UNIVERSITY OF NAPLES FEDERICO II

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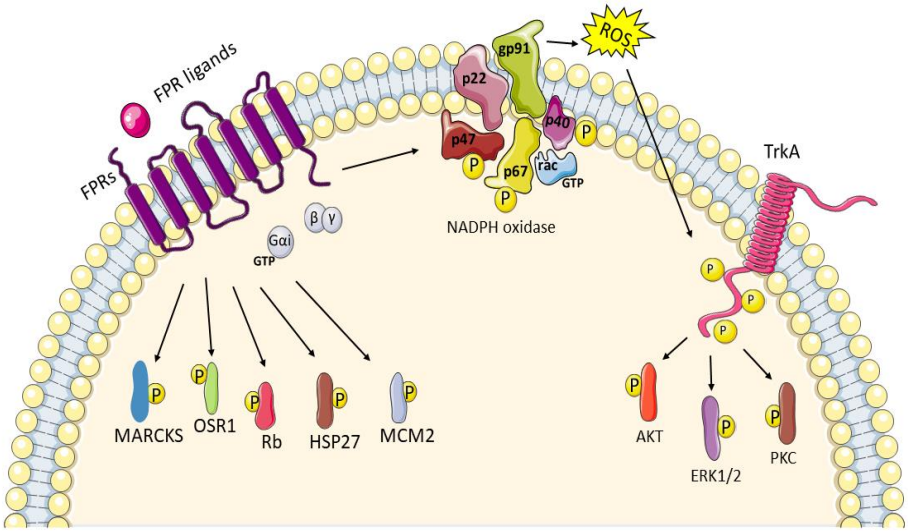
Martina Castaldo

Intracellular signaling cascades activated by Formyl Peptide Receptors



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List of abbreviations

ANXA1	Annexin A1
CSH	Cyclosporine H
DAG	Diacylglycerol
EGFR	Epidermal growth factor receptor
ERK1/2	Extracellular signal-regulated kinase 1/2
FPR1	Formyl peptide receptor 1
FPR2	Formyl peptide receptor 2
FPR3	Formyl peptide receptor 3
GPCR	G protein-coupled receptor
HSP-27	Heat shock protein-27
IP3	Inositol trisphosphate
LXA4	Lipoxin A4
MAPK	Mitogen-activated protein kinase
MARCKS	Myristoylated alanine-rich C-kinase substrate
MCM2	Minichromosome Maintenance Complex Component 2
MMP	Matrix metalloproteinases
NADPH oxidase	Nicotinamide adenine dinucleotide phosphate oxidase
N-fMLP	N-formylmethionyl-leucyl-phenylalanine
NGF	Nerve growth factor
OSR1	Odd-skipped-related 1
PI3K	Phosphoinositide 3-kinase
PIP2	Phosphatidylinositol 4,5-bisphosphate
PIP3	Phosphatidylinositol 3,4,5-trisphosphate
PKC	Protein kinase C
PLCγ	Phospholipase C gamma
PTX	Pertussis toxin
Rb	Retinoblastoma protein
ROS	Reactive oxygen species
TrkA	Tropomyosin receptor kinase A

ABSTRACT

The formyl peptide receptors FPR1, FPR2 and FPR3 are seven transmembrane G_i-protein coupled receptors. They were first identified as mediators of chemotaxis and activation of leukocytes in response to bacterial formylated peptides. To date, expression of FPRs was described also in non-myeloid cells, together with the ability of these receptors to recognize an heterogenous range of ligands of different origin. In the last few years, FPRs activation or their overexpression has been correlated to cell tumorigenicity, inflammation, cell proliferation, invasion and tumour progression. Furthermore, increasing evidence have highlighted the ability of FPRs to transactivate receptor tyrosine kinase (RTKs) through NADPH oxidase-dependent production of reactive oxygen species (ROS).

Herein, we investigated (i) the ability of FPR1 to transactivate the nerve growth factor receptor TrkA, in SH-SY5Y human neuroblastoma cell line; (ii) the involvement of NADPH oxidase-derived ROS in mediating TrkA transactivation; (iii) the downstream signaling cascades triggered by FPR1 stimulation and, in turn, by TrkA transactivation; (iv) the biological effects of FPR1 activation. Western blotting experiments demonstrated that FPR1 stimulation mediates NADPH oxidase-dependent phosphorylation of cytosolic residues Y490, Y751, and Y785 of TrkA, that represent docking sites for Erk, Akt and PKC pathway activation. Cell count assay, neurite outgrowth assay and wound-healing assay indicated that FPR1-mediated TrkA

phosphorylation enhances cell proliferation, growth and migration.

Furthermore, to characterize phosphorylations of intracellular signaling molecules triggered by FPR activation, we performed a TiO_2-based affinity chromatography to obtain an enrichment of phosphoproteins derived from FPR2 stimulation. Mass spectrometry analysis identified 290 differentially phosphorylated proteins and 53 unique phosphopeptides. Phosphorylations of five selected phosphoproteins (HSP-27, MCM2, OSR1, Rb and MARCKS) were further validated by western blotting experiments, confirming their dependence on FPR2 activation. Furthermore, we show that FPR2 stimulation with two anti-inflammatory agonists (Annexin A1 or Lipoxin A4) induces the phosphorylation of selected differentially phosphorylated proteins, suggesting their role in the resolution of inflammation.

Taken together, these data represent a promising resource for further studies on new signaling networks established by FPRs that could lead to the identification of novel molecular drug targets for human diseases.

1. INTRODUCTION

1.1 Formyl peptide receptors

1.1.1 Structure, function, tissue and cellular distribution

Formyl-peptide Receptors (FPRs) belong to the class A rhodopsin-like receptor family of the five main classes of G protein-coupled receptors (GPCRs), the largest family of transmembrane receptors in the human genome (Fredriksson, 2003). FPRs are seven-transmembrane domain receptors structurally characterized by an extracellular N-terminal domain followed by seven hydrophobic transmembrane α-helices (TM-1 to TM-7) connected by three extracellular loops (EL-1 to EL-3) involved in ligand binding, and three intracellular loops (IL-1 to IL-3) involved in G proteins binding, and finally by an intracellular C-terminal domain (Fig. 1). Agonist binding to the extracellular binding site causes receptor conformational changes that are required for receptor activation and intracellular heterotrimeric G protein binding (Latorraca, 2017). Human FPR family includes three members: FPR1, FPR2 and FPR3. FPR1 was first identified in 1976 as a high affinity receptor for N-formyl-Methionine-Leucine-Phenylalanine (N-fMLP) on neutrophils (Le, 2002) and cloned in 1990 by functional screening of a cDNA library from differentiated HL-60 myeloid leukaemia cells. FPR1 gene spans 6kb and encoded a 350 amino acids protein (Boulay,1990; Perez, 1992).

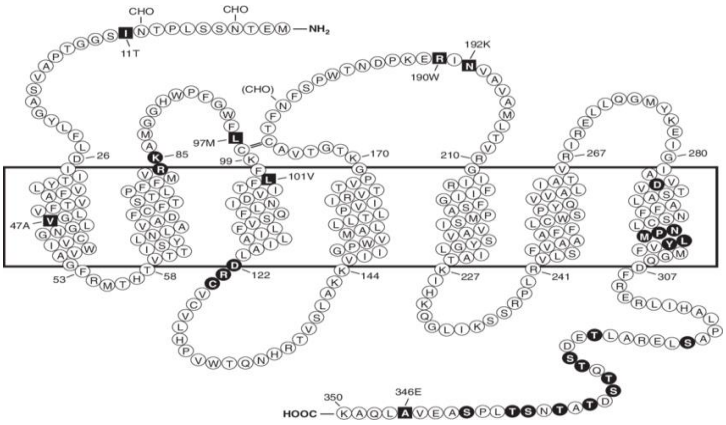


Figure 1. Predicted transmembrane disposition of the human FPR1 (Boulay et al, 1990a).

Low stringency hybridization, by using FPR1 cDNA as probe, allowed the identification of two paralogous genes with high sequence homology with FPR1, initially named FPRL1 and FPRL2. These genes were subsequently named, respectively, FPR2/ALX and FPR3 according to the International Union of Basic and Clinical Pharmacology (IUPHAR) nomenclature based on receptor affinity for different formyl peptides binding (Ye, 2009). Human FPR1, FPR2 and FPR3 are clustered on chromosome 19q13.3-19q13.4, suggesting their origin from a gene duplication event (Fig. 2). FPR2 is a 351 amino acid protein that shares 69% sequence homology with FPR1, whereas FPR3 is a 352 amino acid protein sharing 56% sequence homology with FPR1. Instead, FPR2 and FPR3 share the highest degree of amino acid identity, about 83% (Murphy, 1992).

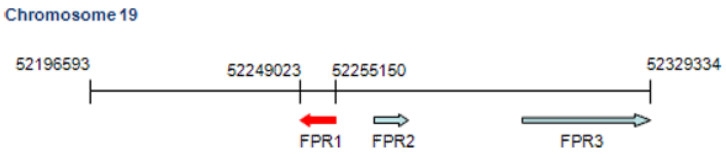


Figure 2. Formyl peptide receptor gene cluster region on chromosome 19p includes FPR1, FPR2 and FPR3 genes (http://atlasgeneticoncology.org).

Orthologs of human FPR genes have been identified in different non-human primates, including mice, rats, rabbits and guinea pigs (Ye, 2009). In mice, eight different FPR genes were identified clustered on chromosome 17 A3.2; mFpr1 shares 76% sequence homology with human FPR1 and different affinity for formylated peptides (Gao, 1993), whereas mFpr2 shares 73% sequence homology with human FPR2 (Takano, 1997).

FPRs are mainly expressed in phagocytic leukocytes, where these receptors play important roles in inflammatory and immune responses, by promoting cell chemotaxis, calcium flux, directed migration, superoxide production, release of proinflammatory cytokines and gene transcription in response to N-formylated peptides (Schiffmann, 1975). FPR1 and FPR2 are expressed in monocytes and neutrophils, while FPR3 expression is restricted only to monocytes. FPR2 expression is lost in immature dendritic cells, whereas FPR1 expression is lost in mature dendritic cells; instead, FPR3 is expressed in both immature and mature dendritic cells.

Besides myeloid cells, FPR1 expression is detected in astrocytes, microglial cells, hepatocytes, endothelial cells, fibroblasts, motor and sensory neurons, vascular smooth muscle cells; FPR1 is also expressed in central and autonomic nervous system. FPR2 has a wider distribution than FPR1, as it is expressed also in epithelial cells, T and B lymphocytes. Tissue expression of FPR1 and FPR2 is also been detected in thyroid, liver, lung, spleen, bone marrow and in neuroblastoma cells (Migeotte, 2006).

1.1.2 Receptor agonists

The first characterized ligand of FPR1 is the N-formylated tripeptide N-fMLP derived from the gram-negative bacterium E. coli. FPR1 is activated by picomolar concentrations of N-fMLP. N-formylated peptides are peptides whose amino acid sequence contains a formyl group added to the amino acid methionine located at the N-terminus. These peptides derive from bacterial or mitochondrial proteins; in fact, both bacterial and mitochondrial protein synthesis is initiated with a N-formyl methionine (Schiffmann, 1975). Therefore, as pattern recognition receptors (PPRs), FPRs are able to recognize bacterial-derived formylated peptides as pathogen-associated molecular patterns (PAMPs) and mitochondrial-derived formylated peptides, released during cellular stress or necrosis, as damage-associated molecular pattern (DAMPs) (Zhang, 2010). FPR2 is defined as low affinity receptor for N-fMLP since it is activated in vitro by micromolar concentrations of N-fMLP and elicits

calcium flux but not chemotaxis. Furthermore, its affinity for formylated peptides depends on their size, hydrophobicity and charge (Murphy, 1992). N-fMLP has no activity on FPR3. Furthermore, FPR1 and FPR2 can be both activated by N-formylated peptides derived from mitochondrial NADH dehydrogenase subunits 4 and 6 and cytochrome c oxidase subunit 1 from mitochondria respiratory chain (Rabiet, 2005).

Despite the high sequence homology of FPRs, differences in their structure are limited to the ligand-binding domain and are causative of different affinity for the same ligand and different selectivity for the wide variety of ligands existing for these receptors. In addition to formylated peptides, FPRs recognize also non-formylated peptides from microbial origin. FPR2 and FPR3 can be activated by Hp(2-20) Helicobacter pylori peptide, stimulating monocyte migration (Betten, 2001). Glycoproteins gp120 and gp41 of human immunodeficiency virus (HIV) contain peptide sequences recognized by FPR1 and, mainly, by FPR2 (Deng, 1999). A glycoprotein sgG-2 peptide of the Herpes simplex virus type 2 induces reactive oxygen species release after FPR1 stimulation in phagocytes (Bellner, 2005). To date, a considerable number of endogenous ligands for FPRs and, in particular, for FPR2 has been identified and associated with human inflammatory diseases. FPR2 ligands include amyloidogenic proteins, counting serum amyloid A (SAA), β-amyloid peptide 42 (Aβ42) and human prion protein fragment ($PrP_{106-126}$). SAA an acute-phase protein, activates FPR2, stimulating migration, metalloproteases and cytokines production in phagocytes. In monocytes, at low concentrations, SAA

stimulates the release of the pro-inflammatory cytokine TNF-α; instead, at high concentrations, SAA induces the release of the anti-inflammatory cytokine IL-10. Furthermore, SAA stimulation increases calcium mobilization, migration, metalloproteases and cytokines expression (Cattaneo, 2013). Aβ42 is produced by the cleavage of the amyloid precursor protein (APP) mediated by β- and γ- secretase enzymes and its accumulation leads to fibrillar aggregation in neurons, causing Alzheimer disease. Aβ42 interaction with FPR2 determines the internalization of Aβ42-FPR2 complex and triggers calcium flux, migration, superoxide production and pro-inflammatory cytokine release from microglial cells, monocytes and macrophages. Low levels of Aβ42 are associated with receptor recycling and Aβ42 degradation, whereas high levels are associated with intracellular complex accumulation, fibrillar aggregation and macrophage death (Yazawa, 2001). Different studies have demonstrated that, in microglial cells, the Aβ1-42-induced signal transduction depends on the physical interaction between FPR1 and FPR2, in association with the macrophage receptor with collagenous structure (MARCO) (Brandenburg, 2010) or with the receptor for advanced glycation end products (RAGE) (Slowik, 2012). $PrP_{106-126}$, a fragment of the prion protein involved in Creutzfeldt-Jacob disease, interacts with FPR2 in microglial cells inducing chemotaxis and pro-inflammatory cytokines production (Le, 2001). Humanin (HN) is a neuroprotective peptide that protects neuronal cells from Aβ42-induced fibrillary formation and death; it acts as ligand of FPR2 and FPR3, probably interfering with Aβ42-FPR2 binding (Ying, 2004). The two neuropeptides vasoactive intestinal

polypeptide (VIP) (El Zein, apr. 2008) and the pituitary adenylate cyclase-activating polypeptide 27 (PACAP27), that belongs to the VIP family (El Zein, mar. 2008), activate FPR2 promoting chemotaxis and inflammation.

uPAR is the receptor for the urokinase-type plasminogen activator (uPA), a serine protease involved in fibrinolysis process; when cleaved by proteases, including uPA, uPAR is able to differentially bind the three FPRs, depending on different residues of the cleaved receptor (Gargiulo, 2005, de Paulis, 2004).

The antimicrobial peptide LL37 is produced by the cleavage of the cathelicidin hCAP18, a neutrophil secondary granule protein that promotes pro-inflammatory responses upon FPR2 binding (Yang, 2000); LL-37-FPR2 interaction induces cell migration, proliferation and superoxide production (Iaccio, 2009, Shaykhiev, 2005).

Annexin A1 (ANXA1), also named Lipocortin-1, is a glucocorticoid-regulated, Ca^{2+}-dependent, lipid-binding 37kDa protein localized in the cytoplasm of neutrophils (Madeeha, 2018). Following neutrophil adhesion to the endothelium, ANXA1 is exposed on cell surface and inhibits trans-endothelial migration (Perretti, 2004). ANXA1 is cleaved by proteases from neutrophil granules; the cleavage determines the release of two peptides derived from its N-terminal domain, Ac2-26 and Ac9-25. ANXA1 and its peptides seems to have different affinity for FPRs and a dual role in inflammation. In fact, at high concentrations, ANXA1 peptides activate FPR1, promoting pro-inflammatory events; otherwise, at low concentrations, these peptides inhibit neutrophil activation and trans-endothelial

migration (Ernst, 2004). Other studies demonstrate that these peptides prompt anti-inflammatory responses by activating FPR2 (Perretti, 2002). An endogenous non-peptide ligand of FPR2 is the lipid metabolite Lipoxin A4 (LXA4), an eicosanoid that derives from arachidonic acid. LXA4 exerts anti-inflammatory and pro-resolving functions, blocking neutrophil infiltration and transmigration across epithelial and endothelial cells (Colgan, 1993), and promoting neutrophil apoptosis and phagocytosis (Godson, 2000). FPR2 is the only FPR able to bind lipid ligands, such as Resolvin D1 (Krishnamoorthy, 2010) and oxidized low-density lipoproteins (oxLDL) (Lee, 2014).

FPR3 is unable to bind formyl peptides and its exact function is still unclear. The only endogenous ligand with high affinity and specificity for FPR3 is the peptide F2L, that derives from the cleavage of human heme-binding protein and is a chemoattractant for monocytes and dendritic cells (Yang, 2002). FPR3 is highly phosphorylated after ligand binding, resulting in inactivation, internalization and localization in small intracellular vesicles (Rabiet, 2011). This suggests that FPR3 could act as a decoy receptor reducing the binding of its ligands to other receptors. In addition to endogenous agonists, screening of random peptide libraries let to the identification of different synthetic agonists for FPRs. The hexapeptide WKYMVm (Trp-Lys-Tyr-Met-Val-D-Met) is the high affinity ligand of FPR2, as it mediates neutrophils and monocytes chemotaxis, cytokine release and NADPH oxidase-mediated respiratory burst (Seo, 1997). WKYMVm binds FPR1 and FPR3 with lower efficiency; in fact,

WKYMVm activates neutrophils through FPR1 only when FPR2 signaling is blocked (Le, 1999).

1.1.3 Receptors antagonists

Given the enormous variety of ligands and the promiscuity of these receptors, FPRs are interesting potential candidate for the development of therapeutic molecules useful in FPR-related diseases.
Several FPR antagonists have been identified and characterized so far. The substitution of the formyl group of N-fMLP with the tertiary butyloxycarbonyl group (t-Boc) creates two FPR1 antagonists, t-Boc-Met-Leu-Phe (tBoc-MLF, Boc1) and t-Boc-Phe-Leu-Phe-Leu-Phe (tBoc-FLFLF, Boc2) (Derian, 1996). At low micromolar concentrations, Boc1 and Boc2 are selective for FPR1, but at high concentrations Boc2 also inhibits FPR2. Cyclosporin H is a cyclic undecapeptide derived form a fungus that represents the most specific FPR1 antagonist, better defined as inverse agonist since its activity is linked to inhibition of N-fMLP binding (Stenfeldt, 2007). The bile acids deoxycholic acid (DCA) and chenodeoxycholic acid (CDCA) are antagonists of FPR1 (Chen, 2000), since they interfere with ligand binding through a steric hindrance mechanism, together with opioid Spinorphin (Yamamoto, 1997). Through the screening of hexapeptide libraries, the peptide WRWWWW (WRW4, Trp-Arg-Trp-Trp-Trp-Trp) was identified as specific and potent FPR2 antagonist (Stenfeldt, 2007).

1.2 Intracellular signaling cascades

FPRs are coupled to the G_i proteins of G-protein family and are sensitive to pertussis toxin (PTX) (Le, 2002), a toxin produced by the bacterium Bordetella Pertussis, that ADP-ribosylates the α-subunit of Gi/o proteins, blocking this subunit into an inactive GDP-bound state, leading to the inhibition of the interaction between the receptor and G-proteins (Burns, 1988). G-proteins are heterotrimeric proteins composed of a Gα subunit with GTPase activity and a βγ dimer. G-proteins are activated by the exchange of GDP with GTP on Gα subunit. G-protein family is composed of four major members: G_s, G_i, $G_{12/13}$ and G_q, that are responsible for triggering different signaling responses. Following ligand binding, FPRs undergo a conformational change that leads to the interaction with G_i proteins and the exchange of GDP with GTP on $G\alpha_i$ subunit that, once in its active GTP-bound state, dissociates from βγ dimer, interacting with a variety of effector molecules. Hydrolysis of GTP causes the return of Gα subunit in its inactive GDP-bound state and the reconstitution of the heterotrimer, ensuring that their activation as a transient event. After dissociation, βγ dimer activates phospholipase Cβ (PLCβ), that catalyses the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2) in inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 mediates calcium release from endoplasmic reticulum, whereas DAG activates protein kinase C (PKC) (Del Prete, 2004). Calcium is an important second messenger whose release is increased in neutrophils following FPR1 activation by N-fMLP stimulation; it mediates

cytoskeletal remodelling through actin polymerization, promoting leukocyte migration (Selvatici, 2006; Zaffran, 1993). Calcium intracellular increase occurs in two phases: a first transient release from intracellular storage sites followed by a secondary influx across the plasma membrane; in fact, calcium release from internal stores induces the opening of the store-operated calcium channel in the plasma membrane (Cavicchioni, 2003).
Moreover, an increase of cAMP has been demonstrated in neutrophils in response to N-fMLP stimulation; cAMP enhancement seems to be dependent from PLC stimulation via the βγ subunits of Gi proteins (Ferretti, 2001).
FPR signaling activates through phosphorylation cascades also the phosphoinositide 3-kinase (PI3K), which convert PIP2 into inositol-3,4,5-trisphosphate (PIP3), that represents the docking site for the interaction with the serine/threonine kinase AKT/PKB, leading to its activation associated with promotion of cell survival, growth, proliferation, cell migration and angiogenesis (Del Prete, 2004). Furthermore, FPRs trigger the activation of the mitogen-activated protein kinases (MAPKs), the NADPH oxidase complex and the phospholipase A_2 (PLA_2) and phospholipase D (PLD) pathways (Le, 2002; Le, 2001; Bae, 2003), promoting chemotaxis, degranulation, superoxide production and cell proliferation (Fig. 3).

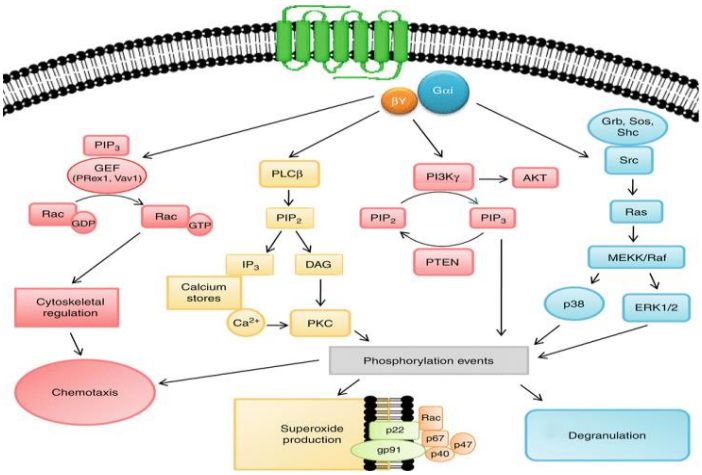


Figure 3. Intracellular signaling cascades triggered by formyl peptide receptors. Agonist binding induces receptor activation and leads to dissociation of Gα and βγ subunits. The released α and βγ subunits activate several downstream signaling pathways, involving GEFs for GTPase activation, PLCβ and PI3Kγ and MAPKs; these proteins mediate multiple cellular functions including chemotaxis, superoxide production and degranulation (Southgate et al, 2012).

1.2.1 PKC activation

PKC proteins are a family of about 11 serine/threonine kinases with a molecular weight of 80kDa that regulate a multitude of different cellular events, such as cell proliferation, differentiation, apoptosis, and motility (Dekker, 1997). PKC family is composed of the classical PKC isoforms α, β1, β2 and γ, the novel PKC isoforms δ, ε, η and θ, and the atypical PKC isoforms ζ and λ or ι (Spitaler, 2004). All the isoforms share a common C-terminal catalytic domain, but differentiate in their N-

terminal regulatory domain, whose structure depends on the interaction with Ca2+, DAG, phosphatidylserine (PS), phorbol ester or other lipids (Fabbro, 1999). In particular, classical PKC isoforms are activated by Ca2+, DAG and PS, whereas novel PKC isoforms require DAG and PS; atypical PKC isoforms are insensitive to both Ca2+ and DAG (Spitaler, 2004).
The activation of PKC is mediated by its transition from the cytosol to the membrane; the membrane recruitment in response to DAG or calcium release is accompanied by the conformational rearrangement of PKC that leads to the loss of auto-inhibitory interactions, activating its kinase activity. The association of PKC with membranes is mediated by its interaction with PIP2 and PS involving the N-terminal regulatory domain (Igumenova, 2015). PKC is an important mediator of cytoskeletal activities, as it is been associated with intermediate filament proteins, microtubule proteins and membrane-cytoskeletal cross-linking proteins (Pettit, 1996). One of the main substrates of PKC is the myristoylated alanine-rich C kinase substrate (MARCKS) protein, which acts as a bridge between plasma membrane and actin cytoskeleton by mediating crosslink of filamentous actin (Hartwig, 1992) and is also involved in leukocyte motility. PKC mediates MARCKS phosphorylation on serine residues that results in the loss of both the calmodulin and actin binding ability of MARCKS, as well as in the loss of MARCKS membrane binding (Arbuzova, 2002). PKC can phosphorylate other cytoskeletal proteins including focal adhesion proteins, such as talin, vinculin and integrins (Fabbri, 1997). PKC family proteins are also involved in the activation of the NADPH oxidase complex through phosphorylation of

its cytosolic subunits in leukocytes stimulated with N-fMLP (Dang, 2001).

1.2.2 MAPK activation

MAPKs are a family of serine/threonine kinase proteins involved in mediating gene expression, mitosis, cell motility, survival, apoptosis and differentiation. The most characterized MAPK proteins are extracellular signal-regulated kinases 1 and 2 (ERK1/2), c-Jun amino-terminal kinases (JNKs) and p38 kinases (Schaeffer, 1999). ERK1 (MAPK3) and ERK2 (MAPK1) are proteins of 44 and 42kDa sharing 83% amino acid identity (Chen, 2001), that are activated in response to mitogen stimuli, such as growth factors, serum, phorbol esters and by GPCR ligands and cytokines (Lewis, 1998). ERK1/2 are activated by the MAPK kinases (MAPKKs) and by extracellular signal-regulated kinases MEK1 and MEK2, through dual phosphorylation on threonine and tyrosine residues located in the Thr-Glu-Tyr (TEY) motif of the activation loop. MAPKKs are in turn phosphorylated and activated by the MAPKK kinase (MAPKKKs) Raf (Moodie, 1994). Raf/MEK/ERK cascade is triggered by the activation of the small GTP-binding protein Ras through the exchange of GDP to GTP mediated by the nucleotide exchange factor SOS (son of sevenless) (Geyer, 1997). When activated, ERKs migrate in the nucleus, where they phosphorylate several substrates such as transcription factors c-Myc, STAT3 and c-Fos (Chen, 1992).

JNK proteins respond to a variety of stress signals including heat shock, osmotic stress, growth factor deprivation, pro-inflammatory cytokines, ischemia and UV irradiation. JNK activation occurs through dual phosphorylation on tyrosine and threonine residues in the Thr-Pro-Tyr (TPY) motif by MAPKKs MEK4 and MEK7 that are in turn activated by MAPKKKs, including MEKK1-4, MLK2 and MLK3 (Kyriakis, 2001). JNK activation determines its translocation into the nucleus and the phosphorylation and activation of the transcription factor c-Jun (Weston, 2002); JNK activates also other transcription factors, including ATF-2, Elk-1, c-Myc, Smad3 and the tumour suppressor p53 (Chen, 2001; Kyriakis, 2001).

The p38MAPKs are generally activated by heat, osmotic and oxidative stresses, ionizing radiations, hypoxia, ischemia, inflammatory cytokines and TNFα receptor signaling (Chen, 2001). To date, four p38MAPK isoforms, α, β, γ and δ have been identified; these isoforms share 60% homology and are involved in cell motility, transcription and chromatin remodelling (Kyriakis, 2001). Furthermore, p38MAPK participates in macrophage and neutrophil functional responses, including respiratory burst activity, chemotaxis and granular exocytosis (Ono, 2000). p38MAPK are subject to dual phosphorylation at the Thr–Gly–Tyr (TGY) motif in their activation loop by the MAPKKs MEK3 and MEK6. While MEK6 activates all p38 isoforms, MEK3 is specific for the p38α and β isoforms (Enslen, 2000). The MAPKKKs involved in phosphorylation of MEK3/6 are MEKKs 1-4, MLK2 and 3, DLK and ASK1 (Kyriakis, 2001). When activated, p38MAPK can be localized in the nucleus (Raingeaud, 1995), but also in

cytoplasm (Ben-Levy, 1998). Once activated p38MAPK phosphorylates different targets, including cytosolic PLA2, the microtubule-associated protein Tau, and the transcription factors ATF1 and -2, MEF2A, Elk-1, NF-kB and p53 (Kyriakis, 2001).
In differentiated HL-60 granulocytes, N-fMLP stimulation of FPR1 triggers a concentration- and time-dependent increase in ERK, JNK, and p38MAPK phosphorylation, that result to be dependent on PTX-sensitive G_i proteins. Furthermore, ERK activation by FPR1 is mediated by PI3K, PLC, and PKC pathways, whereas p38MAPK activation is mediated by PI3K and PLC (Rane, 1997).

1.2.3 NADPH oxidase complex activation

In phagocytes, NADPH oxidase complex plays a key role in controlling inflammation and promoting host defence against pathogens. Neutrophil stimulation with the chemoattractant N-fMLP potently induces the activation of the nicotinamide adenine dinucleotide oxidase (NADPH oxidase) complex; this complex triggers the respiratory burst characterized by a rapid increase in oxygen uptake, glucose consumption and reactive oxygen species (ROS) generation that contribute to pathogen elimination. (Hallet, 1989; Omann, 1987). In fact, NADPH oxidase deficiency is associated with the chronic granulomatous disease (CGD), a disorder characterized by high recurrence of bacterial and fungal infections (Brahm, 2012).

Phagocyte NADPH oxidase (phox) is a multi-enzymatic complex composed of the two membrane-associated proteins $p22^{phox}$ and $gp91^{phox}$ that constitute the flavocytochrome b_{558}, and the four cytosolic proteins $p47^{phox}$, $p67^{phox}$, $p40^{phox}$ and the GTPase Rac1/2 (Groemping, 2005; Vignais, 2002). In resting conditions, membrane and cytosolic subunits are spatially separated; the phosphorylation of cytosolic subunits leads to their association with membrane subunits, NADPH oxidase complex activation and ROS production (Groemping, 2005).

The key event of NADPH oxidase activation is the phosphorylation of $p47^{phox}$ protein. $p47^{phox}$, also known as neutrophil cytosolic factor 1 (NCF1) is a 390 amino acid protein with a molecular mass of about 47kDa. Structurally, $p47^{phox}$ is characterized by an N-terminal phox homology (PX) domain, two SH3 domains, and a C-terminal autoinhibitory region (AIR) and proline-rich region (PRR). In resting conditions, $p47^{phox}$ is in an auto-inhibited conformation characterized by the interaction between AIR and SH3 domains. Phosphorylation of $p47^{phox}$ on different serines located in its C-terminal domain induces a conformational change that allows the binding to $p22^{phox}$, $p67^{phox}$ and the membrane translocation. In particular, SH3 domains of $p47^{phox}$ interact with the PRR of $p22^{phox}$, while the PX domain is responsible of the binding to PIP_2, assuring membrane localization. Furthermore, as organizer subunit, $p47^{phox}$ through its PRR interacts with the SH3 domain of $p67^{phox}$. $p67^{phox}$, also called neutrophil cytosolic factor 2 (NCF2) is the activator subunit that, in turn, interacts through the activation domain (AD) with $gp91^{phox}$, through the NH2-terminal tetricopeptide repeat (TPR)

domains with Rac and through the PB1 domain with $p40^{phox}$ (El-Benna, 2009) (Fig. 4).
Once assembled the complex, the activation consists in the transfer of NADPH electrons to FAD involving the AD of $p67^{phox}$. $FADH_2$ transfers its electron to the iron of the heme of $gp91^{phox}$ and then transferred to the O_2 (Nisimoto, 1999, Vignais, 2002).
$gp91^{phox}$ is characterized by a short cytoplasmic N-terminal sequence, six transmembrane alpha helices that bind two haems and a NADPH and FAD-binding site at C-terminal cytosolic tail (Nisimoto, 1999). The association of $gp91^{phox}$ with $p22^{phox}$ is essential for the stability of the flavocytochrome $b_{558;}$ in fact, when $gp91^{phox}$ and $p22^{phox}$ are present as monomers, they are degraded.
The flavocytochrome b_{558} represents the electron transfer chain, since it mediates the reduction reaction of molecular oxygen (O_2) using cytosolic NADPH as electron donor, to produce superoxide anion (O_2^-), NADP+ and protons. O_2^- is a very unstable free radical and reacts spontaneously or by superoxide dismutase with protons to form hydrogen peroxide (H_2O_2), which is used by myeloperoxidases (MPO) to produce ROS (Hampton, 1998).
$p47^{phox}$ phosphorylation occurs in its C-terminal domain and involves serines from 303 to 379; in particular, serine 303, 304, 359 and 379 phosphorylation are necessary for NADPH oxidase activation (Faust, 1995).

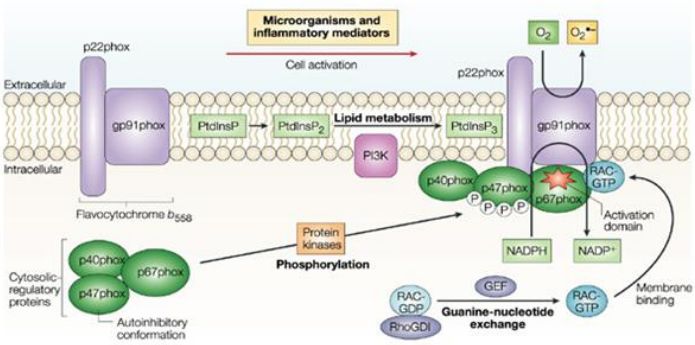


Figure 4. Schematic representation of the NADPH oxidase complex activation. The activation of NADPH oxidase complex is induced by the assembly of cytosolic components p47^{phox}, p67^{phox}, p40^{phox} and Rac with the flavocytochrome b558 components p22^{phox} and gp91^{phox}. Phosphorylation of the autoinhibitory region (AIR) of p47phox by different protein kinases allows membrane translocation of cytosolic subunits and p22^{phox} binding, leading to complex activation and reactive oxygen species (ROS) production (Lambeth, 2004).

p47^{phox} phosphorylation on specific serines can be triggered by different protein kinases such as PKC α, β, δ, and ζ (Dang, 2001a), PKA (El Benna, 1996a), ERK2 and p38MAPK (El Benna, 1996b), protein casein kinase 2 (CKII) (Park, 2001), AKT (Chen, 2003; Hoyal, 2003), p21-activated kinase (PAK) (Martyn, 2005), and Src kinase (Chowdhury, 2005). p67^{phox} phosphorylation on serine or threonine is dependent on PKC (Benna, 1997), ERK1/2, p38MAPK (Dang, 2003) and PAK (Ahmed, 1998) pathways. p40^{phox} phosphorylation is PKC-dependent (Bouin, 1998). Activation of the NADPH oxidase in phagocytes requires the involvement of the GTPase Rac2 or Rac1, members of the Ras superfamily

of GTP-binding proteins. Rac proteins are activated by the exchange of GDP with GTP and are involved in the recruitment of $p67^{phox}$ and assembly of the complex (Hordijk, 2006) (Fig. 4). $gp91^{phox}$, also named NOX2, was first described in neutrophils and macrophages and is often referred as the phagocyte NADPH oxidase since has a wide tissue distribution. Six homologs of the cytochrome subunit of the phagocyte NADPH oxidase, deriving from a gene duplication event, were recently identified: NOX1, NOX3, NOX4, NOX5, DUOX1, and DUOX2. Activation mechanisms and tissue distribution of the different members of the family are markedly different. NOX2 expression is described in thymus, small intestine, colon, spleen, pancreas, ovary, placenta, prostate, and testis (Cheng, 2001), neurons (Serrano, 2003), cardiomyocytes (Heymes, 2003), skeletal muscle myocytes (Javesghani, 2002) and hematopoietic stem cells (Piccoli, 2005).

1.2.4 GPCR desensitization

Many GPCRs undergo desensitization following agonist stimulation, which represents an important regulatory step that prevents receptor iperactivation. The signaling ability of GPCRs is regulated at different levels: in a ligand dose-dependent manner, or by controlling the number of receptors on cell surface, or by regulating the signaling efficiency of receptors (Gainetdinov, 2004).
The main regulatory mechanism of GPCR signaling is the activation-dependent regulation, also known as GPCR homologous desensitization. In this mechanism

the activated GPCRs are substrates for phosphorylation by GPCR kinases (GRKs). The GRK family is composed of seven members, GRK1-GRK7, that present significant sequence homology. GRKs phosphorylate GPCRs on serine and threonine residues localized within either the third intracellular loop or C-terminal domain (Claing, 2002). Once phosphorylated by a GRK, the activated GPCR is a substrate of the β-arrestin protein that recognize both GRK phosphorylation sites on GPCRs and the active conformation of the receptor (Luttrell, 2002). β-arrestin inhibits further G-protein activation by preventing the exchange of GDP to GTP on the Gα-subunit and mediates GPCR internalization through the binding to clathrin and clathrin adaptor protein AP2, leading to receptor recruitment into clathrin-coated pits (Goodman, 1996). In addition to its role in receptor desensitization, recent evidence also supports an important function for GRKs and ß-arrestin as signal transduction mediators, acting as adaptors to facilitate the interaction between GPCRs and signaling molecules such as c-Src, PI3K and MAPK proteins (Luttrell, 2002, Hall, 1999; Shenoy, 2003).

1.3 Receptor tyrosine kinases

Receptor tyrosine kinases (RTKs) are key regulators of different cellular processes, such as proliferation, differentiation, cell survival, metabolism, and cell migration (Grassot, 2003). Structurally, RTKs are single-pass transmembrane receptors characterized by an extracellular ligand-binding domain, a single

transmembrane helix and a cytoplasmic tyrosine kinase (TK) domain. Binding of ligands, such as polypeptides, protein hormones, cytokines and growth factors, induces receptor dimerization and activation of its intrinsic tyrosine kinase activity, leading to the trans-phosphorylation of tyrosine residues located in the cytoplasmic domain. These represent the docking sites for downstream signaling proteins with Src homology 2 (SH2) or phosphotyrosine-binding (PTB) domains, such as the adaptor protein Shc that interacts with the growth factor receptor-bound 2 (GRB2) protein. GRB2, through the SH3 domains, binds Sos that catalyse the exchange of GDP with GTP on Ras, leading to MAPK, PKC, PI3K/AKT pathway activation (Schlessinger, 2000). Several diseases result from genetic mutations or alteration of the activity, cellular distribution, or regulation of RTKs, such as cancers, inflammation, arteriosclerosis and angiogenesis.

1.3.1 Epidermal growth factor receptor EGFR

The epidermal growth factor receptor (EGFR) is a RTK receptor involved in a wide range of biological processes, such as cell division, proliferation, migration, differentiation and apoptosis (Pinkas-Kramarski, 1996). EGFR is the receptor of the epidermal growth factor (EGF), a ubiquitous polypeptide capable of stimulating proliferation of many types of epithelial cells. The EGFR family is composed of four homologous members: EGFR, (erbB1 or HER1), HER2 (erbB2), HER3 (erbB3), and HER4 (erbB4). Their protein structure is

characterized by an extracellular domain (ECD) with two cysteine-rich regions, a single trans-membrane region, a juxtamembrane cytoplasmic domain and an intracellular kinase domain (Oda, 2005). Ligand binding to the receptor ectodomain promotes receptor homodimerization or heterodimerization, that is essential for activation of the intracellular tyrosine kinase domain and, in turn, for its transphosphorylation. Phosphotyrosine residues represent docking site for the activation of downstream signaling pathways including Ras/MAPK, PLCγ1/PKC, PI3K/AKT, and signal transducers and activators of transcription pathway (STAT) pathways (Sato, 2013). STAT3 binding to activated EGFR leads to STAT3 dimerization and translocation into the nucleus, thus regulating gene transcription (Scaltriti, 2006).
EGFR overexpression is associated with different human cancers including glioblastoma, neuroblastoma, non-small cell lung cancer (NSCLC), head and neck cancer and colorectal cancer (Salomon, 1994).

1.3.2 Nerve growth factor receptor TrkA

Tropomyosin-related kinase (Trk) receptor family is composed of three single-pass type I transmembrane proteins, TrkA, TrkB and TrkC, that are encoded by NTRK1, NTRK2, and NTRK3 genes, respectively, and p75NTR, a member of the TNF receptor superfamily, that interacts with all neurotrophins with low and similar affinity.

The structure of Trk receptors is characterized by an extracellular glycosylated domain that contains three leucine-rich repeats, two cysteine repeats and immunoglobulin-C2 (Ig) domains proximal to the transmembrane region and an intracellular tyrosine kinase domain (Ultsch, 1999). Trk are the receptors for neurotrophins, a class of proteins that are involved in promoting cell survival, cell differentiation, neurite outgrowth and synaptic plasticity during central and peripheral nervous system development. Nerve growth factor (NGF) belongs to this class of proteins and was purified as a factor able to support survival of sympathetic and sensory spinal neurons in vitro (Levi-Montalcini, 1987). NGF, brain derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4 (NT-4) are produced as precursor proteins that are processed to mature proteins that associate as homodimers. NGF is the TrkA specific ligand, whereas BDNF and NT-4 are TrkB ligands; NT-3 binds to TrkC (McDonald, 1995). TrkA activation induced by NGF enhances cell proliferation, survival, differentiation, apoptosis, axonal and dendritic growth, organization of the cytoskeleton, membrane trafficking and synapse formation, through the activation of intracellular signaling cascades involving PI3K/AKT, Ras/MAPK/ERK and PLCγ1/PKC pathways (Kawamura, 2007).

TrkA phosphorylation occurs on tyrosines located in the activation loop, Y670, Y674, and Y675, and on two other phosphorylation sites, Y490 and Tyr-785, both of which are located outside the kinase domain. Phosphorylation of Y490 represent a docking site for the activation of MAPK and PI3K/AKT pathways, whereas

phosphorylation of Y785 is associated with PLCγ1/PKC pathway (Obermeier, 1994). Furthermore, Y751 phosphorylation is essential for PI3K docking and activation (Jang, 2007) (Fig. 5). Different evidence demonstrated that Trk receptors result to be overexpressed or iperactivated in numerous cancers, including breast, lung, colon-rectum, gastric cancer, pancreas, prostate, glioblastoma, neuroblastoma, myeloma, and lymphoid tumors (Meldolesi, 2018).

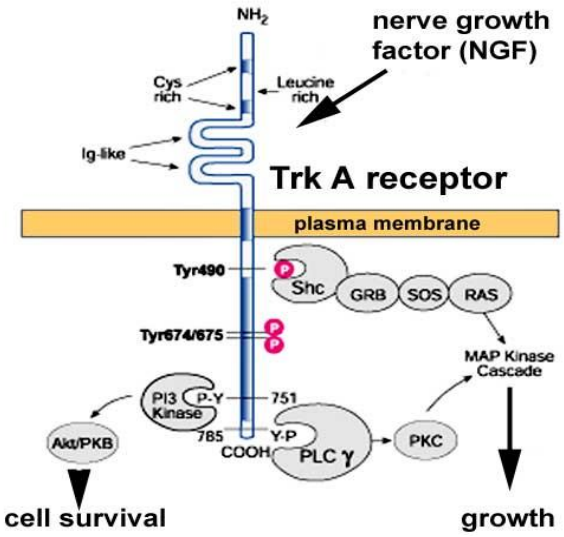


Figure 5. NGF-dependent phosphorylation of TrkA intracellular tyrosines provides docking sites for MAPK, PI3K-Akt and PLC-PKC pathway activation.
(https://cellbiology.med.unsw.edu.au/cellbiology/index.php?curid=1754)

1.4 Receptor tyrosine kinases transactivation

Extracellular stimuli are transduced into intracellular signals through the activation of several classes of receptors. The two main classes of cell surface receptors are GPCRs and RTKs. Originally GPCRs and RTKs were thought to activate diverse signaling cascades, but to date it has been widely demonstrated that there are complex bidirectional cross talk mechanisms between different receptors, responsible of the connection and diversification of signals from different sources. Indeed, protein kinases involved in a specific signaling pathway can phosphorylate components of other signaling pathways (Borroto-Escuela, 2017a).

GPCRs stimulation can enhance RTK tyrosine phosphorylation and signaling activity, coupling the wide diversity of GPCRs with the strong growth-promoting ability of RTKs. This mechanism, named transactivation, was first described in fibroblasts in which GPCR stimulation induces a rapid activation of epidermal growth factor receptor (EGFR) (Daub, 1996). Transactivation of RTKs induced by GPCR stimulation can be mediated by two different mechanisms, depending on RTK ligand involvement.

In the ligand-dependent triple-membrane-passing-signal mechanism, RTK transactivation by GPCRs is elicited by the activation of membrane-bound matrix metalloproteases (MMPs). MMPs are calcium-dependent endopeptidases that are synthetized in an inactive form or zymogen (pro-MMPs), which is then processed to its active form able to cleave extracellular matrix (ECM) components and mediate the release of

active cytokines and growth factors bound to cell membrane through ectodomain shedding (Daub, 1996, Prenzel, 1999). GPCR stimulation by its ligand triggers intracellular signals that activate MMPs that, in turn, mediates the cleavage and the release of mature RTK ligands that transactivate RTKs in an autocrine manner (Prenzel, 1999, Ohtsu, 2006). MMPs, such as MMP-3 and MMP-9, are involved in GPCR-mediated transactivation of EGFR (Uchiyama-Tanaka, 2002), VEGFR (Tanimoto, 2002), PDGFR (Tsai, 2014). In the RTK ligand-independent mechanism, transactivation can require the activation of several downstream second messengers of GPCR signaling, such as Ca^{2+} ions, the protein kinases Src and Pyk, β-arrestin and ROS, responsible of tyrosine phosphorylation and activation of RTKs (Cattaneo, 2014). Src proteins are intracellular tyrosine kinases that are involved in promoting cell growth; c-Src is regulated by binding to Gβγ subunits of GPCRs and can interact with β-arrestin (Luttrell, 2004). Stimulation of CaLu6 cells with the FPR2 agonist WKYMVm induces c-Src phosphorylation and EGFR transactivation (Cattaneo, 2011). In other RTKs, such as in the case of TrkA receptor/neurotrophic receptor tyrosine kinasetype 1 (NTR1), RTK transactivation is not mediated by c-Src activation (Moughal, 2004).

RTK transactivation can be mediated by ROS production induced by NADPH oxidase complex activation. In fact, ROS can activate kinases by altering protein–protein interactions or can inactivate by oxidation of the sulfhydryl groups of the cysteine residues located in the catalytic domains of protein tyrosine phosphatases, activating RTKs. ROS can also

stimulate proteolysis of regulatory proteins inhibiting tyrosine kinase activity (Adrain, 2014).

ROS generated by GPCR-dependent NADPH oxidase activation can result in transactivation of more than one RTK, suggesting a wide and diversified response to GPCR activation (Kruk, 2013). In fact, in human monocytes, FPR1 stimulation with its agonist N-fMLP triggers TrkA and EGFR tyrosine phosphorylation, ROS production, MMP-9 activation and integrin CD11b upregulation (El Zein, 2010). NADPH oxidase-induced EGFR transactivation in Calu-6 cells involves the non-receptor tyrosine kinase c-Src (Cattaneo, 2001). ROS production mediated by NADPH oxidase activation can be triggered by the increase of intracellular Ca^{2+} concentration and PKC activation; in fact, in human fibroblasts stimulated with WKYMVm, Ca^{2+}-dependent PKCα and PKCδ activation is required for $p47^{phox}$ phosphorylation (Iaccio, 2007). NADPH oxidase-dependent ROS production mediates also the FPR2-dependent transactivation of hepatocyte growth factor receptor c-Met in human prostate epithelial PNT1A cells (Cattaneo, 2013). Furthermore, in human umbilical vein endothelial ECV304 cells, FPR1 stimulation by N-fMLP triggers $p47^{phox}$ phosphorylation and the phosphorylation of cytosolic Y951, Y996, and Y1175 residues of VEGFR2 (Cattaneo, 2018).

β-arrestin is also involved in the GPCR-mediated transactivation of different RTKs; for example, agonists of the beta-1 adrenergic receptor trigger β-arrestin-dependent EGFR, AKT and ERK phosphorylation, that is mediated by GRK activation (Noma, 2007).

2. AIM

In the last years, FPRs class has attracted the attention of the scientific community, since these receptors show an heterogenous cell and tissue distribution coupled to their ability to recognize different ligands. Several data suggest that they play further biological roles, such as human inflammatory diseases, neurodegenerative disorders and cancers. To date, the biological functions in non-myeloid cells and the whole signaling network activated by these receptors are still not fully delucidated. Previous studies of my PhD thesis lab demonstrated that FPRs are able to transactivate receptor tyrosine kinases and this trans-phosphorylation is mediated by the generation of reactive oxygen species produced by NADPH oxidase enzymatic complex. In fact, in CaLu-6 and PNT1A cell lines, FPR2 stimulation triggers NOX-dependent EGFR and c-Met transactivation, respectively (Cattaneo, 2011; Cattaneo, 2013), and in human umbilical vein endothelial ECV304 cells FPR1 elicits NADPH oxidase-dependent trans-phosphorylation of vascular endothelial growth factor receptor VEGFR2 (Cattaneo, 2018).

Given the variety of agonists that are associated with nervous system diseases, including b-amyloid peptide, prion protein fragment, humanin and annexin, one of the aims of this research project was to study the biological functions of FPR1 in neuronal cells. In particular, we evaluated the involvement of FPR1 in the transactivation of the nerve growth factor receptor TrkA, one of the most characterized receptor tyrosine kinases in the nervous system, and the role of NOX-dependent ROS in the intracellular signaling. We evaluated the role of FPR-

dependent TrkA transactivation in neuronal survival, migration, differentiation, axon growth, and cell proliferation, and we investigated the intracellular signaling cascades activated by TrkA transactivation.
Furthermore, to characterize phosphorylations of intracellular signaling molecules triggered by FPR activation, this research project also aimed to set up the first global phosphoproteome study for the identification of phosphorylation events triggered by FPR2 in order to deeply characterize the dynamics of signaling networks activated by these receptors.

3. MATERIALS AND METHODS

3.1 Cell lines and reagents

SH-SY5Y cell line (ATCC, Manassas, VA, USA) was cultured in Dulbecco's modified Eagle's medium (DMEM) (Thermo Fisher Scientific, Monza, Italy) containing 15% foetal bovine serum (FBS) (Invitrogen). After reaching 80% confluence, cells were serum-starved for 24 hours, and stimulated with N-fMLP (Sigma) at the final concentration of 0.1μM for 2, 5, or 10 minutes. In other experiments, serum-deprived cells were preincubated for 16 hours with pertussis toxin (PTX) (Sigma) at a final concentration of 100 ng/mL, or with 5μM cyclosporin H (CSH) (Sigma) for 30 minutes, or with 5μM rottlerin (Sigma) for 1 hour, or with 100μM apocynin (Sigma) for 2 hours, or with 10μM GW441756 (Sigma) for 1 hour, before the stimulation with N-fMLP for 5 minutes. CaLu-6 cell line (ATCC, Manassas, VA, USA) was cultured in DMEM (Thermo Fisher Scientific, Monza, Italy) supplemented with 10% foetal bovine serum (FBS) (Invitrogen). Once reached 80% confluence, cells were serum starved for 24 hours, and stimulated for 5 minutes with WKYMVm (Primm, Milan, Italy) at the final concentration of 10μM, or with Lipoxin A4 (Santa Cruz, CA, USA) at the final concentration of 1μM or with Annexin A1 (Abcam) at the final concentration of 10nM. In other experiments, serum-deprived cells were preincubated for 16 hours with 100 ng/mL PTX, or with 10μM WRWWWW (WRW4) (Primm, Milan, Italy) for 15 minutes, or with 50μM PD098059 (Sigma) for 90 minutes, or with 5μM Rottlerin for 1 hour before the stimulation with

WKYMVm. SDS-PAGE reagents were obtained from Bio-Rad (Hercules, CA, USA). Anti-phosphoAkt (S473) (#4060), anti-phosphoP38MAPK (T180, Y182) (#4511), anti-CD133 (#5860), anti-phosphoTrkA(Y490) (#9141) and anti-phosphoTrkA(Y785) (#4168), anti-phosphoHSP-27 (S82), anti-phosphoMCM2 (S139) (#8861) and anti-phosphoRb (S608) (#2181) were from Cell Signaling Technology (Denvers, MA, USA). Anti-phosphoTrkA(Y751) (44-1342G) was from Life technologies. Anti-phosphop47phox(S359) (GTX55429) and anti-phosphoMARCKS (S170) (GTX50348) was from GeneTex (Irvine, CA, USA). Anti-phosphoOSR1 (S339) (#13031) was purchased from Signalway Antibody (Baltimore, MD, USA). Anti-phosphoERK 1/2 (sc-81492), anti-PKCα (sc-8393), anti-PKCδ (sc-937), anti-phosphoPKCδ (T507) (sc-11770), anti-cyclin D (sc-246), anti-cyclin E (sc-248), anti-tubulin (sc-8035), anti-rabbit (sc-2357), and anti-mouse (sc-2005) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

3.2 Protein Extraction and Western Blot Analysis

Whole or membrane lysates proteins were purified from serum-starved SH-SY5Y cells stimulated with 0.1 μM N-fMLP, in presence or absence of the appropriate amounts of selective inhibitors. Whole lysates were obtained by incubation with RIPA buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 1mM EDTA, 0.25% sodium deoxycholate, 1 mM NaF, 10 μM Na3VO4, 1 mM phenylmethylsulfonylfluoride (PMSF), 10 μg/ml aprotinin, 10 μg/ml pepstatin, 10 μg/ml

leupeptin) for 45 min at 4°C. Membrane proteins were purified by incubating SH-SY5Y cells with a buffer containing 10 mM Tris-HCl, 1 mM CaCl2, 150 mM NaCl, 1 mM phenylmethylsulfonylfuoride, 10 µg/mL aprotinin, 10 µg/mL pepstatin, and 10 µg/mL leupeptin (Buffer I). Samples were centrifuged at 400xg for 10 minutes at 4 °C, to obtain a cytosolic (supernatant) and membrane (pellet) fraction. Membrane fraction was washed three times in Buffer I and incubated overnight at 4 °C in constant agitation with a buffer containing 125 mM Tris-HCl, 1 mM PMSF, 1% Triton X100, 10 µg/mL aprotinin, 10 µg/mL pepstatin, and 10 µg/mL leupeptin (Buffer II). Bio-Rad protein assay was used to determine proteins concentration (BioRAD, Hercules, CA, USA). Equal amounts of proteins (40-60 µg, depending on the specific experiment) were separated on 8%, 10% or 12% SDS-PAGE (Biorad), depending on molecular weight of analysed protein. Proteins were elettroblotted onto an immobilion-P PVDF membrane (Thermo Fisher Scientific) and non-specific binding sites were blocked by incubating membranes at room temperature with a solution of 5% non-fat dry milk or 5% bovine serum albumin in Tris Buffered Saline 0.1% Tween for 1 hour. After over-night incubation at 4°C with primary antibodies, membranes were washed and incubated at room temperature for 1 hour with peroxidase-conjugated mouse or rabbit IgG. The expression of targeted proteins was detected by an ECL chemiluminescence reagent kit and visualized by autoradiography. Discover Pharmacia scanner equipped with a sun spark classic densitometric workstation was used to evaluate bands densitometry. The equal amount of loaded protein was determined by reprobing the same filters with an anti-α-tubulin or anti-

CD133 antibody. All western blot experiments are representative of at least four independent experiments.

3.3 Proliferation Assay

$4x10^4$ SH-SY5Y cells/well were seeded in a 24-well plate, serum-starved for 24 hours and treated with 0.1μM N-fMLP, in the presence or absence of the appropriate amounts of selective inhibitors. The number of trypan blue-positive cells were counted at 24, 48 and 72 hours, by direct counting using a Burker chamber. Five independent experiments were performed in triplicate.

3.4 Wound healing Assay

SH-SY5Y cells were cultured until 100% confluences with DMEM containing 15% FBS, at 37°C and 5% CO2. The cell monolayer was scratched with an 80μm diameter sterile tip and the plates were washed with PBS to remove the detached cells. Cells were serum-deprived for 24 hours and incubated with 0.1μM N-fMLP in the presence or absence of the appropriate amounts of selective inhibitors. Four images were captured for the same condition at 0, 24 and 48 hours after the wound by using the Leica AF6000 Modular System and processed by using the Leica LAS AF lite software. Image J software was used to quantify the covered surface from four independent experiments.

3.5 Reactive Oxygen Species Assay

Generation of intracellular ROS was determined by measuring 2′,7′-dichlorodihydrofluorescein-diacetate (H2DCFDA; Sigma) oxidation into the fluorescent 2′,7′-dichlorofluorescein (DCF). 4×10^4 SH-SY5Y cells were seeded in a 12-well plate and cultured at 37°C, 5% CO2 with DMEM supplemented with 15% FBS. Cells were then serum-deprived for 24 hours and stimulated for different times with 0.1μM N-fMLP in the presence or absence of the appropriate amounts of selective inhibitors. Cells were then incubated for 45 minutes at 37°C with 50μM H2DCFDA and oxidization to the fluorescent DCF was analysed by FACS flow cytometer BD Biosciences Accuri C6 Flow Cytometer (BD Biosciences). Five independent experiments were performed in triplicate.

3.6 Neurite Outgrowth Assay

Neurite formation was determined by plating 10^4 cells in a 12-well plate in triplicate and cultured with DMEM supplemented with 15% FBS. Cells serum-starved for 24 hours and then incubated with 0.1μM N-fMLP or with 100ng/ml NGF. Five images/well were recorded and analysed after 24, 48 and 72 hours using ImageJ software plugin NeuronJ from five independent experiments. The length of neurites was measured starting from the soma to the growth cone in each area. Untreated cells were used as controls. The morphometric analysis was performed on the images obtained under inverted-phase-

contrast microscopy (Leica AF6000 Modular System) and processed by using the Leica LAS AF light software.

3.7 Phospho-proteins enrichment

The enrichment of phosphorylated proteins was performed by a phosphoprotein purification kit (Qiagen, Hilden, Germany), accordingly to the manufacturer's instructions. Calu-6 cells were serum starved for 24 hours and treated or not with 10μM WKYMVm for 5 minutes. Whole lysates were incubated with the appropriate amount of Phosphoprotein Lysis Buffer for 30 minutes at 4 °C and gently vortexed every 10 minutes. Lysates were centrifuged at 10000xg for 30 minutes at 4 °C and protein concentration was determined by using a Bio-Rad protein assay (Biorad, Hercules, CA, USA). 2.5 mg of whole lysates were loaded onto Phosphoprotein Purification Column to allow the binding of phosphorylated proteins and the column was washed with the appropriate buffer as described in the manufacturer's instructions. Finally, phosphoproteins were eluted by adding to the column 500μL of Phosphoprotein Elution Buffer. This step was repeated four times and the concentration of the four enriched phosphoprotein fractions was determined. To concentrate samples, an ultrafiltration step was performed by centrifuging the eluted fractions, at 10000xg for 10 minutes.

3.8 Tryptic digestion and sample preparation for MS/MS analyses

Chemicals, tosyl phenylalanyl chloromethyl ketone (TPCK)-treated trypsin were from Sigma Chemical Co. (Milan, Italy). Acetonitrile (CH3CN), formic acid (FA) and water LC-MS grade were from Fisher Scientific Italia (Rodano, Milan, Italy). Aliquots of enriched phosphoprotein samples (~50 μg) from 24-hours serum starved CaLu-6 cells, treated or not with W peptide for 5 minutes, were precipitated by adding pre-chilled acetone (six volumes) for 16 hours at −20 °C. Following centrifugation for 10 min at 8,000×g at 4 °C, protein pellets were resuspended in 100 μL of 50 mM NH4HCO3 pH 8.2. Samples were then subjected to disulphide reduction with 10 mM DTT (1 h at 55 °C) and alkylation with 7.5 mM iodacetamide (20 min at room temperature in the dark). Enzymatic hydrolyses were performed on reduced and alkylated samples by adding TPCK-treated trypsin with an enzyme/substrate (E/S) ratio of 1:100 (w/w) for 3 h and 1:50 for 16 h at 37 °C.

3.9 High-resolution nano LC-tandem mass spectrometry

Mass spectrometry analyses of tryptic digests (2 μg) were performed on a Q-Exactive Orbitrap mass spectrometer equipped with an EASY-Spray nanoelectrospray ion source (Thermo Fisher Scientific, Bremen, Germany) and coupled to a ThermoScientific Dionex UltiMate 3000RSLC nano system (Thermo Fisher Scientific).

3.10 Data processing

The acquired raw files were analysed with Proteome Discoverer 2.1 software (Thermo Fisher Scientific) using the SEQUEST HT search engine. The HCD MS/MS spectra were searched against the Homo sapiens database available in UniprotKB (20,413 reviewed entries, release 2018_01, 31-Jan- 2018) assuming trypsin (Full) as digestion enzyme with two allowed number of missed cleavage sites and a minimum peptide length of six residues. The mass tolerances were set to 10 ppm and 0.02 Da for precursor and fragment ions, respectively. Carbamidomethylation (+57.021464 Da) of cysteine was set as static modification. Phosphorylation of serine, threonine, and tyrosine (+79.966 Da) and acetylation of lysine (+42.011 Da) were set as dynamic modifications. False discovery rates (FDRs) for peptide spectral matches (PSMs) were calculated and filtered using the Target Decoy PSM Validator Node in Proteome Discoverer with the following settings: maximum Delta Cn of 0.05, a strict target FDR of 0.01, a relaxed target FDR of 0.05 and validation based on q-value. Localization and best site probability for variable post-translational modifications within peptides were performed with the ptmRS tool, integrated in Proteome Discoverer26. The Protein FDR Validator Node in Proteome Discoverer was used to classify protein identifications based on q-value. Proteins with a q-value of 0.01 were classified as high confidence identifications and proteins with a q-value of 0.01–0.05 were classified as medium confidence identifications. The resulting protein table was then filtered based on the presence of phosphorylated

peptides. Phosphopeptide changes following WKYMVm treatment were only considered when modifications were identified in at least two out of three replicate injections in treated sample and not identified in replicate injections of untreated sample.

3.11 Bioinformatic analysis

Interaction network analysis of proteins identified by LC-MS/MS were performed by the FunRich open access software.
Molecular functional enrichment of identified proteins based on gene ontology categories was performed by using the Network analyst software.

3.12 Statistical analysis

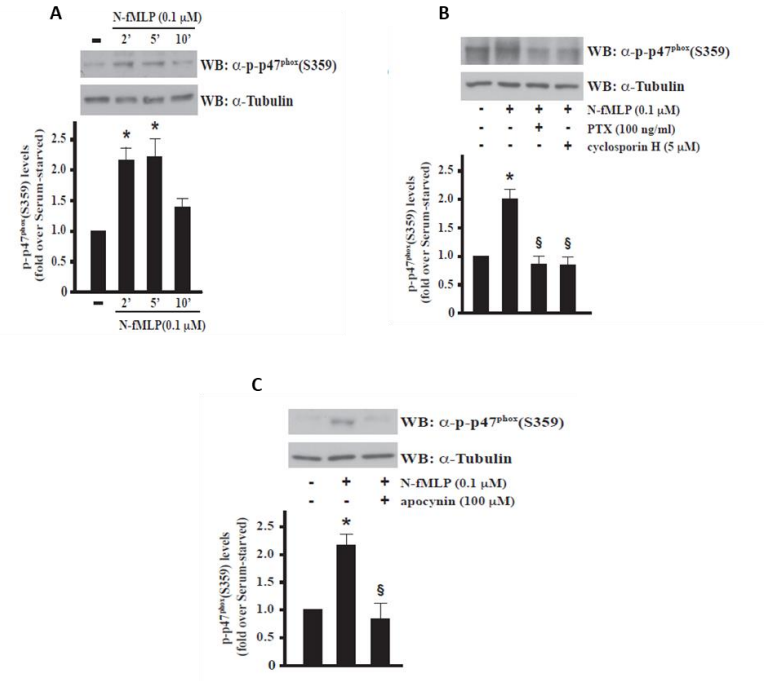
All the data presented are expressed as mean ± Standard Error Mean (SEM) and are representative of three or more independent experiments. For the statistical analyses of experiments in SH-SY5Y cells, the comparisons were made by two-way analysis of variance (ANOVA). In Calu-6 cell experiments, statistical analyses were evaluated by one-way analysis of variance (ANOVA). Differences were considered statistically significant at a p value < 0.05. All the analyses were performed with GraphPad Prism version 7 (GraphPad Software, San Diego, CA, USA).

4. RESULTS

4.1 FPR1 stimulation by N-fMLP induces NOX2 activation in SH-SY5Y cell line

In phagocytes, FPR1 mediates NADPH oxidase-dependent ROS production in response to bacterial N-formyl peptides (Hallet, 1989). Human neuroblastoma SH-SY5Y cell line expresses FPR1 at both transcriptional and translational level (Snapkov, 2016). We tested the hypothesis that FPR1 could induce NADPH oxidase-dependent ROS generation in these cells. NOX2 is expressed in SH-SY5Y cells (Li, 2017) where represents the most important sources of ROS. The key event of NADPH oxidase/NOX2 complex activation is $p47^{phox}$ phosphorylation on a S359 residue, which triggers the membrane assembly of the complex. We demonstrate by western blot experiments that FPR1 stimulation with its agonist N-fMLP, at a final concentration of 0.1µM, induces time-dependent increase in S359 phosphorylation of $p47^{phox}$ (Figure 6A). SH-SY5Y cells were also pretreated for 16 hours with PTX, that ADP-ribosylates $G\alpha_i$ subunit associated with FPR1, or for 30 minutes with 5µM Cyclosporin H, an FPR1 antagonist and western blot experiments show that N-fMLP-induced $p47^{phox}$ phosphorylation is prevented by these pretreatments, demonstrating that $p47^{phox}$ phosphorylation depends on FPR1 stimulation (Figure 6B). Furthermore, preincubation with Apocynin, a specific inhibitor of NADPH oxidase activation and ROS production, prevents N-fMLP-dependent $p47^{phox}$

S359 phosphorylation (Figure 6C). Accordingly with these results, experiments performed by using the ROS detection reagent H_2DCFDA demonstrated that N-fMLP treatment induces NOX2-dependent ROS generation with a maximum at 5 minutes of stimulation (Figure 6D), and that the pretreatment with PTX, Cyclosporin H or Apocynin prevents ROS production (Figure 6E). Taken together, these data demonstrate that, in SH-SY5Y cells, FPR1 stimulation triggers NOX2 activation and ROS production.



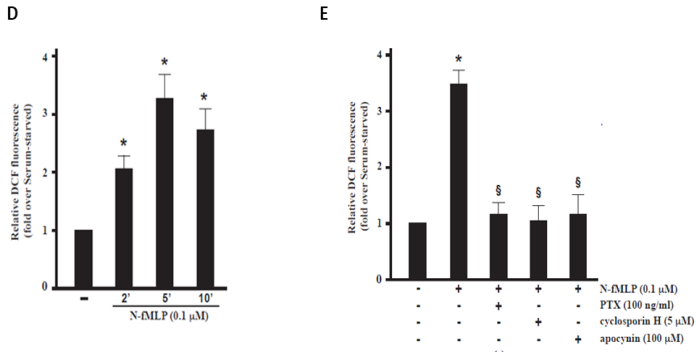


Figure 6. FPR1 stimulation induces NOX2 activation and ROS production. SH-SY5Y cells were serum-starved for 24 hours and (A) stimulated for 2, 5, or 10 minutes with 0.1μM N-fMLP or (B) preincubated with 100 ng/mL PTX (16h), or with 5μM cyclosporin H (30'), or (C) with 100μM apocynin (2h) before stimulation with N-fMLP for 5 minutes. Sixty micrograms of whole lysates (A, B, and C) were incubated with an anti-phosphop47^{phox} (S359) antibody; an anti-α-tubulin antibody was used as a control for protein loading. Band densitometry was evaluated through a scanner equipped with a densitometric workstation. Serum-starved SH-SY5Y cells were (D) stimulated with N-fMLP for 2, 5, or 10 minutes, or (E) preincubated with PTX, or cyclosporin H, or apocynin before the stimulation with 0.1μM N-fMLP for 5 minutes. Detection of ROS was determined by measuring the level of DCF. ⁎$p < 0.05$ compared to unstimulated cells. §$p < 0.05$ compared to N-fMLP stimulated cells.

4.2 FPR1 stimulation by N-fMLP induces NOX2-dependent TrkA transactivation in SH-SY5Y cells

It has been widely demonstrated that GPCRs and RTKs are able to cross-communicate, thus connecting, amplifying and diversifying the canonical signal transduction pathways attributed to one receptor rather than another.
In this study, we tested the hypothesis that FPR1 could induce the transactivation of TrkA, one of the most important RTKs in the nervous system. TrkA is the high affinity receptor for NGF, a neurotrophin that promotes the correct development of the nervous system. Once activated by its ligand, TrkA is phosphorylated in different tyrosine residues. We evaluated the phosphorylation of Y490, Y751 and Y785, the most characterized phosphorylation sites of TrkA following NGF binding. Western blot experiments show that stimulation of SH-SY5Y cells with N-fMLP for 5 minutes induces the phosphorylation of Y490, Y751 and Y785 of TrkA (Figure 7A). The pretreatment with PTX or Cyclosporin H before N-fMLP stimulation prevents these phosphorylations (Figure 7B), confirming that TrkA phosphorylation depends on FPR1 stimulation. The transactivation of a RTK can be mediated by the production of ROS that, at low levels, can act as second messengers, inactivating intracellular protein tyrosine phosphatases, and thus promoting intracellular phosphorylation cascades. The involvement of NOX2-dependent ROS generation in mediating TrkA trans-phosphorylation was analysed by pretreating SH-SY5Y cells with a NADPH oxidase inhibitor, before FPR1 stimulation. As shown in Figure 7C, preincubation with

Apocynin prevents TrkA Y490, Y751 and Y785 phosphorylation, demonstrating that TrkA phosphorylation is mediated by FPR1-induced ROS production.

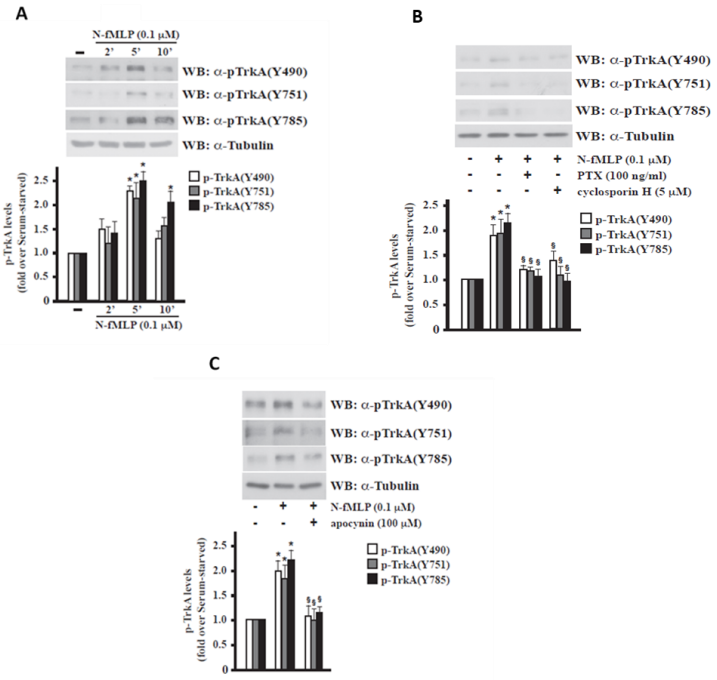


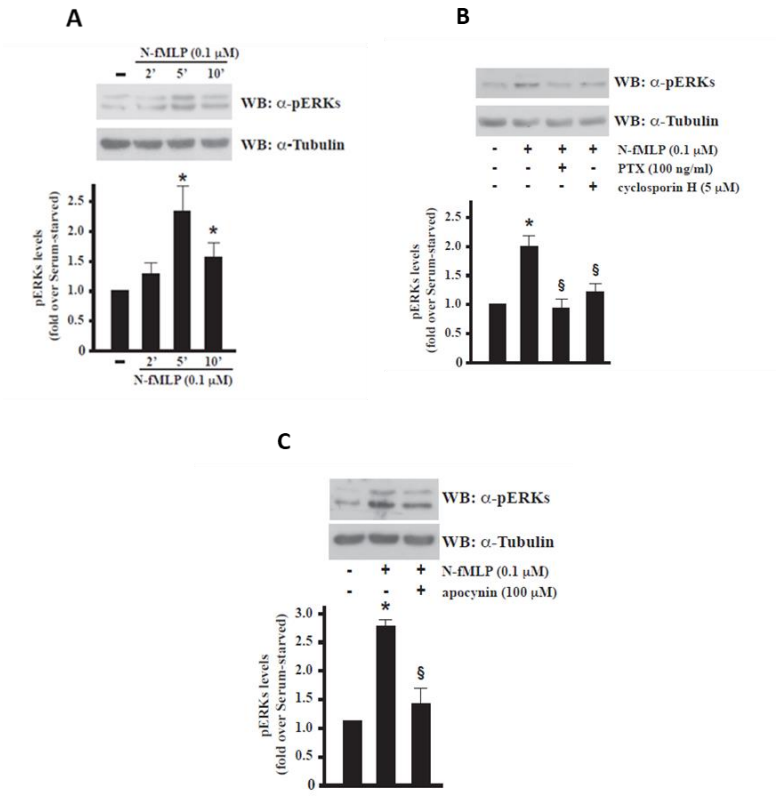
Figure 7. FPR1 stimulation triggers TrkA transactivation. SH-SY5Y cells were serum-deprived for 24 hours and (A) stimulated with N-fMLP for 2, 5, or 10 minutes or (B) preincubated with 100 ng/mL PTX (16h), or with 5μM cyclosporin H (30'), or (C) with 100μM apocynin (2h) before stimulation with 0.1μM N-fMLP for 5 minutes. Fifty micrograms of total protein lysates were resolved on 10% SDS-PAGE and immunoblotted with anti-phosphoTrkA (Y490), or

anti-phosphoTrkA (Y751), or anti-phosphoTrkA (Y785) antibodies. An anti-αtubulin antibody was used as a control for protein loading. Bar graphs show the densitometric analysis performed on phosphorylated bands. All the experiments are representative of four independent experiments. ∗p < 0.05 compared to unstimulated cells. §p < 0.05 compared to N-fMLP-stimulated cells.

4.3 FPR1-induced TrkA transactivation triggers the Ras/MAPK pathway activation in SH-SY5Y cells

TrkA phosphorylation on tyrosine 490 represents a docking site for the binding of the adaptor proteins Shc, GRB2 and SOS, that mediate the activation of the Ras/MEK/ERKs pathway. ERK phosphorylation and activation is associated with cell growth, survival, differentiation and motility. In neuronal cells, ERK1/2 are involved in promoting neurite outgrowth.
Dual threonine and tyrosine residue phosphorylation activates both ERKs, at T202/Y204 for human ERK1 and T185/Y187 for human ERK2. Western blot experiments show that FPR1 stimulation induces a time-dependent-increase in ERK1/2 phosphorylation (Figure 8A), which was prevented by PTX or Cyclosporin H preincubation (Figure 8B). We also preincubated SH-SY5Y cells for 1 hour with the TrkA specific inhibitor GW441756 or with Apocynin. As shown in Figure 8, pretreatment with Apocynin (Panel C) or GW441756 (Panel D), before N-fMLP stimulation, prevents FPR1-dependent ERK1/2 phosphorylation, suggesting that ERK1/2 phosphorylation depends on FPR1 stimulation and on NADPH oxidase-mediated TrkA activation.
NGF-dependent TrkA activation triggers p38MAPK phosphorylation. Our results show that stimulation of SH-SY5Y cells for 5 minutes with N-fMLP induces a

significative increase in p38MAPK phosphorylation, which is prevented by preincubation with PTX or cyclosporin H before N-fMLP stimulation (Figure 8E).



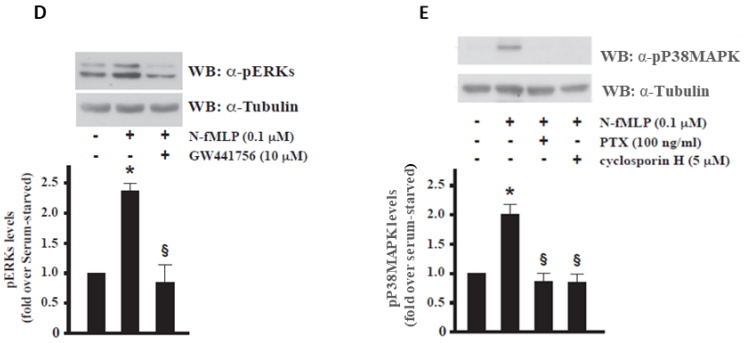


Figure 8. N-fMLP-induced TrkA phosphorylation on Y490 is crucially involved in MAPK activation. Serum-deprived SH-SY5Y cells were (A) stimulated for 2, 5, or 10 minutes with 0.1 μMN-fMLP or (B and E) pretreated with 100 ng/mL PTX (16h) or 5μM cyclosporin H (30'), or (C) with 100μM apocynin (2h), or (D) with 10μM GW441756 (1h), before the stimulation with N-fMLP for 5 minutes. Forty micrograms of whole lysates were incubated with (A, B, C, and D) an anti-phospho-ERK1/2 antibody or (E) with an anti-phospho-P38MAPK (α-pP38MAPK) antibody. An anti-tubulin (α-tubulin) antibody was used as control for protein loading. The data are representative of five independent experiments. Densitometric analysis was performed as described in Materials and Methods. ∗$p < 0.05$ compared to unstimulated cells. §$p < 0.05$ compared to N-fMLP-stimulated cells.

4.4 N-fMLP-dependent phosphorylation of Y490 and Y751 residues of TrkA triggers the PI3K/Akt pathway in SH-SY5Y cells

TrkA phosphorylation on tyrosine 490 or 751 is associated with the activation of the PI3K-Akt pathway. The PI3K-Akt signaling pathway plays a critical role in mediating survival signals in a wide variety of neuronal cells. Full activation of Akt requires serine 473 (S473) phosphorylation. Western blot experiments show that in SH-SY5Y cells, N-fMLP induces a time-dependent increase in Akt phosphorylation on S473, with a maximum after 5 minutes of stimulation (Figure 9A), which was prevented by the pretreatment with PTX or Cyclosporin H, before stimulation with N-fMLP for 5 minutes (Figure 9B). We also show that Akt phosphorylation depends on FPR1-dependent TrkA phosphorylation, as demonstrated by the loss of Akt S473 phosphorylation in SH-SY5Y cells pretreated with the TrkA inhibitor GW441756 (Figure 9D), and is mediated by NADPH oxidase activation, as demonstrated by the loss of Akt S473 phosphorylation following Apocynin pretreatment (Figure 9C).

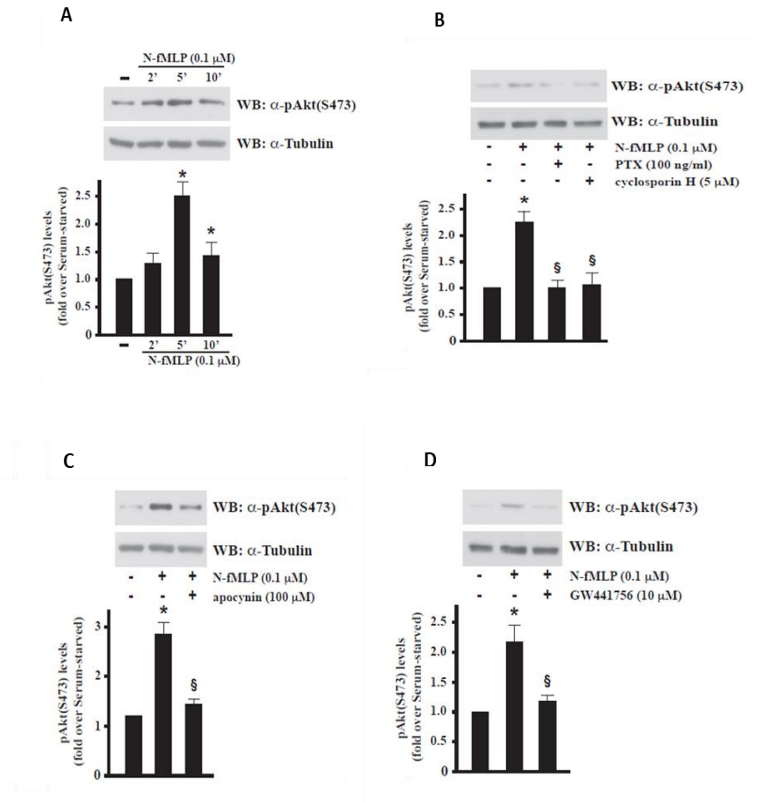


Figure 9. FPR1-mediated phosphorylation of Y751 residue of TrkA provides a docking site for PI3K/Akt pathway activation. SH-SY5Y cells were serum-starved for 24 hours and (A) stimulated for 2, 5, or 10 minutes with 0.1μM N-fMLP or (B) pretreated with 100 ng/mL PTX (16h) or 5μM cyclosporin H (30') or (C) with 100μM Apocynin (2h) or (D) with 10μM GW441756 (1h), before N-fMLP stimulation. Fifty micrograms of whole lysates were immunoblotted with an anti-phosphoAkt(S473) antibody. An anti-αtubulin antibody was used as a control for protein loading. All the experiments are representative of five independent experiments. ∗p < 0.05 compared to unstimulated cells. §p < 0.05 compared to N-fMLP-stimulated cells.

4.5 FPR1-mediated phosphorylation of Y785 residue of TrkA provides a docking site for PLCγ1/PKC pathway activation in SH-SY5Y cells

TrkA phosphorylation on tyrosine 785 represents a docking site for the binding of PLCγ, the enzyme that converts the PIP_2 in DAG and IP_3 which, in turn, trigger an increase of Ca^{2+}, an activator of PKC pathway. Upon activation, PKC translocates from the cytosol to the membrane, where it associates with phosphatidylinositols. In neuronal cells, PKCα is involved in regulation of neuritogenesis and synaptic plasticity and PKCδ is involved in promoting neurite outgrowth. Our experiments demonstrate that stimulation of SH-SY5Y cells with N-fMLP for 5 minutes induces PKCα and PKCδ membrane translocation, which was prevented when cells are preincubated with PTX and Cyclosporin H (Figure 10A). Phosphorylation of PKCδ on threonine 507 is associated with an increase in its phosphotransferase activity. We observed that, in SH-SY5Y cells, stimulation with N-fMLP for 5 minutes induces T507 phosphorylation of PKCδ and that the pretreatment with Apocynin and Rottlerin, a selective PKCδ inhibitor with IC50 values of 3-6 μM, prevents PKCδ T507 phosphorylation (Figure 10B).

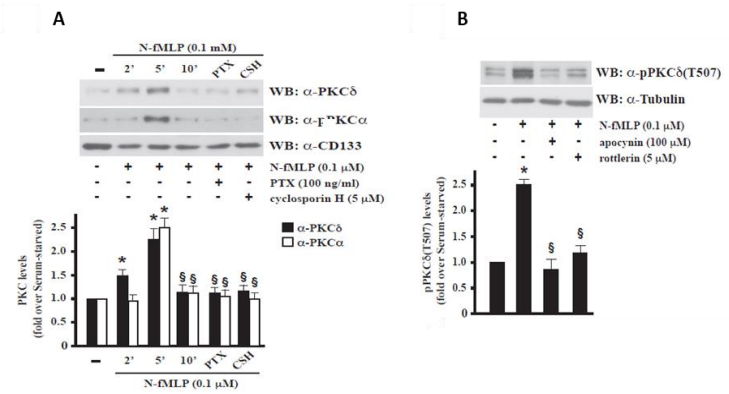


Figure 10. FPR1-mediated TrkA transactivation triggers PLCγ1/PKC pathway activation. SH-SY5Y cells were serum-deprived for 24 hours and (A) stimulated for 2, 5, or 10 minutes with N-fMLP or pretreated with 100 ng/mL PTX (16h) or 5μM cyclosporin H (30') before the stimulation for 5 minutes with N-fMLP. Cells were also preincubated with 100μM Apocynin (2h) or 5μM Rottlerin (1h) before N-fMLP stimulation (B). Fifty micrograms of membrane lysates were immunoblotted with an anti-PKCδ or anti-PKCα antibody (A). Fifty micrograms of whole lysates were immunoblotted with an anti-phosphoPKCδ (T507) antibody (B). An anti-CD133 or an anti- α tubulin antibody was used as control for protein loading. ∗p < 0:05 compared to unstimulated cells. §p < 0:05 compared to N-fMLP-stimulated cells.

4.6 FPR1-mediated TrkA transactivation promotes cell proliferation in SH-SY5Y cells

FPR1 expression and activation are associated with increased tumorigenicity of different cancers. Therefore, we investigated whether FPR1 stimulation could induce cell proliferation. SH-SY5Y cells were serum-starved for 24 hours and stimulated with 0.1μM N-fMLP for 24,

48, and 72 hours. We evaluated the rate of cell proliferation by using Trypan blue dye for counting cells after different times of exposition to the formyl-peptide. Our results indicate that FPR1 stimulation by N-fMLP induces a time-dependent proliferation of SH-SY5Y cells. This is prevented by preincubation with PTX, or cyclosporin H, or GW441756, thus suggesting that it depends on FPR1-dependent TrkA transactivation (Figure 11).

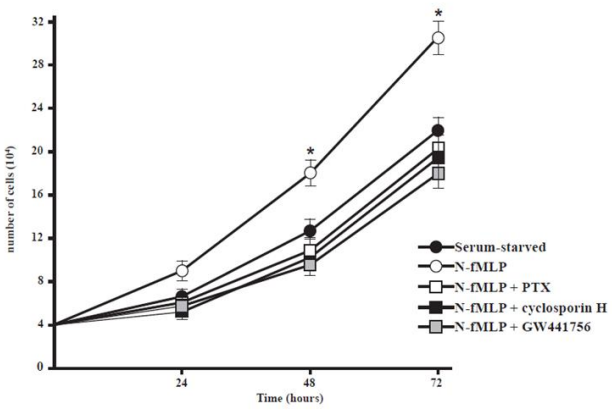


Figure 11. FPR1-mediated TrkA transactivation promotes SH-SY5Y cell proliferation. (a) SH-SY5Y cells were grown in the presence or absence of 0.1μM N-fMLP and preincubated or not with 100ng/mL PTX (16h), or 5μM cyclosporin H (30'), or 10μM GW441756 (1h). The cellular proliferation graph is representative of five independent experiments. Cell count was determined at 24, 48, and 72 h after plating 10^4 cells/well for each condition.

4.7 FPR1-mediated TrkA transactivation promotes cell migration in SH-SY5Y cells

FPR1 expression and activation is associated with migration of leukocytes in response to a large variety of chemotactic factors, and with the invasion ability of different cancers. Thus, we investigated whether FPR1 stimulation could promote cell migration in SH-SY5Y cells by performing and *in vitro* wound healing assay. Cells were serum-starved for 24 hours and stimulated with 0.1μM N-fMLP for 24 or 48 hours. Our results show that FPR1 stimulation increases cell migration, with respect to untreated cells, and that this is prevented by the preincubation with PTX, or Cyclosporin H, or with the TrkA inhibitor GW441756, suggesting that it depends on FPR1-dependent TrkA transactivation (Figure 12).

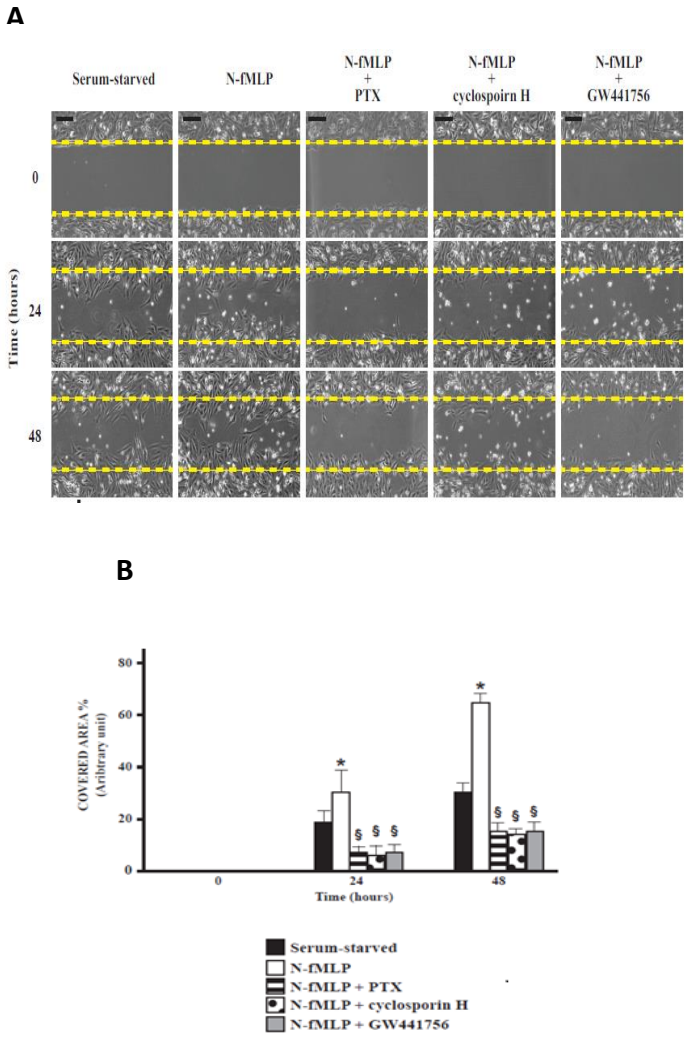


Figure 12. FPR1-mediated TrkA transactivation promotes SH-SY5Y cell migration. Representative images (A) and bar graph (B) quantification of SH-SY5Y cell migration from four independent

experiments. Cells were incubated with 0.1μM N-fMLP or vehicle in the presence or absence of 100ng/mL PTX (16h), or 5μM cyclosporin H (30'), or 10μM GW441756 (1h). Images were acquired at 0, 24, and 48 hours after wound injury (scale bar: 20 μm). ∗p < 0.05 compared to unstimulated cells. §p < 0.05 compared to N-fMLP-stimulated cells.

4.8 FPR1-mediated TrkA transactivation promotes neurite outgrowth in SH-SY5Y cells

TrkA stimulation through the binding of NGF is associated with morphological variations of neuronal cells, such as neurite outgrowth, a process that, through cytoskeletal reorganization, determines dendritic and axonal growth and branching. We examined the ability of FPR1-induced TrkA transactivation to induce neurite outgrowth in SH-SY5Y cells.
These were serum-starved for 24 hours and treated with 0.1μM N-fMLP or with 100ng/mL NGF, as positive control, for 24 or 48 hours. Neurite outgrowth was evaluated as the length of neurites measured starting from the soma to the growth cone. The results demonstrated that N-fMLP stimulation increases neurite outgrowth, when compared to untreated cells and NGF-treated cells (Figure 13).

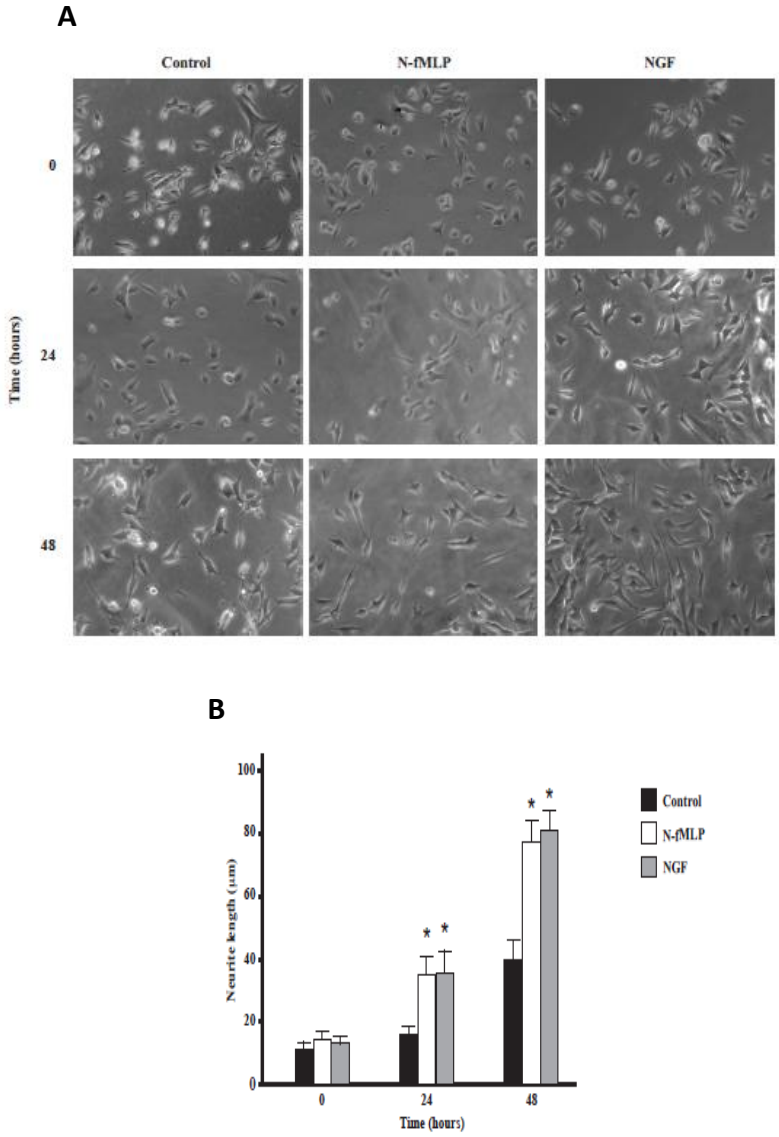


Figure 13. FPR1-mediated TrkA transactivation promotes SH-SY5Y neurite outgrowth. Representative images (A) and bar graph (B)

quantification of SH-SY5Y neurite outgrowth from five independent experiments. Neurite length was measured in untreated SH-SY5Y cells treated or not with 0.1μM N-fMLP or 100ng/mL NFG up to 48 hours. Neurite length was measured at different times (0, 24, and 48 hours). ∗p < 0.05 compared to unstimulated cells. §p < 0.05 compared to N-fMLP-stimulated cells.

4.9 Phosphoproteomic analysis in Calu-6 cells identifies WKYMVm-dependen tprotein phosphorylations

Our previous results demonstrated that in human lung anaplastic carcinoma Calu-6 cells FPR2 stimulation by WKYMVm triggers $p47^{phox}$ phosphorylation, c-Src phosphorylation, EGFR transactivation, ERK1/2 and p38MAPK phosphorylation, and STAT3 phosphorylation (Cattaneo, 2011).
To elucidate the global intracellular phosphorylation cascades induced by WKYMVm stimulation of FPR2, we purified protein extracts from serum-starved Calu-6 cells, treated or untreated with WKYMVm for 5 minutes, to elucidate the global intracellular phosphorylation cascades induced by FPR2 stimulation. Total extracts were used to perform an affinity chromatography based on the titanium dioxide (TiO_2) method for the enrichment of phosphorylated proteins, by using the Qiagen phosphoprotein purification kit. TiO_2 method allows selective interaction of organic phosphates of phosphorylated proteins with porous TiO_2 beads. Subsequently, enriched phosphoproteins were enzymatically digested by using protease trypsin to obtain phosphopeptides. These were used to perform a

nano-liquid chromatography followed by high-resolution tandem mass spectrometry (nano-LC-MS/MS) (Figure 14).

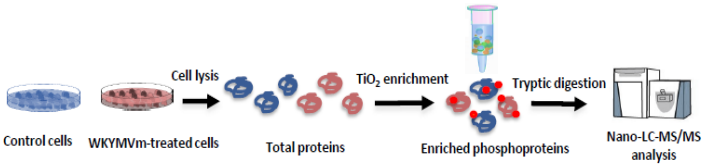


Figure 14. Schematic diagram of experimental workflow. Total proteins were purified from control and WKYMVm-treated CaLu-6 cells for the analysis of phosphoproteome by nano-liquid TiO_2 based-chromatography coupled with high-resolution tandem mass spectrometry.

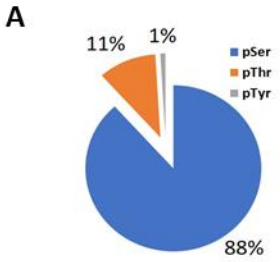
Mass spectrometry analysis allowed the identification of 290 proteins with a total of 510 different phosphopeptides identified, originated from 430 phosphorylation sites. We identified 53 unique phosphopeptides mapping on 40 proteins that result to be phosphorylated exclusively in WKYMVm-stimulated Calu-6 cells, with respect to untreated cells (Figure 15).

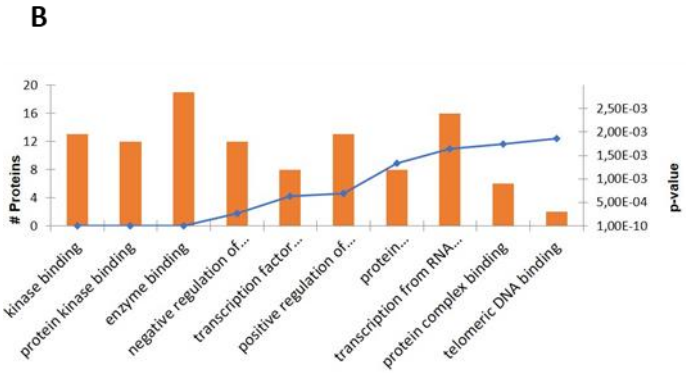
Accessions	Description	Peptide Sequence	Positions	ptmRS Probabilities
O60256-1	PRPP synthase-associated protein 2	[R].LGIAVIHGEAQDAESDLVDGRHSPPMVR.[S]	[205-232]	S23: 100
O95425-3	Isoform SV3 of Supervillin	[R].SPSFGDPQLSPEARPR.[CV]	[261-276]	S10: 100
O95747	Serine/threonine-protein kinase OSR1	[R].LHKTEDGSWEWSDDEFDEESEEGK.[A]	[328-351]	S12: 100
P04792	Heat shock protein beta-1	[R].QLSSGVSEIR.[H]	[80-89]	S3: 100
P06400	Retinoblastoma-associated protein	[K].DREGPTDHLESACPLNLPLQNNHTAADMYLSPVRSPK.[K]	[578-614]	S31: 98.09; S35: 100
P06748	Nucleophosmin	[K].MQASIEKGGSLPKVEAK.[F]	[251-267]	S4: 100
P07910-1	Heterogeneous nuclear ribonucleoproteins C1	[K].MESEGGADDSAEEGDLLDDDDNEDRGDDQLELIK.[D]	[238-271]	S10: 100
P16401	Histone H1.5	[K].KATKSPAKPK.[A]	[185-194]	S5: 100
P23588	eukaryotic translation initiation factor 4B	[R].ERHPSWRSEETQER.[E]	[402-415]	S5: 99.37
P29590	Protein PML	[R].SPVIGSEVFLPNSNHVASGAGEAEER.[V]	[530-555]	S1: 100
P29966	Myristoylated alanine-rich C-kinase substrate	[K].SFKLSGFSFKK.[N]	[163-173]	S8: 100
P31943	Heterogeneous nuclear ribonucleoprotein H 1	[K].HTGPNSPDTANDGFVR.[L]	[99-114]	S6: 100
P38159-1	RNA-binding motif protein,X chromosome	[R].DVYLSPRDDGYSTKDSYSSR.[D]	[204-223]	S5: 100
P42167	Lamina-associated polypeptide 2	[R].AKTPVTLK.[Q]	[206-213]	T3: 100
P49006	MARCKS-related protein	[K].KFSFKKPFK.[L]	[91-99]	S3: 100
P49585	choline-phosphate cytidylyltransferase A	[R].HKAAAYDISEDEED.[-]	[354-367]	S9: 100
P51991-1	Heterogeneous nuclear ribonucleoprotein A3	[R].SSGSPYGGGYGSGGSGSGGYGSRRF.[-]	[355-378]	S4: 100
Q01518-1	adenylyl cyclase-associated protein 1	[R].SGPKPFSAPKPQTSPSPKR.[A]	[295-313]	T13: 99.99; S14: 50; S16: 50
Q01581	hydroxymethylglutaryl-CoA synthase, cytoplasmic	[R].RPTPNDDTLDEGVGLVHSNIATEHIPSPAK.[K]	[469-498]	S27: 100
Q04637-8	Isoform 8 of eIF-4-gamma 1	[R].EAALPPVSPLKAALSEEELEKK.[S]	[1225-1246]	S8: 100
Q13442	28 kDa heat- and acid-stable phosphoprotein	[K].SLDSDESEDEEDDYQQKRK.[G]	[57-75]	S4: 100; S7: 100
Q15424-3	Isoform 3 of Scaffold attachment factor B1	[R].SVVSFDKVKEPR.[K]	[601-612]	S4: 100
Q8IU81	interferon regulatory factor 2-binding protein 1	[R].AGGASPAASSTAQPPTQHR.[L]	[449-467]	S5: 100
Q8NC51-1	PAI1 RNA-binding protein 1	[R].GGSGSHNWGTVKDELTESPKYIQK.[Q]	[217-240]	S18: 96.8
Q8WUD4	Coiled-coil domain-containing protein 12	[R].LKGQEDSLASAVDAATEQKTCDSD.[-]	[143-166]	S23: 100
Q8WW12	PEST proteolytic signal-containing nuclear protein	[K].TLSVAAAFNEDEDSEPEEMPPEAKMR.[M]	[106-131]	S14: 100
Q8WY36	HMG box transcription factor BBX	[R].TADGRVSPAGGTLDDKPKEQLQR.[S]	[838-860]	S7: 99.99
Q92538	BFA-resistant GEF 1	[R].GYTSDIEVYTDHGRPGK.[I]	[1315-1331]	S4: 99.46
Q96A00	Protein phosphatase 1 regulatory subunit 14A	[R].VLSKLQSPSR.[A]	[10-19]	S7: 99.72
Q9HC35	Echinoderm microtubule-associated protein-like 4	[R].ASPSPQPSSQPLQIHR.[Q]	[143-158]	S4: 99.36
Q9UDY2-7	Isoform 7 of Tight junction protein ZO-2	[R].SSEPVQHEESIRKPSPEPR.[A]	[983-1001]	S15: 99.98
Q96FF9	Sororin	[R].SGPRAPSPTKPLRR.[S]	[15-28]	S7: 99.99
Q8NE71-1	ATP-binding cassette sub-family F member 1	[K].KAEQGSEEEGEGEEEEEEGGESKADDPYAHLSK.[K]	[223-255]	S6: 100
		[R].LKKLSVPTSDEEDEVPAPKPR.[G]	[101-121]	S5: 100; T8: 100; S9: 100
Q9GZR7	ATP-dependent RNA helicase DDX24	[K].AQAVSEEEEEEEGKSSSPK.[K]	[78-96]	S5: 100
		[K].AQAVSEEEEEEEGK.[S]	[78-91]	S5: 100
		[R].KAQAVSEEEEEEEGK.[S]	[77-91]	S6: 100
		[K].VVDYSQFQESDDADEDYGRDSGPPTKK.[I]	[10-36]	S10: 100
Q9H6F5	Coiled-coil domain-containing protein 86	[R].APGSPRGQHEPSKPPPAGETVTGGFGAK.[K]	[185-212]	S4: 100
		[R].AGLGSPERPPKTSPGSPR.[L]	[54-71]	S16: 99.99
		[R].RALVEFESNPEETREPGSPPSVQR.[A]	[30-53]	S18: 99.18
Q8WWM7-3	Isoform 3 of Ataxin-2-like protein	[K].EVDGLLTSEPMGSPVSSK.[T]	[582-599]	S13: 100
		[K].STSTPTSPGPR.[T]	[678-688]	S7: 100
P52926-1	high mobility group protein HMGI-C	[R].KWPQQVVQKKPAQEETEETSSQSEAEED.[-]	[82-109]	S24: 100
		[R].KWPQQVVQKKPAQEETEETSSQSEAEED.[-]	[82-109]	T19: 66.67; S20: 66.67; S21: 66.67; S24: 100
		[R].KWPQQVVQKKPAQEETEETSSQSEAEED.[-]	[82-109]	S20: 97.22; S21: 97.22
P19338	Nucleolin	[K].AAAAAPASEDEDDEDDEDDEDDDDDEEDDSEEEAMETTPAKGKK.[A]	[177-220]	S8: 100; S30: 100
		[K].VVVSPTKKVAVATPAK.[K]	[64-79]	S4: 99.99
P49736	DNA replication licensing factor mcm2	[R].GLLYDSDEDEERPARK.[R]	[134-150]	S6: 100
		[R].RGLLYDSDEDEERPARK.[R]	[133-150]	S7: 100
Q9H1E3	Nuclear ubiquitous casein and cyclin-dependent kinase substrate 1	[K].VVDYSQFQESDDADEDYGRDSGPPTK.[K]	[10-35]	S10: 100
		[R].KVVDYSQFQESDDADEDYGRDSGPPTK.[K]	[9-35]	S11: 100
		[R].LKATVTPSPVKGK.[G]	[174-186]	T6: 100; S8: 100

Figure 15. Phosphopeptides uniquely identified in WKYMVm-stimulated CaLu-6 cells. Accession numbers, peptide sequence, positions within proteins, localization and best site probability for phosphorylation within peptides are reported.

4.10 Functional bioinformatic analysis of FPR2 signaling-dependent phosphorylations

The bioinformatic analysis of phosphoproteins obtained from WKYMVm-stimulated Calu-6 cells was performed by using different bioinformatic software. Localization and best site probability for variable post-translational modifications within peptide were obtained using the ptmRS tool of the Proteome Discoverer software by calculating individual probability values for each putatively modified site based on MS/MS data. Characterization of phosphorylated sites highlights the high prevalence of serine phosphorylation (about 88%), with respect to 11% of threonine and 1% of tyrosine phosphorylation (Figure 16A).
The Network analysis software has allowed the classification of proteins on the basis of different functional categories, including kinase binding proteins, enzyme binding proteins, proteins involved in transcription, regulation, and DNA-binding proteins (Figure 16B). The PANTHER (Protein ANalysis THrough Evolutionary Relationships) classification system was used to classify proteins based on protein classes and on biological processes in which proteins are involved. Eight different protein groups were identified through biological processes classification; part of these include proteins involved in metabolic processes, biological process regulation and cellular component organization or biogenesis (Figure 16C). Furthermore, PANTHER analysis characterized six different protein classes, most of which are DNA- or RNA-binding proteins, and cytoskeletal proteins (Figure 16D).


A
11%
1%
88%
pSer
pThr
pTyr


B
Proteins
p-value
20
16
12
8
4
0
2,50E-03
2,00E-03
1,50E-03
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5,00E-04
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kinase binding
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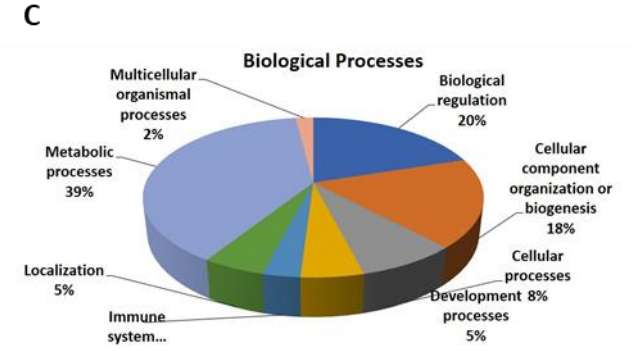
enzyme binding
negative regulation of...
transcription factor...
positive regulation of...
protein...
transcription from RNA...
protein complex binding
telomeric DNA binding


C
Biological Processes
Multicellular organismal processes 2%
Biological regulation 20%
Metabolic processes 39%
Cellular component organization or biogenesis 18%
Cellular processes
Development 8%
processes
5%
Localization 5%
Immune system...

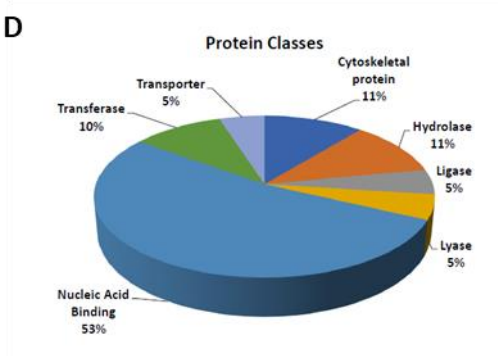


Figure 16. Bioinformatic analysis of phosphoproteins uniquely identified in WKYMVm stimulated CaLu-6 cells. (A) Pie chart of the percentage of phosphorylated amino acids obtained from phosphoproteomic analysis. (B) Bar graph of the of phosphoprotein molecular functions. The number of proteins belonging to the enriched gene ontology term and the p-values are reported on the graph. (C) PANTHER classification of phosphorylated proteins in according to their Biological Processes. (D) PANTHER classification of phosphorylated proteins in according to the Protein Classes.

Furthermore, the FunRich software was used to highlight the biological connections existing among enriched phosphoproteins. This *in silico* analysis generated an interaction network map obtained from the integration of the accession numbers corresponding to the identified phosphoproteins (Figure 17, grey nodes), with accession numbers of proteins that, from literature data, are known to be involved in FPR2-dependent signaling (Figure 17, red nodes), such as EGFR, ERK1/2, PKCs, Akt, STAT3, $p47^{phox}$, and with

phosphoproteins uniquely identified in WKYMVm-treated cells in phosphoproteomic analysis (Figure 17, green nodes). The larger cluster proteins converge on β-arrestin-1 (ARRB1), that exerts an important role in the regulation of GPCR signalling pathways.

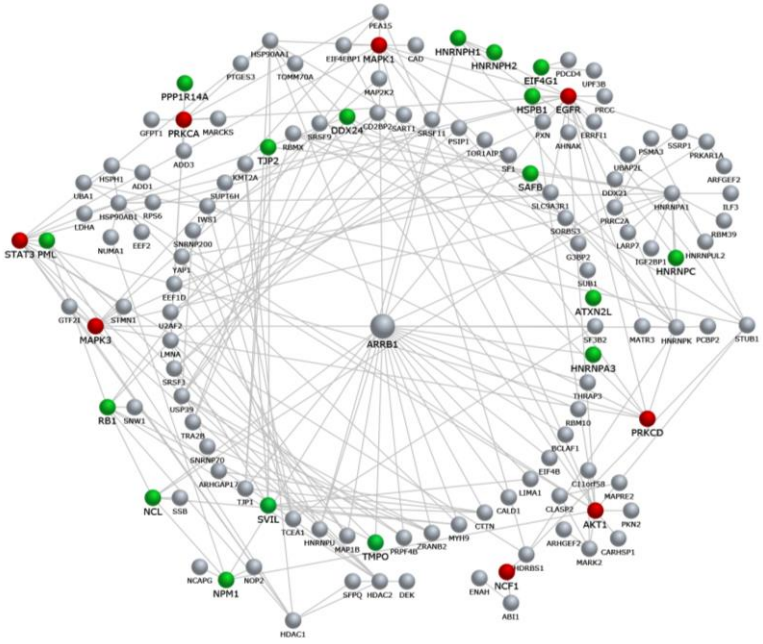


Figure 17. Interaction network analysis of phosphoproteins identified by LC-MS/MS. Phosphoproteins identified by LC-MS analysis are indicated as grey nodes, the subset of proteins uniquely identified upon WKYMVm stimulation as green nodes, and selected phosphoproteins known to be involved in FPR2 signaling as red nodes.

4.11 FPR2 stimulation triggers HSP-27, MCM2, OSR1, Rb and MARCKS phosphorylation

Phosphoproteins identified by mass spectrometry analysis were subsequently validated by performing western blotting experiments. We selected a subset of identified phosphoproteins for further validation on the basis of their involvement in signal transduction pathways activated by FPR2 stimulation, in control of cell cycle and proliferation, in cytoskeletal rearrangements and in cell migration. We investigated the phosphorylation of serine 82 (S82) of Heat Shock Protein-27 (HSP-27), serine 139 (S139) of Mini-Chromosome Maintenance (MCM2), serine 339 (S339) of Oxidative-Stress-Responsive Kinase 1 (OSR1), serine 608 (S608) of Retinoblastoma (RB), and serine 170 (S170) of Myristolated Alanine-Rich C-Kinase Substrate (MARCKS). HSP-27 is a heat shock protein that exerts anti-inflammatory and pro-resolving activities and is activated by p38MAPK and PKC-dependent phosphorylation on a S82 residue. Western blotting experiments showed an increase in HSP-27(S82) phosphorylation in serum-starved Calu-6 cells stimulated for 5 minutes with WKYMVm (Figure 18A). MCM2 is a helicase involved in promoting DNA replication initiation and elongation. Its phosphorylation is involved in the control of cell cycle progression and in checkpoints regulation. We found that WKYMVm stimulation of Calu-6 cells for 5 minutes triggers S139 phosphorylation of MCM2 (Figure 18B). OSR1 is involved in the regulation of cation-chloride cotransporters and is activated by PI3K-Akt-WNK pathway and mTORC2 pathway. Western blotting

analysis showed that FPR2 stimulation triggers Ser339 phosphorylation of OSR1 (Figure 18C). Rb is a tumor suppressor protein involved in cell cycle control. Phosphorylation of Rb on S608 inhibits its binding to the transcription factor E2F, which promotes cell cycle progression when it is dissociated from Rb. WKYMVm stimulation of Calu-6 cells for 5 minutes induces a strong phosphorylation of Rb on S608 (Figure 18D). MARCKS is involved in cytoskeletal reorganization, actin binding, migration and cell cycle regulation. In WKYMVm-stimulated cells, phosphorylation of MARCKS on S170 residue was detected after WKYMVm stimulation (Figure 18E). All these phosphorylations are prevented by pretreatment with the FPR2 antagonist WRW4 or with G_i protein inhibitor PTX (Figures 18A-E).

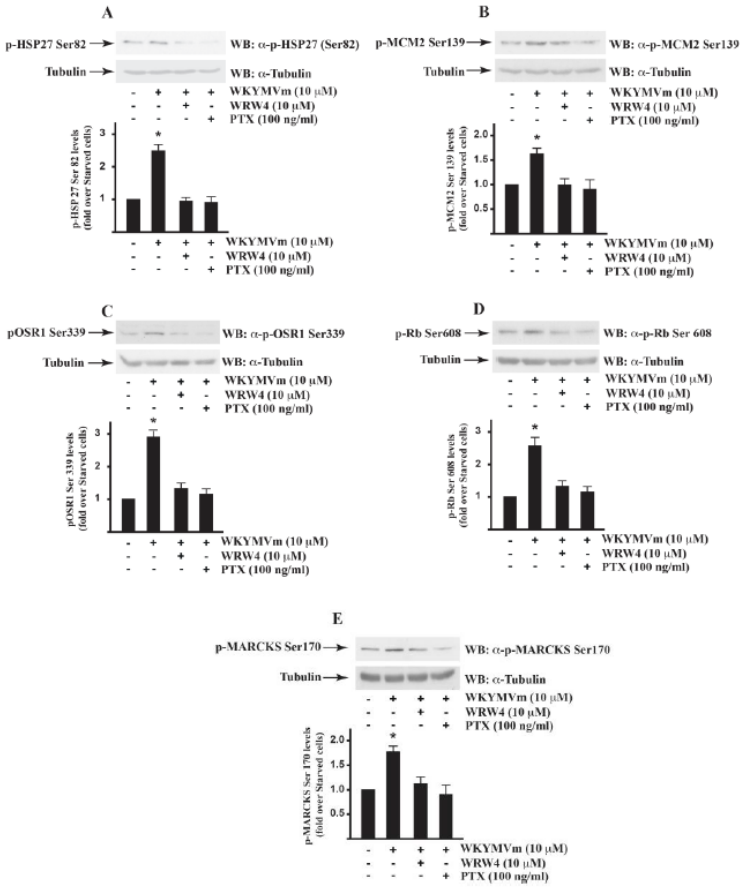


Figure 18. Validation of FPR2-dependent phosphorylations. Western blot and densitometric analyses of at least three independent experiments. Fifty micrograms of whole lysates were purified from 24 hours serum-starved CaLu-6 cells stimulated or not with 10 μM WKYMVm for 5 minutes, in the presence or absence of 10 μM WRWWWW (WRW4) or 100 ng/mL PTX. Lysates were resolved on 10% SDS-PAGE and hybridized with anti phospho-specific antibodies: (A) p-HSP-27 (Ser82); (B) p-MCM2 (Ser139); (C) p-OSR1(Ser339); (D) p-Rb(Ser608) and(E) p-MARCKS (Ser170). An anti-tubulin antibody was used as control for protein loading.

We also used specific inhibitors to determine the interconnection between some of the signaling readouts identified. PKC and MAPK represent two nodes in the interaction network analysis (Figure 17). Therefore, we preincubated cells with Rottlerin (a PKC inhibitor) or PD098059 prior WKYMVm stimulation. In western blot experiments we observed that MARCKS(S170) phosphorylation is prevented by the inhibition of PKCδ (Figure 19A). Moreover, the MEK inhibitor PD098059 prevents both Rb(S608) (Figure 19B) and MCM2(S139) (Figure 19C) phosphorylation induced by WKYMVm stimulation. These results strengthen the notion on the ability of FPR2 to trigger Ras/MAPK cascade, mitogenic stimuli and, in turn, cell cycle progression.

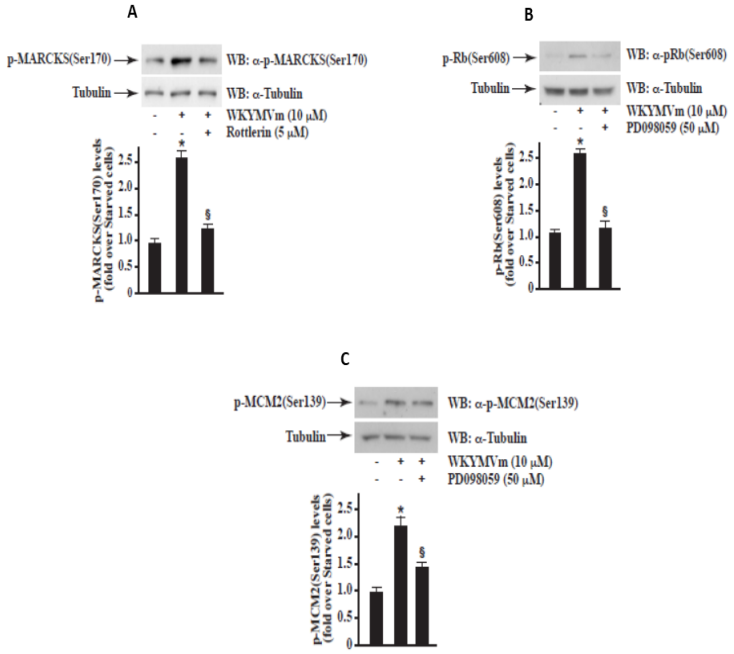


Figure 19. MARCKS (Ser170), MCM2 (Ser139) Rb (Ser608) phosphorylations are mediated by PKC and ERK signaling. Fifty micrograms of proteins were purified from 24 hours serum-starved Calu-6 cells stimulated or not with 10 μM W peptide for 5 minutes, in the presence or absence of 5 μM Rottlerin (A) or 50 μM PD098059 (B, C). Lysates were resolved on 10% SDS-PAGE and hybridized with anti anti p-MARCKS(Ser170) (A), or anti p-Rb(Ser608) (B), or anti p-MCM2(Ser139) (C). An anti-tubulin antibody was used as a control for protein loading. Densitometric analyses was performed on at least three independent experiments.

4.12 FPR2 agonists with anti-inflammatory activity induce HSP-27, OSR1, Rb and MARCKS phosphorylation

FPR2 can transmit both pro- and anti-inflammatory signals. WKYMVm is an hexapeptide derived from a screening of a peptide library, with an anti-inflammatory and immunomodulatory biological activity. We stimulated CaLu-6 cells with two others anti-inflammatory FPR2 ligands, the peptide agonist ANXA1 and the lipidic agonist LXA4, and we observed that both induce HSP-27(S82), OSR1(S339), Rb(S608), and MARCKS(S170) phosphorylation, which was prevented by pretreatment with PTX or WRW4 (Figure 20A-D, Figure 21A-D). However, no significant variation was detected in MCM2(S139) phosphorylation upon ANXA1 or LXA4 stimulation (Figure 20E and Figure 21E), suggesting that MCM2(S139) phosphorylation depends exclusively on WKYMVm stimulation.

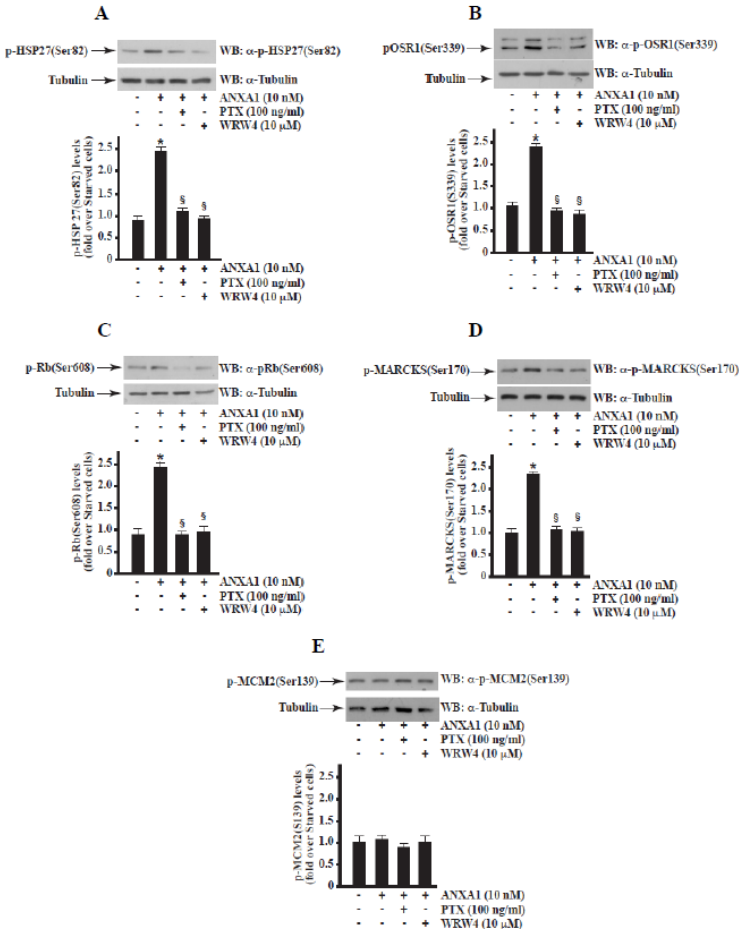


Figure 20. ANXA1 triggers HSP-27(S82), OSR1(S339), Rb(S608) and MARCKS(S170) phosphorylation. CaLu-6 cells were serum-starved for 24 hours and stimulated or not with 10nM ANXA1 for 5 minutes, or preincubated with 10μM WRW4 or 100 ng/mL PTX before ANXA1 stimulation. 50μg of whole lysates were resolved on 10% SDS-PAGE and filters were incubated with anti-p-HSP-27 (Ser82) (A), or anti-p-OSR1 (Ser339) (B), or anti-p-Rb (Ser608) (C) or anti-p-MARCKS (Ser170) (D), or anti-p-MCM2 (Ser139)

antibodies. Proteins were detected by autoradiography and bands were estimated from densitometric scanning. An anti-tubulin antibody was used as a control for protein loading. Results are representative of three independent experiments performed in triplicate.

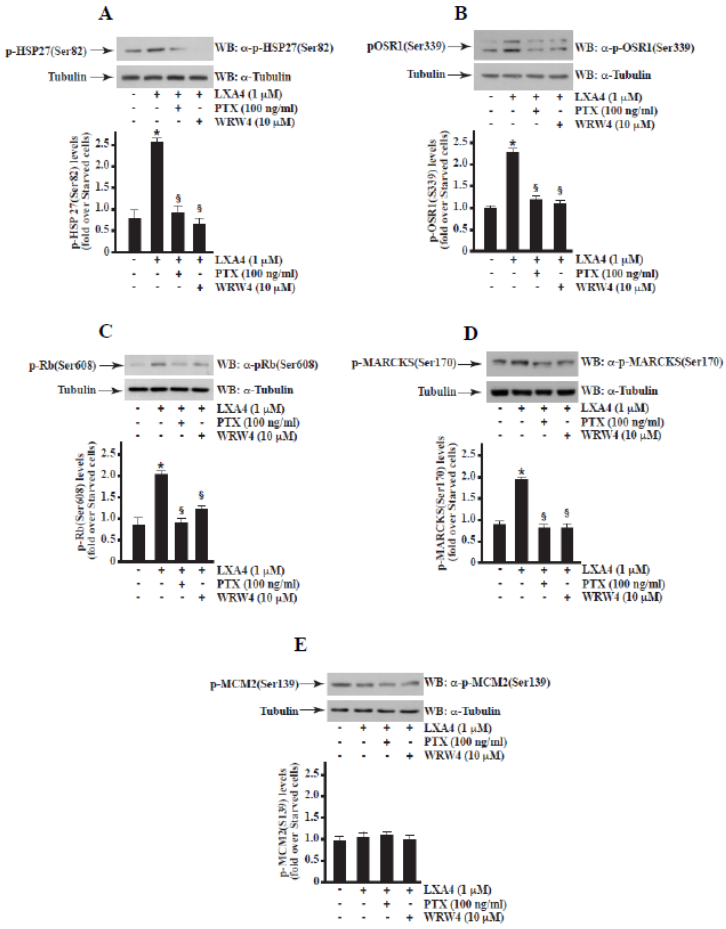


Figure 21. LXA4 triggers HSP-27(S82), OSR1(S339), Rb(S608) and MARCKS(S170) phosphorylation. CaLu-6 cells were serum-starved for 24 hours and stimulated or not with 1μM LXA4 for 5 minutes, or preincubated with 10μM WRW4 or 100 ng/mL PTX before LXA4 stimulation. 50μg of whole lysates were resolved on 10% SDS-PAGE and filters were incubated with anti-p-HSP-27 (Ser82) (A) or anti-p-OSR1 (Ser339) (B) or anti-p-Rb (Ser608) (C) or anti-p-MARCKS (Ser170) (D), or anti-p-MCM2 (Ser139) antibodies. Proteins were detected by autoradiography and bands were estimated from densitometric scanning. An anti-tubulin antibody was used as a control for protein loading. Results are representative of three independent experiments performed in triplicate.

5. DISCUSSION

The project of this thesis focused on the study of the molecular and biological functions of Formyl Peptide Receptors (FPRs), which belong to seven transmembrane Gi-protein coupled receptors. Overall, the results obtained highlighted the ability of this receptors to activate distinct intracellular signaling cascades in response to structurally diverse agonists, without similarity in their amino acid sequence or natural origin, and the existence of an intricate crosstalk with Receptor Tyrosine Kinases (RTKs).

The ability of FPRs to activate NOX enzymes allowed to demonstrate that ROS act as mediators of receptor-receptor interactions. This study has demonstrated that, in human neuroblastoma SH-SY5Y cell line, activation of FPR1 induced by the binding of its agonist N-fMLP triggers NOX2-dependent ROS generation and $p47^{phox}$ phosphorylation. Crosstalk between different receptor classes is a biological phenomenon that allows cells to integrate external stimuli through the activation of dynamic signaling networks. In the nervous system, TrkA is the high affinity receptor for the NGF neurotrophin and our results demonstrate that, in SH-SY5Y cells, FPR1 stimulation triggers the phosphorylation of Y490, Y751, and Y785 of TrkA. The observation that blocking FPR1 or NADPH oxidase activity prevents tyrosine phosphorylations of TrkA and the downstream signaling cascades triggered by TrkA, strongly suggests that TrkA transactivation depends on FPR1 stimulation and NADPH oxidase-dependent ROS generation. In this study we focused on the analysis of the role of ROS generated by NADPH oxidase complex

in FPR1-dependent transactivation of the TrkA receptor. However, we cannot exclude that TrkA transactivation could be also mediated by the activation of intracellular tyrosine kinases or by a ligand-dependent mechanism, based on the metalloprotease-dependent cleavage of pro-ligands of TrkA receptor. Furthermore, NOX2 activation in polymorphonuclear cells also requires $p40^{phox}$ and $p67^{phox}$ phosphorylation, Rac activation and membrane translocation of all the cytosolic components. In this study we have not investigated on molecular mechanisms responsible of their activation. However, $p47^{phox}$ phosphorylation is required for the interaction with the other cytosolic subunits ($p40^{phox}$, $p67^{phox}$ and Rac), for their membrane translocation and, in turn, for NADPH oxidase complex activation (El-Benna, 2009). Therefore, it is likely to assume the involvement of all cytosolic components in NOX2 activation following FPR1 stimulation.

Binding of NGF to TrkA induces ERK1/2 phosphorylation that promotes neurite outgrowth, the process of formation of functional neural circuits during neuronal development and differentiation (Vaudry, 2002). Phosphorylation of tyrosine Y490 of TrkA provides a docking site for the Shc domain and a phosphotyrosine site on Shc recruits Grb2, which is bound to the exchange factor SOS that represents a scaffold for Ras.

Activation of Ras pathway is essential for neuronal differentiation and survival of neuronal cells (Reichardt, 2006). Active Ras triggers intracellular signaling through cRaf, which phosphorylates Mek1/2, which, in turn, phosphorylates ERK1/2 on serine and threonine residues.

In our experiments, FPR1 stimulation triggers ERK1/2 phosphorylation, that is prevented by preincubation with PTX, Cyclosporin H or Apocynin. Ras/MAPK signaling cascades are largely involved in NGF-dependent wound healing and this signaling is triggered by NGF-dependent phosphorylation of Y490 residues of TrkA (Chen, 2014), whose phosphorylation in our experiments is trans-phosphorylated upon FPR1 activation by N-fMLP. ERK pathway is also involved in the extension of neurites from cell bodies and thus induces a differentiated neuronal phenotype. Our in vitro experiments demonstrated that FPR1 stimulation increases neurite outgrowth.

Phosphorylation on Y490 and Y751 of TrkA is associated with the activation of PI3K-Akt pathway, which is essential for the survival of developing neurons and in the initial phase of neurite outgrowth (Nusser, 2002). Our experiments show that FPR1 stimulation triggers Akt phosphorylation on serine 473, which is prevented by PTX, Cyclosporin H and Apocynin pretreatment.

NGF-dependent phosphorylation of Y785 of TrkA represents a docking site for the activation of PLCγ-PKC pathway which regulates neuronal gene expression, synaptic strength and plasticity and neurite outgrowth (Uren, 2014). PLC mediates the production of DAG and IP3 and the increase of intracellular Ca2+. Ca2+ release activates PKC, which translocates on cellular membranes. Our experiments demonstrated that FPR1 stimulation induces membrane translocation of PKCα and PKCδ.

Besides FPR1 expression in non-myeloid cells, high expression of FPR1 has been detected in several types of

cancers. This over-expression is associated with increased tumour aggressiveness and metastastic potential and correlated with tumour advanced stage and poor prognosis. In fact, FPR1 over-expression has been associated with cervical cancer tumorigenesis (Cao, 2018), breast cancer aggressiveness (Vecchi, 2018), invasiveness of colorectal cancer (Li, 2017), and with the development of nervous system tumours, such as neuroblastoma (Snapkov, 2016) and glioblastoma (Boer, 2015). Furthermore, different studies show that TrkA results to be over-expressed or iper-activated in several cancers, including breast, lung, colon-rectum, glioblastoma, neuroblastoma, myeloma, and lymphoid tumours (Meldolesi, 2018). Our experiments show that FPR1 mediates cell proliferation and migration of SH-SY5Y cells and that these biological effects are mediated by TrkA transactivation.

Signaling pathways dependent from the activation of GPCRs, RTKs and MAP kinases are major players in the regulation of cell fate and in the development of human diseases, such as inflammatory, neurodegenerative and cancer diseases, characterized by an alteration of the balance between phosphorylation and dephosphorylation events. Post translational modifications (PTMs) such as phosphorylation regulates a wide number of cellular activities like cell growth, differentiation, migration and apoptosis. The analysis of the global phosphoproteome activated by FPR2 stimulation in Calu-6 cells allows us to further clarify the intracellular networks activated by FPRs.

Mass spectrometry of enriched phosphoproteins obtained by TiO2-based affinity nano-liquid chromatography allows the identification of 290 proteins

and 510 different phospho-peptides associated with FPR2-dependent intracellular phosphorylations.
Phospho-protein functional analysis revealed that the phospho-proteins can be classified into eight groups and that Metabolic process represents the largest group in all the identified phosphorylated proteins. About 33% of the proteins of this group are involved in bio-synthetic processes. The remaining 67% of proteins are involved in cellular metabolic processes. Biological regulation represents the second largest group in all the identified phosphorylated proteins. In particular, here we identified proteins involved in the regulation of cell cycle, cell division, apoptosis and transmembrane transport. Analysis of the protein classes of the uniquely identified phosphoproteins shows that the nucleic acid binding proteins represent the largest group in all the phosphorylated proteins (RNA-binding proteins and DNA-binding proteins). They play crucial roles in transcription, replication, recombination, DNA repair, and other nuclear functions. In this group, we identified transcription and splicing regulatory proteins, proteins involved in the synthesis and maturation of ribosomes and translation initiation factors, cell cycle and DNA replication regulatory proteins, histonic and non-histonic DNA-binding proteins.
We validated by western blotting WKYMVm-dependent phosphorylation of Retinoblastoma protein (Rb), Minichromosome Maintenance Complex component 2 (MCM2), Myristoylated alanine-rich C-kinase substrate (MARCKS), Odd-skipped-related 1 protein (OSR1) and Heat shock protein 27 (HSP27).
Rb is a tumor suppressor protein that negatively regulates cell entry into S phase of cell cycle when it is

bound to the transcription factor E2F. The activity of Rb is regulated by phosphorylation of different serine residues. When phosphorylated on S608, Rb loses its ability to bind and inhibit E2F; hence, E2F is able to promote the G1/S transition (Burke, 2012). Rb was an interesting candidate for this validation since our previous results have demonstrated that FPR2 stimulation triggers Calu-6 cell proliferation (Cattaneo, 2011). We demonstrated that FPR2 stimulation induces Rb phosphorylation on S608 and that this phosphorylation depends on ERK1/2 activation.

MCM2 is a helicase with ATPase activity necessary for DNA replication initiation and elongation that belongs to the MCM2-7 complex (Ishimi, 2018). MCM2 can be phosphorylated on different residues by different protein kinases. Phosphorylation of MCM2 on S139 is reported to be induced by the serine-threonine kinase cell division cycle 7-related protein kinase (Cdc7) which is, in turn, phosphorylated by the protein Dbf4 (Tsuji, 2006). Our results show that MCM2 S139 phosphorylation depends on FPR2 and ERK1/2 activation. We speculate that FPR2 could transactivate EGFR and that the serine/threonine protein kinase Casein kinase II (CK2) phosphorylates and modulates ERK1/2, as well as several members of the EGFR downstream signaling pathways (Li, 2019). Furthermore, EGFR-dependent activation of CK2 leads to the phosphorylation of the enzyme phosphoglycerate kinase 1 (PGK1) that results in its interaction with Cdc7 (Li, 2018).

MARCKS is a membrane PIP2-binding protein substrate of PKC. It connects membranes with cytoskeleton, since it also binds actin and acts bridging PKC signaling with Ca2+-dependent protein calmodulin. Hence, MARCKS

is involved in cell migration, adhesion, actin crosslinking and regulation of cell cycle. Different studies have correlated MARCKS with the physiopathology of airway diseases, including chronic obstructive pulmonary disease, asthma, and lung cancer (Sheats, 2019). MARCKS phosphorylation on S170 is mediated by PKC and prevents MARCKS membrane localization and calmodulin binding (Gallant, 2005). According to the observation that MARCKS can be phosphorylated by PKCα, PKCδ and PKCε (Fujise, 1994), our previous results demonstrated that FPR2 stimulation triggers PKCα and PKCδ activation. Furthermore, we confirmed FPR2-PKC-dependence of MARCKS S170 phosphorylation by using inhibitors of either FPR2 or PKC signaling.

OSR1 is a serine/threonine-protein kinase involved in the regulation of solute carrier family 12 member 8 (SLC12A8), also known as cation-chloride cotransporter 9 (CCC9), that modulates ionic homeostasis in response to alterations in extracellular osmolarity. OSR1 is activated by phosphorylation on S339 triggered by a member of the "with no lysine" (WNK) protein kinase family (Alessi, 2014). The connection with FPR2 signaling is the EGFR-dependent activation of PI3K-Akt pathway that, in turn, activates the WNK-OSR1 cascade; in fact, Akt phosphorylates WNK3 on T60 (Gallolu Kankanamalage, 2018). The mammalian target of rapamycin complex 2 (mTORC2) can also phosphorylate OSR1 on S339 residue (Gagnon, 2010) in a WNK-independent pathway (Gallolu Kankanamalage, 2018).

HSP-27 (HSPB1) is a heat shock protein that acts as chaperone with anti-inflammatory and

immunomodulatory actions, since it can mediate the release of the anti-inflammatory cytokine IL-10 (De, 2000). In fact, it has been demonstrated that the anti-inflammatory FPR2 agonist ANXA1 induces the phosphorylation of p38MAPK, MAPKAP kinase, and HSP-27; on the contrary, HSP27 is not phosphorylated by the pro-inflammatory FPR2 agonists LL-37 and SAA (Cooray, 2013). HSP27 phosphorylation on S82 can be mediated by the P38MAPK/MAPKAP kinase 2/3/5 (Moens, 2013) or PKC pathway (Boivin, 2012). Coherently, our previous results demonstrated that FPR2 stimulation triggers PKC and p38MAPK phosphorylation.

Finally, our data demonstrated that the stimulation of FPR2 with the anti-inflammatory agonists WKYMVm, ANXA1 or LXA4 triggers the phosphorylation of the same intracellular targets, with the exception of MCM2.

6. CONCLUSIONS

Overall, the results obtained in this study demonstrate that FPR1-dependent ROS production exerts a key role in TrkA transactivation, in signalling cascades triggered by this receptor and, in turn, in modulating cell proliferation, migration, and neurite outgrowth. Furthermore, phosphoproteome analysis of FPR2-dependent phosphorylations highlights new signalling networks activated by FPRs and biological processes in which FPRs are involved.

A deeper understanding of signalling pathways responsible of tyrosine kinase transactivation and of intracellular phosphorylations triggered by FPRs will contribute to decipher the role of these receptors in the physiopathology of human diseases, such as inflammation, neurodegenerative disorders and cancers. Further advances on the knowledge of the functioning of FPRs will allow the evaluation and the identification of new molecular targets for the development of drugs able to target FPRs-dependent signalling molecules

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8. LIST OF PUBLICATIONS

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