Università degli Studi del Molise, Località Tappino, 86100 Campobasso, Italy

Introduction

From an evolutionary perspective, the formyl-peptide receptors family has a complex history and the number of family members varies significantly between species. In human there are three genes encoding two functional receptors, FPR (formyl-peptide receptor) and FPRL1 (formyl-peptide receptor-like 1), and the putative receptor FPRL2 (formyl-peptide receptor-like 2) which encodes a putative protein with 56% amino acid sequence identity to human FPR and 83% to FPRL1 [1–3]. Although a number of functional studies of FPRs were performed by using neutrophils and monocytes, the expression of these receptors have been demonstrated in other cell types. For instance, epithelial cells, follicular cells of the thyroid, cortical cells of adrenal, hepatocytes, Kupffer cells, neurons, astrocytoma cell lines, A549 cells, brain and nervous sytem express either or/and both FPR or/and FPRL1 [4–8].

The important biological implications of FPRs in neuronal cells and tissue are illustrated by the identification of host-derived agonists that are associated with various pathophysiological settings. These agonists include β-amyloid peptide (Aβ42) [9], the prion protein fragment $PrP_{106–126}$ [10], humanin [11] and annexin I (ANXA1) [12]. All these molecules elicit pro-inflammatory responses through the use of FPRL1 as a receptor. Aβ42 and $PrP_{106–126}$ are amyloidogenic and are involved in Alzheimer's disease (AD) [13] and prion diseases [10, 14], respectively. FPRL1 has also reported to interact with a lipid metabolite lipoxin A4 (LXA4) in neuronal cells [15], showing an inhibitory effect on the expression of pro-inflammatory chemokines.

Binding to FPRs of these agonists triggers intracellular signaling cascades involved in the regulation of angiogenesis, cell proliferation, protection against apotosis and neuroendocrine functions, such as the inhibition of the

Published online: 02 November 2010

exocytosis. Unlike FPR, the signal transduction pathways mediated by FPRL1 have not been extensively studied. Following activation by ligand, FPRs undergo rapid serine and threonine phosphorylation, and are desensitized and internalized [16, 17]. However, FPRs internalization can occur in the absence of internalization, indicating that desensitization and internalization are controlled by distinct mechanisms [18, 19]. Further studies suggest that FPRs internalization is mediated by mechanisms independent by arrestin, dynamin and clathrin [20].

Recent findings have demonstrated a central role of FPRs also in the regulation of cell cycle in different cell types [20–26]. These receptors act by increasing protein levels of some inhibitors of cyclins/CDK complexes (CKI), such as $p21^{waf1/cip1}$ and $p16^{INK4A}$ and $p27^{kip1}$, in parallel with the inhibition of the expression of cyclins. Here we review and discuss some of the most significant results on: (i) the expression of FPRs in neuronal cells and tissues; (ii) the intracellular signaling triggered by these receptors; (iii) the control of the cell cycle progression exerted by some members of the FPR family.

Formyl-Peptide Receptors

The formyl-peptide receptors FPR, FPRL1 and FPRL2, expressed in human cells, belong to the G protein-coupled receptor (GPCR) family [1]. The FPR gene spans 6 kb with an intronless open reading frame interrupted by an intron in its 5'-untranslated region [27]. Both FPRL1 and FPRL2 are single-copy genes with intronless open reading frames, which co-localize with FPR in a cluster on the chromosomal region 19q13.3 [1]. FPR and FPRL1 were first detected in phagocytic leukocytes, and FPRL2 was first found in monocytes and in dendritic cells [28].

The murine FPR gene family includes at least six members: Fpr1 (mFPR1) codes for the murine orthologue of human FPR; Fpr-rs2 and Fpr-rs1 encode receptors that are strucurally and functionally similar to human FPRL1 [29]: Fpr-rs2 encodes mFPR2, which is a N-formyl-Met-Leu-Phe (N-fMLP) receptor, whereas the product of murine gene Fpr-rs1 is a receptor for lipoxin A4 (LXA4), a lipid derivative of arachidonate metabolism [30]. The other murine FPRs encode putative receptors which ligands have not been yet identified.

FPR binds N-fMLP with high efficiency, whereas FPRL1 is defined as a low-affinity N-fMLP receptor, based on its activation only by micromolecolar concentrations of formyl-peptide. FPRL1 is the only receptor whose ligands include both a formyl-peptide and a lipid, as demonstrated by the observation that it also binds LXA4 [31]. FPRL2 does not respond to formyl-peptides and it was reported to be a low affinity receptor for several FPRL1 agonists [32].

In addition to N-fMLP, several non-formylated peptides, that preferentially activate either or both FPR and FPRL1, have been identified. For instance, the synthetic hexapeptide WKYMVm (W peptide) binds FPRL1 with high efficiency, activating neutrophils and monocytes functions including chemotaxis, mobilization of complement receptor-3, cytokines release and NADPH oxidase activation [33]. We demonstrated that in IMR90 human fibroblasts, binding of WKYMVm to FPRL1, which is expressed in these cells [34], induces superoxide generation consequent to MEK- and PTX-dependent serine phosphorylation and membrane translocation of the regulatory cytosolic NADPH oxidase subunit $p47^{phox}$ [34]. In the same cells WKYMVm activates also selected protein kinase C (PKC) isoforms required for NADPH oxidase-dependent superoxide generation [35].

MMK-1, another non-formylated peptide, is a potent and very specific agonist for FPRL1, as demonstrated by its ability to induce superoxide generation in neutrophils [36] and calcium mobilization in phagocytic leukocytes [37].

In addition to exogenous agonists, many findings have been focused to identify host-derived molecules that interact with formyl-peptide receptors. A clear evidence of an endogenous agonist for FPR is represented by ANXA1 and its N-terminal domains, which specifically and significantly interfere with neutrophil transmigration [38]. Nevertheless, FPRL1 seems to interact with a greater number of host-derived agonists, including the acute-phase protein serum amloid A (SAA) [39], the 42-amino acid form of amyloid β (Aβ42) [9], the prion protein fragment $PrP_{106–126}$, [10], LXA4 [31], and LL-37, an enzymatic cleavage fragment of the cathelicidin [40]. We demonstrated that in IMR90 cells LL-37 triggers FPRL1-mediated induction of superoxide, via NADPH oxidase activation [41]. Finally, humanin, a 14–22 peptide encoded by a gene isolated from an apparently normal region from Alzheimer's disease (AD) brain, shares FPRL1 with Aβ42 on neuronal cells [11].

Formyl-Peptide Receptors Activation

FPR, FPRL1 and FPRL2 are coupled to the Gi family of G proteins, as indicated by the total loss of cell response to their agonists upon exposure to pertussis toxin (PTX) [42, 43]. FPR can also couple to G_o, $G_{\alpha 16}$ and to PTX-resistant G_z [44].

In polymorphonuclear (PMN) cells, binding of N-fMLP to FPR or FPRL1 induces an intracellular complex program that results in cell migration, reorganization of the actin cytoskeleton and NADPH oxidase activation, thereby triggering signal transduction pathways leading to a chemotactic response and superoxide generation, respectively.

After N-fMLP binding, activated heterotrimeric G-protein dissociates into α and $\beta\gamma$ subunits, thereby activating phospholipase C (PLC) and phosphoinositide 3-kinase (PI3K). This, in turn, converts the membrane phosphatidylinositol 4,5-bisphosphate (PIP2) into phosphatidylinositol 3,4,5-triphosphate (PIP3). PLC converts PIP3 in diacylglycerol (DAG) and inositol-1,4,5-triphosphate (IP3), which regulates calcium mobilization from endoplasmic reticulum stores. DAG activates PKC isoforms. PI3K (γ isoform) can also triggers the activation of the PI3K-Akt/PKB pathway. In PMN cells other intracellular effectors coupled to the signaling cascade of FPRs include phospholipases A2 and D (PLD), ERK1/2, lyn, p125FAK, Jun kinase (JNK) and p38MAPK. PKC isoforms, ERKs and p38MAPK are involved in the phosphorylation on multiple serine residues of the regulatory subunit $p47^{phox}$ of NADPH oxidase in vivo and in vitro [45]. CD38, a transmembrane glycoprotein which catalyzes the production of cyclic ADP-ribose from its substrate NAD^+, is an essential and specific transducer of N-fMLP signals in mouse neutrophils, as demonstrated by the observation that neutrophils from $CD38^{-/-}$ mice fail to migrate in response to N-fMLP [46].

Fprs Expression in Neuronal Tissues and Cells

The observation that FPR and FPRL1 have been identified in several other cell types and tissues suggests that they exert functions other than those exerted in PMN cells, probably elicited by endogenous ligands associated with human diseases.

By using immunocytochemical approaches, FPR was detected in human brain, spinal cord, anterior horn cells and hypoglossal nucleus neurons [4]. Cerebellar system, the neuropil, the sensory system, many large reticular activating system neurons, choroid plexus epithelium, ependyma, and vascular smooth muscle stained intensively. Many pyramidal cell neurons, the extrapyramidal motor system, the sympathetic portion of the autonomic nervous system, the parasympathetic system, the hippocampal neurons, many end-plate pyramidal cells, some astrocytes and pial astrocytes moderately stained with the antibody [4]. Schwann cells of the peripheral nervous system stained positively but the oligodendrocytes were negative. Interestingly, FPR antigen was not detectable in microglia, the modified macrophages of the brain [4].

By conducting a high throghput screen for GPCR expressed in mouse vomeronasal organ (VNO), which detects pheromones and other semiochemicals, 5 of 7 members of the FPR family, have been recently identified [47]. The expression patterns of the VNO-FPRs are remarkable similar to those of V1Rs and V2Rs, the two known families of chemoreceptors. Each FPR is selectively expressed in a different small subset of neurons that are highly dispersed in the neuroepithelium, consistently express $G_{\alpha i2}$ or G_0, and appear to lack other chemosensory receptors [47]. These finding suggest that VNO-FPRs are likely to function as chemosensory receptors, probably associated with the identification of pathogens, or of pathogenic states [48].

By RT-PCR analysis, mRNAs for three FPR family members, Fpr1, Fpr-rs1 and Fpr-rs2, have been identified in the mouse hippocampus, hypothalamus, anterior pituitary and adrenal gland [12]. In addition, the hypothalamus and anterior pituitary gland also express Fpr-rs6 and Fpr-rs7 mRNAs [12]. These receptors play a key role in the regulation of neuroendocrine functions mediated by ANXA1. In the pituitary gland, ANXA1 has a well defined role as a cell–cell mediator of the inhibitory effects of glucocorticoids (GC) on the secretion of corticotropin (ACTH). ANXA1 inhibits the evoked release of ACTH and this effect is mediated by Fpr-rs1 or by a closely related receptor [12]. In the mouse brain, which expresses Fpr-rs2, ANXA1 or a short ANXA1 petidomimetic (Ac_{2-26}) exert significant protection in the microcirculation [49]. The ANXA1-mediated cerebroprotection is associated with a marked attenuation of cell adhesion and of markers of inflammation. Ac_{2-26} acts by binding Fpr-rs2, as demonstrated by displacement assays with transfected cells, in vivo experiments with transgenic mice and receptor agonists [49].

The 1321N1 human astrocyte cell line expresses a functional FPRL1 both at mRNA and protein level [15]. In these cells, LXA4, which efficiently binds FPRL1 [31], has an inhibitory effect on the expression of the proinflammatory chemokine IL-8 and adhesion molecule ICAM-1 in response to IL-1β [15]. LXA4 also regulates proliferation and differentiation of murine neural stem cells (NSC) isolated by embryo brains [26]. These cells express Fpr-rs2 and LXA4 signaling triggered by this receptor appears to tightly regulate the expansion and contraction of NSC mass by acting as "accelerator and brake" after pathological events in brain tissue [26]. These results suggest the potential therapeutic utility of LXA4 for a wide range of neuropathological disorders, wherein specifically regulating neurogenesis and neuroinflammation may be beneficial.

FPRL1 is expressed at high levels by inflammatory cells infiltrating senil plaques in brain tissues of AD patients [9], where Aβ42 can induce NADPH oxidase-dependent superoxide production via FPRL1, thus generating an oxidative stress in microglial cells [50]. The internalization of Aβ42 is mediated by FPRL1 and requires PLD in the endocytosis process [51]. By co-immunoprecipitation and fluorescence microscopy, a physical interaction between FPRL1 and MARCO (macrophage receptor with

collagenous structure) has been observed in astrocytes and in microglia [52]. The expression of FPRL1-MARCO complex increases in glial cells playing an important role for binding at cell surface and subsequent internalization of Aβ42 [52]. Stimulation of microglial cell line N9 with IFN-γ induces the expression of high levels of mFPR2 in association with increased cell migration in response to Aβ42 [53]. IFN-γ also increases the endocytosis of Aβ42 by microglial cells via mFPR2 [53]. Furthermore, an Aβ42 conformation-dependent mFPR2 overexpression is observed in mouse primary microglial cells and in murine microglial cell line MMGT12 [54]. This suggests that FPRs expression is not only constitutive but can be also induced by specific agonists.

Humanin was originally identified as an anti-apoptotic peptide that rescued neuronal cells from apoptosis induced by presenilin mutants associated with familial AD and by Aβ42 [55]. The human neuroblastoma cell lines SK-N-MC and SK-N-SK, and the mouse microglial cell line N9 express FPRL1 and the murine orthologue mFPR2, respectively [11]. In these cells the protective effect of humanin from damage by Aβ42 requires the PTX-sensitive activation of FPRL1, which is a functional receptor shared by humanin and Aβ42 [11]. Therefore, the neuroprotective activity of humanin may be attributed to its competitive occupation of FPRL1.

FPRL1 is also involved in proinflammatory processes of prion disorders which, similar to AD, include the infiltration and activation of mononuclear phagocytes in brain lesions. The 21 aminoacid fragment of the aberrant human prion protein, $PrP_{106–126}$ can form fibrils in vitro and elicits a diverse array of inflammatory responses. $PrP_{106–126}$ is an agonist for FPRL1 in glial cells [10] and induces an increase of proinflammatory cytokines, tumor necrosis factor-α and IL-6, which are implicated as neurotoxic mediators. Also in this case, PLD activity is essential for $PrP_{106–126}$-endocytosis [56] and, similar to Aβ42, in astrocytes and microglia the internalization of $PrP_{106–126}$ is mediated by FPRL1 [56]. The identification of FPRL1 as a functional receptor for Aβ42 and $PrP_{106–126}$ provides a molecular link in the chain of proinflammatory responses observed in AD and in prion diseases.

Fprs Signaling in Neuronal Tissues and Cells

In neuronal cells, binding of different agonists to FPRs triggers multiple intracellular signaling pathways necessary to regulate important biological functions. The possible involvement of FPRL1 in the regulation of astrocytosis has major biological implications, because reactive astrocytosis and brain inflammation are pathological features of many neurodegenerative diseases.

A linkage among FPRL1, MAPK, astrocytes activation and the inflammatory response has been described in U-87 cells, where the activation of FPRL1 with the highly potent agonist WKYMVm induces JNK and ERKs phosphorylation [57]. FPRL1-evoked MAPK activation depends on $G_{i/0}$ proteins and Src family tyrosine kinase activation, but is independent of PI3K and PKC. Interestingly, stimulation of FPRL1 in these cells augments the expression of glial fibrillary acidic protein (GFAP) and IL-1α, which are correlated with reactive astrocytosis [57]. Moreover, inhibition of $G_{i/0}$ proteins and JNK completely abolishes both GFAP and IL-1α up-regulations by FPRL1, while blockade of the MEK/ERK cascade exclusively suppress the GFAP production [57]. In the same cells, WKYMVm also promotes $G_{i/0}$-dependent IKK (inhibitor kB kinase) phosphorylation. This requires ERKs, PI3K and cSrc activations, but not p38MAPK, JNK or calcium mobilization [58]. PI3K activates PKB/Akt which, in turn, induces the transcriptional function of NFkB by stimulating the transactivation of RelA/p65 subunit. In U-87 cells a FPRL1-dependent NFkB-driven luciferase expression is observed [58]. Interestingly, cholesterol depletion abolishes IKK phosphorylation, denoting the important role of lipid raft integrity in the FPRL1/IKK signaling [58]. In N-fMLP- stimulated U-87 cells, the activation of signaling molecules triggered by FPR, including ERKs, p38MAPK, JNK and Akt are significantly attenuated by the lipoxygenase inhibitor Nordy [59].

The role of FPRL1 in regulating immune responses is further suggested by the observation that, in 1321N1 astrocytoma cells, LXA4 exerts anti-inflammatory effects, at least in part, via an NFkB-dependent mechanism [15]. In fact, LXA4 inhibits IL-1β-induced degradation of IkBβ and modulates IL-1β activation of NFkB-regulated reporter gene expression via FPRL1 [15].

FPRs have been also implicated in the cellular signaling regulating neuroendocrine functions. In the hypothalamo-pituitary-adrenocortical axis, ANXA1 induces the inibition of corticotrophin-release hormone (CRH)-driven ACTH secretion [60]. The signaling mechanisms used by FPRL1, the ANXA1 receptor, impinge on the CRH-driven prosecretory cascade at a late stage, compromising the SNARE proteins interaction which is critical to exocytosis [60]. At least three molecular mechanisms have been suggested for the neuroendocrine regulation of FPRL1: (i) ANXA1 activates PTX-sensitive and $G_{\alpha 1}$-mediated activation of PLC, increased intracellular calcium concentration and PLD activation; (ii) FPRL1 may couple also to G_z protein which signaling induces a long-lasting blockade of exocytosis in pituitary cells; (iii) FPRL1 signals via $\beta\gamma$ G proteins to impair exocytosis [61].

Signal transduction pathways triggered by FPRs play a key role in neurodegenerative disorders. In rat glial cells

PLD activity is essential for Aβ42 endocytosis and is required for Aβ42-induced ERKs phosphorylation [54]. The central role of ERKs in FPRL1 signaling is further supported by the observation that, in murine microglial cells, Aβ42-induced signal transduction strongly depends on phosphorylation mechanisms mediated by MAPK and that FPRL1 deactivation by antagonists or by siRNA inhibits Aβ42-induced ERKs phosphorylation [52]. The neuroprotective effect of humanin on Aβ42 requires the FPRL1-mediated activation of STAT3 transcription factor and of several tyrosine kinases [62].

FPRs signaling also partecipate in the progression of malignant tumors. In human glioblastoma cell lines U-87 and SNB75, which express FPR [63], N-fMLP induces a rapid and transient phosphorylation of ERKs, JNK, p38MAPK and Akt, as well as of STAT3 both at Tyr705 and Ser 727 residues [63]. N-fMLP-treated cells also show elevated levels of VEGF protein secreted in the medium, augmented DNA synthesis and higher levels of Bcl2. These finding suggest a contribute of FPR to glioma cell motility, proliferation and tumorigenicity. The role of FPR in these events is further supported by its ability to transactivate the Epidermal Growth Factor Receptor (EGFR), exacerbating the malignant behaviour of human glioma cells [64, 65]. In these cells N-fMLP induces PTX-sensitive EGFR phosphorylation at Tyr992 residue which is prevented by a Src tyrosine kinase inhibitor, suggesting that cSrc plays a key role in bridging the signal transduction from FPR to EGFR. These results strongly indicate that both FPR and EGFR play important roles in tumor cell growth and tumorigenesis, and that the two receptors cooperate to potentiate the proliferation of glioblastoma cells. Furthermore, the potential therapeutical implications of these findings suggest that targeting both receptors may yeld superior therapeutic effects compared with targetin either one receptor.

FPRs and Cell Cycle

Many evidences suggest that binding of specific agonists to FPRs inhibit proliferation in different cell lines. LXA4 prevents cell growth of renal mesangial cells induced by leukotriene D4 (LTD4) or platelet-derived growth factor [21]. LXA4 blocks LTD4-induced PI3K activity and triggers MEK1/ERK pathway, through which inhibition of cell proliferation is effected [21, 22].

The inhibitory effects of LXA4 on cell cycle have been also observed in human lung fibroblasts (HLF) stimulated by connective tissue growth factor (CTGF) [23]. In these cells, which express FPRL1, LXA4 supresses CTGF-stimulated phosphorylation of PI3K and PKB/Akt, modulates CTGF-activated phosphorylation of ERKs, down-regulates CTGF-evoked DNA-binding activity of STAT3 and inhibits the expression of cyclin D1. Pretreatment with PTX prevents, and over-expression of FPRL1 enhances, the inhibitory effects of LXA4 on CTGF-induced proliferation of HLF, suggesting that FPRL1 mediates the action of LXA4 on these cells [23].

A similar effect was observed in rat mesangial cells stimulated with TNFα [24]. Here, the TNFα-induced marked increments in mRNA expression and protein synthesis of cyclin E, in parallel with cell proliferation, are down-regulated by LXA4. TNFα also enhances DNA-binding activity of STAT3 and induces PKB/Akt phosphorylation at Thr308 residue, which are prevented by LXA4. Furthermore, TNFα-induced decrement in expression of $p27^{kip1}$ is ameliorated by LXA4 in a dose-dependent manner [24]. The LXA4-mediated accumulation of CDK inhibitors (CKIs) is also observed in human mesangial cells, where LXA4 significantly inhibits PDGF-stimulated proliferation [25]. In these cells lipoxins modulate PDGF-induced decrements in the levels of $p27^{kip1}$ and $p21^{waf1/cip1}$, and promotes nuclear retention of these CKIs [25].

The LXA4–induced signaling pathway also inhibits cell growth of neural stem cells [26]. LXA4-mediated alterations in cell proliferation include several molecules involved in cell cycle and growth, such as EGFR and cyclin E, which are down-regulated, and $p27^{kip1}$, which protein levels are increased [26].

We observed that the exposure to N-fMLP of human glioblastoma cell line U-87, induces $p21^{waf1/cip1}$ and $p16^{INK4}$ accumulation in a time-dependent manner (Fig. 1a). $p21^{waf1/cip1}$ has been shown to have bimodal effects on cell cycle progression and cell proliferation. In fact, $p21^{waf1/cip1}$ inhibitory activity is associated with nuclear localization, whereas cytosolic $p21^{waf1/cip1}$ increases cell cycle transit [66]. Therefore, we investigated its localization and we found that N-fMLP-induced $p21^{waf1/cip1}$ accumulation is predominantly nuclear, thus suggesting its involvement in cell cycle arrest (Fig. 1b). In several cell types the increased protein level of $p21^{waf1/cip1}$ is independent of p53 and is the consequence of the activation of Ras-ERK pathway [67]. Furthermore, many evidences demonstrate that the exposure of cells to oxidants results in the activation of the Ras-MAPK pathway. This effect is reversible, is prevented by antioxidants and is very similar to that provoked by the exposure of resting cells to growth factors [68]. We observed that, in U-87 cells N-fMLP induces ERKs phosphorylation, which is inhibited by the MEK inhibitor PD098059 (Fig. 2a) and by pre-incubation with apocynin, which prevents the translocation of $p47^{phox}$ on the membrane inhibiting the NADPH oxidase complex in phagocytic and non-phagocytic cells [69, 70] (Fig. 2a). The N-fMLP-induced $p21^{waf1/cip1}$ accumulation observed in U-87 cells is also prevented by PD098059 and apocynin

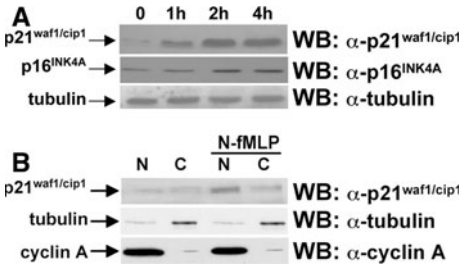

Fig. 1 N-fMLP induces CKIs accumulation. (**a**) U-87 cells (ATCC, Manassas VA) were exposed to 1 μM N-fMLP (PRIMM, Milan, Italy) for the indicated times. Cells were rinsed with phosphate-buffered saline (PBS) buffer and cell lysates were purified as previously described [34]. Protein extracts, containing 20 μg of proteins, were resolved on 10% SDS–PAGE (Bio-Rad, Richmond, CA, USA) and $p21^{waf/cip}$ and $p16^{INK4}$ were detected by using specific antibodies (Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA, sc-397 and sc-65224, respectively). The same filter was reprobed with an anti-tubulin antibody (Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA, sc-8035). (**b**) Cells were exposed to PBS or to 1 μM N-fMLP for 2 h and Nuclear (*N*) and cytosolic (*C*) proteins were purified by using the Nuclear/Cytosol Fractionation kit (BioVision, Inc., USA), accordingly to manufacturer's instructions. Proteins (20 μg) were resolved on 10% SDS–PAGE and subjected to immunoblotting analysis with an anti-$p21^{waf/cip}$ antibody, as previously described [34]. An anti-tubulin antibody and anti-cyclin A antibody (Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA, sc-751) served as a control of cytosolic and nuclear proteins loading, respectively. Antigen–antibody complexes were detected with an ECL chemiluminescence reagent kit (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, UK)

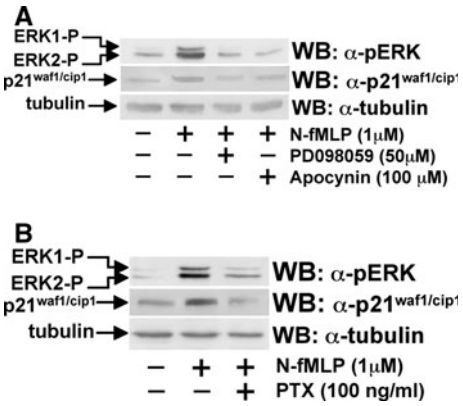

Fig. 2 N-fMLP-induced $p21^{waf/cip}$ accumulation is prevented by PD098059 and Apocynin and is mediated by FPR. (**a**) Cell lysates were purified from U-87 cells exposed to 1 μM N-fMLP for 2 h, or preincubated with 50 mM PD098059 for 90 min (Calbiochem, La Jolla, CA, USA), or with 100 mM Apocynin (Sigma, St. Louis, MO, USA) for 2 h, before stimulation. Twenty micrograms of proteins were resolved on 10% SDS–PAGE and ERKs phosphorylation and $p21^{waf/cip}$ accumulation were detected by western blot with an anti-phosphoERKs (a-pERK, Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA, sc-7383) or an anti-$p21^{waf/cip}$ antibody, respectively. The same filter was reprobed with an anti-tubulin antibody, as a control of protein loading. (**b**) U-87 cells were exposed to 1 μM N-fMLP for 2 h, or preincubated with 100 ng/ml of pertussis toxin (PTX) (Sigma, St. Louis, MO, USA) for 12 h before stimulation. Total proteins (20 μg) were electrophoresed and transferred to immobilion P membranes (Millipore, Bedford, MA). The blot was incubated with an anti-phosphoERKs antibody or an anti-$p21^{waf/cip}$ antibody. An anti-tubulin antibody served as a control of protein loading. The arrows indicate the phosphorylated forms of $p44^{MAPK}$ (ERK1-P) and $p42^{MAPK}$ (ERK2-P), respectively

(Fig. 2a), suggesting that it requires the activation of MEK-ERK cascade and NADPH oxidase-dependent superoxide generation. We also examined the role of FPR in the N-fMLP-induced signalling cascade and we found that preincubation with PTX prevents both ERKs phosphorylation and $p21^{waf1/cip1}$ accumulation, as a consequence of the $G_{\alpha i}$-specific inhibition (Fig. 2b). Taken together our results suggest that in U-87 cells binding of a specific agonist to FPR inhibits cell cycle progression through increased protein levels of $p16^{INK4}$ and PTX-, NADPH oxidase- and MEK-dependent accumulation of $p21^{waf1/cip1}$.

Future Directions

During recent years, much progress has been made in the understanding of the biological roles played by formyl-peptide receptors in the brain and in neuronal cells. FPRs family has complex functional properties, partly due to their high promiscuity, but also because their activation can stimulate several signal transduction pathways depending on the ligand, its concentration and the cell type involved. FPR and FPRL1 can be activated by a wide variety of unrelated ligands that can be also generated during pathological conditions, and respond to synthetic ligands which are very useful for pharmacological studies. In particular, the use of FPRL1 by SAA, Aβ42 and $PrP_{106–126}$ suggests that this receptor may play a crucial role in pro-inflammatory aspects of systemic amyloidosis, AD and prion diseases. On the other hand, LXA4 shows inhibitory effect on the expression of pro-inflammatory chemokines via FPRL1 and may represents a strategy in the treatment of acute and chronic brain inflammation. Future research will need to address the issue of how the same receptor can bind such structurally diverse ligands, ranging from small peptides to large proteins to lipids. The understanding of the molecular mechanisms of interaction and whether the various ligands bind by shared or unique domains, is the assumption for the development of specific antagonists. The role of FPRs in the definition of different biological responses, including the FPRL1-mediated neuroendocrine responses to bacterial/viral infections and to locally generated anti-inflammatory eicosanoids (e.g., lipoxins) requires further investigations.

Further study is also required to define the relationship between the FPR expression and the progression of human primary gliomas and to identify the mechanicistic basis for the control of FPR expression in highly malignant human

glioma cells. Nevertheless, FPR and its signaling pathway may be candidate molecular target for developing novel therapeutics to treat gliomas, including the specific blockade of EGFR signaling depending on FPR transactivation by agonist. Such pharmacological strategies presumably produce less pronunced side effects than approaches based on direct EGFR inhibition by antagonists or tyrosine kinase inhibitors.

Binding of specific agonists to FPRs triggers several intracellular signaling cascades in neuronal cells, including PI3K-Akt, Ras-ERK, PKC and STAT pathways which play key role in angiogenesis, cell proliferation, protection against cell death and in the tight regulation of NADPH oxidase activity. The complete dissection of the intracellular signaling pathways triggered by different agonists will clarify the role of formyl-peptide receptors in neuronal cells in both human physiology and disease.

Acknowledgments We are greatful to the eminent scientist Prof. Abel Lajtha for being founder and Editor in Chief of Neurochemical Research, now in its 34th year. This work was supported by grants from Ministero dell'Università e della Ricerca Scientifica e Tecnologica PRIN 2007 "Attivazione dei recettori per formil-peptidi e regolazione della NADPH ossidasi in linee cellulari tumorali umane non fagocitiche". We thank Jean Ann Gilder (Scientific Communication srl) for text editing.

References

1. Bao L, Gerard NP, Eddy RL Jr et al (1992) Mapping of genes for the human C5a receptor (C5AR), human FMLP receptor (FPR), and two FMLP receptor homologue orphan receptors (FPRH1, FPRH2) to chromosome 19. Genomics 13:437–440
2. Murphy PM, Ozcelik T, Kennedy RT et al (1992) A structural homologous of the N-formyl-peptide receptor. Characterization and chromosomal mapping of a peptide chemoattractant receptor family. J Biol Chem 267:7637–7643
3. Ye RD, Cavanagh SL, Quehenberger O et al (1992) Isolation of a cDNA that encodes a novel granulocyte N-formyl peptide receptor. Biochem Biophys Res Comm 184:582–589
4. Becker EL, Forouhar FA, Grunnet ML et al (1998) Broad immunocytochemical localization of the formylpeptide receptor in human organs, tissues and cells. Cell Tissue Res 292:129–135
5. McCoy R, Haviland DL, Molmenti EP et al (1995) N-formyl-peptide and complement C5a receptors are expressed in liver cells and mediate hepatic acute phase gene regulation. J Exp Med 182:207–217
6. Rescher U, Danielczyk A, Markoff A et al (2002) Functional activation of the formyl peptide receptor by a new endogenous ligand in human lung A549 cells. J Immunol 169:1500–1504
7. Le Y, Hu J, Gong W et al (2000) Expression of functional formyl peptide receptors by human astrocytoma cell lines. J Neuroimmunol 111:102–108
8. Sozzani S, Sallusto F, Luini W et al (1995) Migration of dendritic cells in response to formylpeptides, C5a, and a distinct set of chemokines. J Immunol 155:3292–3295
9. Le Y, Gong W, Tiffany HL et al. (2001) Amyloid (beta)42 activates a G-protein-coupled chemoattractant receptor, FPR-like-1. J Neurosci 21, RC123:1–5
10. Le Y, Yazawa H, Gong W et al (2001) The neurotoxic prion peptide fragment Prp(106–126) is a chemotactic agonist for the G protein-coupled receptor formyl-peptide receptor-like1. J Immunol 166:1448–1451
11. Ying G, Iribarren P, Zhou Y et al (2004) Humanin, a newly identified neuroprotective factor, uses the G protein-coupled formylpeptide receptor-like 1 as a functional receptor. J Immunol 172:7078–7085
12. John CD, Sahni V, Mehet D et al (2007) Formyl peptide receptors and the regulation of ACTH secretion: targets for annexin A1, lipoxins, and bacterial peptides. FASEB J 21:1037–1046
13. Lambert MP, Barlow AK, Chromy BA et al (1998) Diffusible, non fibrillar ligands derived from Abeta 1–42 are potent central nervous system neurotoxins. Proc Natl Acad Sci USA 95:6448–6453
14. Brown DR, Schmidt B, Kretzschmar HA (1996) Role of microglia and host prion protein in neurotoxicity of a prion protein fragment. Nature 6:15–24
15. Decker Y, McBean G, Godson C (2009) Lipoxin A4 inhibits IL-1beta-induced IL-8 and ICAM-1 expression in 1321N1 human astrocytoma cells. Am J Physiol Cell Physiol. 296:C1420–C1427
16. Ali H, Richardson RM, Tomhave ED et al (1993) Differences in phosphorylation of formylpeptide and C5a chemoattractant receptors correlated with differences in desensitization. J Biol Chem 268:24247–24254
17. Tardif M, Mery L, Brouchon L et al (1993) Agonist-dependent phosphorylation of N-formylpeptide and activation peptide from the fifth component of C (C5a) chemoattractant receptors in HL60 cells. J Immunol 150:3534–3545
18. Prossnitz ER (1997) Desentization of N-formyl peptide receptor-mediate activation. J Biol Chem 272:15213–15219
19. Hsu MH, Chiang SC, Rd Ye et al (1997) Phosphorylation of the N-formyl peptide receptor is required for receptor internalization but not chemotaxis. J Biol Chem 272:29426–29429
20. Bennett TA, Maestas DC, Prossnitz ER (2000) Arrestin binding to G protein-coupled N-formyl peptide receptor is regulated by the conserved "DRY" sequence. J Biol Chem 32:24590–24594
21. McMahon B, Stenson C, McPhillips F et al (2000) Lipoxin antagonizes the mitogenic effects of leukotriene D4 in human renal mesangial cells. Differential activation of MAP kinases through distinct receptors. J Biol Chem 275:27566–27575
22. Mc Mahon B, Mitchell D, Shattock R et al. (2002), Lipoxin, leukotriene, and PDGF receptors cross-talk to regulate mesangial cell proliferation. FASEB J 10.1096/fj.02-0416fje
23. Wu SH, Wu XH, Lu C et al (2006) Lipoxin A4 inhibits proliferation of human lung fibroblasts induced by connective tissue growth factor. Am J Respir Cell Mol Biol 34:65–72
24. Wu SH, Lu C, Dong L et al (2005) Lipoxin A4 inhibits TNF-alpha-induced production of interleukins and proliferation of rat mesangial cells. Kideny Int 68:35–46
25. Mitchel D, Rodgers K, Hanly J et al (2004) Lipoxins inhibit Akt/PKB activation and cell cycle progression in human mesangial cells. Am J Pathol 164:937–946
26. Wada K, Arita M, Nakajima A et al (2006) Leukotriene B4 and lipoxin A4 are regulatory signals for neural stem cell proliferation and differentiation. FASEB J 20:1785–1792
27. Perez HD, Holmes R, Kelly E et al (1992) Cloning of the gene coding for the human receptor for formyl-peptides. Characterization of a promoter region and evidence for polymorphic expression. Biochemistry 31:11595–11599
28. Durstin M, Gao JL, Tiffany HL et al (1994) Differential expression of members of the N-formylpeptide receptor gene-cluster in human phagocytes. Biochem Biophys Res Commun 201:174–179
29. Hartt JK, Barish G, Murphy PM et al (1999) N-formylpeptides induce two distinct concentration optima for mouse neutrophil

chemotaxis by differential interaction with two N-formylpeptide receptor (FPR) subtypes. Molecular characterization of FPR2, a second mouse neutrophil FPR. J Exp Med 190:741–747
30. Takano T, Fiore S, Maddox JF et al (1997) Aspirin-triggered 15-epi-lipoxin A4 (LXA4) and LXA4 stable analogues are potent inhibitors of acute inflammation: evidence for anti-inflammatory receptors. J Exp Med 185:1693–1704
31. Fiore S, Maddox JF, Perez HD et al (1994) Identification of a human cDNA- encoding a functional high-affinity lipoxin A(4) receptor. J Exp Med 180:253–260
32. Le Y, Oppenheim JJ, Wang JM (2001) Pleiotropic roles of formyl peptide receptors. Cytokine Growth Factor Rev 12:91–105
33. Bae YS, Kim Y, Kim Y et al (1999) Trp-Lys-Tyr-Met-Val-D-Met is a chemoattractant for human phagocytic cells. J Leukoc Biol 66:915–922
34. Ammendola R, Russo L, De Felice C et al (2004) Low- affinity receptor-mediated induction of superoxide by N-formyl-methionyl-leucyl-phenylalanine and WKYMVm in IMR90 human fibroblasts. Free Rad Biol Med 36:189–200
35. Iaccio A, Collinet C, Montesano Gesualdi N et al (2007) Protein kinase C-α and–δ are required for NADPH oxidase activation in WKYMVm-stimulated IMR90 fibroblasts. Arch Biochem Biophys 459:288–294
36. Karlsson J, Stenfeldt AL, Rabiet MJ et al (2009) The FPR2-specific ligand MMK-1 activates the neutrophil NADPH-oxidase, but triggers no unique pathway for opening of plasma membrane calcium channels. Cell Calcium 45:431–438
37. Hu JY, Le Y, Gong W et al (2001) Synthetic peptide MMK-1 is a highly specific chemotactic agonist for leukocyte FPRL1. Leukc Biol 70:155–161
38. Walther A, Riehmann K, Gerke V et al (2000) A novel ligand of the formyl peptide receptor: annexin I regulates neutrophil extravasation by interacting with the FPR. Mol Cell 5: 831–840
39. Su SB, Gon W, Gao JL et al (1999) A seven-transmembrane, G protein-coupled receptor, FPRL1, mediates the chemotactic activity of serum amyloid A for human phagocytic. J Exp Med 189:395–402
40. De Y, Chen Q, Schmidt AP et al (2000) LL-37, the neutrophil granule- and epithelial cell-derived cathelicidin, utilizes formyl peptide receptor-like 1 (FPRL1) as a receptor to chemoattract human peripheral blood neutrophils, monocytes, and T cells. J Exp Med 192:1069–1074
41. Iaccio A, Cattaneo F, Mauro M et al (2009) FPRL1-mediated induction of superoxide in LL-37-stimulated IMR90 human fibroblast. Arch Biochem Biophys 481:94–100
42. Gierschik P, Sidoropoulos D, Jakobs KH et al (1989) Two distinct Gi-proteins mediate formyl peptide receptor signal transduction in human leukemia (HL-60) cells. J Biol Chem 264:21470–21473
43. Wentel-Seifert K, Arthur JM, Liu HY et al (1999) Quantitative analysis of formyl peptide receptor coupling to g(i)alpha(1), g(i)alpha(2), and g(i)alpha(3). J Biol Chem 264:21470–21473
44. Tsu RC, Lai HWL, Allen RA et al (1995) Differential coupling of the formyl peptide receptor to adenylate cyclase and phospholipase C by the pertussis toxin-insensitive Gz protein. Biochem J 309:331–339
45. Iaccio A, Angiolillo A, Ammendola R (2008) Intracellular signaling triggered by formyl-peptide receptors in nonphagocytic cells. Curr Sign Transd Ther 3:88–96
46. Partida-Sanchez S, Cockaine DA, Monard S et al (2001) Cyclic ADP-ribose production by CD38 regulates intracellular calcium release, extracellular calcium influx and chemotaxis in neutrophils and is required for bacterial clearance in vivo. Nat Med 7:1209–1216
47. Liberles SD, Horowitz LF, Kuang D et al (2009) Formyl peptide receptors are candidate chemosensory receptors in the vomeronasal organ. Proc Natl Acad Sci USA 106:9842–9847
48. Rivière S, Challet L, Fluegge D et al (2009) Formyl peptide receptor-like proteins are a novel family of vomeronasal chemosensors. Nature 459:574–577
49. Gavins FN, Dalli J, Flower RJ et al (2007) Activation of annexin1 counter-regulatory circuit affords protection in the mouse brain microcirculation. FASEB J 21:1751–1758
50. Tiffany HL, Lavigne MC, Cui YH et al (2001) Amyloid-beta induces chemotaxis and oxidant stress by acting at formylpeptide receptor 2, a G protein-coupled receptor expressed in phagocytes and brain. J Biol Chem 276:23645–23652
51. Brandenburg LO, Konrad M, Wruck C et al (2008) Involvement of formyl-peptide-receptor-like-1 and phospholipase D in the internalization and signal transduction of amyloid beta 1–42 in glial cells. Neuroscience 156:266–276
52. Brandenburg LO, Konrad M, Wruck CJ et al (2010) Functional and physical interactions between formyl-peptide-receptors and scavenger receptor MARCO and their involvement in amyloid beta 1–42-induced signal transduction in glial cells. J Neurochem 113:749–760
53. Chen K, Iribarren P, Huang J et al (2007) Induction of the formyl peptide receptor 2 in microglia by IFN-gamma and synergy with CD40 ligand. J Immunol 178:1759–1766
54. Heurtaux T, Michelucci A, Losciuto S et al (2010) Microglial activation depends on beta-amyloid conformation: role of the formylpeptide receptor 2. J Neurochem 114:576–586
55. Hashimoto Y, Niikura T, Tajima H et al (2001) A rescue factor abolishing neuronal cell death by a wide spectrum of familial Alzheimer's diseases genes and Aβ42. Proc Natl Acad Sci USA 98:6336–6341
56. Brandenburg LO, Koch T, Sievers J et al (2007) Internalization of PrP106–126 by the formyl-peptide-receptor-like-1 in glial cells. J Neurochem 101:718–728
57. Kam AY, Tse TT, Kwan DH et al (2007) Formyl peptide receptor like 1 differentially requires mitogen-activated protein kinase for the induction of glial fibrillare acidic protein and interleukin-1alpha in human U87 astrocytoma cells. Cell Signal 19: 2106–2117
58. Kam AY, Liu AM, Wong YH et al (2007) Formyl peptide-receptor like-1 requires lipid raft and extracellular signal-regulated protein kinase to activate inhibitor-kappa B kinase in human U87 astrocytoma cells. J Neurochem 103:1533–1566
59. Chen JH, Yao XH, Gong W et al (2007) A novel lipoxygenase inhibitor Nordy attenuates malignant human glioma cell response to chemotactic and growth stimulating factors. J Neurooncol 84:223–231
60. John CD, Gavins FN, Buss N et al (2008) Annexin-1 and the formyl peptide receptor family: neuroendocrine and metabolic aspects. Curr Opin Pharmacol 8:765–776
61. Andric SA, Zivadinovic D, Gonzales-Iglesias AE et al (2005) Endothelin-induced, long lasting, and Ca^{++} influx-independent blockade of intrinsic secretion in pituitary cells by Gz subunits. J Biol Chem 280:26896–26903
62. Hashimoto Y, Suzuki H, Aiso S et al (2005) Involvement of tyrosine kinase and STAT3 in humanin-mediated neuroprotection. Life Sci 77:3092–3104
63. Zhou Y, Bian X, Le Y et al (2005) Formylpeptide receptor FPR and the rapid growth of malignant human gliomas. J Natl Cancer Inst 97:823–835
64. Huang J, Hu J, Bian X et al (2007) Transactivation of the epidermal growth factor receptor by formylpeptide receptor exacerbates the malignant behavior of human glioblastoma cells. Cancer Res 67:5906–5913

65. Huang J, Chen K, Chen J et al (2010) The G-protein-coupled formylpeptide receptor FPR confers a more invasive phenotype on human glioblastoma cells. Br J Cancer 102:1052–1060
66. Sherr CJ, Roberts JM (1999) CDK inhibitors: positive and negative regulators of G1-phase progression. Genes Dev 13:1501–1512
67. Russo T, Zambrano N, Esposito F et al (1995) A p53-independent pathway for activation of WAF1/CIP1 expression following oxidative stress. J Biol Chem 271:4138–4142
68. Ammendola R, Ruocchio MR, Chirico G et al (2002) Inibition of NADH/NADPH oxidase affects signal transduction by growth factor receptors in normal fibroblasts. Arch Biochem Biophys 397:253–257
69. Zhang Y, Chan MMK, Andrews MC et al (2005) Apocynin but not allopurinol prevents and reverses adrenocorticotropic hormone-induced hypertension in the rat. Am J Hypertens 18: 910–916
70. Barbieri SS, Cavalca V, Eligini S et al (2004) Apocynin prevents cyclooxygenase 2 expression in human monocytes through NADPH oxidase and glutathione redox-dependent mechanisms. Free Rad Biol Med 37:156–165